Microbial transformation of starch to Lactic acid derivatives from potato processing industries

A thesis
submitted in fulfillment of the requirement
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DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

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September, 2014

Dedicated to my parents 

Certificate

THAPAR UNIVERSITY

Certified that the thesis **"Microbial Transformation of Starch to Lactic acid derivatives from Potato Processing Industries"** which is submitted by **Ms. Seema**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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I hereby declare that the work which is being presented in this thesis **"Microbial Transformation of Starch to Lactic acid derivatives from Potato Processing Industries"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Abhijit Ganguli**, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

(Seema)

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Abstract

This study aimed at producing high amounts of lactic acid and its derivatives from a cheap agro processing industry waste i.e. potato starch waste (PSW) in a direct fermentation utilizing a potential GRAS microorganism. For this, various fermented food samples were screened for isolating starch hydrolyzing lactic acid bacteria. A homofermentative strain, *Lactococcus lactis*, capable of potato starch hydrolysis was isolated from pickled yam. Maximum lactic acid production (8.5±0.5 g/L) by this strain was observed at optimal conditions of pH 5.0, temperature 30ºC and incubation time 48 hrs. in shake flask fermentation in minimal media and 10.1±0.5 g/L in modified MRS media. To refine L-Lactic acid production, a second order polynomial equation with significant R^2 (0.9037) showed considerable correspondence between the predicted (13.25 g/L L-lactic acid) and experimental values (15.60 g/L L-lactic acid) after 48hrs. at pH 5.5 and temperature 30ºC. Besides, the production of L- lactic acid, a reduction in chemical oxygen demand, biochemical oxygen demand and suspended solids of 76.9%, 62% and 77% respectively was also achieved in the process in Potato starch waste.

To maximize bioconversion and cell stability, high cell density systems such as immobilized cells as well as dialysis sac based bioreactor was fabricated. The maximum lactic acid concentration (18.9 g/L), L-lactic acid productivity and yield of 0.79 g/L/h and 1.48 g/g starch was 1.2 and 2.4 times higher respectively in bioreactor than shake flask fermentation in PSW at pH and temperature of 5.5 and 30ºC with operability for upto 3 cycles. L-Lactic acid obtained after fermentation was 89% pure as observed by HPLC which could be further esterified to produce lactic acid esters due to their wide applications in food, cosmetics and pharmaceutical industries. L-lactic acid recovered after fermentation was esterified to produce esters, namely, ethyl lactate and methyl lactate with

a yield of 0.69 g/g starch and 0.79 g/g starch respectively. Overall the results conclusively suggest an economically viable process for a microbial conversion of potato starch waste to the production of L-lactic acid and its derivatives. A comparison of economies vis-a-vis similar or related process as well as commercially available products indicate this process to be prospective as the cost reduces to 10 times and 2.4 times in in ethyl lactate and methyl lactate respectively.

Keywords: dialysis sac bioreactor, ethyl lactate, L-lactic acid, *Lactococcus lactis*, methyl lactate, potato starch waste

List of Publications[1]

Publications in peer reviewed journals

- **Seema Bhanwar**, Meenakshi Bamnia, Moushumi Ghosh and Abhijit Ganguli (2013). Use of *Lactococcus lactis t*o enrich sourdough bread with γ-aminobutyric acid. **International Journal of Food Sciences and Nutrition.** 64(1):77-81. Doi: 10.3109/09637486.2012.700919. Epub 2012 Jul 6.

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- **Seema Bhanwar** and Abhijit Ganguli, 2010. Co-production of dextransucrase and γ-aminobutyric acid (gaba) by *Lactococcus lactis. 51st* Association of Microbiologists of India, Dec. 14-17, BIT, Mesra, Ranchi.
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[1]Related to present thesis work

List of Abbreviations

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
Bsh	Bile salt hydrolase
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
FAO	Food agricultural organization
GI	Gastrointestinal tract
GRAS	Generally recognised as safe
HPLC	High performance liquid chromatography
KOH	Potassium hydroxide
LM	Light microscopy
LAB	Lactic Acid Bacteria
Min	minutes
MRS	De Man, Rogosa and Sharpe
NCBI	National centre for biotechnology information
OD	Optical Density
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
ppm	Parts per million
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
Sec	Seconds
SEM	Scanning electron micrography
TLC	Thin Layer Chromatgraphy
WTO	World Trade Organization
PSW	Potato Starch Waste
COD	Chemical Oxygen Demand
BOD	Biochemical Oxygen Demand

List of Symbols

%	Percentage
µ	Micron
µg	Microgram
µl	Microlitre
bp	Base pair
C	Carbon
d	Days
Da	Dalton
g	Gram
H	Hydrogen
H2O2	Hydrogen peroxide
hrs	Hours
kb	Kilo base
KDa	Kilo Dalton
kV	Kilo volt
L	Litre
M	Molar
mA	Milli ampere
mg	Milligram
Min	Minutes
mL	Milliliter
rpm	Revolution per minute
Sec	Seconds
U	Unit
V	Volt
v/v	volume by volume
w/v	Weight by volume
α	Alpha
β	Beta

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Chapter 1

Introduction

Introduction

Agro based industries produces large volumes of wastes, both solids and liquid; these wastes pose increasing disposal and potential severe pollution (High BOD or COD) problems and represent a loss of valuable biomass and nutrients. Beside their pollution and hazard aspects, in many cases, food processing wastes have a potential for conversion into useful products of higher value as by-products e.g. biogas, ethanol, fibers and different organic acids such as lactic acid, or even used as raw material for other industries. Organic waste materials that are generated particularly by food processing industries utilizing agricultural produces have great potential to be used as substrates for fermentation to produce various useful and valuable by products. For instance, potato processing plants generate an appreciable amount of starch in waste water streams; additionally potatoes which do not fit the standard quality criterion are discarded. They therefore could be utilized cheaply as a potential substrate for microorganisms producing intermediate volume of high value organic acids like lactic acid.

1.1 Lactic acid

Lactic acid (2-hydroxypropionic acid or 2-hydroxypropanoic acid), CH_3-CHOHCOOH, is the most widely naturally occurring carboxylic acid. Lactic acid has a chiral atom and occurs naturally in two optical isomers. One is known as L-(+) lactic acid or (*S*)-lactic acid and the other, its mirror image, is D-(-) lactic acid or (*R*)-lactic acid (Fig. 1.1). Since elevated levels of the D-(-) lactic acid are harmful to human beings, optically pure L-(+) lactic acid is biologically important and preferred isomer in pharmaceutical and food industries (Akerberg *et al.,* 1998; Hofvendahl and Hägerdal, 2000; Tashiro *et al.,* 2011; Shi *et al.,* 2013). Lactic acid, being present in almost every form of life, has important function for energy supply in tissues in human and animals. Lactic acid is considered as

generally recognized as safe (GRAS) for use as food additives by the regulatory agencies like FDA in USA.

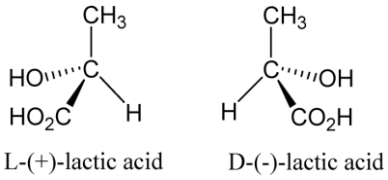

Fig. 1.1 Chemical structures of L-(+)-lactic acid and D-(-)-lactic acid

It has diverse practical applications: as a preservative, pH regulator and taste-enhancer in food industry; for implants and suture in the medical practice; as a reagent for polylactic and polyacrylic acids synthesis for biodegradable polymers; as a mordant in textile industry; tanning material in leather; and other chemical industries (Vickroy 1985; Wee *et al.*, 2006; John *et al.,* 2007). The natural occurrence of lactic acid in human body makes it very useful as an active ingredient in cosmetics (Wee *et al.,* 2006). Food industries account for approximately 85% of lactic acid applications whereas industrial applications account for only 15% of the demand. (John *et al.,* 2007). It has many other applications: it is used as descaling agent, solvent, cleaning agent, slow acid-releasing agent and humectants in a variety of technical processes. It is also used as flavoring or buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants (Datta *et al.,* 1995; John *et al.,* 2007). In fact, the demand for lactic acid has been increasing considerably, owing to the promising applications of its polymer as an environment-friendly alternative to petrochemicals plastics. The lactic acid polymers and co-polymers, offer tremendous advantages like biodegradability, thermoplasticity, high strength etc., and thus have potentially huge markets. Lactic acid fits wide application for the

manufacturing of large-volume oxygenated chemicals such as propylene glycol, propylene oxide, acrylic acid, and acrylate esters, and other chemical intermediates such as lactate ester plasticizers. In view of these applications, the production of lactic acid has increased considerably.

Lactic acid can be produced commercially by either chemical synthesis or biotechnological fermentation. The most commonly used method of chemical synthesis provides the racemic mixture of D- and L-lactic acid and mainly based on the hydrolysis of lactonitrile, a derivative of petrochemicals, by strong acids. Other possible routes for chemical synthesis of lactic acid include oxidation of propylene glycol, reaction of acetaldehyde, carbon monoxide, and water at elevated temperatures and pressures, base-catalyzed degradation of sugars, hydrolysis of chloropropionic acid, and nitric acid oxidation of propylene. These routes are neither technically feasible nor economically viable processes for industries (Datta *et al.,* 1995; Akerberg *et al.,* 1998; Tsao *et al.,* 1999; Yin *et al.,* 1997; Hetenyia *et al.,* 2011).

A biotechnological process can yield either a mixture of two isomers in different proportions, or form a single isomer alone (L-(+)- or D-(-)-lactic acid), which depend on the microorganism, substrate, metabolism control and growth conditions used. Biotechnological routes have significant advantages over chemical synthesis. Firstly, the use of cheap raw materials such as molasses, starchy waste, cellulosic and other carbohydrate rich materials (Anuradha *et al.,* 1999; Vishnu *et al.,* 2000). As raw material cost is one of the major factors considered in economic production of lactic acid, the media composition plays a vital role in the improvement of such a process. Secondly, besides high product specificity, it produces an optically pure L-(+)- or D-(−)-lactic acid with low production temperature, low energy consumption and ease of recovery (Pandey *et al.,* 2001; John *et al.,* 2007). Thirdly, the biological production of lactic acid has

received a significant interest as it offers an alternative to environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources.

Fermentative production of lactic acid from biomass has been extensively reviewed (John *et al.,* 2007; Datta *et al.,* 1995; Wee *et al.,* 2006). Various inexpensive raw materials such as starchy and cellulosic materials and molasses, whey, lactose containing waste generated from the dairy processing industry (Pauli and Fitzpatrick, 2002; Podlech *et al.,* 1990) and mussel processing waste containing glycogen (Pintado *et al.,* 1999) are exploited for lactic acid production. Among these, cellulosic materials and starch (produced by plants as reserve material) are currently receiving a great deal of attention because they are cheap, abundant, and renewable (Wee *et al.,* 2006). The main plants cultivated for the production of starch are cereals, especially corn, followed by wheat and potato (de Baere 1999). The carbon source for microbial production of lactic acid can be either sugar in pure form such as glucose, sucrose, lactose etc. or sugar-containing materials such as molasses, whey, sugarcane bagasse, cassava bagasse, and starchy materials from potato, tapioca, wheat, barley, and carrot (Pandey et al. 2001; Anuradha et al. 1999).

1.2 Raw material for lactic acid production

For the biotechnological production of lactic acid to be feasible, cheap raw materials are necessary, as already discussed. In view of this, various starchy raw materials such as corn by *Enterococcus faecalis* RKY1 (Oh et al., 2005), *Lactobacillus amylophilus* GV6 (Vishnu et al., 2000) and *Lactobacillus amylovorous* ATCC 33620 (Hofvendahl and Hagerdal, 1997) and wheat starch by *Enterococcus faecalis* RKY1 (Oh et al., 2005) and *Lactococcus lactis* subsp. *lactis* ATCC 19435 (Xiaodong et al., 1997) to name a few were used for lactic acid production. Though these raw materials are a good source of starch, carbohydrates and minerals but the major disadvantage is that the use of these agricultural

crops directly for the production of lactic acid increases the cost of production. Besides these starchy raw materials, another agricultural crop, potatoes are an excellent substrate for growth of microorganisms as they possess carbohydrate, protein and mineral constituents.

Potato starch waste (PSW) generated after potato processing like washing and blanching in industries have the potential to furnish microorganisms as an energy source (Natu, 1991; Tsao, 1999; Richter, 1998). Since potato starch waste is generated industrially at a large scale from various snack food industries and potato processing industries. PSW is abundantly and cheaply available. Therefore it is treated to produce value added products. It not only decreases the cost of production but also protects the environment from pollution. Various biotechnological methods have been developed for lactate generation from a cheap, abundant and renewable substrate as the potato starch. However, the main hindrance in starch utilization is the starch saccharification - a step that could be performed separately prior to fermentation or in parallel with the lactic acid synthesis (Hofvendahl *et al.,* 1999). Conventional fermentative production of lactic acid from starch material requires a pretreatment process that involves gelatinization and liquefaction, which is carried out at temperatures 90°C - 130°C for 15 minutes, followed by long time enzymatic saccharification to glucose at higher temperature, and subsequent conversion of glucose to lactic acid by fermentation. To overcome this disadvantage, one-step process of lactic acid production from starch was achieved by the use of lactic acid bacteria possessing extracellular amylase activity such as *Lactobacillus plantarum* A6 (Giraud *et al.,* 1991), *Lactobacillus manihotivorans* (Morlon-Guyot *et al.,* 1998), *Lactobacillus amylophilus* (Vishnu *et al.,* 2002), *Lactobacillus amylovorus* (Zhang and Cheyran, 1991) and *Streptococcus bovis* (Narita *et al.,* 2004). *Lactococcus lactis* is quite desirable for

lactic acid industry, because it is homofermentative, secretes only L (+)-lactic acid and is highly productive (Hofvendahl and Hahn-Hagerdal, 1997).

Lactic acid bacteria, such as *Lactobacillus amylovorous* ATCC 33620 (Yun et al., 2004), *Lactobacillus plantarum* MTCC 1407 (Panda and Ray, 2008), *Lactobacillus casei* (Palaniraj and Nagarajan, 2012) and some filamentous fungi like *Rhizopus arrhizus* and *Rhizopus oryzae* (Huang et al., 2003) are the major microbial sources involved in the utilization of PSW and production of lactic acid (Litchfield 1996). Lactic acid, as already discussed, have a large number of applications in food, pharmaceutical and cosmetic industry.

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) are characterized by a relatively simple sugar fermentation pathway that results in the formation of lactic acid. LAB are naturally found in nutrient-rich environments such as plant, fermented food, milk, meat, and intestinal tracts of human and animals (Hofvendahl and Hagerdal, 2000). Numerous investigations have been carried out on LAB producing lactic acid including *Lactobacillus, Lactococcus, Streptococcus* etc. as mentioned above but the microorganism used in this study needed to be homofermentative, i.e. produce only lactic acid. Alternately if the LAB have probiotic properties i.e. have GRAS status alongwith being amylolytic then it will provide dual application in using these bacteria for the consumption of starch based fermented foods for the production of lactic acid that will be industrially acceptable to be used as such in food industries. So, various potato starch hydrolysing LAB isolates were isolated from fermented starch based food samples in this study for lactic acid production.

Lactococcus lactis is one of the most used lactic acid bacterium by the dairy industry. It participates in the manufacture of fresh and semi-hard cheeses, cream, butter and sour

milks, giving the specific taste and texture of the products (Samarzija *et al.,* 2001). Widespread in fermented foods, it could also be isolated from certain sources containing starch: fermented cereal beverages such as maize pozol, non-alcoholic drink bushera and sorghum beer (Dıaz-Ruiz *et al.,* 2003; Muyanja *et al.,* 2003; Sawadogo-Lingani *et al.,* 2007); sorghum, maize, or wheat sourdough (Hamad *et al.,* 1997; Rocha and Malcata, 1999; Corsetti *et al.,* 2001), cooked fish products and the starchy meal poi made from the taro plant (Ostergaard *et al.,* 1998; Brown and Valiere, 2004). In spite of the starch abundance in these inhabitant niches, very few of the isolated lactococci were found to possess amylolytic properties (Ostergaard *et al.,* 1998; Dıaz-Ruiz *et al.,* 2003; Muyanja *et al.,* 2003). The isolation of a natural strain *L. lactis* subsp. *lactis* B84 capable of complete starch saccharification and produce lactic acid by studying the gene expression of the key enzymes responsible for the starch hydrolysis has also been reported (Petrov *et al.,* 2008). Fermentation broth containing lactic acid also contains impurities such as residual sugar, nutrients, colour and other organic acids, apart from cell mass. These impurities need to be removed for obtaining pure lactic acid. In order to purify and reduce the cost, many studies concerning lactic acid separation have been conducted using different separation techniques such as reactive extraction, adsorption, electro dialysis and esterification and reactive distillation. However, purification of lactic acid obtained from fermentation is difficult due to low vapor pressure of lactic acid, its self-esterification, and other impurities such as glucose and organic acid (acetic acid and butyric acid).

1.4 Lactic acid derivatives: esters

Lactic acid, its salts and esters are consumed primarily in food and beverages as an acidulant and preservative, in industrial applications, pharmaceuticals and personal care products. A great number of lactate esters are known in particular the main ones being methyl, ethyl and n-butyl esters. They are used in pharmaceutical, cosmetic industries and

used as solvents for varnishes, nitrocellulose and polyvinyl compounds. Methyl, ethyl and propyl lactates are water-soluble while butyl lactate is only slightly soluble. The lower esters are prepared by direct esterification while the lactic esters of higher alcohols are prepared from methyl or ethyl lactate, by trans-esterification with the appropriate alcohol. Esterification is currently the only downstream process which separates other organic acids from lactic acid, for instance, ethyl lactate and ethyl acetate etc. Therefore the aim of this research is to focus at developing a technology for un-catalyzed production of lactic acid esters, namely, ethyl and methyl lactate from lactic acid with wet ethanol and concentrated lactic acid while simultaneously achieving high selectivity toward ethyl and methyl lactate.

Ethyl lactate is the active ingredient in many anti-acne preparations used in many other applications including inks, flavourings, coatings, silicone oil and grease removal, and in the semiconductor industry, used as a "green" solubilizing agent. It is a replacement solvent for *N*-methylpyrrolidone, toluene, acetone, and xylene and also approved by the US Food and Drug Administration as an adjuvant solubilizing ingredient for flavors and pharmaceutical dyes. It is a non-ozone-depleting chemical, poses no hazard as an air pollutant, and is non-carcinogenic and noncorrosive. Methyl lactate is used to make flavours, fragrances, and dyes, but is also a common chemical base for producing many pharmaceuticals.

Objectives

Thus far there have been numerous investigations and developments of methodologies for the production of lactic acid including the use of cheap raw materials like whey, molasses, corn steep liquor and also potato starch wastewater by either direct fermentation or enzyme catalyzed fermentations. There have also been reports on various purification

processes like electrodialysis, ultrafiltration and distillation processes for recovery of purified lactic acid but no systematic studies have been conducted on development of industrially feasible, economically viable and sustainable process for the production of lactic acid and its derivatives from a cheap agro industrial waste. The following objectives were designed keeping in view the current gaps in research as follows:

- Identification and selection of amylolytic LAB
- Evaluation of starch degradation and characterization of potential isolates
- Kinetic analysis of lactic acid production from starch
- Optimization of cultural parameters for maximal lactic acid production
- Optimization of procedure for conversion of lactic acid to lactic acid derivatives.

Chapter 2

Review of Literature

Background

The purpose behind designing a microbial process for production of lactic acid, ethyl and methyl esters, is a multidisciplinary insight that uses cheap raw material for the generation of economically viable and environmentally robust and safe process for industrially important products. Various major components involved in the process are discussed in this chapter. Additionally a critical approach into the uses of various technologies like immobilization, dialysis and esterification, their limitations leading to fabrication of a new method for an economically sustainable process to ease product recovery is discussed.

2.1 Lactic acid

Lactic acid (2-hydroxypropanoic acid) is the most widely occurring natural organic acid in many foods, or as a product of *in situ* microbial fermentation (as in sauerkraut, yogurt, buttermilk, sourdough breads, and many other fermented foods). It was first discovered in sour milk by Scheele in 1780, who initially considered it a milk component. In 1789, Lavoisier named this milk component “acide lactique”, which became the possible origin of the current terminology for lactic acid. In 1857, however, Pasteur, Lister and Delbrueck discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms (Benninga 1990; Gregor 1999; Boontawan 2010).

2.1.1 Chemical structure and property of lactic acid

Lactic acid occurs naturally in two isomeric forms, D-(-)- and L-(+)-lactic acids. L-Lactic acid is a mirror image of D-lactic acid which could be soluble in water (Narayanan *et al*., 2004). It exhibits low volatility and has chemical formula of $C_3H_6O_3$. L(+)-Lactic acid

differs from D(-)-lactic acid in its effect on polarized light. Since lactic acid has high reactivity due to the presence of both hydroxyl (-OH) and carboxyl (-COOH) groups, in solution, it can lose a proton from the acidic group, reproduce the lactate ion, $CH_3CH(OH)COO^-$. The lactate ion could be precipitated with salt solution such as $Ca(OH)_2$ and $CaCO_3$ (Narayanan *et al*., 2004). Excess $CaCO_3$ is added to the supernatant to neutralize the acid produced and produce a calcium salt of the acid in the broth. The broth containing calcium lactate could improve purification method of lactic acid fermentation (Datta *et al*., 1995).

Both enantiomers of lactic acid have the same physical properties. The melting point of L(+)-lactic acid is reported at 52.8°C, and that of racemic lactic acid is 16.8 to 25.5°C. The pure isomers form colourless monoclinic crystals with a melting point of 54°C, a synthetic racemic lactic acid prepared by mixing equal quantities of the D(-)- and L(+)-isomers melts in the range of 28 to 33°C. Lactic acid is a weak acid that both isomers and the racemic mixture have the same dissociation constants and pKa (Lockwood *et al*., 1965). The presence of two functional groups, hydroxyl and carboxyl, permits a wide variety of chemical reactions for lactic acid. The primary classes of these reactions are oxidation, reduction, condensation, and substitutions.

2.1.2 Markets and Applications

L-Lactic acid is a versatile chemical having a wide range of applications in food, pharmaceutical, leather and textile industries. It also functions as the monomer for the biodegradable plastics (John *et al*., 2007). Some of the applications of lactic acid are as follows:

2.1.2.1 Biodegradable plastics

Biodegradable poly-L-lactic acid (PLLA or PLA) is a biodegradable polymer approved for use in food packaging in several countries, particularly U.S.A., European Union countries, and Japan (Narayanan *et al*., 2004). There is an increased interest in degradable plastics because of environmental concerns over disposal of plastics. Conventional plastic materials are not easily degraded in the environment because of their high molecular weight and hydrophobic character (Kharas *et al*., 1994). PLLA might become a potential environmentally friendly substitute of non-biodegradable plastics derived from petrochemicals (Lu *et al*., 2009). PLLA is an aliphatic polyesters and belongs to the α-hydroxy group which is the most readily biodegradable thermoplastics material (Kharas *et al*., 1994). L-Lactic acid contains both hydroxyl and carboxylic acid functional groups. This substance can thus undergo self-esterification to form a cyclic dimer, L-lactide and a linear polymer, poly (L-lactide) (Lockwood *et al*., 1965). The conversion of L-lactic acid to high-molecular weight PLLA is achieved by either ring-opening polymerization & polycondensation or chain extension and grafting.

PLLA is a semi crystalline polymer (approximately 37% crystallinity) having a glass transition temperature of 60-65°C and a melting temperature of approximately 175°C (Middleton and Tipton, 2000). PLLA has a good tensile strength, low extension and a high modulus (approximately 4.8 Giga Pascal, GPa) (Nair and Laurencin, 2007), is sensitive to heat, especially at temperatures higher than 190°C (Kharas *et al*., 1994). However, being more hydrophobic than polyglycolide, the degradation rate of PLLA is very low. It has been reported that high molecular weight PLLA can take between 2 and 5.6 years for total resorption *in vivo* (Bergsma *et al*., 1995; Middleton and Tipton, 2000). The rate of degradation, however, depends on the degree of polymer crystallinity as well as the porosity of the matrix.

2.1.2.2 Food industry

The major use of L-lactic acid is in food and food-related applications accounting for 85 and 15% of the demand, respectively. As a food acidulant, lactic acid has a mild acidic taste in contrast to other food acids. Lactic acid is non-volatile, odourless, and is classified as generally recognized as safe (GRAS) for use as a general purpose food additive by the FDA in the U.S.A. and other regulatory agencies elsewhere. It is a very good preservative and pickling agent for sauerkraut, olives, and pickled vegetables. It is used as acidulant, flavouring, pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods such as candy, bread and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants. An emerging new use for lactic acid or its salts is in the disinfection and packaging of carcasses, particularly those of poultry and fish, where the addition of aqueous solutions of lactic acid and its salts during the processing increased shelf life and reduced the growth of anaerobic spoilage organisms such as *Clostridium botulinum*. A large fraction (>50%) of the lactic acid for food-related uses goes to producing emulsifying agents used in foods, particularly for bakery goods (Datta *et al*., 1995).

2.1.2.3 Pharmaceutical and cosmetic applications

The water-retaining capacity of lactic acid makes it suitable for use as moisturizer in cosmetic formulations. The ability of lactic acid to suppress the formation of tyrosinase is responsible for its effects like skin lightening and rejuvenation. As humectants, the lactates are often superior to natural products and more effective then polyols (Datta *et al*., 1995; John *et al.,* 2007). Ethyl lactate is the active ingredient in many anti-acne preparations. L-Lactic acid is used in pharmaceutical industry as a very important ingredient.

Pharmaceutical industry shows preference for L-lactic acid because the D-isomer is not metabolized by human body. Lactic acid and its salts have been mentioned for various medical uses. L-(+)-lactic acid is used as excipient in pharmaceutical drugs due to its stabilizing effect on proteins and its natural anti-bacterial properties. Lactic acid solutions have pH range 3.5-4.2 which makes them perfect for elution of antibodies off Protein-A sorbents. It is also reported that pure lactic acid is one of the main components for IV resuscitation after blood loss due to trauma, surgery, or a burn injury. Lactic acid is used in various medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications (Wee *et al*., 2006).

2.1.2.4 Other industries

Lactic acid is also used in some other industries. For example, technical-grade lactic acid is extensively used in leather tanning industries as an acidulant for deliming hides and in vegetable tanning. Lactic acid is used as descaling agent, solvent, cleaning agent, slow acid-releasing agent and humectants in a variety of technical processes (John *et al*., 2007). L-Lactic acid could be potentially used for the manufacturing of large-volume oxygenated chemicals such as propylene glycol, propylene oxide, acrylic acid, and acrylate esters, and other chemical intermediates such as lactate ester plasticizers. The advances made in hydrogenolysis technology can be further developed and integrated to make propylene glycol from lactic acid in the future (Datta and Henry, 2006). Also, lactic acid is used as a natural anti-bacterial agent in disinfecting products.The applications in food and non-food sectors of lactic acid are summarized in Table 2.1.

2.2 Lactic Acid Bacteria

Lactic acid bacteria (LAB) include various major genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB.

Table 2.1 Industrial Applications of Lactic acid in Different Sectors

FOOD	**pH regulation**	To extend the shelf life. Lactic acid based acidulants are for instance used in salads and dressings.
	Flavoring	Enhanced and protected meat flavor. The mild acidic flavor is used in candy, soft drinks, beer, soups, dairy products etc.
	Antimicrobial action	Controlling pathogenic bacteria such as *E. coli, C. botulinum, L. monocytogenes* in foodstuff.
	Pickling agent	Pickled vegetables, olives and sauerkraut are conserved in lactic acid containing brine.
	Emulsifying agents	Particularly used in bakery goods
	Animal feed supplement	Stabilize gastro-intestinal function of dairy, beef, pork, poultry and pet animals.
NON-FOOD	**Pharmaceuticals**	Electrolyte solutions e.g. for artificial kidney machines, lactate salts of magnesium, zinc and iron lactate for deficiency therapy.
	Cosmetics	pH-regulator in shampoo and soap, moisturizing and antiwrinkling agent in many skin care products, anti-tartar agent in toothpastes and mouthwashes, skin lightening and rejuvenating agent in moisturizers.
	Solvents	Paint & Ink thinners, high-purity rinsing solvents for electronics, detergents, metal complexing agent and cleaning solutions with strong degreasing ability.
	Biodegradable polymers – Poly(lactic acid) (PLA)	Surgical sutures, implantable medical devices, pharmaceutical controlled drug delivery systems, and especially packaging materials, films, fibers, and yarns for manufacturing of clothing.

Other genera included in LAB are *Aerococcus, Microbacterium, Propionibacterium* and *Bifidobacterium* (Carr *et al*., 2002; Holzapfel *et al*., 2001).

LAB were first isolated from milk (Carr *et al*., 2002; Metchnikoff, 1908; Sandine *et al*., 1972) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris *et al*., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan *et al*., 2002). Fermented food naturally contains LAB (Caplice and Fitzgerald, 1999) and they have also been detected in soil, water, manure and sewage (Holzapfel *et al*., 2001). LAB such as *Lactobacillus* sp., *Lactococcus lactis,* and *Streptococcus thermophiles* are few examples that inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (Heller 2001; O'Sullivan *et al*., 2002). Metabolites of LAB have been reported to be used as biological preservatives in food packaging materials (Pirttijarvi *et al*., 2001; Scannell *et al*., 2000). Lactic and organic acid production decreases the pH of the growth environment which is responsible for the antimicrobial effect of LAB (Caplice and Fitzgerald, 1999; Kuipers *et al*., 2000). Low pH induces organic acids to become lipid soluble and diffuse through the cell membrane into the cytoplasm. LAB also produces acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodrígues *et al*., 2003), some of which may act as antimicrobials.

2.2.1 Taxonomy

Lactic acid bacteria (LAB) are Gram-positive cocci or rods, anaerobic, microaerophilic, or aero-tolerant; and catalase negative. They produce lactic acid as the major end product during fermentation of carbohydrate(s). Generally, LAB are mesophilic microorganisms

that grow in the temperature range of 10 to 45°C. However, some LAB are reported to be thermophilic and grow at 45°C. Some can grow at pH 3.2, some at high as 9.6, and most can grow in the pH range of 4.0-4.5 (Axelsson, 2004). The classification of LAB into different genera is largely based on their cell morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration, acid or alkaline tolerance, chemotaxonomic markers such as fatty acid composition, constituents of the cell wall, and phylogenetic relationships (Axelsson, 2004). The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis on phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE) are regarded important for specific characterization and detection of LAB strains (Gevers *et al*., 2001; Holzapfel *et al*., 2001).

2.2.2 Cell morphology

Cell morphology is important in identifying LAB genera. The bacteria can be divided into 2 groups: rods (*Lactobacillus* and *Carnobacterium*) and cocci (*Aerococcus*, *Alloiococcus*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*) (Axelsson, 2004). Cell morphology of the cocci have spherical cells ranging in diameter from 0.1-4.0 µm, which occur singly or in pairs, chain and tetrads. Some cocci that are sometimes oval or even short rods and occur as coccobacilli, are classified in the genus *Leuconostoc*. The Gram-positive rod group are long, slender rods to coccobacilli, which are variable in size

and range from 0.5-1.2×1.0-11.0 μm, and cells are arranged in chains (Yubon Pikul-ngoen, 2010).

2.2.3 Health benefiting LAB

LAB are regarded as a major group of probiotic bacteria (Collins *et al*., 1998; Metchnikoff, 1908; Schrezenmeir and de Vrese, 2001; Tannock, 1998). Various definitions of probiotic bacteria have been proposed over the years. Probiotics are "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989). Salminen *et al*. (1999) proposed that probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. Probiotics are regarded as health-benefiting bacteria and mainly reported are lactobacilli, lactococci and bifidobacteria (Rolfe 2000; Tuohy *et al*., 2003). LAB constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism (Fernandes *et al*., 1987). The mechanism of probiotics is believed to be fermentation (Gibson and Fuller, 2000; Metchnikoff 1908).

LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gibson and Fuller, 2000; Gorbach, 1990; Jay, 2000) and also synthesize enzymes, vitamins, antioxidants and bacteriocins (Fernandes *et al*., 1987; Knorr, 1998). With these properties, intestinal LAB constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990). Health benefitting LAB have been found in gastrointestinal tract and also in vaginal epithelium. Vaginal LAB strains have ability to self-aggregate in a process mediated by surface proteins or lipoproteins (Boris *et al*., 1998). Both aggregation and adhesion in LAB may

favour the vaginal epithelium through the formation of a bacterial film (Nitisinprasert *et al.*, 2006) contributing to the exclusion of pathogens from the vaginal mucosa (Boris *et al*., 1998). *Lactobacillus rhamnosus* strain GG and *Lactobacillus reuteri* ING1 have been shown to exhibit disease-specific adhesion to intestinal tissue (Ouwehand *et al*., 2003).

2.2.4 Exploitation of probiotic LAB

Probiotics are live microorganisms that are used as dietary supplements with the aim of benefiting the health of consumers by positively influencing the intestinal microbial balance. *Bifidobacterium* and *Lactobacillus* species have been the focus of probiotic interest since a large population of these bacteria in the intestinal tract is generally considered to be indicative of a healthy microbiota. These bacteria are increasingly being included as functional ingredients, particularly in dairy products such as yogurts and fermented milks, as evidence accumulates that they have beneficial effects on human health (Crittenden *et al*., 2001).

The methods for selection of probiotic bacterial strains are well defined. Host specificity, GRAS status, colonization, antimicrobial activity, and desirable metabolic activity are generally agreed upon (Collins *et al*., 1998; Reid *et al*., 2003; Tannock, 1998). A large number of LAB are isolated form food and non-food samples and characterized for their probiotic properties with production of additional valuable products till date. For instance, *Lactobacillus plantarum* (IB-1) and *Lactococcus lactis* (IB-2) strains isolated from fermented Idli batter could tolerate high bile salt concentration and low pH and produce Vitamin B12 and β-galactosidase (Iyer *et al*., 2011). The ultimate test for probiotic functionality is a double blind, placebo-controlled and randomised human study (Gibson and Fuller, 2000). The demonstration of probiotic activity of a given strain requires a well - designed, double blind, placebo-controlled host-specific study also showing resistance to

technological processes, i.e. viability and activity throughout processing phases (Dunne *et al*., 2001). Each potential probiotic strain must be documented independently without extrapolating any data from closely related strains and employing only well defined strains, products and study populations in trials.

The use of LAB as a probiotic requires a safety assessment. The characterization and functional properties of the strains should be well studied and documented (Holzapfel *et al*., 2001; Mahdhi *et al*., 2011). Generally recognized health-promoting properties are non-pathogenic behavior, the ability to persist within the GI tract and adhesion, and the ability to modulate immune responses (Dunne *et al*., 2001; Gibson and Fuller, 2000; Holzapfel *et al*., 2001; Reid *et al*, 2003). Gibson and Fuller (2000) pointed out the importance of considering the possible side effects of probiotics on the consumer, e.g. bloating or blocking the normal functional gut transit.

2.2.5 Selection Criteria for Probiotics

A successful potential probiotic strain is expected to have a number of desirable properties (Table 2.2). A potential probiotic strains does not need to fulfill all such selection criteria (Quwehand, *et al*. 1999) but major criterion discussed below should be accomplished.

Bile and Acid Tolerance

Bacteria that are used as probiotic strains are present in the intestinal tract via the mouth. Probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity as it enters the food system through mouth. As the food containing probiotic culture reaches the stomach and enter the upper intestinal tract which contain bile, here strains should have the ability to resist the digestion processes. It is reported that time of the food intake to digestion takes three hours. Strains need to be resistant to the stressful conditions of the

stomach (pH 1.5-3.0) and upper intestine which contain bile (Chou and Weimer 1999, Cakır 2003). As discussed, the criterion for probiotic strain is resistance to acid and bile. Bile acids are synthesized in the liver from cholesterol and sent to the gall –bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids undergoes some chemical modifications (deconjugation, dehydroxylation dehydrogenation and deglucuronidation) due to the microbial activity.

Escherichia coli subspecies, *Klebsiella* spp., and *Enterococcus* spp. are the main target for conjugated and deconjugated bile acids *in vitro.* The deconjugated acid forms are more effective on Gram positive bacteria (Dunne, *et al.* 1999, Cakır 2003). Dairy industry uses *Lactobacillus acidophilus* as probiotic starter strain. Chou and Weimer (1999) tried to isolate acid and bile resistant variants of *Lactobacillus acidophilus* and reported that these strains were capable of growth in medium at pH 3.5 containing 0.2% mixed bile salts.

In a study a large culture collection of LAB (200 strains of *Lactobacillus* and *Bifidobacterium*) of NZDRI was screened to select strains to use as probiotics based on their ability of resistant to bile (0, 0.5 and 1% w/v) and acid (pH 1-3) and four of them selected. Three of them were from dairy origin and the last one was from human origin. They were compared with the two commercial probiotic strains namely *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* LA-1. They showed tolerance to the above conditions and among them, the strain from human origin showed higher tolerance. These strains were identified as *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN067, *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* HN019 (Prasad, *et al*. 1998).

Table: 2.2 Selection criteria for probiotics

Probiotic Strain Properties	**Remarks**
Human origin for human usage	Although the human probiotic *Saccharomyces boulardii* is not human origin, this criterion is important for species dependent health effects.
Acid and bile tolerance	Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity
Adhesion to mucosal Surface	Imortant to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization.
Safe for food and clinical use	Identification and characterization of strains accurately, documented safety. No invasion and no degradation of intestinal mucus.
Clinically validated and documented health effects	Minumum effective dosage has to be known for each particular strain and in different products. Placebo controlled, double-blinded and randomized studies have to be run.
Good technological Properties	Survival in products if viable organisms are required, phage resistance, strain stability, culturable in large scales, oxygen resistance, have no negative effects on product flavour.

Antimicrobial Activity

Antimicrobial activity is a major selection criteria for probiotics as it aims at the enteric undesirables and pathogens (Klaenhammer and Kullen 1999; Mahdhi *et al*., 2011) by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Quwehand and Vesterlund 2004, Cakir 2003; Ratanapibulsawat *et al.*, 2005; Alemu *et al.,* 2006). Different species produce different antimicrobial substances, for instance, *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuterin;

subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocin acidophilucin A (Quwehand and Vesterlund 2004; Melaku *et al*., 2005). Production of bacteriocins is highly affected by factors such as the species of microorganisms, ingredients and pH of medium, incubation temperature and time. Nisin, produced by *L. lactis* subsp. *lactis* is well-known bacteriocin and is allowed for use in food preparations (Cakır 2003).

Antimicrobial activity of *Lactobacillus salivarus* UCC118 was noted against *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, Escherichia coli, Lactobacillus, Streptococcus, Bifidobacterium* and *Lactococcus*. The study showed that *Lactobacillus salivarus* UCC118 is significantly capable of inhibiting *in vitro* growth of both some Gram positive and some Gram negative bacteria such as *Lactobacillus fermentum* KLD, *B. longum, B. bifidum, Bacillus subtilus, B. cereus, Bacillus thuringiensis, Enterococcus faecalis, Enterococcus faecium* etc. although it is not effective against some of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* etc. species (Dunne, *et al*. 1999). Chuayana *et al* (2003) isolated potential probiotic bacteria from some milk products and determined their possible antimicrobial activities. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Serratia marcescens* and *Candida albicans* were used as indicator microorganisms. After the study, the results showed that, Yakult and Ski D' Lite probiotics inhibited all the tested indicator microorganisms, Nestle yogurt probiotics were bactericidal for *Staphylococcus aureus* and *Pseudomonas aeruginosa* but inhibitory for *Salmonella typhi*, Neslac probiotics killed E. coli and *Salmonella typhi* while they were only inhibitory for

Staphylococcus aureus and *Candida albicans,* Gain probiotics inhibited *Candida albicans* (Chuayana, *et al*. 2003).

In another research, potential probiotic lactobacilli strains (*Lactobacillus reuteri, Lactobacillus plantarum, Lactobacillus mucosae, Lactobacillus rossiae* strains) (from pig feces), used as additives in pelleted feeding, were examined according to their antibacterial activity against to *Salmonella typhimurium* ATCC 27164, *Escherichia coli, Clostridium perfringens* 22G, *Staphylococcus aureus* ATCC 25923, *Bacillus megaterium* F6, *Lactobacillus innocua* DSM 20649 and *Brachyspira hyodysenteriae* ATCC 27164. Generally the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *Brachyrospira hyodysenteriae* ATCC 27164. The study showed that, neutralization and treatment with catalase affected the antibacterial activity marginally (De Angelis *et al*. 2006).

2.2.6 Starch-utilizing and lactic acid-producing bacteria

Starch is one of the most common storage compounds, and generally consists of 25% amylose which is a linear polymer constituting 15% to 20% starch, and 75% amylopectin which is a large branched molecule and is a major component of starch (Moat *et al*., 2002; (Sajilata *et al*., 2006). Amylose is a long and unbranched chain of glucose in α-(1,4) linkage whereas amylopectin is a highly branched form of starch in which the backbone consists of glucose chains in α-(1,4) linkage with α-(1,6) linkages at the branch points. Amylose can be hydrolyzed by α-amylase which cleaves the α-(1,4) linkages to yield a mixture of α-glucose and α-maltose. Amylose is also hydrolyzed by β-amylase producing β-maltose. These enzymes also hydrolyze amylopectin to yield glucose, maltose, and a branched core, but it is not completely degradation. The α-(1,6) linkage in branch is hydrolyzed by α-(1,6)-glucosidase. Thus, the combined action of α-(1,6)-glucosidase and

α-amylase is required to completely degrade amylopectin to glucose and maltose (Sansit, 2004). Lactic acid fermentation with emphasis on the use of starch or starchy substrates have been extensively studied. In most cases, starch cannot be used by LAB directly, and the large starch macromolecules are converted into glucose molecules by treatment with acid or enzymes. Bioconversion of polysaccharide carbohydrate materials to lactic acid can be made much more effective by coupling the enzymatic hydrolysis of substrates and microbial fermentation of the derived glucose, which has been successfully employed for lactic acid production from raw starch materials (Reddy *et al*., 2008). The hydrolysis occurs in two steps. First, the insoluble starch has to be liquefied at high temperatures (80-85°C) using an amylase. In the second step, the liquefied starch is degraded to glucose by a glucoamylase at medium temperatures (55°C) (Venus, 2006).

Amylolytic LAB can ferment different types of amylaceous raw material, such as potato starch (Giraud *et al*., 1991; Petrov *et al*., 2008), corn starch (Nakamura, 1981; Zhang and Cheryan, 1991; Mercier *et al*., 1992; Vishnu *et al*., 2000; Narita *et al*. 2004; John *et al*., 2007), sago starch (Shibata *et al*., 2007), sorghum flour, wheat flour, cassava flour, rice flour and barley flour (Vishnu *et al*., 2002) due to the ability of their α-amylases to partially hydrolyze raw starch. Amylolytic LAB utilize starchy biomass and convert into lactic acid in a single step fermentation. Although amylolytic LAB are able to simultaneously hydrolyze and ferment starch to lactic acid, however, only a few amylolytic LAB have been reported on their lactic acid production ability such as *Lactobacillus plantarum* (Giraud *et al*., 1991; 1994; Panda and Ray, 2008), *Lactobacillus manihotivorans* (Guyot *et al*., 2000; Ohkouchi and Inoue, 2006), *Lactobacillus amylophilus* (Yumoto and Ikeda, 1995; Vishnu *et al*., 2000; 2002; John *et al* , 2007; Yen and Kang, 2010), *Lactobacillus amylovorus* (Zhang and Cheryan,1991; 1994; Linko *et al*.,

1996; Nagarjun *et al*., 2005), *Lactobacillus fermentum* Ogi E1 (Santoyo *et al*., 2003), *Lactococcus lactis* subsp. *lactis* (Petrov *et al*., 2008), *Enterococcus faecium* (Shibata *et al*., 2007), and *Streptococcus bovis* (Narita *et al*., 2004).

2.3 Raw material selection

It is very difficult to choose appropriate feedstock for the economically viable production of lactic acid. Chemical composition of the feedstock and structure of the carbon source is essential to be known for implementing appropriate pretreatment and product recovery processes as well as identification and selection for right fermenting microorganism(s). The production cost for lactic acid mainly depends on initial cost of substrate and the purification processes. The separation and purification processes are the most expensive part of fermentative lactic acid production which is especially pronounced with the most impure substrates. The costs for product purification should be significantly reduced when refined materials are used for production.

A number of different renewable resources have been found to have potential as a feedstock for production of lactic acid (Hofvendahl and Hahn-Hagerdal 2000). Among these renewable materials, glucose and starch have been more widely used in previous studies because of higher lactic acid yields obtained. However, as the substrate cost is one of the major operational costs, representing 30–40% of total production costs (Akerberg and Zacchi 2000), use of cheap and widely available raw starch materials, especially waste starch, is an economical approach for lactic acid production. Waste potato starch is an abundant byproduct in the potato processing industry. Jin *et al*. (2003) used waste potato starch as a substrate to produce lactic acid using *Rhizopus* spp, and the feasibility of lactic acid production using waste potato starch was proved in their research.

Approximately 3.5 billion tons of agricultural residues are produced per annum in the world. The use of a specific carbohydrate feedstock depends on its price, availability, and purity. Although agro-industrial residues are rich in carbohydrates, their utilization is limited due to low protein content and poor digestibility by the presence of cellulosic residues (John *et al*., 2007). Some agricultural residues that are potential substrates for lactic acid production are lignocellulose/ hemicellulose hydrolyzates (Karel *et al*., 1997), cottonseed hulls, corn cob, corn stalks (Vickroy, 1985), beet molasses (Goksungur and Guvenc, 1999; Kotzamanidis *et al*., 2002), sugarcane press mud (Xavier and Lonsane,1994), cassava bagasse (Rojan *et al*., 2005; John *et al*., 2006a, b), cellulose (Venkatesh, 1997), carrot processing waste (Pandey *et al*., 2001), molasses spent wash (Sharma *et al*., 2003), corn fiber hydrolysates (Saha and Nakamura, 2003), and wheat bran (John *et al*., 2006c; Naveena *et al*., 2005a, b).

Besides agro residues, other reasonably cheap raw materials such as starchy and cellulosic materials, whey, and molasses have been used for lactic acid production (Hofvendahl and Hahn-Hägerdal, 2000). Among these, starchy and cellulosic materials receive a great deal of attention, because they are cheap, abundant, and renewable (Richter and Berthold, 1998). The starchy materials used for lactic acid production include sorghum flour (Richter and Träger, 1994; Vishnu *et al*., 2002), wheat flour (Hofvendahl and Hahn-Hägerdal, 1997; Oh *et al*., 2005), corn starch (Vishnu *et al*., 2000; Narita *et al*. 2004; Oh *et al*., 2005; John *et al*., 2007), cassava starch (Xiaodong *et al*., 1997; Vishnu *et al*., 2002), sago starch (Shibata *et al*., 2007), potato starch (Giraud *et al*., 1991; Yun *et al*., 2004; Petrov *et al*., 2008), rice flour (Vishnu *et al*., 2002; Yun *et al*., 2004), and barley (Linko and Javanainen, 1996; Vishnu *et al*., 2002). These materials have to be hydrolyzed into fermentable sugars before fermentation, because they consist mainly of α(1,4)- and α(1,6)-

linked glucose (Richter and Träger, 1994; Hofvendahl and Hahn-Hägerdal, 1997; Oh *et al*., 2005). This hydrolysis can be carried out simultaneously with fermentation. Some industrial waste products such as whey and molasses are of interest as common substrates for lactic acid production. Whey is a major byproduct of the dairy industry, and it contains lactose, protein, fat, and mineral salts. For complete utilization of whey lactose, it is necessary to supplement whey with an additional nitrogen source (Hofvendahl and Hahn-Hägerdal, 2000). Schepers *et al*. (2002) supplemented whey with yeast extract for rapid production of lactic acid with *Lactobacillus helveticus*. There have been several attempts to produce lactic acid from whey by batch culture of *L. casei* (Büyükkilci and Harsa, 2004). Molasses is a waste product from the sugar manufacturing process, and it usually contains a large amount of sucrose (Hofvendahl and Hahn-Hägerdal, 2000). *Lactobacillus delbrueckii* and *Enterococcus faecalis* have been used for lactic acid production from molasses (Kotzanmanidis *et al*., 2002).

Inexpensive raw materials are necessary for the feasible economic production of lactic acid because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should not only be of low cost, but with low levels of contaminants and low toxic materials capable of being fermented with little or no pre-treatment resulting in rapid production rate and high yield with little or no by-product formation and be available throughout the year (Wee *et al*., 2006; John *et al*., 2007). Therefore, potato starch waste was selected in the present study for the production of lactic acid and its derivatives due to being low priced and its ease of year round availability.

2.4 Production of lactic acid

Lactic acid can be manufactured by either chemical synthesis or microbial fermentation; both are used for commercial production.

2.4.1 Chemical synthesis

All chemical synthesis routes produce only the racemic lactic acid. The commercial process is based on lactonitrile, which used to be a byproduct from acrylonitrile synthesis. Lactonitrile is produced by combining of hydrogen cyanide reaction and occurs at atmospheric pressures. The crude lactonitrile is then recovered and purified by distillation and is hydrolyzed to lactic acid by using either concentrated hydrochloric or Sulfuric acid, producing the corresponding ammonium salt as a byproduct.

There are other possible routes for chemical synthesis of lactic acid, for example: oxidation of propylene glycol; reaction of acetaldehyde, carbon monoxide, and water at elevated temperatures and pressures; hydrolysis of chloropropionic acid (prepared by chlorination of propionic acid), and nitric acid oxidation of propylene. A critical review of the above data indicates technical or economical non viability of these processes (Datta *et al*., 1995).

$CH_3CHO + HCN \rightarrow CH_3CHOHCN$

Lactonitrile

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

Ammonium sulphate

$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOH + H_2O$

Lactic acid Methanol Methyl lactate

$CH_3CHOHCOOH + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$

2.4.2 Microbial fermentation

Microorganisms that produce lactic acid can be divided into two groups: bacteria and fungi. Zhou *et al*. (1999) reported that *Rhizopus oryzae* ATCC 52311 utilize glucose aerobically to produce only L-lactic acid. However in industrial fermentations, the use of various species of *Lactobacillus* is preferred owing to higher rates of metabolism and increased yields. Stereo specific lactic acid can be easily made by carbohydrate fermentation depending on the microbial strain being used, it can be described by the reactions (Narayanan, 2004).

$$C_6H_{12}O_6 + Ca(OH)_2 \rightarrow 2(CH_3CHOHCOO^-)Ca^{2+} + 2H_2O$$

Carbohydrate Calcium hydroxide Calcium lactate

$$2(CH_3CHOHCOO^-)Ca^{2+} + H_2SO_4 \rightarrow 2CH_3CHOH + CaSO_4$$

Calcium sulphate

$$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O$$

Methyl lactate

$$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$$

The broth containing calcium lactate is filtered to remove cells, carbon treated, evaporated and acidified with sulphuric acid to get lactic acid and calcium sulphate. The insoluble calcium sulphate is removed by filtration; lactic acid is obtained by hydrolysis, esterification, distillation and hydrolysis. The stereospecificity of the lactate dehydrogenase and the presence of a Lactate racemase determines whether D (-) /L (+)/DL mixture would be produced. There are two specific routes for fermentation depening upon the microorganisms (Shuler, 2003).

2.4.2.1 Homofermentative lactate fermentation:

Homofermentative LAB like *Lactococcus sp., Streptococci sp., Enterococcus sp* produce pure or almost pure (90%) lactate. They metabolize glucose via the fructose-bis phosphate pathway and produce 1 molecule of lactate from 1 molecule of glucose.

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$$

2.4.2.2 Heterofermentative lactate fermentation:

Heterofermentative lactate fermentation is species specific for instance, *Leuconostoc sp., Lactobacillus sp, Lactobacillus fermentum.* In this fermentation, lactic acid bacteria produce 1 molecule of lactate along with 1 molecule of ethanol and 1 molecule of carbon dioxide (or acetic acid)

$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2 \text{ (or } CH_3COOH)$$

Lactate dehydrogenase is a key enzyme in lactic acid fermentation by most LAB. Most bacterial species possess only one lactate dehydrogenase gene (Rodtong and Ishizaki, 2003). L-Lactic acid producing bacteria contain L-lactate dehydrogenase (L-LDH) which is a key enzyme in converting pyruvate to L-lactic acid.

Fermentation

Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used process for lactic acid production. Higher lactic acid concentrations may be obtained in batch and fed-batch cultures than in continuous cultures, whereas higher productivity may be achieved by the use of continuous cultures (Hujanen and Linko, 1996; Holvendahl and Hahn-Hägerdal, 2000). For example, *Enterococcus faecium* No.78 was selected for L-lactic acid production using sago starch in batch and continuous fermentations (Shibata *et*

al., 2007). Linko and Javanainen (1996) proposed that during fermentation of the batch process, the bacterium fermented sugar to create pyruvate which was then converted to L-lactic acid via L-lactate dehydrogenase under anaerobic condition. L-Lactic acid could also be produced from starchy substrates by the simultaneous saccharification and fermentation (SSF). Such a combined process of converting enzymically liquefied starch to glucose with α-amylase and glucoamylase, and glucose to lactic acid by LAB, would be expected to decrease the total process time, and the capital and operating costs (Linko and Javanainen, 1996). The process of lactic acid production from starch was achieved by the use of amylolytic LAB possessing extracellular amylase activity. Very few reports are available on isolation of amylolytic LAB for single step fermentation of inexpensive complex carbohydrates (starch) to lactic acid. Use of efficient amylolytic lactic acid-producing bacteria eliminated saccharification costs of substrate thereby reducing the production cost (Reddy *et al*., 2008).

Simultaneous Saccharification and Fermentation (SSF)

The bioconversion of carbohydrate materials to lactic acid may be rendered more effective by coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived sugars into a single step, known as "simultaneous saccharification and fermentation". SSF eliminates the need for complete hydrolysis of carbon substrates prior to the fermentation. In the SSF process, enzymatic hydrolysis, cell growth and microbial production occur simultaneously. A direct benefit of the SSF is a decrease in the inhibition caused by mono or di-saccharide accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs (Anuradha *et al*., 1999). Many factors such as pH, temperature, substrates and product concentration of glucose and lactic acid can affect the

efficiency of the SSF (Akerberg *et al*., 1998; Anuradha *et al*., 1999; Linko *et al*., 1996; Moldes *et al*., 2001). A disadvantage associated with SSF is the difference in cultivation conditions such as temperature and pH required for saccharification versus fermentation (Stenberg *et al*., 2000). Therefore, characterization of the microbial and biochemical kinetics and determination of the optimal process conditions which enhances SSF is of importance for optimal lactic acid production. SSF has been successfully used for lactic acid production from raw starch materials with lactic acid producing *Lactobacillus* and *Lactococcus* species (Vishnu *et al*., 2002; Zhang *et al*., 1994).

Conventional biotechnological production of lactic acid from starch materials requires a pretreatment process by gelatinization and liquefaction, which is carried out between 90 and 130 ºC for 15 min followed by enzymatic saccharification to glucose, and subsequent conversion of glucose to lactic acid by fermentation (Anuradha *et al*., 1999; Linko *et al*., 1996). In contrast, SSF integrates the saccharification and fermentation steps. Fig. 2.1 shows the conventional process (A) and SSF process (B) for lactic acid production. Lactic acid production from lignocellulolytic agricultural biomass using lactic acid bacteria has been explored (Abe and Takagi, 1991; Venkatesh, 1997). Simultaneous saccharification and fermentation at a low wheat flour concentration was simulated to investigate the influence of the enzyme concentration on the lactic acid production rate. It was observed that the enzyme concentration could be decreased from 6 µl/g to 1 µl/g starch without the saccharification being rate-limiting, provided that sufficient nutrients were available. Thus, for enzyme concentrations higher than 1 µl/g starch the fermentation is rate-limiting.

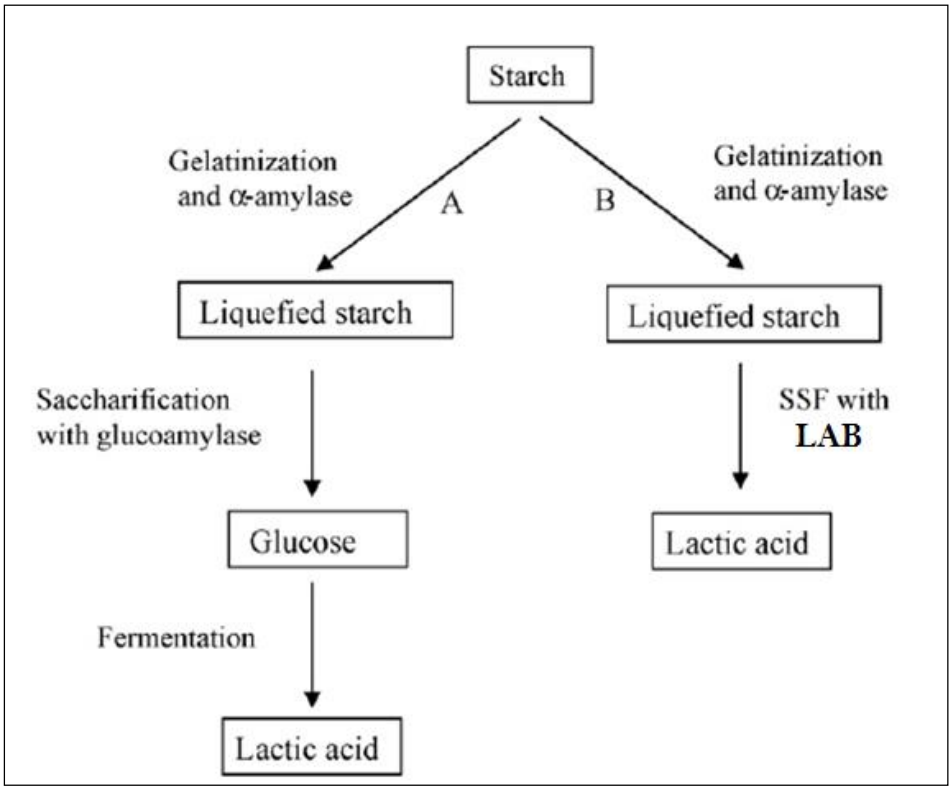

Fig.2.1. Comparison of conventional and SSF process for lactic acid production

Simulation showed that production of lactic acid with SSF decreases the requirement for enzymes, thus decreasing the cost (Hofvendahl *et al*., 1999). The first study on lactic acid production by SSF of cellulosic raw materials was reported by Abe and Takagi (1991), and since then some more articles have reported on this subject (Schmidt and Padukone,1997; Parajo *et al*.,1997; Moldes *et al*., 2000).

Direct lactic acid fermentation

Lactic acid can be produced by the fermentation of sugars or sugar containing hydrolyzates or the single step conversion of starchy or cellulosic wastes by direct conversion using amylolytic lactic acid producing microorganisms or by the simultaneous hydrolysis and fermentation with concomitant addition of saccharifying enzymes and inoculum together. Generally, hydrolyzate is used instead of refined sugars which can be utilized for the submerged fermentation or solid-state fermentation (Rojan *et al*., 2005;

John *et al*., 2006a). The reducing sugar concentration in the hydrolyzate affects fermentation, as bacterial cells cease to produce lactic acid when the sugar concentration is high. The conversion of starch or cellulose to sugar consumes energy during liquefaction or saccharification and increases the cost of production. Woiciechowski *et al*. (2002) studied the hydrolysis of cassava bagasse starch by acid and enzymatic hydrolysis. They reported that both methods were quite efficient when considering one or the other parameter like the percentage of hydrolysis, time and cost of the chemicals and energy consumption. Although acid hydrolysis is time saving and cost effective, a mandatory acid neutralizing step increases salts and microbial growth in the medium alongwith the production of lactic acid.

Though enzymatic hydrolysis is deemed to be superior since it yields a high percentage of reducing sugars from cassava bagasse, the enzymatic hydrolysis of 150 kg cassava bagasse required US$ 2470.99 as cost of enzymes and power for long time saccharification. Direct fermentation reduces the cost of energy consumption for the liquefaction and saccharification and thus it is cost effective and time saving process for the lactic acid production, thus the relevance of direct lactic acid fermentation. Direct fermentation has been successfully employed for lactic acid production from raw starch materials and in the recent past, bacteria including *Lactobacillus* and *Lactococcus* species are used to produce lactic acid effectively (Hofvendahl *et al*., 1999; Akerberg *et al*., 1998; Wee *et al*., 2004; Naveena *et al*., 2005; Vishnu *et al*., 2000; Ayad *et al*., 2004).

Besides various raw materials, fermentative production of lactic acid from biomass has been reviewed on microbiological production and biotechnological production of L-lactic acid (Datta *et al*., 1995; Wee *et al*., 2006; John *et al*., 2007). Development of production strains which ferment starch to lactic acid in a single step is necessary to make the process

economical. A few bacteria have been reported so far for direct fermentation of starch to lactic acid (Reddy *et al*., 2008). *Streptococcus bovis* 148 was found to directly produce lactic acid from starch with maximum lactic acid concentration of 14.2 g/L (Narita *et al*., 2004); direct lactic acid production by *Lactobacillus manihotivorans* LMG18011 using soluble starch and food wastes as substrates resulted in 19.5 g L(+)-lactic acid from 200 g food wastes (Ohkouchi and Inoue, 2006); *Lactobacillus plantarum* produced lactate yield of 0.81 g/g substrate (Giraud *et al*., 1994) and *Lactobacillus amylophilus* JCM 1125 produced 53.4 g of lactic acid/l using 100 g/L liquefied starch (Yumoto and Ikeda, 1995). Lactic acid production by *Lactobacillus plantarum* NCIM 2084 was 72.9 g/L with 100 g/L of liquefied starch (Krishnan *et al*., 1998). *Lactobacillus amylophilus* NRRL B4437 produced 29 g/L lactic acid from 45 g/L of corn starch and *Lactobacillus amylovorus* converted 120 g/L liquefied starch to 92.5 g/L lactic acid (Zhang and Cheryan, 1991; Mercier *et al*., 1992). *Lactobacillus amylovorus* utilized raw corn starch, rice starch and wheat starch medium to produce lactic acid with a productivity of 10.1, 7.9 and 7.8 g lactic acid/l respectively, but had lower productivities of 4.8 g/L and 4.2 g/L on cassava and potato starch in basal medium respectively.

When fermentative production of lactic acid directly from starch was studied in a batch fermentor using *Lactobacillus amylovorus*, 96.2 g/L of lactic acid was produced from an initial liquefied starch concentration of 120 g/L starch in 20 h while 92.5 g/L of lactate was produced from the raw starch of the same concentration in 39 h (Zhang and Cheryan, 1991). *Lactobacillus amylovorus* (Nakamura, 1981) and *Lactobacillus amylophilus* GV6 actively ferment starch to lactic acid when compared with other amylolytic strains of lactic acid bacteria (Cheng *et al*., 1991; Zhang and Cheryan, 1994; Vishnu *et al*., 1998).

2.4.2.3 Factors affecting L-lactic acid fermentation

Parameters including microorganism, carbon and nitrogen sources, fermentation mode, pH, and temperature affect the fermentative L-lactic acid production. The potential production of the acid is compared in terms of L-lactic acid concentration, yield, and productivity. Also byproduct formation and isomer of L-lactic acid have been reported (Holfvendahl and Hahn-Hagerdal, 2000). The amount of L-lactic acid produced by LAB is strongly influenced by cultures and fermentation conditions. Some main factors affecting the L-lactic acid production are as follows:

Chemical factors

Since LAB typically have complex nutritional requirements due to their limited ability to synthesize their own growth factors such as B vitamins and amino acids. They require carbon and nitrogen sources in the form of carbohydrates, amino acids, vitamins, and minerals (Axelsson, 2004; Wee *et al*., 2006). There are several growth-stimulation factors that have a considerable effect on the production rate of lactic acid (Wee *et al*., 2006). Compositions of the medium (carbon and nitrogen sources minerals and growth factors) are known to have impact on L-lactic acid production. Carbon sources are mainly carbohydrates utilized for L-lactic acid manufacture which are generally derived from sucrose (from syrups, juices and molasses), lactose (from whey), maltose (produced by specific enzymatic starch conversion processes), glucose (from starch conversion processes). Many investigators have reported on the capability of several bacterial strains to produce L-lactic acid by using molasses, whey, starchy, and cellulosic materials. The starchy materials used for lactic acid production include wheat, corn, sago, potato, rye, sweet sorghum, tapioca, rice, and barley as a carbon source.

A number of nitrogenous materials like whey permeate, yeast extract, malt sprouts, malt combing nuts, grass extract, peptones, beef extract, casein hydrolysate, corn steep liquor, soybean hydrolysate with supplementation of vitamins to supplement carbohydrate sources to give fast and heavy growth have been studied. However, yeast extract seems to be the most effective supplement (Narayanan *et al*., 2004). Presumably due to principal growth factor for LAB, some low-cost nutrients such as soy protein hydrolyzate (Hsieh *et al*., 1999), byproducts from malting industry (Hujanen and Linko, 1996; Pauli and Fitzpatrick, 2002; Fitzpatrick *et al*., 2003), bacterial extract (Rivas *et al*., 2004; Gao *et al*., 2006), ram horn protein hydrolyzate (Kurbanoglu and Kurbanoglu, 2003), fish wastes (Martone *et al*., 2005; Gao *et al*., 2007), corn steep liquor (Rivas *et al*., 2004; Lee, 2005; Oh *et al*., 2005), whey protein hydrolyzate (Fitzpatrick and O'Keeffe, 2001), red lentil flour and baker's yeast (Altaf *et al*., 2005, 2006, 2007a, and 2007b), and rice bran (Yun *et al*., 2004; Gao *et al*., 2008) have been used for lactic acid production.

Physical factors

The main physical factors reported to influence lactic acid fermentation are pH and temperature; pH is an easily manipulated variable in the process and it has a very strong impact on the cell response and metabolism. From the production standpoint, pH control is absolutely required to achieve the lactic acid concentrations essential for an economical process. In general, LAB can tolerate pH values between 3.4 and 8.0, but growth and production mostly occur between pH 5.4 and 6.4, with the optimum pH being strain-dependent (Kharas *et al*., 1994). The optimum pH for cell growth and lactic acid production of *Lactobacillus plantarum* was shown to be between 5.0 and 6.8 (Yumoto and Ikeda, 1995; Fu and Mathews, 1999; Ray *et al*., 2009) whereas, *Lactobacillus manihotivorans* had optimum pH at 5.0-6.0 (Guyot *et al*., 2000; Ohkouchi and Inoue,

2006) and *Streptococcus bovis* grew at pH between 5.8-9.6 with the optimum pH at 5.5-6.0 (Narita *et al*., 2004; Yuwono and Kokugan, 2008). For *Lactobacillus amylophilus* (Vishnu *et al*., 2000) and *L. lactis* (Bai *et al*., 2004) could produce lactic acid at pH 6.5. *Lactococcus lactis* subsp. *lactis* could produce lactic acid at pH 6.0 (Petrov *et al*., 2008), whereas *Enterococcus faecium* was produced lactic acid of pH 6.5 (Shibata *et al*., 2007).

Most LAB grow best between 37 and 42°C (Kharas *et al*., 1994). Effects of temperature on L-lactic acid production are highly variable, and depend on the strain being used and the experimental conditions. The effect of temperature on the production of L-lactic acid has only been studied in a few reports. The temperature giving the highest productivity was in some cases lower than the temperature resulting in high L-lactic acid concentration and yield (Hofvendahl and Hahn-Hägerdal, 2000), whereas in others, the same temperature gave the best results in all categories (Hujanen and Linko, 1996). For *Lactobacillus amylophilus*, which grows in a range of 15°C to 45°C (Hammes and Vogel, 1995), the optimal temperatures were 25°C and 35°C for the maximum productivity and yield, respectively. *Lactobacillus amylophilus* showed its optimum temperature at 30°C (Yen and Kang, 2010). For *Lactobacillus casei* and *Lactobacillus paracasei*, the optimal temperatures were reported to be between 37°C and 44°C (Richter and Träger, 1994), which is contradictory to the information that the strains grow at 15°C but not at 45°C (Hammes and Vogel, 1995). For strain *L. casei* LA-04-1, its optimal temperature was reported at 42°C (Ding and Tan, 2006). In agreement with previous observations, *Lactococcus lactis* and *Lactobacillus rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C, respectively (Hujanen and Linko, 1996). For strain *Lactococcus lactis* subsp. lactis, the optimal temperature was reported at 33°C (Petrov *et al*., 2008). For the optimal temperature was reported to be 37°C of *Lactobacillus*

casei (Linko and Javanainen, 1996), *Lactobacillus amylophilus* (Vishnu *et al*., 2000), *Lactobacillus helveticus* (Aeschlimann and Von Stockar, 1990), *Lactobacillus amylovorus* (Shibata *et al*., 2007) and *Lactococcus lactis* (Bai *et al*., 2004). For strain *Enterococcus faecium* was produced lactic acid at 30°C (Shibata *et al*., 2007). For *Streptococcus*, its generally grows within the range 20-42°C (Hardie and Whiley, 1995). A strain of *Streptococcus bovis*, the optimal temperature was reported to be 37-39°C (Narita *et al*., 2004; Yuwono and Kokugan, 2008).

2.5 Role of enzyme

Enzyme plays a very crucial role in the fermentation of starch to lactic acid as the major enzymes involved in starch degradation is mainly α- or β-amylases. Recovery and recycling of enzymes (Gonzalez *et al*., 1989; Ramos and Saddler, 1994) or utilization of enzymes until exhaustion of their activity (Moritz and Duff, 1996) are strategies that have been applied in this field. For a given media with fixed enzymatic activity, the maximization of the product concentration is an indirect method for improving the efficiency of enzyme utilization. High concentrations of hydrolysis products have been obtained using packed-bed, column reactors (Gonzalez *et al*., 1989), but additional problems occur when using stirred reactors, owing to limitations in mixing and mass transfer. Moritz and Duff (1996) proposed to avoid this kind of problems by using substrate concentrations below 10% (w/v). Multi-step feeding is an interesting way for overcoming the inconveniences derived from using high solid concentrations in stirred reactors (Moldes *et al*., 2000).

2.6 Response surface methodology (RSM) for optimization of cultural parameters for Lactic acid production

Often biotechnological processes need to be optimized condition under which a certain process attains the optimal results. In other words, the levels of the design parameters at which the response reaches its optimum level must be determined. Statistical methods provide an alternative methodology to optimize a process by considering the mutual interactions among the variables and to give an estimation of the combined effects of these variables on the final result (Hanrahan and Lu, 2006). One such methodology is Response Surface Methodology (RSM). RSM is a powerful statistical method based on the multivariate non-linear model and is useful for the evaluation and understanding the interactions of the various parameters affecting the process. This multivariate approach has advantages over the conventional methods in terms of maximum response in a bioprocess (Dwevedi et al., 2009; Lee *et al*., 2006; Potumarthi *et al*., 2008).

RSM can be used to evaluate the relative significance of several factors in the presence of complex interactions. It is a powerful technique for testing multiple-process variables because fewer experimental trials are needed as compared with the study of one variable at a time. RSM enables selection of the levels for the applied factors to obtain the desirable, smallest or largest, value of the response function in a reduced number of experiments (Hanrahan and Lu, 2006). This technique includes primary screening of variables by the application of "one variable at a time" method or Plackett-Burman design (Xin *et al*., 2005; Preetha *et al*., 2007; Zhang *et al*., 2007) as well as using a design for fitting the chosen model (Myers and Montgomery, 1995). The most popular approach is based on full factorial central composite design (CCD), which enables one to estimate the

coefficients of a second-order model (Puri *et al*., 2010; Ruchir *et al*., 2010; Polak-Berecka *et al*., 2011).

Conventional methods are based on 'one variable at time' (OVAT) approach, which provides information regarding that variable alone. Furthermore, such methods suffer from disadvantages such as being more time consuming, requiring large number of experiments, being uneconomical, and most importantly, lacking the mutual interactions among the variables. RSM quantifies the relationship between the controllable input parameters and the obtained response surfaces (Raissi *et al*. 2009). At first, some ideas are generated concerning which factors or variables are likely to be important in response surface study. It is usually called a screening experiment.

The objective of factor screening is to reduce the list of candidate variables so that subsequent experiments will be more efficient and require fewer runs or tests. The purpose of this phase is the identification of the important independent variables. Further, the main aim is to determine if the current settings of the independent variables result in a value of the response that is near the optimum. If the current settings or levels of the independent variables are not consistent with optimum performance, then the experimenter must determine a set of adjustments to the process variables that will move the process toward the optimum. This phase of RSM makes considerable use of the first-order model and an optimization technique called the method of steepest ascent. This step begins when the process is near the optimum. At this point such a model is expected that will accurately approximate the true response function within a relatively small region around the optimum. Because the true response surface usually exhibits curvature near the optimum, a second-order model (or perhaps some higher-order polynomial) should be used. Once an appropriate approximating model has been obtained, this model may be analyzed to

determine the optimum conditions for the process. All variables are assumed to be measurable, the response surface can be expressed in the form of the second degree polynomial equation as:

$$Yi=\beta 0+\Sigma\beta iXi+\Sigma\beta iiXi^2+\Sigma\beta ijXiXj,$$

Where Yi is the response variable, XiXj are input variables which influence the response variable Y; βo is the interception coefficient; βi is the ith linear coefficient; βii is the ith quadratic coefficient and βij is the ijth interaction coefficient.

The mathematical models were evaluated for each response by means of multiple linear regression analysis (Murthy *et al*. 2000; Gohel *et al*. 2005; Ratnam *et al*. 2005). Modeling was started with a quadratic model including linear, squared and interaction terms. The significant terms in the model were found by analysis of variance (ANOVA) for each response. Significance was judged by determining the probability level that the F-statistic calculated from the data is less than 5%. After model fitting is performed, residual analysis is conducted to validate the assumptions used in the ANOVA. This analysis included calculating case statistics to identify outliers and examining diagnostic plots such as normal probability plots and residual plots. Maximization and minimization of the polynomials thus fitted is usually performed by desirability function method, and mapping of the fitted responses is achieved using computer software such as Design Expert. Most applications of RSM are sequential in nature and can be carried out in the following phases: This sequential experimental process is usually performed within some region of the independent variable space called the operability region or experimentation region or region of interest.

RSM has been successfully applied for optimization of media and culture conditions in many cultivation processes for production of amino acids, ethanol and enzymes (Lieu *et al*., 2005; Boyacil *et al*., 2005). Several LA-based fermented foods have been developed from sweet potato such as Lacto-pickle (Panda *et al*., 2007), lacto juice (Panda and Ray, 2007) and curd (Panda *et al*., Mohapatra *et al*., 2007) using *Lactobacillus plantarum* MTCC 1407 as starter culture. L(+) Lactic acid fermentation was studied by *Lactobacillus rhamnosus* sp. under the effects of pH control and a lowcost nutritional medium (sugarcane juice and corn steep liquor-CSL) (de Lima *et al*., 2010). However, observing the imperative role of RSM in various biotechnological processes, the technique was employed for the optimization of various parameters for the maximum production of lactic acid during the present work.

2.7 Strategies/ processes for enhancing lactic acid production

2.7.1 Whole cell immobilization

Performance of enzymes and whole cells in commercial applications can often be dramatically improved by immobilization of the biocatalysts, for instance by their covalent attachment to or adsorption on solid supports, entrapment, encapsulation and cross-linking (Gisby *et al*. 1987; Taghvaei *et al*. 2000). Immobilization of cells or enzymes helps in their economic reuse and development of continuous bioprocesses have been used in a variety of applications such as biotransformations, biosensors, production of ethanol, degradation of phenol etc (Phadtare *et al*. 2004; Cetinus *et al*. 2003). Immobilized biocatalysts – whole microbial cells (viable or non-viable) or their enzymes have distinct benefits over employing free cells in bioprocesses (Krasaekoopt *et al*., 2003; Plessas *et al*., 2005; Busto *et al*., 2006; Birgisson *et al*. 2007). Cell immobilization provides a physical incarceration or localization of microbial cells or enzymes to a certain defined space for

the preservation of certain desired catalytic activity and subsequent continuous operation stability (Karel *et al*. 1985). Industrial application of immobilized enzymes has been limited due to several factors like low stability, low recovery, low yield and expensive steps involved in isolation and purification of enzymes. Therefore, whole cell immobilization has been employed and offers many advantages over immobilized or free enzymes and free microbial cells. This method is economical, offers continuous operational stability and carries out multi-step cofactor requiring bioconversion (Haiau *et al*. 1997; Babu & Panda, 1991) with minimum downstream processing, decreased product inhibition, simplified biocatalyst recovery, high reusability of cells, relative ease of product separation, high volumetric productivity and reduced susceptibility of cells to contamination (Bernal 2007). Microbial cells and their enzymes (both in free form and immobilized forms) have been employed for the development of strategies for the enhanced production of lactic acid. The use of immobilized cell technology to improve lactic acid fermentation processes has been tried by several research workers (Norton *et al.*, 1994; Kanwar *et al*., 1995). *Lactobacillus helveticus, Streptococcus salivarius, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus casei, Rhizopus oryzae* and *Pediococcus halophilus* are the important microorganisms used for the production of lactic acid by immobilized cells. Similar to other microbial fermentations, entrapment in calcium alginate or k -carrageenan is the method of immobilization widely followed. Many workers have reported shrinkage and decreased mechanical strength of alginate beads during lactic acid fermentation.

2.7.2 Dialysis sac bioreactor

The separation, concentration and purification of molecular mixtures are major problems in chemical industries. Efficient separation processes are needed to obtain high grade

products in the food and pharmaceutical industries to supply communities and industry with high quality water and to remove or recover toxic or valuable components from industrial effluents. For this task, a multitude of separation techniques such as distillation, precipitation, crystallization, extraction, adsorption, and ion-exchange are used today. More recently, these conventional separation methods have been supplemented by a family of processes that utilize semipermeable membranes as separation barriers. (Strathmann *et al*., 2006)

Membranes and membrane processes were first introduced as an analytical tool in chemical and biomedical laboratories. They developed very rapidly into industrial products and methods with significant technical and commercial impact (Lonsdale, 1982; Ho *et al*., 1992; Drioli *et al*., 2001; Baker, 2004, Strathmann, 2004). Today membranes are used on a large scale to produce potable water from sea and brackish water, to clean industrial effluents and recover valuable constituents, to concentrate, purify or fractionate macromolecular mixtures in the food and drug industries, and to separate gases and vapors in petrochemical processes.

The membranes used in the various applications differ widely in their structure, in their function and the way they are operated. The characteristic property of a membrane is its *permselectivity* which is determined by differences in the transport rates of various components in membrane matrix. It is different from *permeability* in which measures the rate at which a given component is transported through a membrane under specific conditions of concentration, temperature, pressure, and/or electric field is measured. The use of different membrane structures and driving forces has resulted in a number of rather different membrane processes such as reverse osmosis, micro-, ultra- and nano filtration,

dialysis, electrodialysis, Donnan dialysis, pervaporation, gas separation, membrane distillation etc. to name a few.

These above mentioned processes are very useful and energy efficient compared to other separation processes depending upon the application for which it is used. For example, electrodialysis or reverse osmosis is used in water desalination but on a large scale distillation, being economical, is preferred in comparison to electrodialysis or reverse osmosis.

Membrane based processes are used where enzyme production, extraction and purification and valuable organic acid product concentrates are desired. The biomass generated in the dialysis membranes can be used as direct nutritious feedstock for the animals. The large scale industrial utilization of membranes began around 1970 with water desalination and purification to produce potable and high quality industrial water. Membrane Dialysis Bioreactors have been developed and used on a large scale for various purposes like producing large scale axenic culture of *Symbiobacterium thermophilum* (Ueda *et al*., 2002), for treatment of wastewater treatment based on biological-activated sludge process and membrane filtration (Radjenovic *et al*., 2008), bioremediation of toxic chromium from effluent by *Pseudomonas aeruginosa* A2Chr (Ganguli and Tripathi, 2002).

2.8 Extraction and purification of L-lactic acid from fermentation medium

The extraction and purification of L-lactic acid from fermentation broth are important for obtaining L-lactic acid. Several methods are available for the purification of lactic acid from fermentation media. Lactic acid is removed from its fermentation broth by a series of separation steps such as precipitation, filtration, acidification, purification using activated carbon, evaporation and crystallization (Yi *et al*., 2008). The classical methods are based

on precipitation, extraction or distillation (Lazarova and Peeva, 1994; Vaccari *et al*., 1993). The extraction and purification steps consists of a series of successive precipitation with $CaCO_3$, butanol esterification, purification by carbon columns, ion exchange, and evaporation (Datta *et al*., 1995). Recently, various attempts have been carried out to recover the lactic acid simultaneously as it is formed. Reactive Extraction is a viable alternative for separation of lactic acid from the fermentation broths. Lactic acid separation by extraction was studied with trioctyl amine in methyl isobutyl ketone (Choudhury and Swaminathan, 1998), tripropylamine dissolved in isoamyl alcohol (Uslu *et al*., 2009), 1-decylaldehyde in tri-n-decylamine (Gao *et al*., 2009). Hano *et al*. (1993) studied the reactive extraction of lactic acid from the fermented broth. They indicated that *in situ* extraction was possible with the use of di-noctyl-amine and with adjustment of the fermentation broth to a pH 5.0 by ammonia. Iyer and Lee (1999) attempted to extract lactic acid simultaneously with the use of a two-zone fermentor-extractor system. The system was operated under a fed-batch mode with *in situ* removal of lactic acid by solvent extraction.

Kim *et al*. (2000) proposed the recovery process of lactic acid using two distillation columns. They used two Oldershaw columns and reboilers for fractionation of methanol and reactions. Sun *et al*. (2006) involved extraction and purification of lactic acid from fermentation broth by esterification and hydrolysis method. Eggeman and Verser (2005) studied the extraction by acidification, esterification and hydrogenolysis. Nanofiltration membranes and ion exchange resins were occasionally coupled with the bioreactor for *in situ* removal of lactic acid (Wee *et al*., 2006). Electrodialysis fermentation with ion exchange membranes is often used for *in situ* removal of lactic acid (Nomura *et al*., 1998). Min-Tian *et al*. (2005) had previously developed a continuous electrodialysis fermentation

system for the production of lactic acid. In their study, the system of electrodialysis fermentation with a level meter was the most efficient system, and a higher yield could be obtained if the glucose concentration in the broth could be controlled to remain at a lower level. Reactive extraction can selectively remove lactic acid from the fermentation broth, and may combine with a modified two-phase electro-electrodialysis (Yi *et al*., 2008). Ion exchange used in bioseparation for lactic acid recovery based on ion exchange have been reported. Evangelista and Nikolov (1996) recovered lactic acid from fermentation broth using weak base anion exchangers (MWA-1, IRA-35, VI- 15). The pH was maintained below the Pka of lactic acid for its adsorption, with the fermentation broth being acidified using a cation exchange resin. Ye *et al*. (1996) also proposed a process for lactic acid production combining a membrane bioreactor and membrane filtration. Lactate was recovered from clarified fermentation broth using an anion exchanger (Amberlite IRA-400).

The operating procedure was similar to that described by Srivastava *et al*. (1992) as they used the same ion exchanger. Vaccari *et al*. (1993) recovered lactic acid from clarified fermentation broth using an anionic resin (Amberlite IRA-420). The eluate from the anionic resin contained ammonium lactate, which was treated with a strong cation-exchange resin (Amberlite IR-120), yielding a lactic acid solution that was subsequently concentrated. Cao *et al*. (2002) studied lactic acid recovery using a strongly basic ion exchanger (Amberlite IRA- 400). They worked with lactic acid solutions and fermentation broths at different pH values (2 and 5). Ataei and Vasheghani-Farahai (2008) recovered lactic acid from fermentation broth using an anionic resin (Amberlite IRA-400) and cationic resin (Amberlite IRA-120) at pH 6.1. González *et al*. (2006) reported the purification of lactic acid from fermentation broth using weak anion exchanger (Lewatit

S3428) and treating with a strong cation resin (Lewatit S2568H) at pH below the pKa of lactic acid (3.86).

2.8.1 Purification techniques of lactic acid

When lactic acid is produced from agroresidues, it is entailed with many impurities and various separation and purification techniques have to be employed to obtain pure lactic acid. Few of them are described below:

2.8.1.1 Membrane separation

Membrane separation process involves use of membranes for purifying the solutions. The mechanism of membrane separation is shown in Fig. 2.2. The IUPAC definition describes membranes as: 'structure, having lateral dimensions much greater than its thickness, through which mass transfer may occur under a variety of driving forces'. Mulder (1991) defined a membrane as a selective barrier between two phases.

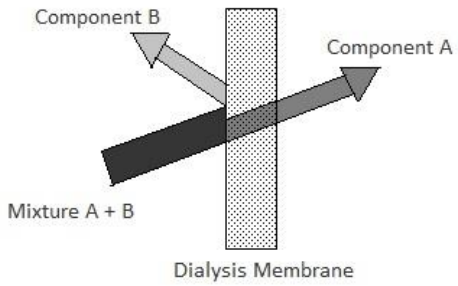

Fig. 2.2 Concept of membrane separation.

Membranes come in many forms: porous or non-porous, polymeric or ceramic, natural or artificial. The selectivity which is their main advantage depends both on the size of the pores and on the membrane material. Some kind of external driving force like a pressure gradient, concentration gradient or electrical field acting across the membrane is required

for a separation to take place. Table 2.3 lists some typical membrane processes and their driving forces.

Table 2.3 Overview of different membrane processes and the corresponding driving forces and typical pore sizes. [a]Not directly pressure driven

Membrane Process	Driving Force	Typical Pore size
Microfiltration	Pressure	0.05 – 10 µm
Ultrafiltration	Pressure	1 – 100 nm
Nanofiltration	Pressure	0.1 – 2 nm
Reverse Osmosis	Pressure[a]	Nonporous
Gas Separation	Pressure[a]	Nonporous (or < 1 µm)
Pervaporation	Pressure[a]	Nonporous
Dialysis	Concentration gradient	1-5 nm or nonporous
Electrodialysis	Electrical field	Nonporous
Liquid Membranes	Concentration gradient	Nonporous (liquid)
Membrane Distillation	Temperature	0.2 – 1 µm

From the table 2.3 it is observed that in dialysis and electrodialysis, transport is driven by concentration differences (Garde, 2002), rather than by pressure or electrical potential differences across the thickness of the membrane (Koros *et al*. 1996). Dialysis is probably best known from treatment of blood in *hemodialysis* where undesired metabolites and toxic by-products such as urea and creatine are transported across a membrane between the blood and a refreshing solution under the action of a concentration gradient. In a

special type of dialysis, called *Donnan dialysis*, ion exchange membranes are used, which primarily facilitate transport of charged molecules, e.g. lactate. Electrodialysis is a membrane-based separation process in which ions are driven through an ion-selective membrane under the influence of an electric field (Koros *et al*. 1996). A more detailed description of the membrane processes is given in the subsequent sections.

Membranes are porous and can be synthesized from organic (polymer) and inorganic materials and allows passing of solvents across them through a concentration gradient. The membrane material is not important for the separation as such, it is the dimension and shape of the pores and the porosity mainly determines the selectivity and flux, respectively. However, the choice of material affects phenomena such as adsorption and chemical stability. The choice of material is primarily based on preventing fouling and on recovery of the membranes (by chemical agents) after fouling (Mulder 1996). A list of materials used for membrane manufacturing is shown in **Table 2.4.**

Table 2.4. Membranes materials and properties (Mulder 1996)

Properties	Material
Hydrophobic polymeric membranes (high protein binding)	Polytetrafluoroethylene (PTFE, Teflon) Poly(vinylidene fluoride) (PVDF) Polypropylene (PP)
Hydrophilic polymeric membranes (Low protein binding)	Polyethylene (PE) Cellulose esters Polycarbonate (PC) Polysulfone/poly(ether sulfone) (Psf/PES) Polyimide/poly(ether imide) (PI/PEI) (Aliphatic) polyamide (PA) Polyetheretherketone (PEEK) Alumina (Al_2O_3)
Ceramic (inorganic) membranes (Medium protein binding)	Zirconia (ZrO_2) Titania (TiO_2) Silicium carbide (SiC)

The tendency of the material to bind to certain organic substance such as proteins is related to the hydrophobicity of the membrane material. When a membrane is hydrophilic a layer of water covers the membrane, preventing the bonding until a certain limit of the adsorption of proteins on the membrane surface.

2.8.1.2 Electrodialysis

Electrodialysis is an electromembrane process in which charged species are transported through ion permeable membranes from one solution to another under the influence of an electrical potential gradient. A conventional set up is shown in Fig 2.3. Since the membranes have the ability to selectively transport ions having positive or negative charge and reject ions of the opposite charge, concentration, removal, or separation of electrolytes can be achieved.

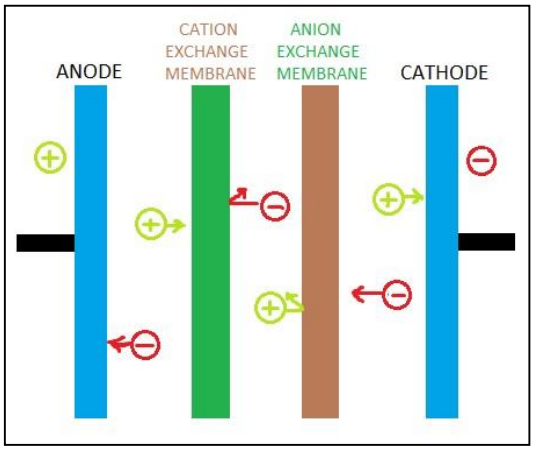

Fig. 2.3 Conventional electrodialysis setup

The charged species must be mobile, and the separation media must be able to transfer electrical current with relatively low resistance. Electrodialysis is used widely for production of potable water from sea or brackish water, electroplating rinse recovery, desalting of cheese whey, production of ultrapure water etc. Conventional electrodialysis, or desalting electrodialysis, is carried out by applying a voltage drop across a membrane

stack with alternating cation-, (CEM) and anion exchange membranes (AEM), forming concentration, and dilution chambers

2.9 Lactic Acid Derivatives: Esters

A great number of lactic acid derivatives like lactate esters (particularly methyl, ethyl propyl and n-butyl esters) are studied so far. These esters have major applications in food, pharmaceutical and cosmetic industries (Ullmann 1985). Methyl, ethyl and propyl lactates are water-soluble while butyl lactate is only slightly soluble.

Several methods have been used to prepare esters of lactic acid; these methods can be classified into the following main groups:

A: Direct esterification of lactic acid and alcohol.

B: Transesterification of one ester into another by reaction with alcohol.

C: Conversion of a metal lactate or ammonium lactate into an ester by treatment with alcohol.

D: Reaction of a metal lactate with an alkyl halide.

2.9.1 Esterification

Esters are most commonly prepared by the reaction of carboxylic acid and an alcohol with the elimination of water. The rate at which different alcohols and acids are esterified as well as the extent of the equilibrium reaction are dependent on the structure of the molecule and types of functional substituents of the alcohols and acids. The primary alcohols are esterified most rapidly and completely, i.e, methanol gives the highest yield

and the most rapid reaction. Under the same conditions the secondary alcohols react much more slowly and afford lower conversions to ester products.

Esterification of a carboxylic acid with an alcohol is extremely slow at ambient temperatures. However, heating the reaction mixture of carboxylic acid and alcohol to the atmospheric boiling point of the mixture generally does not provide for a suitable practical rate of esterification. The major concern for practical applications is increasing the reaction rate and establishing conditions which will allow obtaining higher conversions (Pipus *et al*., 2000). Since the esterification of an alcohol and an organic acid involves a reversible equilibrium these reactions usually do not go to completion. Conversions approaching 100% can often be achieved by removing one of the products formed, either the ester or the water, provided the esterification reaction is equilibrium limited are not rate limited. A variety of distillation methods can be applied to remove ester and water from the esterification reaction. The concentrations present at equilibrium depend on the characteristics of the alcohols and esters involved (Dietz *et al*., 1947).

Esterification of the carboxyl group is an important reaction which is used to recover and purify lactic acid from impure solutions or to produce the ester as the desired end product (Kirk-Othmer 1991). Esterification of lactic acid with alcohols can be performed in number of different ways; the choice of the experimental conditions depends on the alcohol to be esterified. A classical method consists of heating a mixture of lactic acid and alcohol in a sealed tube, generally to about 150ºC. This method has been used to prepare methyl lactate and ethyl lactate. However this method cannot be regarded as satisfactory as the yields are generally low (Holten 1971). Formation of esters depends, as mentioned above, on the reversible reaction and the yield of ester can therefore be improved when one of the products, ester or water, is removed from the system while the reaction is going

on. Removal of ester has been brought about by distillation or solvent extraction but removal of water is more useful since very high yields can be obtained in this way (Dietz 1947). Catalytic esterification of alcohols and acid in the vapor phase has received attention because the conversions obtained are generally higher than in the corresponding liquid phase reactions. Therefore the most effective method for the preparation of lactate esters of lower alcohols is passing vapors of the alcohol through the lactic acid previously heated to a temperature above the boiling point of the alcohol. Series of experiments have been made by using this method with methanol and ethanol (Filachione & Ficher 1946; Sepitka 1963, 1964). Esterification has been performed in small and medium scale experiments, as well as in pilot scale. It can be done in batch-wise experiments, as well as in continuous working systems in which lactic acid and alcohol flow counter currently, the ester is carried out of the apparatus by the excess of alcohol vapors, which then re-circulated. In the process, lactic acid can be converted almost quantitatively into the esters, and loss of alcohol is small due to the recirculation. Thus, continuous methods have been recommended for the purification of crude lactic acid; the purified acid is then recovered by hydrolysis of the ester. However, the equilibrium point of the reaction is not altered by the catalyst, only the rate of esterification is increased.

Chapter 3

Material & Methods

3.1 Chemicals, reagents and media

All chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Standards of Lactic acid (D or L form) were purchased from Sigma Aldrich (St. Louis, MO, USA). Chemicals of molecular biology grade were procured from New England Biolabs (UK).

The microbiological medium used for culturing lactic acid bacteria (LAB) were De Man, Rogosa Sharpe (MRS) medium, modified MRS medium, Potato starch (Sigma, MO, USA) and soluble starch was procured from HiMedia (Mumbai, India). Potato Starch Waste (PSW) was a kind gift from potato chips manufacturing unit of Pepsi (Patiala, India) and L-lactic acid kit was purchased from Randox Laboratories Ltd. (Finsbury, London, UK) and D-lactic acid kit was purchased from Megazyme International Ireland (Ireland). All the standard microbial cultures were procured from Microbial Type Culture Collection (MTCC, Chandigarh) and American Type Culture Collection (ATCC, USA).

3.2 Collection of samples & screening of microorganisms

A total of 180 samples of fermented fruits, vegetables and beverages comprising of pickled mango, pickled yam, pickled garlic–chilli, pickled chilly, potato leaves mixed hukuti mass and bhaatijaanr mixed hukuti mass (fish) samples (Table 3.1) were obtained from local markets of Northern and North Eastern states of India. All samples were collected in pre-sterile zip locked poly-bags (HiMedia, Mumbai), transported on ice and processed within 6h of receipt in the laboratory.

3.3 Isolation and Screening of potential amylolytic lactic acid bacteria

The strains of LAB were isolated and screened (Dhillon et al., 2007) based on their starch utilizing potential. Morphological characteristics of the colonies that exhibited starch degradation on starch agar plates were determined by Gram's staining. The isolates were stored at -20ºC as glycerol stocks. The isolates were inoculated in 5 ml of MRS broth in a test tube at 37ºC for 24 ± 2 h in an anaerobic jar. The cultures were centrifuged and the pellets were washed twice with 0.85% saline, resuspended in fresh MRS and stored in cryo-vial containing 40% glycerol at -20ºC. Working cultures were revived in MRS or M17 at least three times prior to any experiment.

Table 3.1 No. of Samples collected for isolation of bacteria

S.No.	Source Name	No. of samples examined
1	Mango pickle	30
2.	Garlic chilli pickle	30
3.	Chilly pickle	30
4.	Yam Pickle	30
5.	Potato leaves mixed hukuti mass	30
6.	Bhaatijaanr mixed hukuti mass	30
	Total samples	**180**

3.3.1 Qualitative starch hydrolysis

The isolates were evaluated for their starch hydrolyzing potential by growing them in the MRS medium, modified MRS medium with soluble or potato starch as the sole source of carbon at 37ºC for 24-48 h. The amylolytic isolates were screened based on their potential to utilize starch and produce a zone of clearance for the starch-iodine complex after the

respective plates were flooded with Gram's iodine (ASM Microbe Library, 2012; Giraud et al., 1993).

Microscopic analysis

The surface of starch molecules were studied with the help of light microscopy (Bozic et al., 2010) and SEM (Florencio et al., 2000).

Light Microscopy

For light microscopy, 1 mL of the fermentation medium (constituting of starch and *L. lactis* cells) was centrifuged at 6,000 rpm for10 min at 4ºC and washed twice with pure ethanol. The pellet containing grains of potato starch and culture were mounted on glass slide, air dried and then stained with iodine solution. The slides were examined under light microscopy using Nikon microscope with 10X magnification, Photographs were acquired using digital CCD Nikon camera.

Scanning Electron Microscopy

The fermentation medium (modified MRS media) (constituting of starch and *L. lactis* cells) was collected in eppendorf and centrifuged. After centrifugation (5500 x *g*, 10 min, 4°C), the pellet was washed with PBS. For scanning electron microscopy (SEM), the pellet was fixed with 3% paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M Sodium phosphate buffer (pH 7.4), post fixed for 2 h with 4% (w/v) OsO_4 in 0.1 M sodium phosphate buffer (pH 7.4), washed 2 times with 0.1 M sodium phosphate buffer (pH 7.4), and dehydrated in 20%, 40%, 60%, 80%, 90% and 100% ethanol. The samples were dried in a critical-point dryer and coated with gold. The specimens were then examined with a Scanning Electron Microscope (Model: JSM-7100F JEOL, Japan) (Drosinos et al., 2005) for the structural changes over a period of time from 0-48 h.

3.3.2 Quantitative starch hydrolysis

The isolates from qualitative analysis were subjected as well to quantitative analysis for starch hydrolysis. For this, the individual isolates were grown on the MRS media or modified MRS medium with starch as the sole source of carbon for 24 h at 37ºC. An aliquot of 2 mL was withdrawn at 4 h intervals and starch degradation profile was established. For this, 1mL of supernatant in each aliquot (100 times diluted), 1mL of Gram's iodine was added, mixed well and observed spectrophotometrically at 585 nm for measuring the absorbance (Giraud et al., 1993). The concentrations were calculated from a standard curve (Appendix). Maximum starch utilizing strain(s) were used for further experiments.

3.4 Enzyme production (α-amylase)

The starch degradation by strain(s) was evaluated for their α-amylase potential on modified MRS medium by using soluble starch or potato starch. α-amylase was monitored by the disappearance of the blue color of the medium by starch-iodine method (ASM Microbe Library, 2012; Giraud et al., 1993) corresponding to the starch utilization.

For examining α-amylase production, modified medium of MRS containing 20 g/L of soluble starch or potato starch was used as sole carbon source in respective modified MRS media for 24 h. The enzymatic activity was determined at different pH (4.0 -9.0; 0.1 mol/L citrate-phosphate buffer) and temperatures (25-75ºC). Aliquots were then withdrawn periodically and centrifuged at 8000 rpm for 5 min and supernatant was analyzed for enzyme activity. The extracellular amylase activity was assayed by measurement of the iodine complexing ability of starch as previously described (Giraud et al. 1993). Briefly, α-amylase activity was measured by incubarting 0.1 mL of appropriately diluted enzyme solution with 0.8 mL of a solution containing 1.2% of soluble starch in 0.1 mol/L citrate

phosphate buffer, pH 5.5 at 55ºC. The reaction was stopped by the addition of 0.1 mol/L H_2SO_4. After incubation, residual starch contents after different lengths of time were determined colorimetrically at 620 nm by adding 0.1 ml of the reaction mixture to 2.4 ml of an iodine solution containing (g 1-1 in distilled water): KI, 30; I,, 3, diluted to 4%. One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described above.

3.4.1 Crude enzyme preparations

Crude enzyme was prepared from intracellular as well as extracellular fractions from cultures (one litre culture centrifuged at 3,000-16,000 g for 20 min at 4ºC). Protein concentrations in un concentrated fractions ranged from 5 µg/mL to 10 µg/mL. Fractions were concentrated 50 to 100-fold by freeze drying using lyophilizer (Thermo Scientic Corporation, Germany). Cells were harvested by centrifugation, washed twice in 50 mM Tris-HCl (pH 7.5), and resuspended in a small volume of the same buffer. Cell extracts were prepared by sonicating the cells with 10 1-min pulses in the presence of 0.1-mm-diameter glass beads, with cooling on ice. Extracts were centrifuged in a microcentrifuge to remove beads and cell debris. Protein concentrations were determined by using Bradford (1976) protein assay reagent with bovine serum albumin as a standard Cell extracts were estimated for protein by SDS-PAGE followed by activity assays for amylase by Native PAGE (Christine and Russell, 1998) as described below. α-amylase production was further estimated by the method of Giraud et al. (1993) as described under section 3.4.

3.4.2 SDS-PAGE

SDS-PAGE was carried out for the determination of presence of proteins in culture supernatant and cell free extracts by the method of Soomro and Masud (2006). A volume of 15 mL of each sample was loaded on gel and was run on mini gel electrophoresis at 100

V for 2 h and stained in a solution containing 0.1 % (by mass per volume) Coomassie blue, 10 % (by volume) acetic acid and 40 % (by volume) methanol. Destaining was performed in a solution containing 10 % (by volume) acetic acid and 45 % (by volume) methanol. The protein molecular mass marker (100 to 140 kDa; Fermentas, USA) was used as standard.

3.4.3 Native PAGE

Concentrated supernatant of extracellular fractions and intracellular fractions were subjected to PAGE under non-denaturing conditions using 1.5-mm 4% stacking gels, 6.5% separating gels, and a Biorad minigel system. Samples were prepared by adding concentrated fraction (120 µL) to 80 µL of sample buffer (20 µL 0.5 M Tris-HCl, pH 6.8, 40 µl of 60% glycerol, 20 µL of 0.05% bromphenol blue). Samples were then applied to 1.0-mm minigels and electrophoresis was carried out at 200 V for approximately 55 min, using a running buffer consisting of 25 mM Tris and 192 mM glycine, pH 8.3. Gels were stained for amylase activity by incubating them for 7 min in a buffer solution (50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, 1% soluble starch), rinsing briefly with water, and then flooding with Lugol's iodine solution (5 g of I_2, 10 g of KI in 100 mL water) to reveal bands of clearing.

3.4.4 β-amylase assay

To check whether other hydrolytic enzymes were induced in starch hydrolysis besides α-amylase, β-amylase was determined according to the method of Bernfeld (1955) wherein the rate at which maltose is released from starch is measured by its ability to reduce 3,5-dinitrosalicylic acid. One unit releases one micromole of β-maltose per min at 25°C and pH 4.8 under the specified conditions. 0.5 mL of enzyme solution was taken in test tubes and incubated at 25°C for 3-4 minutes to achieve temperature equilibration. At timed

intervals, 0.5 mL starch solution (at 25°C) was added and incubated exactly for 3 minutes followed by 1 mL dinitrosalicylic acid color reagent to each test tube. The tubes were heated in a boiling water bath for 5 minutes and cooled to room temperature followed by addition of 10 mL reagent grade water. It was mixed well and absorbance was read at 540nm against a blank (containing 0.5mL reagent grade water instead of enzyme solution). The enzyme produced was determined from standard curve as micromoles maltose released (Appendix).

3.4.5 β-cyclodextrin glucosyl transferase activity

β-cyclodextrin glucosyl transferase (β-CGTase) is an enzyme produced by some strains of bacteria on starch hydrolysis (Pakzad et al., 2005). It hydrolyses starch to produce β-cyclodextrin by transglycosylation (cyclization) activity. The conventional method for detection of β-CGTase activity is based on detecting starch hydrolysis by iodine staining. This method reveals all amylolytic enzymes, but cannot discriminate them. For specific detection of β-CGTase activity and its product i.e. β-cyclodextrin polyacrylamide gel was opted to distinguish between β-CGTase and α-amylase (Pakzad et al., 2005).

CGTase preparation: The concentrated supernatant containing enzyme was collected after centrifugation at 10,000 x g for 20 min. The proteins of this supernatant were precipitated with saturated ammonium sulfate. Dialysis against phosphate buffer (pH 8) was done to solubilize the proteins. Then the dialyzed solution was double concentrated by polyethylene glycol.

Electrophoresis: Non-denaturing discontinuous PAGE was performed with 10% polyacrylamide gels according to Davis but in slab gel. In two identical sets of three consecutive wells, samples of β-CGTase preparation, α-amylase, and glucoamylase were

applied, respectively. The gel was washed with 0.2 M phosphate buffer (pH 8) and was cut between the two sets and each one was subjected to a different staining method.

Iodine staining method: One half of the gel was incubated in 3% soluble starch at 37ºC for 30 minutes. Then the gel was washed with distilled water and stained with a solution containing 0.1% I_2 in 1% KI. Development of a clear band in the blue context of the gel is indicative of amylolytic activity.

Phenolphthalein indicator gel method: Prior to completion of the electrophoresis, the indicator gel was prepared by mixing 0.24 g soluble starch, 0.14 g agar in 16 mL of 0.2 M phosphate buffer (pH 8). After preparing the mixture, 0.5 mL of 0.4% phenolphthalein was added and the whole mixture was cooled about 50ºC. The indicator gel was poured uniformly on the second half of the polyacrylamide gel and was allowed to solidify. Following a 5-minute incubation at 37ºC, the indicator gel was flooded with a 0.1% sodium carbonate solution until the content of the gel turned into red except for a colourless band indicative of β -CGTase activity.

Coomassie blue staining method: After visualization of β-CGTase activity, the indicator gel was gently removed from the top of the polyacrylamide gel and discarded. Polyacrylamide gel was subjected to conventional Coomassie blue staining method.

3.4.6 Effect of pH on enzyme production

The effect of pH on enzyme production was investigated by adjusting the initial pH of media to 4, 5, 7, 9. The media was inoculated by overnight grown isolate and incubated at 37°C for 24 h Samples were withdrawn and the enzyme activity was determined as described in the enzyme assay under section 3.4.

3.4.7 Effect of Temperature on enzyme production

The effect of temperature was investigated at different temperatures (25, 30, 35, 40, 45, 55, 65 and 75°C). The samples were withdrawn and promptly chilled in ice and the α-amylase activity was determined as described in the enzyme assay under section 3.4.

3.5 Acid production

Lactic acid production was tested on MRS agar plates by using Bromocresol purple (BCP, 0.006%) as pH indicator by the method described by Lee and Lee (2008). Briefly, overnight grown culture of lactic acid bacteria in MRS broth was collected by centrifugation, washed with saline, and dispersed in saline to give an optical density of 1.0 at 600 nm. After serial dilution, an aliquot of diluted sample was inoculated on MRS-BCP agar plate using spreading method. Following inoculation, plates were incubated at 37ºC for no longer than 48 hours. After incubation, colonies exhibiting yellow zone around them were confirmed as acid producing strain(s).

3.6 Selection of L-lactic acid producing isolates

After cultivating bacterial isolate(s) in 10 mL of MRS broth or modified MRS broth containing 2% potato starch media at 37°C for 24 h, cells were removed from the broth by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.45 µm membrane filter (Millipore, Bedford, USA) and estimated for L-lactic acid using L-lactic acid kit (Randox Laboratories, Finsbury, UK) and D-Lactic acid by D-Lactic acid kit (Megazyme, Ireland). The isolate(s) capable of producing L-lactic acid in high concentrations were selected.

3.7 Morphological and biochemical studies

Morphological and biochemical characteristics of prospective bacterial cultures were carried out as described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

3.7.1 Gram staining

The Gram's staining was performed as detailed by Gram (1884). Briefly, bacterial smear from actively growing cells were spread and heat fixed on a glass slide. The smear was flooded with crystal violet and Gram's iodine with intermittent incubation of 30 sec respectively. The extra stain was removed with a washing with alcohol. The bacterial smear was further counterstained with safranin for 30 sec followed by a washing with distilled H_2O. Finally, samples were visualized under microscope at different magnification i.e. the morphology of strains was studied upto 1000x magnification under microscope (Nikon Eclipse e-200 LED, USA).

3.7.2 Catalase test

Microorganisms which can survive in aerobic environments produce catalase which breaks hydrogen peroxide (H_2O_2) into water and molecular oxygen. A small amount of log phase culture was grown, smeared on microscopic slide along with the addition of few drops of H_2O_2 (3%). The microorganism showing bubbling indicates positive result which depicts the evolution of O_2. Absence of bubbles or rare bubbling evidences a negative response.

3.7.3 Oxidase test

A reagent (N,N,N',N'-tetra-methyl-p-phenylenediamine dihydrochloride) (1 drop) was added onto the bacterial cultures on respective MRS agar plates. Positive results were confirmed when the reagent had turned the bacteria violet to purple immediately.

3.7.4 Sugar fermentation profiles

The sugar fermentation profiles of the isolates were observed in basal medium for API 50 CH (API System; BioMerieux, Marcy I'Etoile, France). The API 50 CH strips consist of 50 microtubes containing dehydrated carbohydrates and its derivatives (heterosides, polyalcohols, uronic acids). Overnight grown cultures were inoculated with the bacterial suspension in the microtubes which rehydrates the substrates. The fermentation was confirmed by the occurrence of a colour change in the tube which was caused by the anaerobic production of acid (detected by the pH indicator present in the medium).

3.7.5 Gas production

The homofermentative and heterofermentative characterization of the bacterial isolates was determined by detecting CO_2 production with glucose test. The log phase bacterial isolates (1%) were inoculated in citrate free MRS broth with inverted Durham tubes. Gas production in Durham tubes was observed during 5 days incubation time on incubation at 37°C.

3.7.6 Arginine hydrolysis

Hydrolysis of arginine was estimated in terms of ammonia released. Overnight grown bacterial isolates (1%) were cultured in MRS supplemented with arginine when incubated at 37°C for 24-72 h. The ammonia released was detected in 100 µl samples followed by

the addition of an equal volume of Nessler's reagent *(Cronobacter sakazakei* MTCC 19111 is used as positive control).

3.7.7 Carbohydrate Fermentation

A total of eight carbohydrates namely glucose, mannitol, sorbitol, maltose, lactose, mannose, galactose, raffinose and arabinose were used to detect the fermentation profile of bacterial isolates. All the reactions were performed by using 96-well microtitre plates in triplicates containing fermentation medium with different sugars (10% w/v) and log phase cells. The suspended cells (200 µl) and glucose (50 µl), containing bromothymol blue (0.05g/l) solution were used as positive control while suspended cells (200 µl) alone, were used as negative control. Following incubation for 24 h at 37°C of the samples, the colour development and the turbidity was observed spectrophotometrically at 620 nm in an automated microplate reader (Bioscreen C, Oy Growth Curves Ab Ltd., Finland).

3.8 Enzyme activity: API-ZYM kit assay

The enzyme (19 enzymes) profile of the isolates was carried out by using API ZYM kit according to manufacturer's instructions (API System; BioMerieux, Marcy I'Etoile, France). In brief, the isolates were grown overnight at 37°C on MRS media. The supernatant was diluted to a protein content of 1 mg/mL in 0.85% saline (Bio-Merieux, France). From this sample mixture, 40 µl were added to each well and incubated for 4 h at 37°C. The reaction was determined from the colour development following the incubation for 5 min under bright light after adding ZYM-A and ZYM-B solutions & results were recorded according to the manufacturer's instructions using the scale from 0 to 5+ based on the visual colour intensity.

3.9 Genotypic characterization

3.9.1 Isolation of genomic DNA from bacteria

The genomic DNA of selected strains were isolated by the guanidinium isothiocyanate procedure explained by Pitcher *et al.* (1989). Five mL of overnight grown bacterial cultures were centrifuged (17,860 x *g*, 10 min) and the pellet was resuspended in 1.5 mL 1x TE containing 0.5% NaCl and in 100 µl TERMLS solution sequentially and incubated at 37°C for 1 h. The peptidoglycan of the bacterial isolates was lysed followed by protein denaturation by adding 500 µl GES solution to the suspension and gently mixing by inversion. The preparations were incubated on ice for 5 min followed by the addition of 250 µl of 7.5 M ammonium acetate, intermittent mixing by gently inversion for 10 min and centrifugation at 15,000 x *g* for 10 min. The upper phase harbouring the DNA was carefully removed and was subjected to DNA precipitation by the addition of 460 µl ice cold 2- propanol and centrifugation at 15,000 x *g* for 10 min at 4°C. The pellet was washed twice with 400 µl ethanol and was vacuum dried (Vacuum-drier, Eppendorf, Hamburg, Germany) at 45°C for about 3-5 min, resuspended in 200 µl of 10 mM Tris-HCl (pH 8) and stored at – 80°C.

3.9.2 PCR amplification for 16S rRNA of isolate

The selected bacterial isolates were further subjected to 16S rDNA gene amplification with Genamp PCR system (Applied Biosystem, USA) using the primers 16S seqfw (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S seqrev (5'-CTAGCGATTCCGACTTCA-3'). The primers correspond to positions 8 to 27 and 1511 to 1491 of the 16S rDNA gene of *E. coli* (Weisburg et al., 1991). The cycler program was selected as: 95ºC for 5 min, 35 cycles of 94ºC/1 min, 55ºC /1 min, 72ºC/ 2 min followed by final elongation at 72ºC/5 min for the denaturation, annealing and extension of the target DNA sequence in a 50 µl

reaction volume containing 250 ng of genomic DNA as template, 10 per mol of each primer, 2.5 unit of Taq DNA polymerase (Bangalore Genei, India), 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris –HCL, 50 mM of each dNTP was added and final volume was adjusted to 50 µl with de-ionised water. The PCR products were purified using quantum prep PCR clean columns (BioRad, USA). Determination of nucleotide sequence of the PCR purified fragments was performed using the ABI PRISM® BigDye™ Terminator cycle sequencing kit and the automatic DNA sequencer ABA PRISM at the DNA sequencing service of Bangalore Genei, India.

3.9.3 Analysis of sequence data

The sequenced 16S rDNA sequences for the bacterial isolates were analyzed to detect the presence of possible chimeric artefacts generated by PCR by using CHECK-CHIMERA program (Maidak *et al.,* 2001). The 16S rDNA gene sequences of bacterial isolates were compared with the similar gene sequences available in GenBank/EMBL databases using nBLAST program (Altschul *et al.,* 1997). The sequences of closely related strains were aligned using multiple alignments CLUSTAL W program (Thompson *et al.,* 1997). The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendrograms were constructed by neighbor joining method by the use of MEGA 5 package (Tamura *et al.,* 2007).

3.10 Growth kinetics

The strain(s) of LAB showing both starch degradation and lactic acid production was grown on MRS plates by the method of four quadrant streaking using a sterile inoculating loop. For growth kinetics, a single well isolated colony was selected from the plate and inoculated in 10 mL of sterile MRS in a test tube to grow it overnight (20 h) in order to generate a growing and activated inoculums. 1% inoculum (3.0×10^4 CFU/mL cells) was

used for inoculation in the culure medium (MRS) and incubated at 37°C with shaking at 150 rpm. One milliliter of aliquots in triplicate samples were withdrawn aseptically at regular intervals of one hour centrifuged at 8,000 rpm for 5 min and resuspended the cells in 1 mL of 0.85% saline. Cell density was estimated by measuring the optical density of cell suspension at 600 nm spectrophotometrically (U-2800, Hitachi, Japan). Growth curve was determined based on the increase in optical density at 600 nm (OD 600) over time of liquid cultures grown in MRS media. Specific growth rate was calculated from the slope of the natural log of OD 600 measurements corresponding to the linear portion of the curve during exponential growth phase. All experiments were conducted in triplicate. The average slope was used the mathematical calculation of generation time and the standard deviation for the replicates is reported.

3.11 Probiotic characterization of *Lactococcus lactis* subsp. *lactis*

3.11.1 Gastric juice tolerance

The isolated strain was tested for their ability to resist low pH. The pH value of gastric acid varies in the range of about 1.5-4.5 in a period of 2 h, depending on the entering time and the type of gastric contents. In the present study, pH 3 was used as a representative gastric pH value (Prasad et al., 1998). The ability of selected isolate to survive under gastric conditions was examined according to Casey et al. (24) with minor modifications. Overnight grown culture was washed with phosphate-buffered saline (PBS, pH 7.0), resuspended in synthetic gastric juice (pH 1.85 adjusted using HCl) and incubated at 37^0C for 3 hours. The artificial gastric juice consisted of (g/l): 3.5 D-glucose 1.28 NaCl, 0.6 KH_2PO_4, 0.11 $CaCl_2$, 0.239 KCl, 0.2 Ox bile, 0.1 lysozyme and 0.013 pepsin. Samples were withdrawn at regular intervals of 2 h, serially diluted in PBS and enumerated on MRS agar to enumerate the viable cells.

3.11.2 Bile salt tolerance

As the mean intestinal bile concentration is believed to be 0.03% to 0.3% (w/v) (Prasad, et al., 1998), the residence time of food in small intestine is approximately 4 h, the experiment was carried out at this concentration of bile for 4 h. MRS medium containing 0.3% and 3% bile (Oxoid) was inoculated with active overnight grown cultures (incubated for 16-18 h). Survival of the strains were then examined by monitoring growth for 4 hours after inoculation and viable colonies were enumerated for every hour with pour plate technique and by monitoring OD at 620nm and plotted against CFU/mL by calculating with the graph optimized for CFU/mL and OD **(Appendix).**

3.11.3 Antagonistic activity

3.11.3.1 Production of H_2O_2

Overnight grown cultures (10 µl) were spotted onto ABTS-agar plates (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) and incubated anaerobically at 37°C for 72 h. After anaerobic incubation, plates were exposed to the atmosphere. Oxidative coloration of ABTS by H_2O_2 was visible as a violet halo surrounding the colony of H_2O_2 producer lactic acid bacteria, indicating H_2O_2 production (Marshall, 1979).

3.11.3.2 Antimicrobial activity

The agar spot test as described by Schillinger and Lucke (1987) was used for screening the antagonistic activity of the selected strain against a variety of indicator strains: *Staphylococcus aureus* ATCC 9144, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterolitica* ATCC 9610, *Salmonella typhimurium* ATCC 19585 and *Listeria monocytogenes* ATCC 19111, *Escherichia coli* O157:H7 ATCC 43895. Briefly, 10 µl of overnight culture was spotted onto modified MRS agar (2 g/l glucose and 13 g/l agar) and

incubated at 37°C for 24 h. These plates were over-layered with MRS soft agar (7.5 g/l agar) inoculated with ca. 1 x 10^8 CFU/mL of indicator strains. The agar spot test method of Uhlman (1992) was used to test for the inhibition zones for antagonistic activity were due to the bacteriocin production or as a result of acid inhibition. Briefly, cell-free neutralized supernatants were obtained from overnight producer cultures grown in MRS broth at 37°C. After centrifuging the culture at 7,200 x g for 10 min, the supernatants were neutralized with sterile 5 M NaOH and then boiled for 5 min to inactivate residual viable cells. The supernatants were tested against the same indicator strains as mentioned above.

3.11.4 Bile-salt hydrolase (BSH) activity

The isolates were screened for BSH activity by spotting 10µl aliquots of overnight cultures on MRS agar plates supplemented with 0.5 % (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma, USA) and 0.37 g/L of $CaCl_2$ (Schillinger *et al.*, 2005). Plates were incubated anaerobically at 37°C for 72 h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of bacteria. Isolates were grouped into one of the three arbitrary classes based on the diameter of the precipitation zones on BSH screening medium according to Mathara *et al.* (2008): low BSH activity if the isolate demonstrated precipitation zone up to 10 mm; medium BSH activity if the isolate demonstrated precipitation zone of 11 to 15 mm; high BSH activity if the isolate demonstrated precipitation zone greater than 16 mm.

3.11.5 Antibiotic resistance

The antibiotic susceptibility of the isolate was determined towards antibiotics, namely, vancomycin, gentamycin, ampicillin, penicillin, chloramphenicol, erythromycin, clindamycin, ciprofloxacin, and tetracycline. The minimum inhibitory concentration (MIC, µg/mL) values used to determine whether strains were susceptible or resistant were

those as suggested by National Committee for Clinical Laboratory Standards (NCCLS, 1998). The assay was done using the method described by Andrews (2001). Stock solutions of selected antibiotics were prepared in MRS broths. For preparation of the test inoculums, the 16 h active cultures of isolate(s) were adjusted to 0.5 McFarland standards (10^7 to 10^8 CFU/mL) by adding sterile distilled water. 50 µl of test inoculums were inoculated into each dilution and incubation was done for 24 h at 37°C in the presence of 10% CO_2

3.11.6 Microbial adhesion to solvents (MATS)

MATS was measured according to the method originally proposed by Rosenberg et al. (1980) and recently modified by Bellon-Fontaine et al. (1996). Briefly, bacteria were harvested in the stationary phase by centrifugation at 5,000 x g for 10 min, washed twice, and resuspended to an optical density of 0.4 at 400 nm (A_0) (approximately 10^8 CFU/mL cell density) in 0.1 M PBS (pH 6.2). To 1.2 mL of cell suspension, 0.2 mL of solvent was added. After a 10-min preincubation at room temperature, the two-phase system was mixed on a vortex for 2 min. To allow complete phase separation of the mixture, the aqueous phase was removed after 15 min and its optical density at 400 nm (A_1) was measured. The percentage of microbial adhesion to solvent was calculated as:

$$(1 - A_1/A_0) \times 100$$

Three different solvents were tested in this study: n-hexadecane, which is a polar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. Only microbial adhesion to n-hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent, as noted above. The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded

as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacteria, respectively (Pelletier et. al., 1997)

3.11.7 Resistance to 0.4% (v/v) phenol

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria, leading to the formation of phenols (Suskovic *et al.*, 1997). These compounds may exert a bacteriostatic effect against some LAB strains. Thus, testing for the resistance to phenol may generate further information on the potential for survival of these in gastrointestinal conditions (Xanthopoulos *et al.*, 2000). The ability of selected LAB strain to grow in the presence of phenol was tested by inoculating cultures (1% of overnight culture) in MRS broth with and without 0.4% phenol. Serial dilutions were spread-plated (100 µl aliquots) onto MRS agar at time 0 and after 24 hours of incubation at 37^0C to enumerate surviving bacteria.

3.12 Optimization of culture conditions for Lactic acid production

To obtain the efficient L-lactic acid production, culture conditions for cultivation of the selected isolate(s) were investigated in Modified MRS and minimal media as follows: initial pH of the culture medium, concentration of starch, cultivation temperatures and inoculum sizes.

3.12.1 Incubation Time

The time of incubation plays an important role in production of any metabolite or a product. Overnight grown culture of *L. lactis* in MRS was inoculated in the fresh medium, incubated for 96 h and sample withdrawn every 24 h, to estimate optimum time for maximum lactic acid production.

3.12.2 Inoculum size

The amount of inoculum size could affect growth and L-lactic acid production. Various inoculum sizes was therefore studied for the production of L-lactic acid by the selected L-lactic acid-producing isolate(s) in the suitable medium at various percent (v/v) inoculum sizes; 0.5, 1, 2 and 3%. L-lactic acid was estimated by HPLC at a regular interval of 24 h as described in section 3.14.2.

3.12.3 Potato starch concentration

Carbon source is a crucial factor affecting L-lactic acid production. Based on the results of starch hydrolysis tests, potato starch was tested as the main carbon source in medium at various concentrations (10, 20 and 30 g/L) to achieve the optimal concentration for Lactic acid production.

3.12.4 Initial pH

The effect of initial pH of media on starch utilization and lactic acid production was examined by adjusting initial pH of medium as 4, 4.5, 5, 5.5, 6, 6.5 and 7. The respective pH was adjusted with NaOH (0.1N) and HCl (0.1N) in 100mL of sterile MRS medium. For pH optimization at different temperatures medium was inoculated with 1 mL of overnight grown culture (1% inoculum level) in separate flasks. Lactic acid in culture supernatant was estimated thereafter by HPLC at a regular interval of 24 h as described in section 3.14.2.

3.12.5 Temperature

For temperature optimization, the standard temperatures used were 25, 30, 37, 45°C. The optimization was carried out for over 96 h Media were inoculated with 1% inoculum and

kept at specified temperature and in each case lactic acid concentration was estimated at regular interval of 24 h as described in section 3.14.2.

3.12.6 Nitrogen Source

Nitrogen source is an important component for the growth of microorganism. Therefore, four organic nitrogen sources ($CO(NH_2)_2$, yeast extract, peptone and beef extract) and two inorganic nitrogen sources ($(NH_4)_2SO_4$ and NH_4NO_3) were employed in this study. Samples were withdrawn at regular intervals of 24 h and estimated for Lactic acid production as explained below in section 3.14.2.

3.13 Statistical optimization of cultural parameters for lactic acid production:

Response Surface Methodology-Central Composite Rotatable Design (RSM-CCRD)

Response Surface Methodology (RSM) with Central Composite Rotatable Design (CCRD) was employed to optimize the levels of the screened factors for the enhanced production of L-lactic acid. The effect of three independent variables i.e starch concentration, pH, temperature for the lactic acid production by *L. lactis* subsp. *lactis* were studied at 5 different levels (-2, -1, 0, +1, +2). A statistical software Design Expert Version 8.7.1.0 (State Ease, Minneapolis, MN) with central composite rotatable design (CCRD) was used to generate the experimental trials and to augment experimental data with points to fit into a polynomial model. To inspect the independent and combined effect of significant variables for lactic acid production, a factorial CCRD leading to a total number of 20 experiments was employed. A general form of the second degree polynomial equation is

$$Yi = \beta_0 + \sum \beta iXi + \sum \beta iiXi^2 + \sum \beta ijXiXj$$

where Yi is the response variable, XiXj are input variables influencing the response variable Y, βo is the interception coefficient, βi is the ith linear coefficient, βii is the ith quadratic coefficient, and βij is the ijth interaction coefficient. Regression analysis was performed for the data obtained from the design experiments in triplicate, followed by the estimation of the second order polynomial coefficients, to estimate the responses of the dependent variable (Y). The performance of the regression equation was evaluated by determining the multiple coefficients of correlation R and the determination coefficient of correlation R^2. *F*- test was employed to evaluate the statistical significance of the quadratic polynomial. The response surface plots were employed for determining the optimum level of the significant variables for maximal lactic acid production.

3.14 L-Lactic Acid Extraction and Purification

L-Lactic acid produced was purified by the method as described by Benthin and Villadsen (1995) with minor modifications. Cells and residual starch were removed by centrifugation at 10,000×g for 15 min at 4°C (Beckman Coulter Inc, U.S.A.). A solution of $CaCl_2$ (0.5 mol Ca/mol lactic acid) was added to the medium for protein precipitation followed by heating. The solution was concentrated to one-fourth by evaporation at 85°C followed by crystallization at 4°C for 24 h. The solution was centrifuged at 10,000 rpm for 5 min and the supernatant was treated with activated carbon for 10 min and finally filtered to obtain colourless lactic acid.

The colourless product (acid) obtained was further tested for purity and identity preliminary by TLC as described by Lee et al (2001) and confirmed by HPLC.

3.14.1 Thin layer chromatography (TLC)

The chromatographic procedure was carried out in a rectangular chamber (10 x 24 x 24cm) by the method described by Lee et al (2001). The percentage composition of the

solvent system was acetone–water–chloroform–ethanol–ammonium hydroxide (60:2:6:10:22). A series of 10% (w/v, or v/v) standard solutions containing equal concentration of lactic acid, acetic acid, butyric acid, succinic acid, and citric acid, respectively, were prepared. The organic acid chromatograms were sprayed with an indicator solution of 0.25 g of methyl red and 0.25 g of bromophenol blue in 100 mL of 70% methanol. The dried TLC plates were sprayed with the indicator solution, and the color was developed by brief heating (1–3 min) in a hot dry oven (165°C).

3.14.2 High performance Liquid chromatography (HPLC)

Aliquots of the fermentation medium were taken every 2h to determine lactic acid concentration. Samples were filtered through a membrane filter (0.45 µm pore size, Millipore, Germany) and detected by HPLC system 10AVP (Shimadzu, Japan), Novapak C18 column (250 x 4.6 mm, 5µm particle size, 100A° unit pore size) with class VP software and UV visible detector The mobile phase used was 5mM Sulfuric acid using an isocratic elution with a flow rate of 0.6 mL/min. The detection of lactic acid was set at λ=210 nm. The calculation of lactic acid was made from the peak area registered at specific retention time for lactic acid, and considering the regression curve factor (Petrov et al., 2008).

3.15 Simulation studies with potato starch waste

3.15.1 Characterization of industrial potato starch waste (PSW)

The approximate analysis of potato starch waste including total solids, total insoluble solids, suspended solids, volatile suspended solids, COD, total nitrogen contents were determined as per APHA methods for water and wastewater testing (2005). Starch was estimated according to Iodine method (Giraud et. al., 1993) and reducing sugars by Miller (1959).

3.15.2 Starch utilization and Lactic acid production under optimized conditions

Starch utilization and Lactic acid production by *L. lactis* cells in potato starch waste (PSW) was estimated by the methods of Giraud et al (1993) and Petrov et al. (2008) respectively as described above.

3.16 Enhanced Lactic acid production

3.16.1 Immobilization

The log phase cells of overnight grown culture of *L. lactis* were immobilized by entrapment using different matrices like agar and sodium alginate (Sigma, St Louis, MO, USA) as explained by Tapingkae et al. (2010). A sterile standard buffer containing a range of 0.5 % to 4 % (w/v) of sodium alginate and agar were prepared respectively at 50°C and were mixed with 2.5 mL of buffer containing 1 g of wet weight of cell. Sterile syringes (2 mL) were then filled with the cell-alginate and cell-agar solutions individually and 0.5 mL was dropped through a 0.45 mm x 12 mm needles into 0.2 M chilled $CaCl_2$ solutions respectively. Each set of beads were cured when kept in shaking condition in a 0.3 M $CaCl_2$ solution over night at 4°C. Several different cell loads of 5, 10, 20, 30, 40, 50, 60 mg/mL were also used for immobilization with variable bead diameters of 1-4 mm, during the maximum starch utilization in PSW using immobilized cells.

The immobilized whole cells were evaluated for their lactic acid production in PSW. The log phase revived culture of *L. lactis* with variable cell loads were used in immobilized forms (agar and Na-alginate) in an Erlenmeyer flask containing 100 mL PSW and incubated at 30ºC. The regular aliquots of the sample from all the sets of free and immobilized (agar and Na-alginate) were aseptically withdrawn at a regular intervals of 24 h, till 96 h. The aliquots were centrifuged at 10,000 rpm for 5 min at 4ºC. The pellet was then resuspended in 1 mL of saline (0.85% NaCl) and the optical density was recorded at a

wavelength of 600 nm. The lactic acid production was estimated as already described before.

3.16.2 Cell mass and viability

Cell mass and viability of immobilized cells was estimated as outlined by Rao et al. (2009). Cell growth was determined spectrophotometrically at 600 nm and converted to cell count using a conversion factor (one unit at 600 nm was equivalent to 10^5 CFU/mL, which corresponded to 28 mg protein that was calculated by plotting standard graphs). In case of all immobilized conditions (agar and Na-alginate), 5 beads were dissolved in 2 mL of phosphate buffer (pH 7.0) and cells were collected by centrifugation at 5000 rpm at 37°C and were used for cell mass measurement, by serial dilution plate count method on MRS agar plates when incubated at 37°C for 24 h. At the end of each batch, the cell densities in the beads were enumerated using similar method to study the total cell loss upon repeated use. Viable cell counts were performed in duplicates and expressed in CFU/mL of immobilized beads.

3.16.3 Development of a Dialysis Bioreactor for enhanced Lactic acid production

Dialysis tubing (Cellulose, MWCO 12000, Sigma-Aldrich, USA) was thoroughly washed in sterile triple-distilled water and clipped at one end to produce a sac. An overnight growing cell culture was centrifuged to pellet down the cells to obtain wet weight of log phase *L. lactis* cells (0.1mg/mL) which correspond to 5.7 x 10^8 CFU/mL. The cell pellets were re-suspended in 4 mL of fresh sterile buffer (PBS, pH 6.8) medium and this culture entrapped in the dialysis tubing and incubated at 30ºC so as to ensure the PSW medium (containing approximately 2% starch) exchange of the dialysis sac. Aliquots were withdrawn from medium at every 24 h till 96 h of incubation to check for the amount of

starch degradation, amylase activity and L-lactic acid concentration. The experiment was conducted aseptically to avoid any contamination.

3.16.4 Fed Batch fermentation experiments and reusability of cells

The production of lactic acid and starch utilization was estimated after every 24 h. As the starch from the media gets depleted, the media was withdrawn from the dialysis sac bioreactor and fresh media was added to the dialysis sac bioreactor to check the reusability of dialysis sac entrapped cells for lactic acid production. The viability and stability of the cells entrapped in dialysis sac was checked by diluting them in Phosphate Buffer Saline (pH 7.2) plating them on MRS agar plates. Lactic acid production was estimated as described previously.

3.16.5 Statistical analysis

All analyses were done in triplicate and the statistical comparison of data was performed by ANOVA to reveal significant differences for each parameter among species. The correlation coefficient (r) and P value were used to show correlations and their significance. A probability value of $P < 0.05$ was adopted as the criteria for significant differences.

3.17 Production of lactic acid derivatives: esters

Esterification of ethyl, methyl and propyl alcohols, ethylene glycol, and glycerol with various acids, such as chloro- or bromoacetic, or pyruvic or lactic acid by the use of a third component such as benzene, toluene, hexane, cyclohexane, or carbon tetrachloride to remove the water produced is quite common. A mixture of 1 mol 80% lactic acid and 2.3 mol 95% ethyl alcohol was added to a volume of benzene equal to half that of the alcohol (ca 43 mL), and the resulting mixture was refluxed for several hours. When distilled, the

overhead condensate separates into layers. The lower layer was extracted to recover the benzene and alcohol, and the water was discarded. The upper layer is returned to the column for reflux. After all the water was removed from the reaction mixture, the excess of alcohol and benzene is removed by distillation, and the ester was fractionated to isolate the pure ester (Donoghue, 1991). The esters obtained were further analyzed using Gas Chromatography as described by Benedict et al (2003).

3.17.1 Gas Chromatography

In order to identify the derivatives, Gas Chromatography was used (Benedict et al., 2003). According to the method, two 5 mL samples were withdrawn from the fermentation medium and one was immediately cryofrozen (-185 °C) to stop the reaction in the sample and then transferred to a laboratory freezer (-80°C). The frozen sample was later analyzed to estimate the concentrations of ethanol, ethyl lactate, lactic acid and water using a gas chromatograph (GC). A Shimadzu GC-14A GC equipped with an AOC-1400 autosampler and an AOC-17 autoinjector was used to estimate concentrations of alcohol, alkyl lactate, and water in the reaction mixture. The GC had a flame ionization detector (FID) and was connected to a Supelco 30 m capillary column. The autoinjector was fitted with a 0.5 µL SGE syringe, with the injection volume being 0.4 µL. FID utilized 99.999% pure helium as the carrier and reference gas, with 99.999% pure hydrogen and dry air being the flame sources for the FID. The chromatography column (length 30 m, o.d. 0.32 mm, and film thickness 0.25 µm) was a water-tolerant Supelcowax 10, bonded phase medium-polarity poly (ethylene glycol) capillary column. A 1 m guard column was installed at the inlet to the capillary column to trap nonvolatile species such as lactic acid and protect the column from contamination. All reaction samples were analyzed by GC in quadruplicate by splitting each sample into two vials and setting the auto-injector for 2 injections per sample vial. Acetone was the internal standard (IS), and all calibration standards and all

reaction samples were diluted 1:1 with IS. To ensure that no residuals remained in the injector port or the column, acetone vials were placed between all sample vials and all samples were analyzed at the same conditions.

Chapter 4

Results & Discussion

4.1 Isolation & Screening of LAB

LAB were isolated from a total of 180 diverse fermented samples including pickled mango, pickled yam, pickled garlic–chilly, pickled chilly, potato mixed hukuti mass and bhaati-jaanr mixed hukuti mass samples so as to obtain potential starch degrading bacteria. Fermented products such as meat, pickles, vegetables, beverages and bakery products have been reported to be a good source for amylolytic LAB like *Lactococcus lactis* subsp. *lactis*, *Lactobacillus sanfrancisco, Lactobacillus sp.* (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris *et al*., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan *et al*., 2002; Ali 2011). Therefore, a diverse range of traditional non-dairy foods were selected for isolating amylolytic LAB.

A total of 210 isolates of LAB were isolated from their natural habitats of above mentioned samples which were obtained from local market of northern and north eastern India. Out of total 210 isolates, 174 isolates were Gram-positive rods and 36 isolates were Gram-positive cocci which occurred singly, in pairs, or in short chains. Their colony morphology was observed after incubating them on MRS agar medium for up to 24 h. Circular and irregular colonies with entire and undulate margins; and flat, low convex, convex and umbonate were observed. These colonies included punctiform, small, moderate and large colonies with 0.1-4.0 mm in diameter. Isolates were subsequently tested for their starch degrading potential using MRS media containing 2% potato starch (modified MRS) as sole carbon source.

4.1.1 Screening of amylolytic lactic acid bacteria

Of the 210 isolates, only 63 bacterial isolates were able to utilize potato starch in MRS-Starch agar plate, incubated at 37ºC for 48 h. These 63 isolates could utilize the potato starch tested, as inferred from clear zones surrounding bacterial colonies (0.1-1.7 cm in diameters), resulting from starch-iodine reaction (Xiao *et al.*, 2006). This method was preferred over others since other screening procedures used for detection of amylase producing microorganisms are time consuming and inconvenient for direct isolation of intact cells (Akpan *et al.*, 1999). Qualitative determination on MRS-starch agar plate containing potato starch revealed that all the 63 isolates were able to degrade potato starch (data not shown). Further, starch hydrolysis was also determined quantitatively for potato starch using method explained by Giraud *et al.* (1993). The selected, maximum starch hydrolyzing isolates were then further characterized morphologically, biochemically & by molecular methods. Of the sixty three isolates that were able to hydrolyze potato starch, only three namely LA-56, LA-57 and LA-60 could utilize potato starch at a comparatively higher percentage (50%, 55% and 85% respectively) (Table 4.1). The starch degradation by the microorganism might depend upon the structure of the starch and the production of enzymes acting upon surface of starch granules by a starch (substrate)-binding domain by the microorganism (Florencio et al., 2000).

4.2 Morphological and Biochemical Characterization

The selected 63 isolates were found to be short, smooth, convex, single or paired square bacilli or coccus, Gram positive and formed opaque creamy colony without any pigmentation (Table 4.1). The colony morphology of the three strains selected, based on their starch hydrolyzing potential, on MRS agar was off white, spherical or rod, with

Table 4.1 Morphological and Biochemical characterization of the isolates

S. no	Isolate No.	Gram's Reaction	Potato Starch hydrolysis (%)	Shape	Catalase test	Carbohydrate fermentation								Source
						Ga	Ma	La	So	Man	De	Ar	Ra	
1	LA 1	+	30	Rods	-	+	-	+	-	-	+	-	-	MP
2	LA 2	+	24	Rods	-	+	-	-	-	-	+	-	-	MP
3	LA 3	+	27	Cocci	-	+	+	+	-	-	-	-	-	MP
4	LA 4	+	10	Rods	-	+	-	-	-	-	+	-	-	MP
5	LA 5	+	9	Rods	-	+	-	-	-	-	+	-	-	MP
6	LA 6	+	10	Cocci	-	+	+	+	-	-	+	-	-	MP
7	LA 7	+	12	Rods	-	-	-	+	-	-	+	-	-	MP
8	LA 8	+	16	Rods	-	+	-	+	-	-	-	-	-	MP
9	LA 9	+	15	Rods	-	-	-	-	-	-	+	-	-	MP
10	LA 10	+	17	Rods	-	-	-	-	-	-	+	-	-	MP
11	LA 11	+	15	Rods	-	-	-	-	-	-	-	-	-	GCP
12	LA 12	+	18	Rods	-	-	-	-	-	-	+	-	-	GCP
13	LA 13	+	20	Rods	-	+	-	+	-	-	-	-	-	GCP
14	LA 14	+	25	Rods	-	+	-	+	-	-	+	-	-	GCP
15	LA 15	+	27	Rods	-	+	-	+	-	-	-	-	-	GCP
16	LA 16	+	28	Cocci	-	-	-	-	-	-	-	-	-	GCP
17	LA 17	+	35	Rods	-	+	-	-	-	-	-	-	-	GCP
18	LA 18	+	29	Cocci	-	+	-	+	-	-	+	-	-	GCP
19	LA 19	+	11	Rods	-	+	-	+	-	-	+	-	-	GCP
20	LA 20	+	15	Rods	-	+	+	+	-	-	+	-	-	GCP
21	LA 21	+	16	Cocci	-	-	-	-	-	-	-	-	-	GCP
22	LA 22	+	19	Rods	-	-	-	-	-	-	+	-	-	GCP
23	LA 23	+	13	Rods	-	+	+	+	-	-	-	-	-	GCP
24	LA 24	+	23	Rods	-	-	-	-	-	-	+	-	-	CP
25	LA 25	+	9	Rods	-	-	-	-	-	-	-	-	-	CP
26	LA 26	+	10	Rods	-	-	-	-	-	-	+	-	-	CP
27	LA 27	+	10	Rods	-	-	-	-	-	-	+	-	-	CP
28	LA 28	+	10	Rods	-	+	+	+	-	-	+	-	-	CP
29	LA 29	+	18	Rods	-	+	+	+	-	-	+	-	-	CP
30	LA 30	+	19	Rods	-	+	-	+	-	-	+	-	-	CP
31	LA 31	+	22	Cocci	-	-	-	-	-	-	-	-	-	CP
32	LA 32	+	12	Rods	-	+	+	+	-	-	+	-	-	CP
33	LA 33	+	24	Rods	-	-	-	-	-	-	-	-	-	CP
34	LA 34	+	26	Rods	-	-	-	-	-	-	+	-	-	CP
35	LA 35	+	25	Rods	-	-	-	-	-	-	-	-	-	CP
36	LA 36	+	25	Rods	-	-	+	+	-	-	+	-	-	CP
37	LA 37	+	20	Rods	-	-	-	+	-	-	-	-	-	CP
38	LA 38	+	27	Cocci	-	-	-	-	-	-	+	-	-	CP
39	LA 39	+	28	Rods	-	+	-	-	-	-	+	-	-	CP
40	LA 40	+	17	Rods	-	+	+	+	-	-	-	-	-	BJHM
41	LA 41	+	18	Cocci	-	-	+	-	-	-	+	-	-	BJHM
42	LA 42	+	27	Rods	-	-	-	-	-	-	-	-	-	BJHM
43	LA 43	+	29	Rods	-	+	-	+	-	-	+	-	-	PLHM
44	LA 44	+	30	Rods	-	-	+	-	-	-	-	-	-	PLHM
45	LA 45	+	35	Rods	-	+	-	+	-	-	+	-	-	PLHM
46	LA 46	+	48	Rods	-	-	-	-	-	-	-	-	-	YP
47	LA47	+	43	Rods	-	+	+	+	-	-	+	-	+	YP
48	LA 48	+	47	Rods	-	-	+	-	-	-	-	-	-	YP
49	LA 49	+	44	Rods	-	-	+	-	-	-	-	-	-	YP
50	LA 50	+	22	Rods	-	+	+	+	-	-	-	-	-	YP
51	LA 51	+	21	Rods	-	+	-	-	-	-	+	-	-	YP
52	LA 52	+	29	Rods	-	+	-	-	-	-	-	-	-	YP
53	LA 53	+	36	Rods	-	-	+	-	-	-	+	-	-	YP
54	LA 54	+	42	Cocci	-	-	+	-	-	-	+	-	-	PLHM
55	LA 55	+	41	Cocci	-	+	+	+	-	-	-	-	-	PLHM
56	LA 56	+	50	Cocci	-	+	-	+	-	-	+	-	-	PLHM
57	LA 57	+	55	Cocci	-	+	-	+	-	-	+	-	-	BJHM
58	LA 58	+	41	Cocci	-	+	-	+	-	-	+	-	-	BJHM
59	LA 59	+	42	Cocci	-	-	+	-	-	-	-	-	-	YP
60	LA 60	+	85	Cocci	-	+	+	+	-	-	+	-	+	YP
61	LA 61	+	35	Cocci	-	+	-	-	-	-	+	-	-	YP
62	LA 62	+	36	Cocci	-	+	+	-	-	-	-	-	-	YP
63	LA 63	+	32	Cocci	-	-	+	-	-	-	-	-	-	YP

MP: Mango Pickle; CP: Chilly Pickle; GCP: Garlic chilly pickle; YP: Yam pickle; PLHM: Potato leaves hukuti mass; BJHM: Bhaatijaaanr hukuti mass; Ga: Galactose; Ma: maltose; La: Lactose; So: sorbitol; Man: mannose; De: dextrose; Ar: Arabinose; Ra: Raffinose

smooth edges and sticky and raised from center with arrangement of singles cells to clusters and chains.

4.3 Phenotypic characterization of the strain

The three selected strains were identified to the species-level by both phenotypic and genotypic methods as described. The strains that stemmed from potato leaves mixed hukuti mass, bhaati-jaanr mixed hukuti mass and yam pickle were selected for identification. The bacteria were coccus-shaped, produced L-lactate or DL lactate, were able to ferment fructose, sucrose and galactose and did not produce gas from glucose fermentation (Table 4.2).

4.4 Enzyme assay

In API-ZYM assay, the three isolates were tested for the production of enzymes which were grouped as strong (from 3 to 5+) or weak (from 1 to 2+) based on the intensity of colour development in their respective cupules. LA-56 and LA-57 showed positive activity for 10 and 11 enzymes respectively. LA-56 showed strongest activity for leucine arylamidase and valine arylamidase followed by α-galactosidase and acid phosphatase, napthol-ASBI-phosphohydrolase, α-fucosidase and exhibiting the lowest activity for α-chymotrypsin, α-glucosidase and N-acetyl-β-glucosaminidase. The enzyme profile of LA-57 demonstrates strongest activity for β-galactosidase followed by cystine acrylamidase, esterase lipase (C8), valine arylammidase, acid phosphatase, napthol-ASBI-phosphohydrolase, α-galactosidase and the lowest activity with α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase (Table 4.3). LA-60 showed positive activity for 11 enzymes, the strongest activity was observed for alkaline phosphatase, acid phosphatase, leucine arylamidase, β-galactosidase, esterase (C4), lipase (C14), β-

glucosidase, α-glucosidase, whereas lowest activity was observed for naphthol-AS-BI-phosphohydrolase and esterase lipase. No activity for N-acetyl-β-glucosaminidase, trypsin, α-glucuronidase, α-mannosidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase and α-fucosidase was detected in the assay. None of the isolates were shown to produce β-glucuronidase, the carcinogen enzyme.

4.5 Molecular characterization of the bacterial isolates

The 16S rRNA sequence of the selected isolates were obtained by sequencing the 16S rRNA amplicon with the primers 16Sseqfw (5'- AGAGTTTGATCCTGGCTCAG-3') and 16Sseqrev (5'-CTAGCGATTCCGACTTCA-3'). To identify the bacterial isolate, they were subjected to 16S rRNA amplification using primers, and about 1.5 Kb amplicon were observed (Fig. 4.1) in all the three isolates. 16S rRNA PCR products were sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers. Sequences of the cultures were deposited in the GenBank database with Accession number JN618456 for isolate LA-60 (Bhanwar *et al.*, 2013a), JN620210 (Isolate LA-56) and JN620216 (Isolate LA-57) respectively (Table 4.4, 4.5, 4.6).

4.5.1Sequence alignment

The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. The homologies among the sequences varied from 97-99% between isolates. The phylogenetic tree (Fig. 4.2) constructed with sequences of representative bacteria revealed that all the isolates clustered with *Lactococcus* sp.

Table 4.2 Phenotypic characterization of the isolates

S.No.	Characteristics	Isolate (LA 56)	Isolate (LA 57)	Isolate (LA 60)
1	Cell Shape	**Spherical**	**Spherical**	**Spherical**
2	Cell Arrangement	**Singly/ paired/ short chains**	**Singly/ paired/ short chains**	**Singly/ paired/ short chains**
3	Cell Size (µm)	**0.29-0.45 µm**	**0.32-0.55 µm**	**0.38-0.51 µm**
4	Gram Staining	+	+	+
5	Spore forming	**No**	**No**	**No**
6	Aerobic growth	+	+	+
7	Anaerobic Growth	+	+	+
8	Catalase Test	-	-	-
9	OxidaseTest	-	-	-
10	Motility	**Non motile**	**Non motile**	**Non motile**
11	Gas from D-Glucose	-	-	-
12	Growth 15/45 (°C)	+/-	+/-	+/-
13	Growth at 0% NaCl	+	+	+
	3.0% NaCl	+	+	+
	4.0% NaCl	-	-	+
	6.5% NaCl	-	-	-
	8.0% NaCl	-	-	-
14	Hydrolysis of			
	Starch	+	+	+
	Skim Milk	+	+	+
	Gelatin	-	-	-
	Arginine	+	+	+
15	Lactic acid configuration	**D/L-form**	**D/L-form**	**L-form**
16	Sugar fermentation Profile			
	Fructose	+	+	+
	Gentibiose	-	-	-
	Galactose	+	+	+
	Lactose	+	+	+
	Maltose	-	-	+
	Mannitol	-	-	-
	Mannose	-	-	-
	Salicine	-	-	+
	Sucrose	+	+	+
	Arabinose	-	-	-
	D-Xylose	-	-	-
	Trehalose	-	-	-
	Raffinose	-	-	+
17	Vogues Praskeur	+	+	+
18	Nitrate reduction	-	-	-
19	H_2S	-	-	-

Table 4.3 Enzyme profile of the isolates

Enzyme	Enzyme activity*		
	LA 56	LA 57	LA 60
Control	0	0	0
Alkaline phosphatase	0	2	4
Esterase (C4)	0	0	4
Esterase lipase (C8)	0	3	2
Lipase (C14)	0	0	3
Leucine acrylamidase	5	0	4
Valine arylamidase	5	3	3
Cystine acrylamidase	0	4	0
Trypsin	0	0	0
α-Chymotrypsin	2	0	0
Acid phosphatase	3	3	3
Napthol-ASBI-phosphohydrolase	3	3	2
α-Galactosidase	4	3	0
β- Galactosidase	0	5	4
β-Glucuronidase	0	0	0
α-Glucosidase	2	2	3
β-Glucosidase	0	2	3
N-acetyl-β-glucosaminidase	2	2	0
α-Mannosidase	0	0	0
α-Fucosidase	3	0	0

*Units of activity are expressed as nanomoles of substrate hydrolyzed under the assay conditions

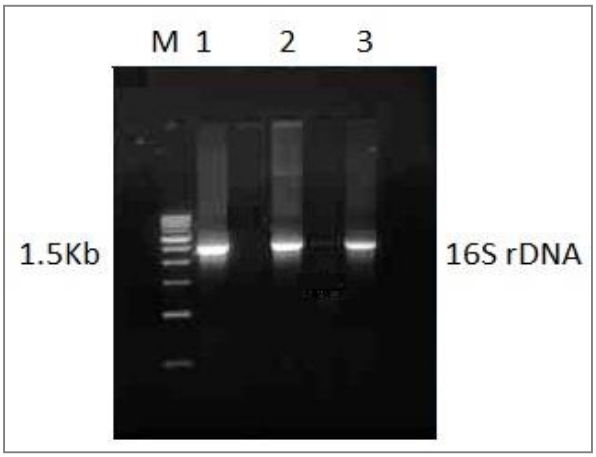

Fig 4.1 16S rDNA amplification of bacterial isolates. M: Marker, Lane 1: LA-56; Lane 2: LA-57; Lane 3: LA-60

Table 4.4 Aligned Sequence Data of LA 60 (*Lactococcus lactis)*

LOCUS JN618456 1300 bp DNA linear BCT 23-NOV-2011
DEFINITION Lactococcus lactis strain LAHKT 2 16S ribosomal RNA gene, partial sequence.
ACCESSION JN618456
VERSION JN618456.1 GI:357540866
KEYWORDS .
SOURCE Lactococcus lactis
ORGANISM Lactococcus lactis
Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Lactococcus.
REFERENCE 1 (bases 1 to 1300)
AUTHORS Singh,M.K., Seema, Singla,R. and Ganguli,A.
TITL E Isolation and characterization of beneficial microbes from traditional food of India
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1300)
AUTHORS Singh, M. K., Seema, Singla, R. and Ganguli, A.
TITLE Direct Submission
JOURNAL Submitted (25-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
source 1..1300
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/strain="LAHKT 2"
/isolation_source="yam pickle"
/db_xref="taxon:1358"
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/collected_by="Mukesh K. Singh"
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/product="16S ribosomal RNA"
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 121 acatttggaa acgaatgcta ataccgcata aaaactttaa acacaagttt taagtttgaa
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Table 4.5 Aligned Sequence Data of LA 56 (*Lactococcus lactis)*

LOCUS JN620210 1498 bp DNA linear BCT 15-NOV-2011
DEFINITION Lactococcus lactis strain HKT 16S ribosomal RNA gene, partial sequence.
ACCESSION JN620210
VERSION JN620210.1 GI:356817712
KEYWORDS
SOURCE Lactococcus lactis
ORGANISM Lactococcus lactis
Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae;
Lactococcus.
REFERENCE 1 (bases 1 to 1498)
AUTHORS Mukesh,S.K. and Abhijit,G.
TITLE Direct Submission
JOURNAL Submitted (10-AUG-2011) Department of Biotechnology and
Environmental Sciences, Thapar University, Patiala, Punjab 147004,
India
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1441 gatcagcacg ccgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacacca

Table 4.6 Aligned Sequence Data of LA 57 (*Lactococcus lactis)*

LOCUS JN620216 1496 bp DNA linear BCT 15-NOV-2011
DEFINITION Lactococcus lactis strain HKBT-9 16S ribosomal RNA gene, partial sequence.
ACCESSION JN620216
VERSION JN620216.1 GI:356817849
KEYWORDS
SOURCE Lactococcus lactis
ORGANISM Lactococcus lactis
Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Lactococcus.
REFERENCE 1 (bases 1 to 1496)
AUTHORS Mukesh,S.K., Purbi,B.A. and Abhijit,G.
TITLE Direct Submission
JOURNAL Submitted (19-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India
FEATURES Location/Qualifiers
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1261 aggaaggtgg ggatgacgtc aaatcatcat gccccttatg acctgggcta cacacgtgct
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The complete 16S rRNA sequence analysis revealed that bacterial isolates had 98% similarity with the sequences of *Lactococcus lactis* inNCBI database. The 16S rRNA gene sequences of bacterial isolates determined in this study were deposited in the GenBank of NCBI data library under accession number JN620210, JN620216 and JN618456, for LA-56, LA-57 and LA-60 respectively. The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTAL W (Thompson *et al.*, 1994). The resulting multiple alignments were optimized visually and the evolutionary distance were calculated by Kimura 2 parameter.

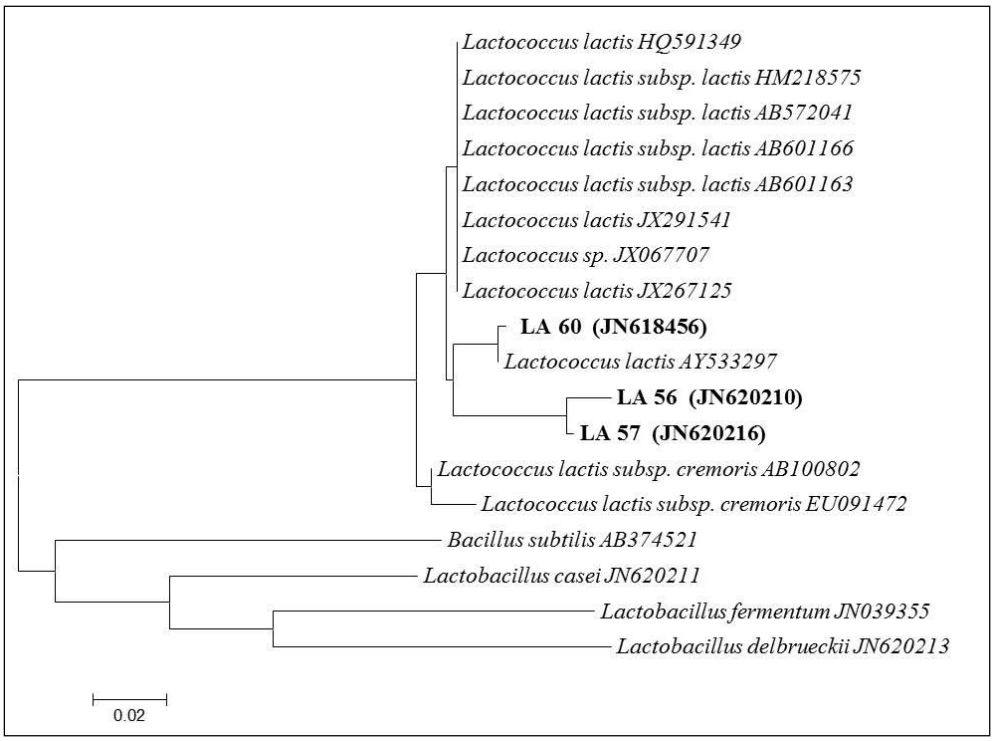


Fig. 4.2 Neighbor-joining tree based on bacterial 16S rRNA sequence data from different isolates of current study along with sequences available in GenBank database. *Bacillus subtilis* was used as out group taxa.

Phylogenetic dendrogram were constructed by neighbor-joining method using MEGA 5 package (Tamura *et al.*, 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. The complete 16S rRNA sequence analysis revealed that the bacterial isolates had 97% to 99% similarity with the sequences

of NCBI database. The isolate LA 56, isolated from potato leaves mixed hukuti mass, was identified as *Lactococcus lactis* strain HKT (GenBank accession number JN620210). On the basis of phylogenetic data, isolate LA 57, isolated from Bhaati-jaanr mixed hukuti mass was identified as *Lactococcus lactis* strain HKBT-9 (GenBank accession number JN620216). Thus, based on morphological, physiological, biochemical properties and 16S rDNA sequence results, the isolated strain LA-60 was assigned as *Lactococcus lactis.*

4.6 Potato Starch

Potato starch is mainly composed of 20-25% amylose (linear chain of glucose units joined by glycosidic bonds) and 75-80% amylopectin (branched chain of glucose units). The mechanism of starch degradation involves breakdown of glycosidic bonds and depending on the relative location of the bond under attack as counted from the end of the chain, the products of starch hydrolysis are dextrin, maltotriose, maltose, and glucose, etc. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction* because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di- and tri-saccharides. This process is called *saccharification*, due to the formation of oligosaccharides.

According to this mechanism, starch is simultaneously degraded to oligosaccharide and hydrolyzed to glucose by the amylase or glucoamylase, oligosaccharide is converted to monomeric glucose by amyloglucosidase, and glucose is catabolized primarily to lactic acid, cell mass and carbon dioxide by a fermentative microorganism. In this simultaneous saccharification and fermentation process (SSF), the saccharification and fermentation steps are integrated, i.e., enzymatic hydrolysis, cell growth and microbial production occur simultaneously. This method eliminates the need for a complete hydrolysis step prior to

the fermentation step. Another direct benefit of the SSF is a decrease in the inhibition caused by glucose accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs. Therefore, SSF was preferred, as *L. lactis* has the ability to convert starch to lactic acid directly.

Starch degradation by lactic acid bacteria involves production of various enzymes mainly amylases that are known to degrade starch to simple sugars, where α-amylase is the principal enzyme that is involved in breakdown of starch to maltose. In the present study, α-amylase produced was extracellular and was found to be inducible of starch, as the media lacking starch and containing other 'C'- source (e.g. glucose) failed to produce α-amylase. Amylase production is known to be induced by a variety of carbohydrate, nitrogen compounds and minerals (Dey *et al.*, 2001, Muralikrishna *et al.*, 2005). In order to achieve high enzyme yield, efforts are made to develop a suitable medium for proper growth and maximum secretion of enzyme, using an adequate combination of carbohydrates, nitrogen and minerals (Goyal *et al.*, 2005; Sodhi *et al.,* 2005).

4.7 Biochemical mechanism of starch degradation

Fungal and bacterial amylases act quite efficiently on gelatinized starches, producing either maltose (α-, β-amylases) or free glucose (amyloglucosidase or glucoamylases). Less often microbes can produce amylolytic activity acting on native or non-pregelatinized starch granules (Florencio *et al.*, 2000). The clear zones produced on starch agar medium presumably unveil that the starch degrading amylase has been produced. Pakzad et al (2005) have reported that though starch hydrolysis is mainly associated with production of α-amylase, but hydrolysis of starch may also be brought about by β-cyclodextrin glycosyl transferase (β-CGTase) which hydrolyses starch to produce β-cyclodextrin by

transglycosylation (cyclization) activity. Since starch hydrolysis may be catalyzed by several enzymes *viz.*α-amylase, β-amylase or β-cyclodextrin glycosyl transferase (β-CGTase) (Beier *et al.*, 2000) as they belong to glycosyl-hydrolase family 13, therefore, the enzymes produced were analyzed for the identity of α-, β-amylases or β-cyclodextrin glycosyl transferase (β-CGTase) production by the selected isolate.

4.7.1 Enzyme Production

Starch agar is a differential medium that tests the ability of an organism to produce the extracellular enzymes (exoenzymes), presumptively α-amylase and oligo-1,6-glucosidase that are secreted out of the bacteria and diffuse into the starch agar. These enzymes hydrolyze starch by breaking the glycosidic linkages between glucose subunits and allow the products of starch hydrolysis to enter the cell (Lal and Cheeptham, 2012).

The concentrated extracellular (supernatant of the culture) and intracellular protein fraction of cells obtained from MRS-potato starch medium were first estimated for protein by Bradford (1960) method and then examined by loading into SDS-PAGE (Fig. 4.3) followed by enzyme activity staining on Native PAGE (Fig. 4.4). One single band in supernatant fraction and three bands in intracellular fractions were observed in SDS-PAGE. Three bands were observed in intracellular fraction of cells and only a single band in supernatant fraction corresponding to 108 kDa as the standard α-amylase following enzymatic staining for amylolytic activity in Native PAGE. α-Amylase was produced in modified MRS medium with potato starch as substrate and reached maximum levels within 48 h. These results suggested that potato starch was hydrolysed by α-amylase produced extracellularly by *L. lactis.*

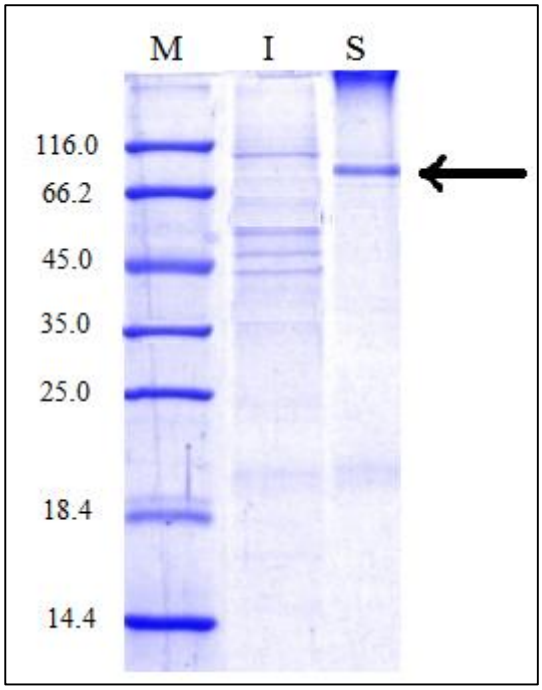


Fig. 4.3 SDS PAGE of intracellular (I) and extracellular (S) fractions with protein marker (M) Lane I: intracellular protein fraction of cells from MRS potato starch media; Lane M: 1 KDa protein marker; Lane S: extracellular protein fraction of cells with MRS potato starch media.

Fig. 4.4 Iodine staining: Clear bands are indicative of α-amylase activity on Native Gel. Lane 1: Supernatant from MRS media with Glucose; Lane 2: standard α-amylase; Lane 3: intracellular fraction of cells from MRS potato starch media; Lane 4: extracellular fraction of cells with MRS potato starch media.

Most of the α-amylase was produced after the end of the period of exponential growth i.e. at the initiation of stationary phase and was absent in MRS medium with glucose, suggesting that starch was the inducer of amylase production. The highest level of amylase (2.54 U/ml) was produced in cultures with starch as the substrate within 48 h. As evident from zymographs and activity assay results, no amylase activity in the culture supernatant of MRS medium (with glucose as substrate) was observed. It is therefore likely that starch or oligosaccharides from starch induced amylase production.

The amylase binds to uncooked starch granules, a feature strongly correlated with the hydrolysis of raw starch, and it has a temperature optimum near the low end of the mesophilic range (20-45ºC) of amylases (Smith and Zahnley, 2005). These properties make the amylase best suited for applications at ambient to slightly above ambient temperatures, where some industrial fermentations occur.

4.7.2 Involvement of β-cyclodextrin glycosyl transferase (β-CGTase) in starch hydrolysis

The conventional method for detection of β-CGTase (β-cyclodextrin glycosyl transferase) activity is based on detecting starch hydrolysis by starch-iodine method. This method is non-specific since all of starch-degrading enzymes, regardless of their specific reactions, show similar results and thus are indistinguishable. However, the phenolphthalein indicator gel method is based on the detection of the specific product of β-CGTase, that is, β-cyclodextrin (Pakzad *et al.*, 2005). β-cyclodextrin forms inclusion complex with phenolphthalein and results in decoloration of the dye. However, β-CGTase produced a negative result when stained with phenolphthalein indicator gel method (Fig. 4.5a). In order to confirm the presence of α-amylase and absence of β-CGTase in phenolphthalein indicator gel method, Coomassie blue staining was used. Fig.4.5b indicates the presence of

α-amylase enzyme as the β-CGTase enzyme failed to produce the colorless positive band with phenolphthalein indicator gel (Fig. 4.5a) indicative of β-CGTase activity, instead, it produced pink color band indicative of α-amylase activity, suggesting it to be devoid of β-CGTase enzyme.

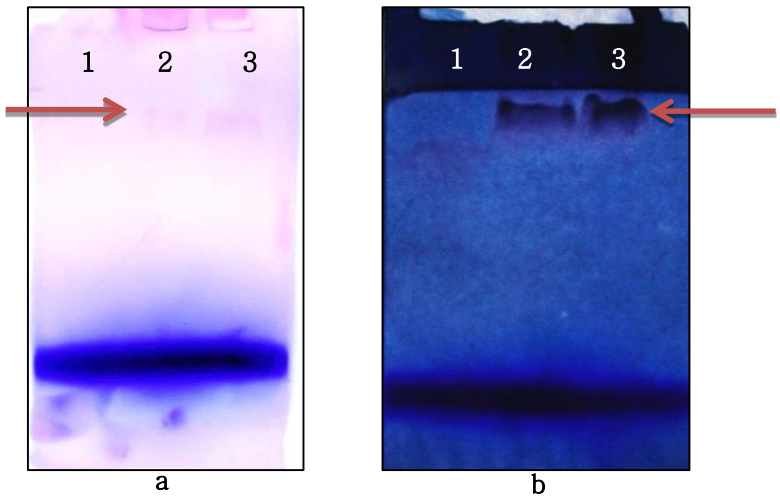


Fig. 4.5a) Phenolphthalein staining. No sharp clear band is observed which is indicative of cyclodextrin production. Pink bands were observed indicative of α-amylase. Lane 1: supernatant with glucose as substrate; Lane 2, α-amylase; Lane 3: supernatant with starch as substrate.

b) Coomassie blue staining. Lane 1, supernatant with glucose as substrate; Lane 2, α-amylase; Lane 3: supernatant with starch as substrate.

To ascertain the biochemical principle responsible for starch hydrolysis, the extracellular fraction was assayed in modified MRS medium containing potato starch (2%) treated with phenylmethylsulfonyl fluoride (PMSF) and heat. The untreated extracellular fraction demonstrated a typical kinetics upon assaying in the medium whereas PMSF or heat treated extracellular as well as intracellular fraction failed to exhibit starch hydrolysis (Fig. 4.6). in accordance with the activity staining experiments reported before, there was almost negligible activity in cell bound or intracellular fractions and no cell-bound amylase activity (Fig. 4.7).

The above results conclusively proved that the enzyme produced upon starch hydrolysis

by *L. lactis* to be α-amylase. These results suggested a clear non-involvement of β-amylase or β-CGTase by *L. lactis* in starch hydrolysis.

4.7.3 Starch Degradation Profile

The surface of native potato starch granules were smooth and intact with the diameter between 12-20μm. Under light microscopy (LM) it was evident that the structure of starch displayed smooth and intact surface in the initial stages (0-8h) and as the fermentation progressed, the number of starch granule became more or less deformed and destroyed by starch degrading amylase enzyme produced by *L. lactis* by the end of 24-48 h. (Fig. 4.8a). The results obtained by LM were confirmed by Scanning Electron Micrographs (Fig. 4.8b).

The results illustrates the initial aspect of the starch granules being smooth and after 24 h fermentation with *L. lactis* the starch granules became rougher and perforated after 48 h, several starch granules displayed large cavities and their lamellar organization was distinctly observed these observations were in congruence with those of Bozic *et al.* (2011) and Sarian et al. (2012)..

4.7.4 Effect of pH on α-amylase production

The effect of pH was assessed by inoculating *L. lactis* into modified MRS media (MRS with 2% potato starch) at different initial pH (4-7). The crude enzyme was extracted by ammonium sulphate precipitation from supernatant and intracellular fraction of the cells from media and the activity of α-amylase enzyme produced was recorded

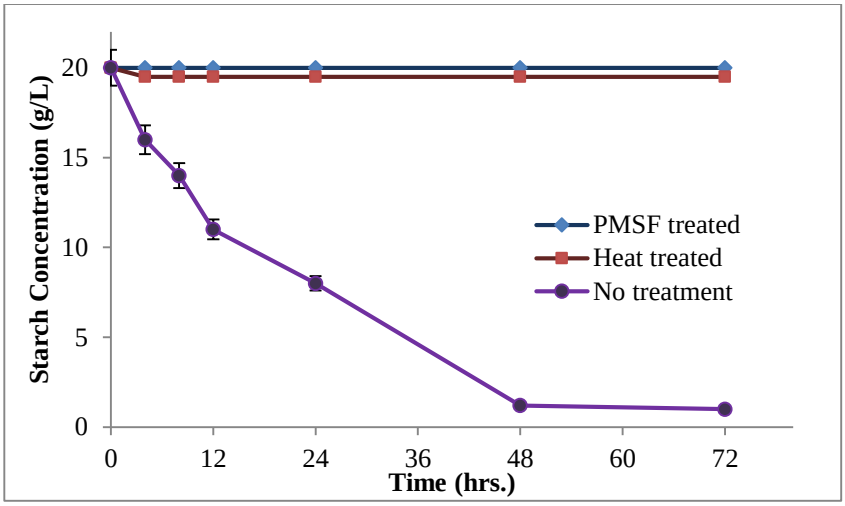


Fig. 4.6 Starch degradation activity by PMSF treated (◆), Heat treated (■) and untreated (●) extracellular protein fraction.

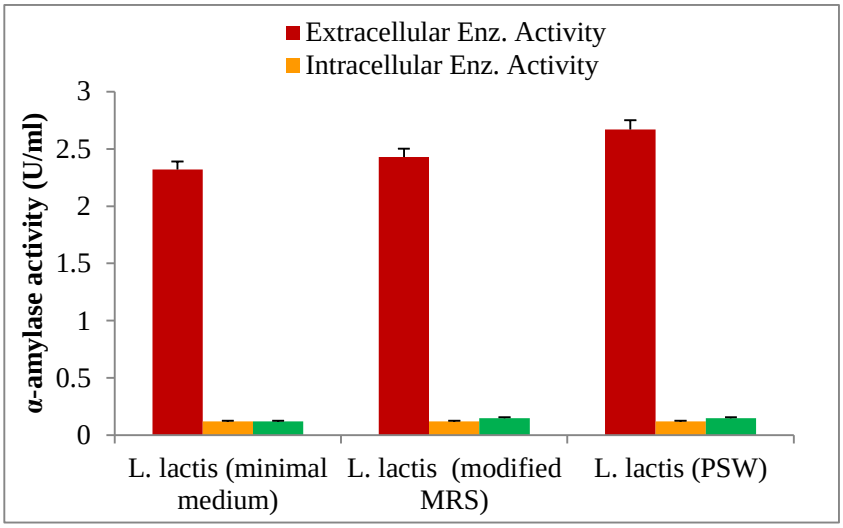


Fig. 4.7 Production of α-amylase in minimal medium, modified MRS and PSW. The values shown represent mean from triplicate experiments. Bars represent standard error

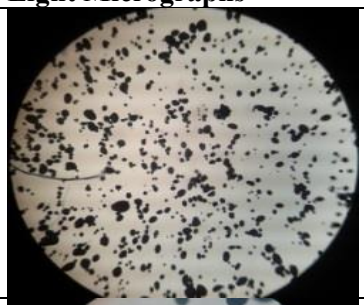
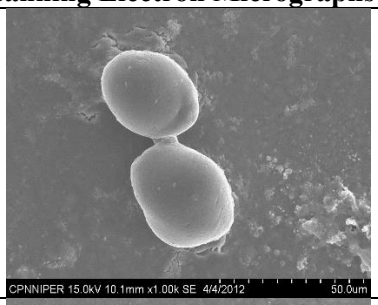
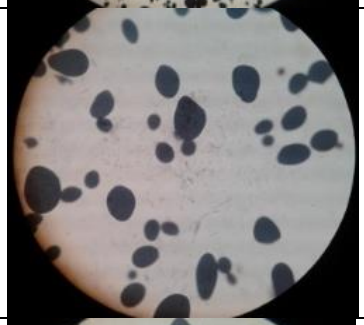
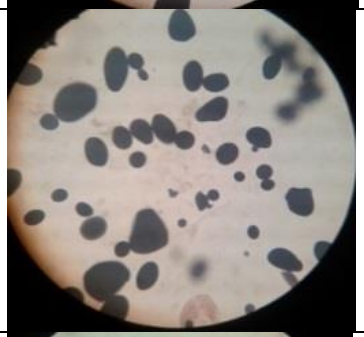
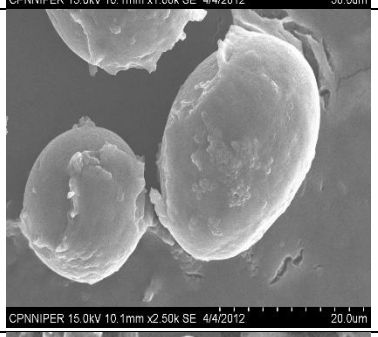
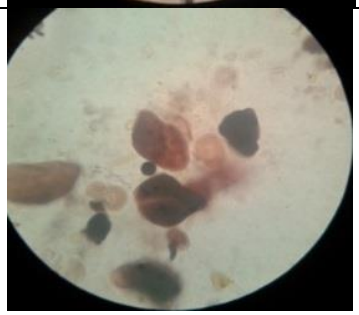
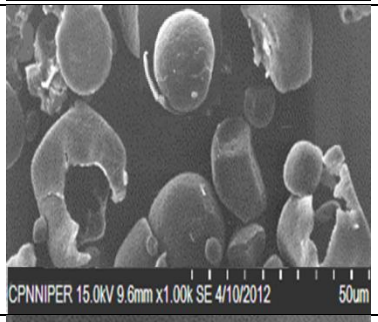
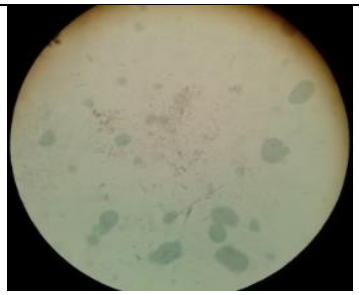
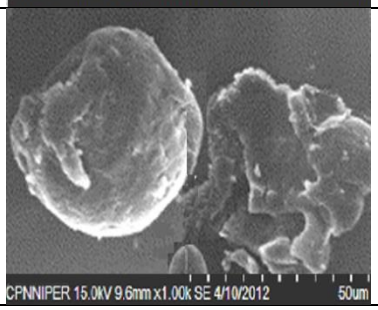
Time (h)	Light Micrographs	Scanning Electron Micrographs
0		
8		
12		
24		
48		
	a	b

Fig. 4.8. The structural shift of starch granules from intact, smooth surfaced & more in number at 0 h over to deformed and lesser in number at 48 h as observed in **a) Light Microscopy** and **b) Scanning Electron Microscopy**

A differential enzyme production (α-amylase) in only extracellular fraction was observed at a pH range of 4-7 (Fig. 4.7 & 4.9) which resulted in an optimum pH of 5.5 for the highest production of enzyme (2.54 U/mL).

Wallenfels and Weil (1960) suggested that decline in enzyme activity above and below of optimum pH may be possible due to the formation of an improper ionic form of the substrate or enzyme (or both), inactivation of the enzyme or from a combination of these effects (Wallenfels *et al.* 1972).

4.7.5 Effect of temperature on α-amylase production

Enzyme activity was assessed by inoculating *L. lactis* in modified MRS medium and incubating at different temperatures (25ºC - 75ºC). Enzyme synthesis occurred at all the temperatures but maximum activity was observed at 55°C (Fig. 4.10). For α-amylase, the specific activity at 55°C was found to be 2.54 U/mL of soluble protein. PSW showed maximum activity as compared to modified MRS and minimal media in extracellular fractions. The optimal pH and temperature for highest α-amylase activity were 5.5 and 55°C respectively.

The above data suggest production of α-amylase is favoured at optimal pH and temperature of 5.5 and 55°C respectively and is inducible of starch concentration. The results are in agreement to *Leuconostoc* sp. (Lindgren and Refai 1984), *Lactobacillus. amnylovorus* and *Lactobacillus amylophilus* (Pompeyo *et al.* 1993) and *Lactobacillus plantarum* A6 amylase optimal pH and temperature were in the range 5-0-6.0 and 40-50ºC, respectively (Giraud *et al.* 1991). For *Lactobacillus cellobiosus,* the optimum temperature was 50ºC whereas the optimum pH was neutral (7.3) (Sen and Chakrabarty, 1984).

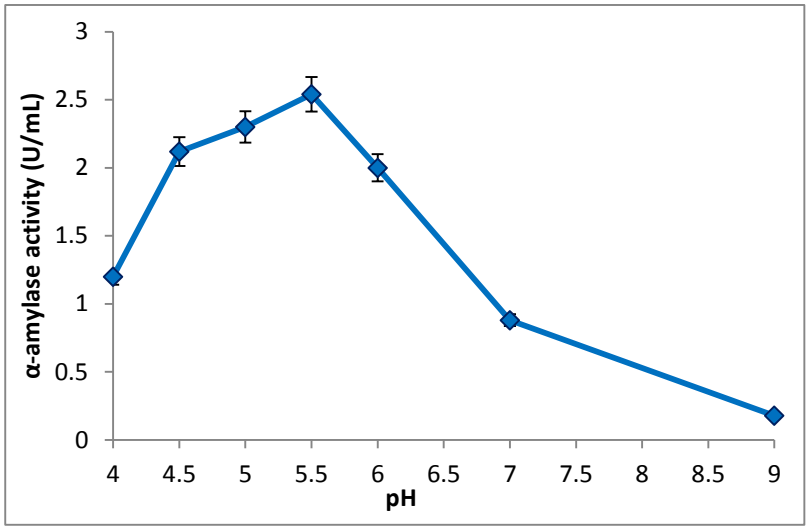


Fig. 4.9 Effect of pH on α-amylase production. The values shown represent mean from triplicate experiments. Bars represent standard error

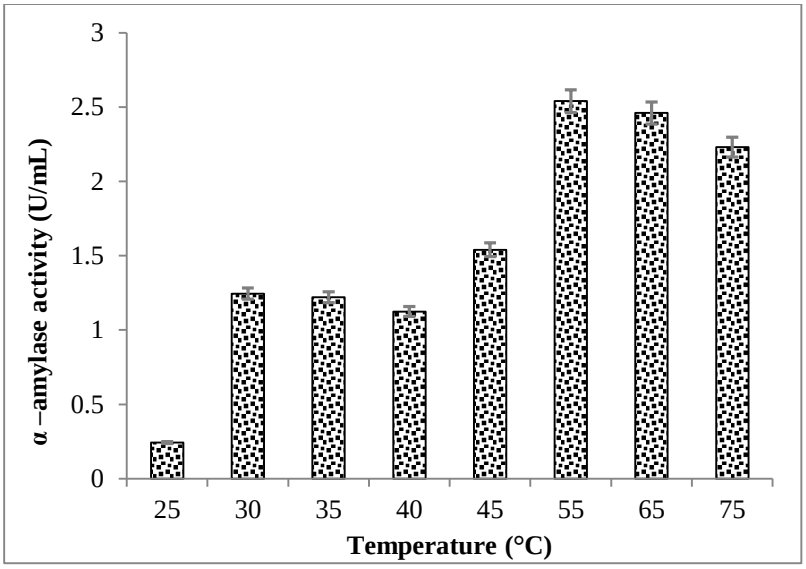


Fig. 4.10 Effect of tempertaure on α-amylase production. The values shown represent mean from triplicate experiments. Bars represent standard error

It was observed that with the depletion of starch with progressive culture growth to log phase, the production of α-amylase increased correspondingly. The strain was found to be α-amylase enzyme producer with 20 g/L potato starch induced in MRS media as 2.54 U/mL (Fig. 4.11).

4.8 Growth Kinetics

The growth curve of *Lactococcus lactis* is characterized by lag, exponential, stationary and death phase. The lag phase continued for 2-3 h; the bacteria adapted to the growth medium following the lag phase where the maximum division rate was observed, this phase continued for 14-16 h as evident in Fig. 4.12.

The stationary phase continued for about 24 h begins after the log phase; here the cell growth and death are in equilibrium in the culture. The transition of the stationary phase from the exponential phase is gradual because the growth rate of the culture declines even before the substrate has been consumed significantly. After 24 h had elapsed the culture showed a decline in growth due to scarcity of the nutrients, accumulation of acid and other metabolic waste products. The phase started 40-48 h after the incubation and continued thereafter. The growth curve observed in MRS medium with starch as carbon source was almost similar to the one obtained with MRS media containing glucose in the terms of specific growth rate (μ_{max}) and highest cell density obtained which accounted to be 0.0268 h^{-1} and 3.5 x 10^6 CFU/mL in MRS starch medium respectively and 0.0216 h^{-1} and 3.8 x 10^6 CFU/mL in the MRS glucose medium respectively. These results suggest the ability of *L. lactis* to survive and grow with starch as carbon source making this isolate a potential starch hydrolyzing strain.

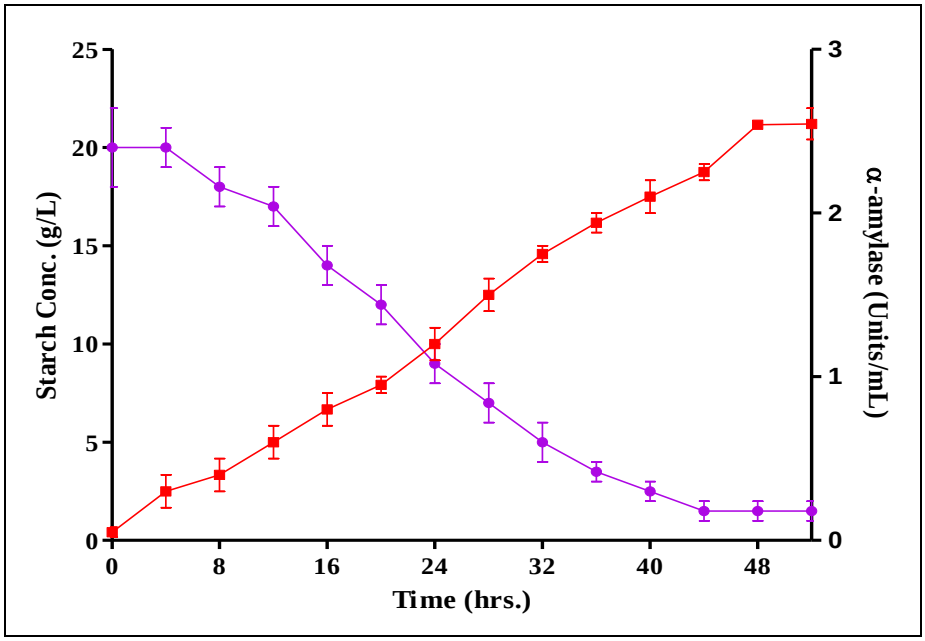


Fig. 4.11 Production of α-amylase (■) by subsequent starch degradation (●) during fermentation for 48 h. The values shown represent mean from triplicate experiments. Bars represent standard error

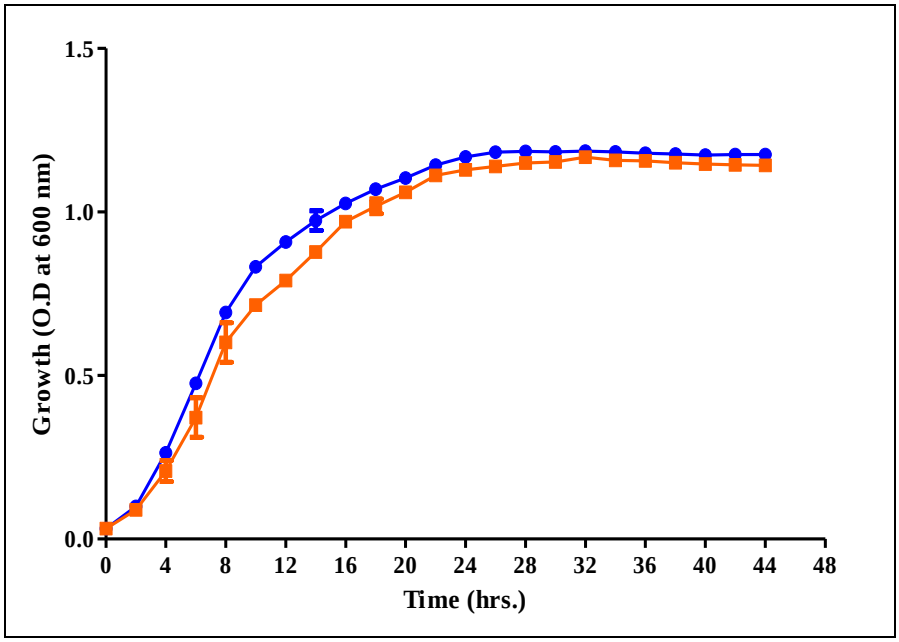


Fig. 4.12 Growth kinetics of *Lactococcus lactis* in MRS media (●) and MRS media (■) with Starch as C-source.

The use of LAB in foods and food supplements has a long history and are considered as GRAS organisms. Bacterial strains belonging to the genera *Lactobacillus and Bifidobacterium, Lactococcus* and *Enterococcus* are widely used as probiotics (Jafari *et al.*, 2011). In fact, GRAS cultures are preferred for high value low volume chemicals destined for human application (Bhanwar *et al.*, 2013b)

Parallel studies with *L. lactis* indicated it to possess beneficial especially probiotic attributes. Therefore, we envisaged that a complete characterization for probiotic property will broaden a scope of application of the product in pharmaceutical and food industry as well as enable utility of the bacterial biomass following fermentation.

4.9 Probiotic Attributes of *Lactococcus lactis*

4.9.1 Tolerance to gastric juice

One of the major selection criteria for probiotic strains is being resistant to low pH (Quwehand, *et al.* 1999, Çakır 2003). Gastrointestinal tract has varying acidic levels ranging from as low as 1.5 to 4.5 pH. Stomach and the regions after stomach have the highest acidity and in order to be used as beneficial adjuncts, *Lactococcus lactis* must be able to survive these harsh conditions and colonise in the gut and therefore tolerate acidity. Since, to reach the small intestine they have to pass through the stressful conditions of stomach (Chou and Weimer 1999, Cakır 2003). Although in the stomach, pH can be as low as 1.0, pH 3.0 has been preferred in most *in vitro* assays. This is due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad, *et al.* 1998).

The isolate *Lactococcus lactis* possessed the ability to survive under pH 2.0 and the viable cells could still reach 10^8 CFU/mL following incubation for 3 h (Fig. 4.13). This indicates

the probability of *L. lactis* to survive low pH under gastric conditions. The effect of the successive passages through artificial juices on the viability of the *L. lactis* is depicted in Fig. 4.13; showing notable survival (99%) in simulated acid gastric juice. The slight decline in the live counts of isolate may be due to the effect of gastric juice (determined after 1 h incubation with simulated acid gastric juice), because in the following stages of incubation, cell counts increases approx. 0.9 log units. The results indicated tolerance of *L. lactis* towards simulated gastric juice.

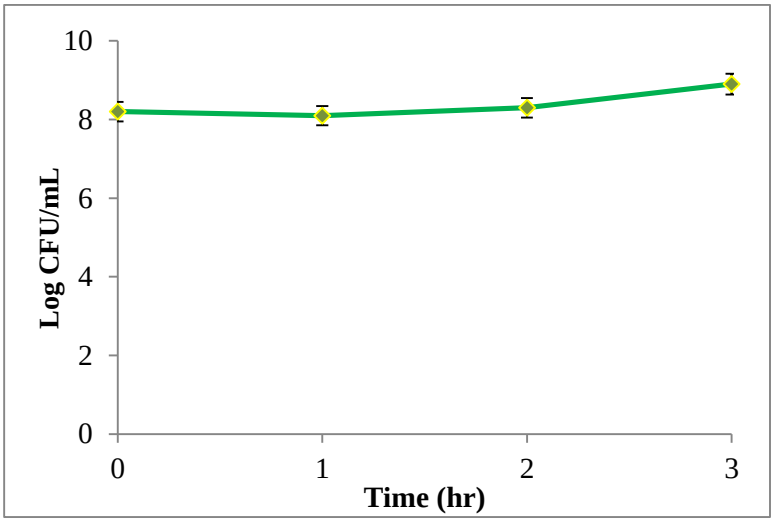


Fig. 4.13 Effect of gastric conditions on *L. lactis.* The values shown represent mean from triplicate experiments. Bars represent standard error

4.9.2 Bile tolerance

The strain was screened for its ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the range of bile concentration is believed to be 0.3% - 3% w/v and the residing time is suggested to be 4 h (Prasad, *et al.* 1998). Results of bile salt tolerance, depicted in Fig. 4.14, suggested that the isolate possessed good capacity to resist bile salts as the viable cells increased in bile salt concentration at 0.1% and it decreased gradually from 0.5% to 3.0% and then stabilized at 10^8 CFU/ml when grown for 4 h. Tolerance to bile salts is considered to be a prerequisite

for colonization and metabolic activity of bacteria in the small intestine of the host (Marteau *et al.*, 1997).

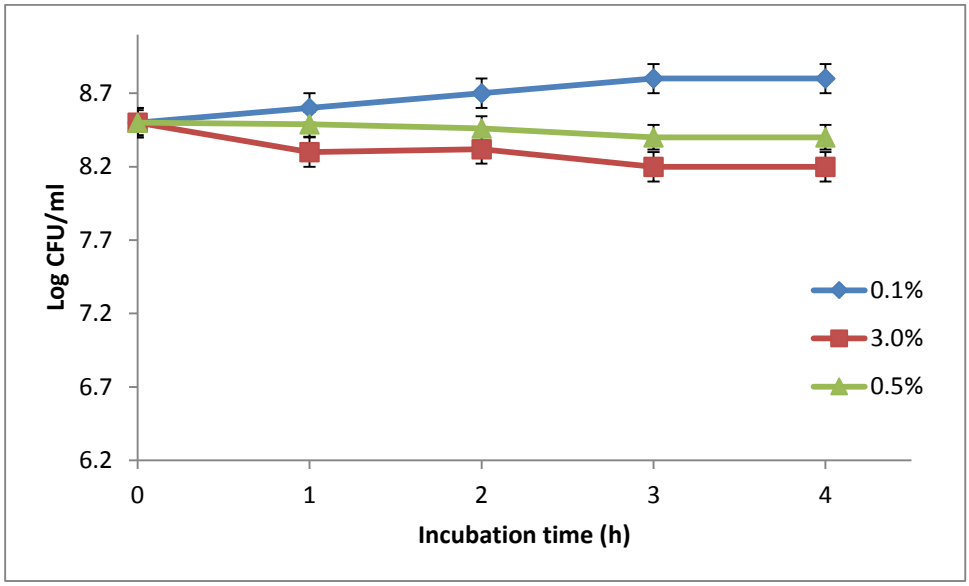


Fig. 4.14 Effect of bile concentration on survival of *L. lactis*. The values shown represent mean from triplicate experiments. Bars represent standard error

Therefore, while evaluating the potential of using LAB as effective probiotics, it is deemed necessary to evaluate their ability to resist the effects of bile acids (Lee *et al.*, 1995).

4.9.3 Bile salt hydrolase (Bsh) activity

To test the ability of the selected strain to hydrolyse the sodium salt of taurodeoxycholic acid, Bsh activity was evaluated. The screened isolate displayed BSH activity by providing the white precipitation zone around the colonies on plate assay when compared with *Lactobacillus acidophilus* ATCC 43121 and *Enterococcus faecium* E179 (controls; results not shown). The isolate demonstrated high Bsh activity by expressing precipitation zone

diameter greater than 15mm (Fig. 4.15). It was noted that the strain also exhibited high resistance to duodenum juice containing 0.5% bile salts (Fig. 4.14), which may be connected with this Bsh activity as suggested by De Boever *et al.* (2000).

4.9.4 Antagonistic activity against pathogens

The free-cell neutralized supernatant of *Lactococcus lactis* inhibited the growth of all pathogenic strains when agar spot method was used. Results for antagonistic activity of the isolate is as shown in Table 4.7. In the agar spot test, *Aeromonas hydrophila* ATCC 35654, *Salmonella typhimurium* ATCC 19585, *E. coli* 0157:H7 ATCC 43895, *Staphylococcus aureus* ATCC 9144 and *Yersinia enterolitica* MTCC 840 indicator strains showed weak to strong inhibition (zone of inhibition of more than 1 mm from edge of producer colony up to 10 mm (Table 4.7) (Schillinger and Lucke 1987; Uhlman *et al.*, 1992).

The inhibitory activity of *Lactococcus lactis* might be due to the production of a heat-stable bacteriocin, because the heated and neutralized cell free supernatant of the producer culture also exhibit antimicrobial activity when compared to live cells in the agar spot test. Therefore, the inhibitory activity observed could be explained by the production of bacteriocin and organic acids. Hydrogen peroxide could hypothetically also act as an inhibitory substance, but the incubation of the plates under anaerobic conditions discards this as cause for the observed inhibition.

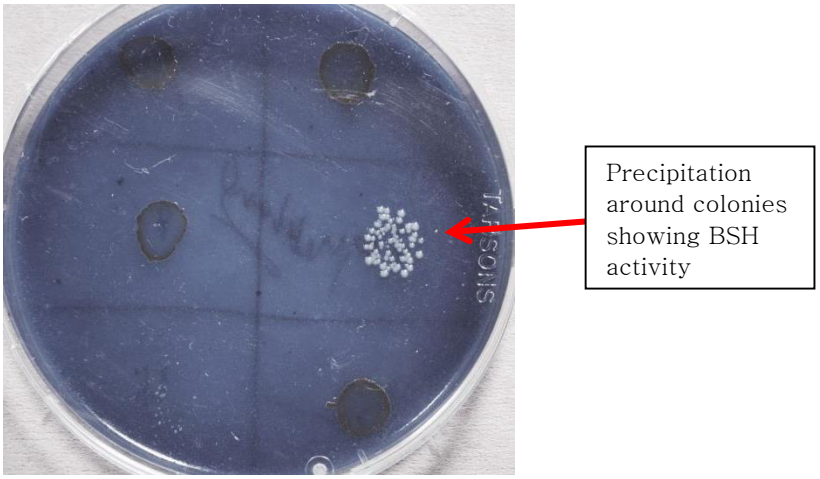


Fig. 4.15 Production of precipitates on agar medium by *Lactococcus lactis* exhibiting BSH activity

Table 4.7 Antagonistic activity of *L. lactis*

Organism	Isolate
***Aeromonas hydrophila*, MTCC 646**	+++
***Salmonella typhimurium* ATCC 19585**	+++
***Escherichia coli* 0157:H7 ATCC 43895**	++
***Staphylococcus aureus* ATCC 9144**	+++
***Yersinia enterolitica* MTCC840**	+

ATCC American type Culture Collection, MTCC Microbial Type Culture Collection
+, inhibition zone <10 mm; ++, inhibition zone >11-19 mm; +++, inhibition zone >20 mm

4.9.5 Antibiotic resistance

Antibiotic susceptibility of LAB is one of the crucial criteria for the safety of potential probiotics since bacteria used as probiotics may serve as host of antibiotic resistance genes which can be transferred to pathogenic bacteria. Selected *Lactococcus lactis* strain was resistant to gentamycin, vancomycin as well as to chloramphenicol and ciprofloxacin. In contrast, it was susceptible to erythromycin, ampicillin, penicillin, tetracycline and clindamycin (Table 4.8), from the MIC breakpoint values as suggested by National Committee for Clinical Laboratory Standards (NCCLS, 1998).

Table 4.8 Antibiotic susceptibilities of *L. lactis*

Antibiotics	MIC (µg/ml)	NCCLSbreakpoints (µg/ml)
Vancomycin	≤1.2	4.0
Gentamycin	≤5.5	32.0
Ampicillin	≤0.12–0.24	2.0
Penicillin	≤0.5–1.0	2.0
Chloramphenicol	≤2.0–4.0	8.0
Clindamycin	≤0.15	4.0
Erythromycin	≤0.50	2.0
Ciprofloxacin	≤1.0–3.0	4.0
Tetracycline	≤1.5	4.0

4.9.6 Adhesive properties

4.9.6.1 Microbial adhesion to solvents (MATS)

Three different solvents were employed by using the MATS method to evaluate the hydrophobic/hydrophilic cell surface properties of *Lactococcus lactis* and their Lewis acid-base (electron donor and acceptor) characteristics. First, direct measurements of the cell surface hydrophobicity and hydrophilicity were carried out by the partitioning of cells between aqueous and n-hexadecane at a high ionic strength of 0.1 M (pH 6.2). Only microbial adhesion to n-hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent, as noted above. The values of MATS obtained with chloroform and ethyl acetate were recorded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacteria, respectively (Bellon-Fontaine *et al.* 1996).

A comparatively less percentage (36.7%) of bacteria which adhered to this polar solvent (n-hexadecane), demonstrated a hydrophillic surface. Microbial adhesion to chloroform showed an overall strong affinity (72.3%) of *Lactococcus lactis* to this acidic solvent and electron acceptor (Table 4.9). These higher values of adhesion were compared with those

obtained for n-hexadecane because both solvents possess the same van-der Waals properties. The important difference observed was due to the implication of Lewis acid-base interactions resulting from the electron donor and basic character of bacterial strain.

The data obtained for ethyl acetate, which is a strongly basic solvent and electron donor, produced results contrary to those encountered with chloroform: the bacterial adhesion to this third solvent was low (24.5%). These results confirmed the non-acidic character of *L. lactis* strain.

4.9.7 Phenol resistance (0.4%)

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols (Suskovic *et al.* 1997). Resistance to phenol was tested as an additional indicator for survival under intestinal conditions (Xanthopoulos *et al.* 1997) for the screened probiotic candidate strain *L. lactis* which survived *in vitro* intestinal passage as described above. These results implied that the strain was less sensitive to phenol and tolerated phenol at 0.4% for 24 h, as no decrease from initial cell numbers were noted (approx.. log 8.1–8.5) (Table 4.10).

In this preliminary screening of the antimicrobial capacity of the strains, five different strains of the gastrointestinal pathogens namely, *S. typhimurium, Escherichia coli, Aeromonas hydrophila, Yersinia enterolitica* and *Staphylococcus aureus* were selected as indicator strains. Interestingly, *L. lactis* isolated from pickled yam had a strong antagonistic activity against *Salmonella*, *Staphylococcus* and *Aeromonas sp.*

In this experimental design, 0.05%–0.3% bile concentration were used as it corresponded to that found in the human intestinal tract, and as 0.3% bile is the maximum concentration that is present in healthy men (Graciela *et al.*, 2001). Therefore, prior to the selection of

probiotic bacteria for human consumption should endure 0.3% bile concentration (Gilliland *et al.*, 1984). *L. lactis* was able to tolerate up to 0.3% of bile concentrations. The viable cell count of *L. lactis* found were 1.5 x 10^5 (CFU/ml) at 0 hours and 1.59x10^{10} (CFU/ml) at 24 h (cells were plated from artificial gastric juice at concentration (Gilliland *et al.*, 1984)). These results indicate that there were no losses of viability of cell in simulated gastrointestinal condition. From this experiment, the results indicated that in GIT environment the gastric juice had minor or possibly no adverse effect on our isolated probiotic bacteria. Bacterial culture plated on MRS agar medium from simulated gastric juice (pH 2.2) showed two morphologically different colonies at 0 and 24 h. Overall, the above results emphasize the potential of this strain as a functional starter in probiotic formulations especially containing starch.

4.10 Production of Lactic acid by *L. Lactis* in PSW

In order to have an economically competitive fermentation process for lactic acid production, an inexpensive and abundant carbon source should be used. There have been various attempts to produce lactic acid efficiently from inexpensive raw materials. As most LAB require a wide range of growth factors including amino acids, specific minerals, vitamins, fatty acids, purines and pyrimidines for their growth and biological activity (Li *et al.*, 2006), a raw material rich in these nutrients is desirable to be used. PSW, as discussed earlier, has earlier been explored extensively for the recovery of starch and lactic acid (Mironescu, 2011;Afifi, 2011), as potatoes are composed of 800 g/kg water, 170 g/ kg carbohydrate, 20 g/kg protein, 1 g/kg fat and 9 g/kg vitamins, inorganic minerals, and trace metals (Pennington, 1985) and therefore have the potential to furnish microorganisms with carbon in the form of starch and sugars; nitrogen and sulfur from protein; inorganic minerals (phosphorus, calcium); trace metals (iron, magnesium, zinc

and copper); and vitamins (B1, B2,C, niacin)(Huang *et al.*, 2003). Therefore, potato starch effluent used in this study was analyzed for its compositional characteristics (Table 4.11) to evaluate its potential to be used as a complete medium for lactic acid production by *L. lactis*. The selected strain was evaluated to produce lactic acid from PSW in the present study. Culture conditions for the maximum production of lactic acid were optimized in minimal media and modified MRS media respectively (containing potato starch as sole Carbon source) by altering various intrinsic and extrinsic parameters to attain the best combination of the parameters.

4.11 Optimization of culture conditions for lactic acid production

It was desirable to optimize the culture variables for maximum lactic acid production from potato starch. Therefore, cultural conditions including initial inoculum size, potato starch concentration, nitrogen source, initial pH of medium and incubation temperature for culturing the selected lactic acid-producing isolate were optimized in minimal media (Appendix) and modified MRS (Appendix) for maximum lactic acid production.

4.11.1 Effect of Incubation time

The time of incubation plays an important role in the optimization of cultural parameters for the production of lactic acid. The sample was withdrawn from minimal media and modified MRS media every 24 h to check the production of lactic acid.

Table 4.9 MATS for *Lactococcus lactis*

Solvent	Nature of solvent	% of adhesion (±SD)
n-hexadecane	Apolar	36.7 ± 2.1
Chloroform	Monopolar, acidic	72.3 ± 2.8
Ethyl acetate	Monopolar, basic	24.5 ± 1.5

Means ± standard deviations of two measures of at least three separate experiments.

Table 4.10 Ability of *Lactococcus lactis* to grow in the presence of phenol 0.4%

Viable count[a] (log10 CFU/ml)					
MRS Broth			MRS Broth + phenol 0.4%		
0 hours	24 hours	Increase[b]	0 hours	24 hours	Increase[b]
8.09±0.06	9.96± 0.11	1.87	8.05±0.43	8.63±0.09	0.58

[a] log mean counts of two trials (average ± s.d.), Increase[b] = $\log_{10}$(final population)-$\log_{10}$(initial population)
n = 3; observations comes from three replicate assays; data are represented as mean ± SD

Table 4.11 Composition and characteristics of potato starch waste water

Parameter	Before treatment (g/L)	After treatment (g/L)	REFERENCES
Total Solids	45 ± 2.0	32 ± 2.0	IS3025 part 18 1989
Total insoluble solids	5.9 ± 0.5	2.6 ± 0.5	IS3025 part 16 1984
Total soluble solids	30.5 ± 1.0	28 ± 1.5	IS3025 part 16 1984
Starch	18 ± 1.5	1.5 ± 0.5	Giraud et al, 1993
Reducing Sugars	1.3 ± 0.8	0.1 ± 0.01	DNS method
COD	26 ± 1.0	6.0 ± 0.5	APHA 21st EDITION 5220 B
BOD	40 ± 2.0	15 ± 1.0	APHA 21st EDITION 5220 B
Total Kjeldhal Nitrogen	1.5 ± 0.2	0.3 ± 0.05	IS3025 part 34 1988

The lactic acid concentration gradually increased with an increase in the incubation time (Fig. 4.16). The maximal concentration of lactic acid (8.9 g/L) in minimal medium and

modified MRS medium (10.3 g/L) was observed at 2nd day i.e. after 48 h of incubation and there was no further increase with an increase in incubation time. These results are comparable to that obtained with *Lactobacillus amylophilus* GV6 (Naveena *et al.* 2005; Altaf *et al.*, 2006) & *Lactobacillus delbrueckii* (John *et al.*, 2006), which had an optimal period of incubation of lactic acid production after 5 days and *Lactococcus lactis* subsp. *lactis* B84 (Petrov *et al.*, 2008) after 6 days.

4.11.2 Effect of Inoculum size

The amount of inoculum for lactic acid production was investigated by cultivating *L. lactis* in optimized media with adjusting the size of inoculum in the range of 0.5% - 3% of 1.096 OD (corresponding to 3.3 x 10^6 CFU/mL cells). Results indicated that the concentration of lactic acid produced at 0.5% inoculum was significantly less ($p<0.05$) and at 2% and 3% of inoculum size, there was no significant increase in lactic acid production over 1%. Thus, 1% inoculum size (approximately 3.0 x 10^4CFU/mL) was selected as optimum for the production of lactic acid. The highest lactic acid production of 8.3 g/L in minimal medium and 10.0 g/L in modified MRS medium (Fig. 4.17) was observed by the strain. Few reports have described the effect of initial inoculum size on the production of lactic acid. *Lactobacillus plantarum* MTCC 1407 when added at five levels (1, 2, 3, 4, and 5%) of inoculum volumes, 2% inoculum size was found to be the best for lactic acid production whereas inoculum above 2% had an adverse effect (Ray *et al.*, 2009). However, in the case of *Lactobacillus casei,* it produced lactic acid when 10% inoculum was added (Linko and Javanainen, 1996). In case of semi-solid fermentation, the inoculum level varies according to the initial sugar or starch content used in the fermentation (John *et al.*, 2007).

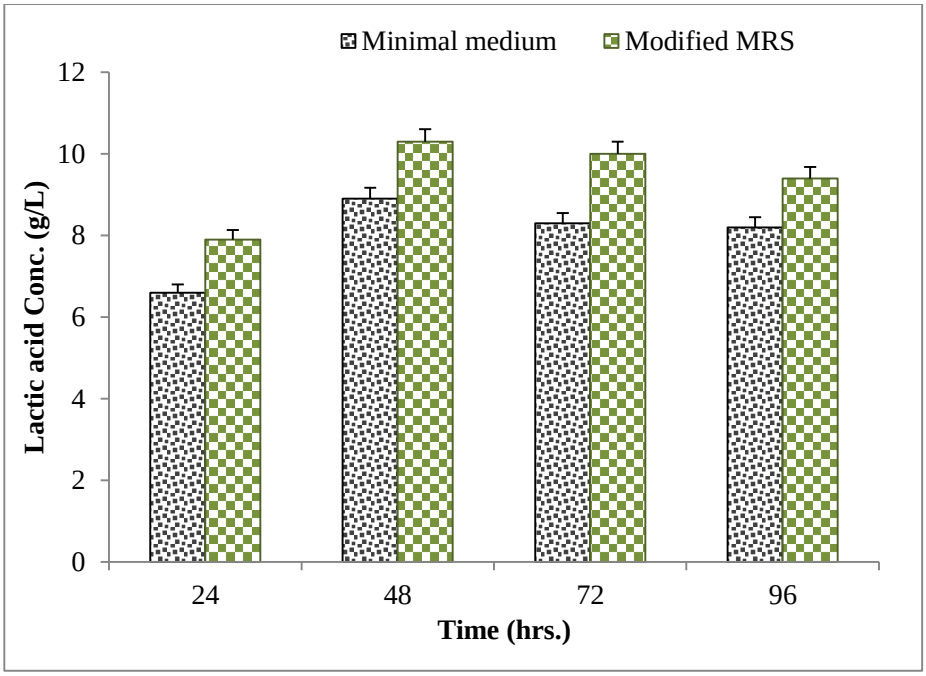


Fig. 4.16 Effect of incubation time on lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

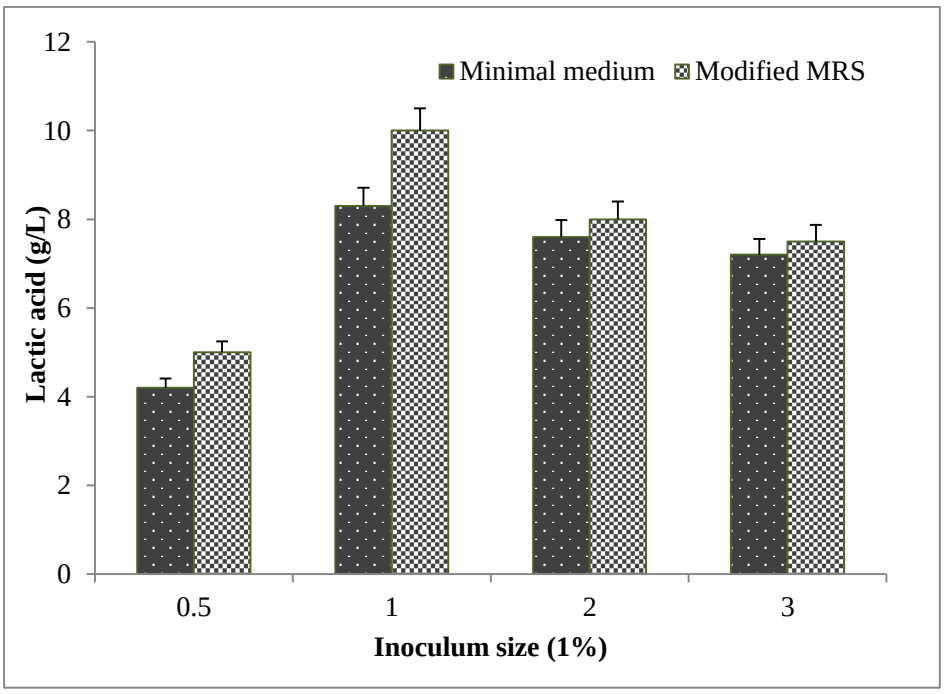


Fig. 4.17 Effect of inoculum size on lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

As the growth of the microorganisms in simultaneous saccharification and fermentation (SSF) depends on the substrate: moisture ratio in correlation with environmental factors like pH and temperature, John *et al.* (2006) reported a similar inoculum volume level in lactic acid production from agro-wastes using *Lactobacillus delbrueckii* as an inoculant. The highest lactic acid yield (98%) was achieved in 47 h from barley starch (130 g/L) simultaneously liquefied, saccharified, and fermented. In a well-balanced simultaneous liquefaction, saccharification, and fermentation using barley starch with the 10% inoculum size compared to 20% inoculum, lactic acid production from with the 20% inoculum was also much slower.

4.11.3 Effect of potato starch concentration

It was found that *L. lactis* was able to utilize potato starch when grown in minimal medium and modified MRS medium containing potato starch. Glucose was replaced with potato starch at various concentrations (10, 20, 30 g/L), to obtain the optimal concentration for lactic acid production. Initial cell count of isolate, *L. lactis,* was approximately 10^6 CFU/mL.

Following 48 h., the isolate produced the highest L-lactic acid concentration at 20 g/L (dry weight) of potato starch amended to MRS broth or minimal medium (Fig. 4.18). When potato starch was added at 10 g/L to fermentation medium, amounts of L-lactic acid produced was lower. Lactic acid productivity decreased above potato starch concentration above 20 g/L. This could probably be attributed to substrate inhibition in batch fermentations. The highest L-lactic acid concentration of 8.9 g/L (Fig. 4.18) in minimal medium and 11.2 g/L in modified MRS was observed from strain with 20 g/L starch.

Yen and King (2010) reported results of batch operation with 20, 40, and 60 g/L sweet

potato starch resulting in 21.62, 29.09, and 37.16 g/L of lactic acid by *Lactobacillus amylophilus* BCRC 14055 at 30°C. *Lactococcus lactis* subsp. *lactis* B84, was capable of utilizing soluble potato starch as a sole carbon source and produced L-lactic acid (5.5 g/L) from completely hydrolyzing 18 g/L starch in batch fermentation at 33°C, agitation 200 rpm and pH 6.0 for 6 days (Petrov *et al.*, 2008).

Direct production of lactic acid from 20 g/L of corn starch yielding the maximum lactic acid concentration of 14.73 g/L from *Streptococcus bovis* (Narita *et al.*, 2004) and 16.6 g/L lactic acid from 20 g/L sago starch by *Enterococcus faecium* No. 78 has been reported (Shibata *et al.*, 2007). The strain could produce lactic acid higher than *L. amylovorus* JCM 1126 and *L. manihotivorans* JCM 12514, which produced lactic acid at concentrations of 14.3 and 11.0 g/L respectively.

4.11.4 Effect of Nitrogen Source

LAB can utilize the hydrolysate of wheat starch, corn starch and potato starch (Hofvendahl and Hahn-Hagerdal, 1997; Akerberg *et al.*, 1998; Akerberg and Zacchi, 2000; Hofvendahl *et al.*, 1999), but these bacteria require a high level of nutrient supplementation including nitrogen source, amino acids, vitamins and microelements (Hofvendahl and Hahn-Hagerdal, 1997; Amrane and Prigent 1998; Nancib *et al.*, 2005), fatty acids, purines and pyrimidines for their growth and biological activity (Li *et al.*, 2006).

To meet these needs, generally yeast extract, beef extract and peptone has been reported to be an important medium component for lactic acid fermentation. A very less quantity of yeast extract have been reported to fulfill the nitrogen requirement of a microorganism growth (Nancib *et al.*, 2005). Six nitrogen sources were tested for the optimization of

lactic acid production *viz.* $CO(NH_2)_2$, peptone, beef extract, $(NH_4)_2SO_4$, NH_4NO_3 and yeast extract in modified MRS medium and minimal media at varying concentrations ranging from 0-10 g/L with defined C:N ratio each time after cultivating *L. lactis* at varying pH and temperature conditions to yield maximum lactic acid. Five nitrogen sources *viz.* ($CO(NH_2)_2$, peptone, beef extract $(NH_4)_2SO_4$ and NH_4NO_3) did not significantly increase lactic acid production. Yeast extract, however, at a very low concentration ($\leq$ 0.5g/L) could induce lactic acid production as discussed below.

4.11.4.2 Yeast extract

Yeast extract is most commonly used as a growth factor which provides complex nutrients as nitrogen source for lactic acid bacteria (Nancib *et al.,* 2005). However, yeast extract is an expensive material to be used in the industrial processes. The yeast extract at initial concentrations of 0-5 g/L were added to modified MRS and minimal medium. Results indicated that yeast extract concentration at as low as 0.5g/L supported *L. lactis* growth (Fig. 4.19) in minimal media as well as modified MRS.

The highest L-lactic acid concentration was obtained in modified MRS medium (10.5 g/L) with 3.0 g/L yeast extract and in minimal medium (8.9 g/L) with 0.5g/L yeast extract and no significant ($p<0.05$) increase further was observed. These media also provided higher bacterial counts ($1.0\text{-}4.1\times10^6$ CFU/ml) than the medium containing 3.0 or 5.0 g/L of yeast extract (9.3×10^5 CFU/mL). In addition, the media containing yeast extract gave better growth of isolate than the media without yeast extract, suggesting yeast extract to be crucial for the bacterial growth.

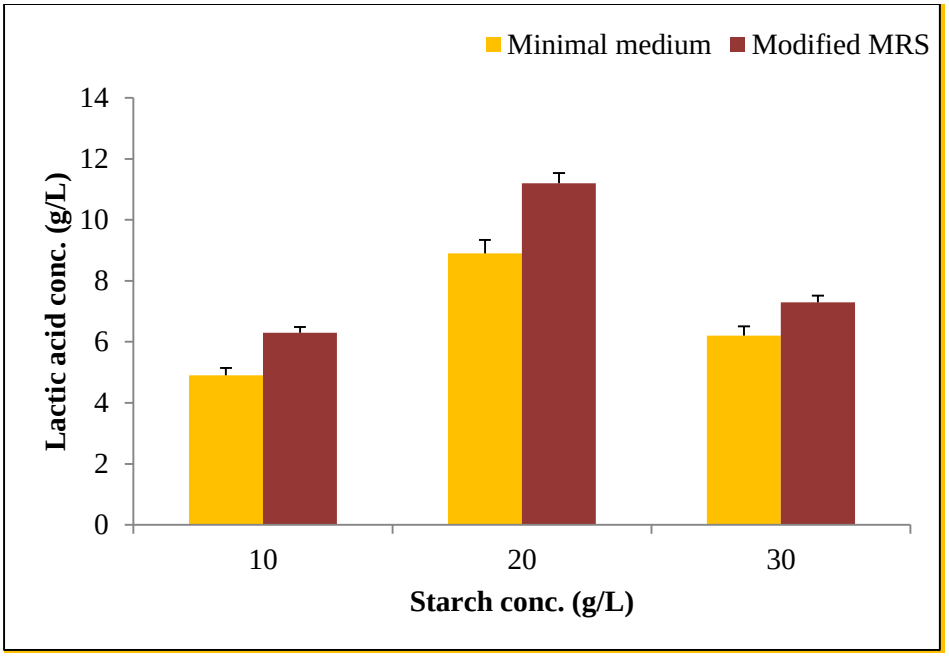


Fig. 4.18 Effect of potato starch concentration on lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

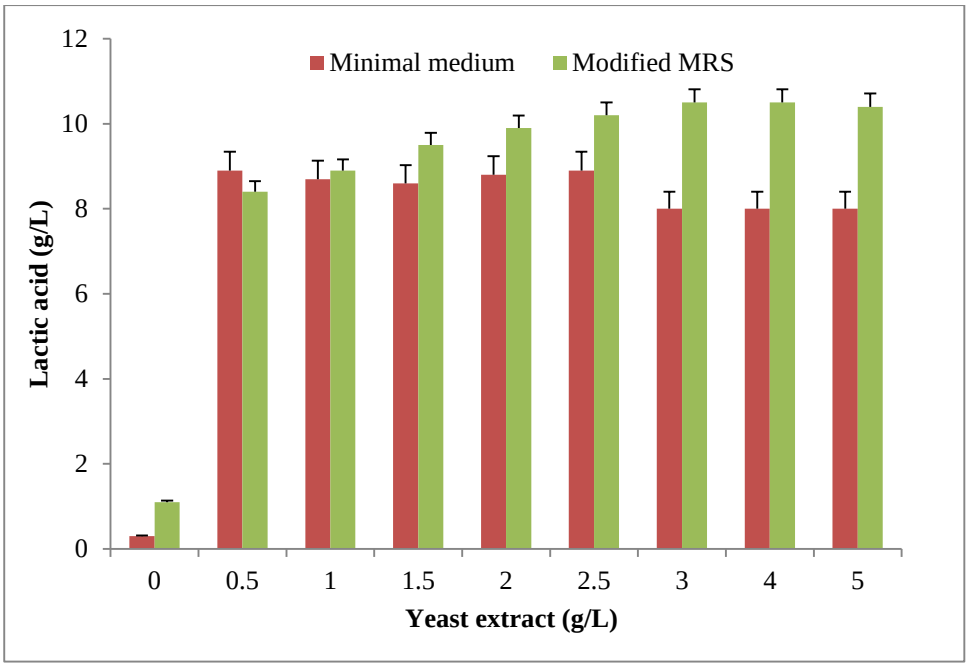


Fig. 4.19 Effect of yeast extract concentration on Lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

The addition of yeast extract upto 3 g/L in media gradually increased lactic acid production and addition of yeast extract to potato starch waste might also increase the lactic acid yield. This might be a useful proposition in future but then cost factor will also be involved. Since the purpose of this study was to produce economically feasible lactic acid from cheap agrowaste, no addition of nitrogen source was made to PSW as it contained nitrogen as described in Table 4.11.

4.11.5 Initial pH of the culture medium

To obtain the maximum L-lactic acid production the initial pH of the medium was varied from pH 4.0 to 7.0. The isolate could produce L-lactic acid in the medium in the entire pH range (Fig.4.20) to produce lactic acid economically by direct bioconversion from starchy substrates, lactic acid productivity in relation to initial pH (which could influence the saccharification of starch) was investigated. The optimum initial pH was found to occur between 5.0and 6.0. Below pH 5.0, strain could not completely convert potato starch to lactic acid. This observation supported the presumption that hydrolysis of starch failed to occur at pH 5.5-8.0. With the initial pH of medium at <5.0, the production of lactic acid was slightly lower.

It has been suggested that in *Lactococcus lactis* subsp. *lactis* C2, the utilization of starch is not affected at pH 4.5 but the uptake of carbohydrate could be inhibited by increased proton levels (Yokota *et al.*, 1995). However, the rapid drop in culture pH < 5.0 during logarithmic growth phase should be prevented, since it causes inhibition of lactic acid production. In the present study, the highest L-lactic acid concentration of 8.6g/L in minimal medium and 10.1 g/L in modified MRS (Fig. 4.20) produced by strain *Lactococcus lactis* respectively was observed. Therefore, the initial pH of medium at 5.0 was deemed to be appropriate for further optimization. The effect of pH on lactic acid

production has been studied by cultivating the isolates *Lactobacillus plantarum*, *L Lactobacillus amylophilus* GV6 and *L. lactis* BME5-18M at pH between 5.0 and 7.0. The optimum pH for cell growth and lactic acid production of *Lactobacillus plantarum* was showed to be between 5.0 and 6.8 (Yumoto and Ikeda, 1995; Fu and Mathews, 1999; Ray *et al.*, 2009). *Lactobacillus amylophilus* GV6 (Vishnu *et al.*, 2000) and *L. lactis* BME5-18M (Bai *et al.*, 2004) could produce lactic acid at pH 6.5. *Lactobacillus manihotivorans* LGM18010T had an optimum pH at 6.0 (Guyot *et al.*, 2000) whereas *Lactobacillus manihotivorans* LGM18011T exhibited the maximum activity for growth at pH 5.0 (Ohkouchi and Inoue, 2006).

Lactococcus lactis subsp. *lactis* B84 could produce lactic acid at pH 6.0 (Petrov *et al.*, 2008). *Enterococcus faecium* No.78 was produced lactic acid at pH 6.5 (Shibata *et al.*, 2007). Narita *et al.* (2004) reported that *Streptococcus bovis* 148 to grow at pH between 5.8 and 9.6 with its optimum pH at 6.0, whereas *S. bovis* JMC 5802 optimum pH for lactic acid production by *S. bovis* JMC 5802 is reported to be 5.5 (Yuwono and Kokugan, 2008). The selected strains grew and showed high L-lactic acid concentration in the medium adjusted to pH at 5.0. It could be used as starter culture for lactic acid production

4.11.6 Effect of Cultivation temperature

The optimal temperature for L-lactic acid production was determined by cultivating the isolate at incubation temperatures varying from 25 to 45°C based on the range of their growth temperatures. Results showed that the isolate had ability to produce L-lactic acid on cultivation in optimal medium at 25-30°C. Above 30°C, L-lactic acid concentration decreased. The strain could produce the maximum L-lactic acid concentration of 8.3 g/L when cultivated at 30°C (Fig. 4.21) in minimal media and 10.1 g/L in modified MRS medium. Thus, the optimal temperature for L-lactic acid production of the selected isolates

was 30°C. The temperature stimulating the highest productivity was in some cases lower than the temperature resulting in the highest lactic acid concentration and yield. For *Lactobacillus amylophilus*, the optimal temperatures were 25 and 35°C for the maximum productivity and yield respectively (Hammes and Vogel, 1995). *Lactobacillus amylophilus* BCRC 14055 showed the optimum temperature for lactic acid production at 30°C (Yen and King, 2010). For *L. casei* and *Lactobacillus paracasei* the optimal temperature for lactic acid production lay between 37 and 44°C respectively (Richter and Trager, 1994).

In agreement with previous observations, *Lactococcus lactis* and *Lactobacillus rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C respectively (Hujanen and Linko, 1996). For *Lactobacillus plantarum* MTCC 1407, it produced lactic acid at 35°C (Ray *et al.*, 2009). The optimal temperature was reported to be 37°C for *Lactobacillus casei* NRRL B441 (Linko and Javanainen, 1996), *Lactobacillus amylophilus* GV6 (Vishnu *et al.*, 2000), *Lactobacillus helveticus* (Aeschlimann and Stockar, 1990), *L. lactis* BME5-18M (Bai *et al.*, 2004), and *Streptococcus bovis* 148 (Narita *et al.*, 2004) whereas the optimal temperature for lactic acid production by *S. bovis* JCM 5802 was at 39ºC (Yuwono and Kokugan, 2008). For *Lactococcus lactis* subsp. *lactis* B84 the optimal temperature for lactic acid production was reported to be 33°C (Petrov *et al.*, 2008).

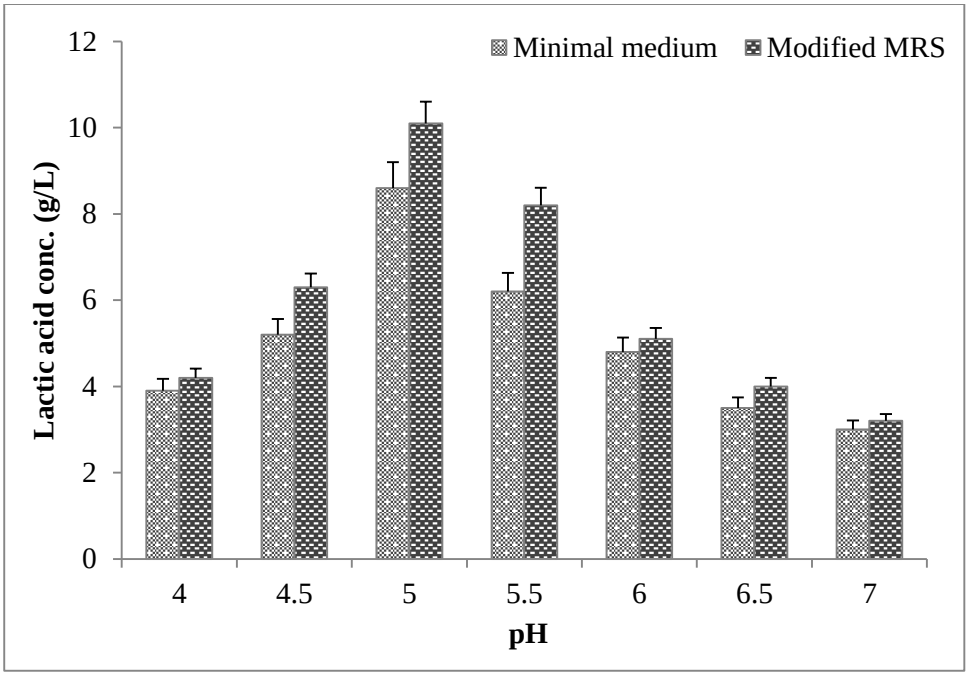


Fig. 4.20 Effect of pH on lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

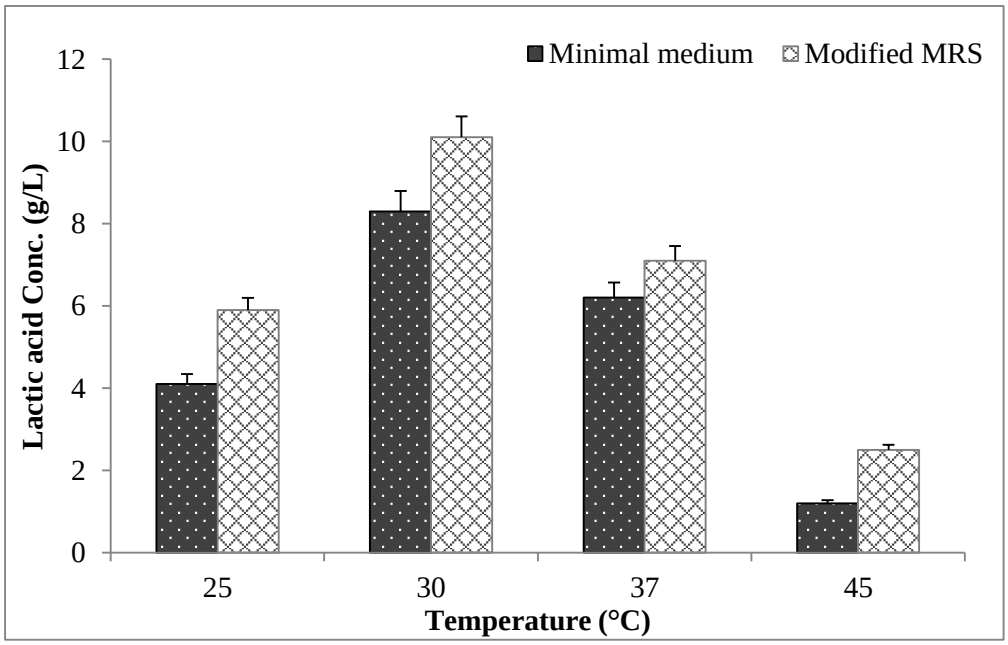


Fig. 4.21 Effect of cultivation temperature on lactic acid production. The values shown represent mean from triplicate experiments. Bars represent standard error

The strain *Enterococcus faecium* No.78 produced lactic acid at 30°C (Shibata *et al.*, 2007). Therefore, these observations suggested that temperature was a critical parameter and need to be controlled for achieving maximum lactic acid production.

4.12 Response surface modeling for maximum Lactic acid production

RSM with CCRD was selected to optimize the significant variables (starch concentration, pH and temperature) for lactic acid production. The coded levels for selected variables are presented in Table 4.12. The mean predicted and experimental values for the lactic acid production values are presented as a response, as detected by central composite rotatable design experiments in various combinations of various levels of the significant variables (Table 4.13). The ANOVA results express that the regression is extremely significant (99% confidence level) and presents a tremendous determination coefficient (R^2 = 0.9037), which depicts that 90.37% of variability observed in the data could be explained by polynomial equation. The second order regression equation resulted after the analysis of variance (ANOVA) explains the levels of lactic acid production as a function of values of the starch concentration (2%), pH (5.5) and temperature (30°C) (Table 4.14).

The final estimated response model equation in terms of lactic acid production (Y) was:

Lactic Acid Production (Y) (Actual factors) = -12.68845 + 0.63097*A + 10.89156*B - 1.31224*C + 2.76667*A*B + 0.21500*A* C + 0.42333*B*C - 5.41112*A^2 - 2.43637*B^2 - 0.015220* C^2

Lactic Acid Production(Y) (Coded factors) = +8.57+ 1.46*A +1.86*B +1.30*C +1.04* A*B +0.54*A* C +1.59* B * C -1.35* A^2 -1.37* B^2 -0.38* C^2

where the coded factors A,B,C are starch concentration, pH and temperature respectively.

ANOVA of the model for lactic acid production indicates the “F” value of 10.42 and values of "Prob > F" less than 0.0500 deduces that the model was significant. A value of

R^2 (0.9037) closer to 1 denotes a very high significance of the model. A higher reliability of the experiment is usually indicated by lower value of coefficient of variation (CV) i.e 21.42 which indicates that the experiments performed were reliable. The "Adequate Precision" measures the signal to noise ratio and a ratio greater than 4 is desirable and a ratio of 11.947 indicates an adequate signal. The "Lack of Fit F-value" of 118.46 implies the Lack of Fit is significant.There is only 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise.The responses obtained from the CCRD were fitted to a second order polynomial equation to elucidate the dependence of production of lactic acid on the optimum level of the significant variables (Fig 4.22 a-c). As shown in Fig. 4.22a the highest value of lactic acid production was obtained when the cells were exposed to a concentration of 2% starch at temperature of 30^{o}C for 48 h.

The optimum operating conditions of temperature and pH for maximum lactic acid production was 30^{o}C and 5.5 respectively. The response surface plotted in Fig. 4.22b between starch concentration and temperature and the trend of the surface plot of temperature versus pH in Fig. 4.22c depicted the similar activity of lactic acid production. In fact, the close interaction of starch concentration, pH and temperature was quite evident. Thus, the response shown was based on the interaction of the significant variables observable in a descending order of AC, BC and AB along with A, B, C, A^2, B^2 being significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The predicted response was approximately 13.25 g/L, whereas the experimental response (based on the best predicted combinations of significant variables showing maximum response in CCRD) was approximately 15.60 g/L confirming (Fig. 4.23) the validity of the model.

Table 4.12 Levels of variables tested in CCRD

Variables		Coded values				
		-1	0	+1	-alpha	+alpha
|A	Starch concentration%	1	1.50	2	0.66	2.34
B	pH	4	4.75	5.5	3.49	6.011
C	Temperature	20	25.00	30	16.59	33.40

Table 4.13 Design matrix (coded and actual values) using CCRD of RSM

Runs	A-Starch conc (%)	B-pH	C-Temp	Lactic Acid Production (g/L)	
				Actual	Predicted
1	1.50 (0)	4.75(0)	16.59(-2)	6.2	5.31
2	1.50 (0)	4.75(0)	25.00(0)	8.7	8.57
3	1.50 (0)	4.75(0)	25.00(0)	8.4	8.57
4	1.50 (0)	4.75(0)	25.00(0)	8.5	8.57
5	1.50 (0)	4.75(0)	33.41(+2)	7.9	9.69
6	1.50 (0)	4.75(0)	25.00(0)	8.6	8.57
7	1.50(0)	4.75(0)	25.00(0)	8.9	8.57
8	1.00(-1)	4.00(-1)	30.00(+1)	3.6	2.37
9	1.00(-1)	5.50(+1)	20.00(-1)	3.5	2.49
10	1.50(0)	3.49(-2)	25.00(0)	2.2	1.57
11	2.34(+2)	4.75(0)	25.00(0)	6.5	7.2
12	2.00(+1)	5.50(+1)	30.00(+1)	15.6	13.25
13	1.00(-1)	4.00(-1)	20.00(-1)	2.3	4.02
14	1.00(-1)	5.50(+1)	30.00(+1)	7.5	7.19
15	1.50(0)	6.01(+2)	25.00(0)	6.3	7.83
16	2.00(+1)	5.50(+1)	20.00(-1)	5.8	6.4
17	1.50(0)	4.75(0)	25.00(0)	8.5	8.57
18	0.66(-2)	4.75(0)	25.00(0)	2.1	2.3
19	2.00(+1)	4.00(-1)	30.00(+1)	3.9	4.28
20	2.00(+1)	4.00(-1)	20.00(-1)	4.1	3.78

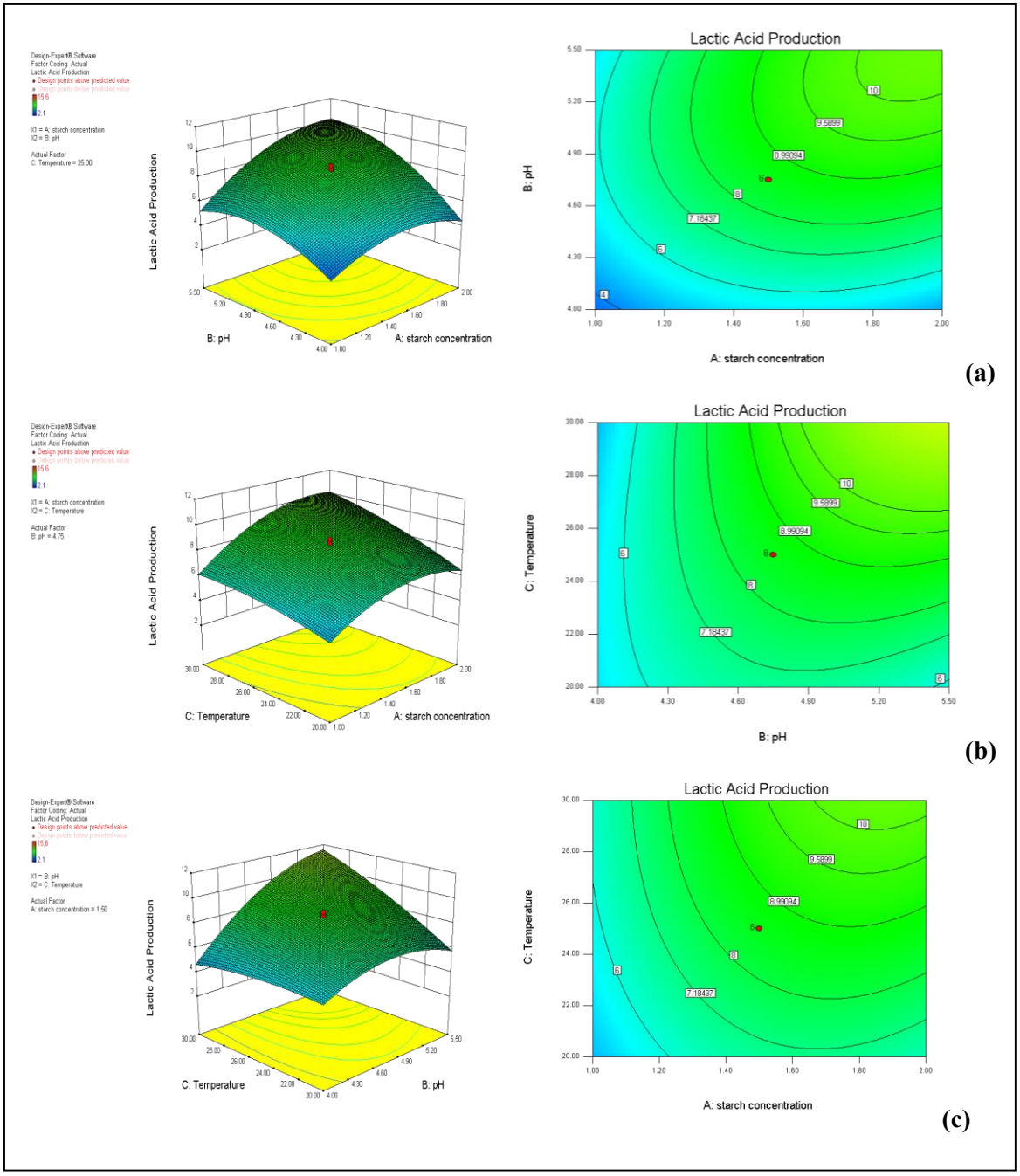
[a]All the responses in the 20 runs were performed in triplicates. The calculated values were significant($p<0.001$)

Table 4.14 ANOVA of the experimental model

Source	Sum of Squares	df	Mean Square	F Value	p-Value Prob > F	
Model	179.28	9	19.92	10.42	0.0005	*Significant*
A-starch concentration	29	1	*29*	*15.17*	*0.003*	
B-pH	47.22	1	*47.22*	*24.71*	*0.0006*	
C-Temperature	23.09	1	*23.09*	*12.08*	*0.006*	
AB	8.61	1	*8.61*	*4.51*	*0.0098*	
AC	2.31	*1*	*2.31*	*1.21*	*0.2973*	
BC	20.16	*1*	*20.16*	*10.55*	*0.0188*	
A2	26.37	*1*	*26.37*	*13.8*	*0.004*	
B2	27.07	*1*	*27.07*	*14.16*	*0.0037*	
C2	2.09	*1*	*2.09*	*1.09*	*0.0207*	
Residual	19.11	10	1.91	-	-	
Lack of Fit	18.95	*5*	*3.79*	*118.46*	*< 0.0001*	*Significant*
Pure Error	*0.16*	*5*	*0.032*			
Cor Total	198.39	19				

4.13 Statistically optimized Lactic acid production

The optimized conditions from statistical model revealed that L-lactic acid production achieved was 15.3 g/L after 48 h at 30ºC at an initial pH of 5.50 with 2% starch concentration. The production after modeling was almost two times higher than the actual results obtained with 1% starch concentration and the pH and temperature conditions being the same. The kinetics of L- lactic acid production shows that the starch degradation is directly proportional to the production of L-lactic acid production (Fig. 4.23).



4.22 (a-c) Response surface 3D plots and contour plots showing the combined effects of the significant variables in terms of Lactic acid production; (a) Interaction effect between pH and starch concentration; (b) Interaction effect between temperature and starch concentration; (c) Interaction effect between temperature and pH

4.14 Scale up Production of L-lactic acid from potato starch effluent

Using the optimized conditions above, L-Lactic acid production was carried out in a 5.0 L controlled fermentor (Fermac 560, Electrolab, UK) containing 3.0 L fermentation medium, potato starch effluent, containing 20 g/L (dry weight) of potato starch. The cultivation temperature and pH were automatically controlled at 30°C and pH 5.5 (as optimized) and the agitation speed was maintained at 200 rpm for 48 h An inoculum (1% v/v) containing approximately 3.0 x 10^4 CFU/mL was inoculated into the medium as optimized above. Fermentation medium was withdrawn every 4 h and analyzed for measurement of bacterial growth, pH of the medium, residual substrate and lactic acid concentration. L-lactic acid production started after 4 h of inoculation and continuously increased until 48 h reaching maximum at 15.6 g/L with a productivity and yield of 0.325g/L/h and 0.78 g/g of starch respectively (Table 4.15).

The profile of cell growth, lactic acid production and substrate consumption was recorded (Fig. 4.23) and it was observed that bacterial growth was related to the increase in lactic acid concentration. The maximum cell growth was obtained as 1.47×10^{13}CFU/mL for 24 h and it was observed that 90% potato starch in the medium was consumed by the end of 48 h and results also indicated that available substrate limited lactic acid production. Our results are in accordance with *Enterococcus faecium* No. 78 which directly produced lactic acid (16.6 g/L) from 20 g/L of sago starch at 30°C and pH 6.5 (Shibata *et al.*, 2007) with purity of 98.6%. In the direct lactic acid fermentation from starch, continuous culture has hardly been reported. Recently, continuous culture system with high cell density of *Enterococcus faecium* showed higher lactic acid productivity (3.04 g/L.h) than those of batch culture (1.10 g/L.h) and conventional continuous culture (1.56 g/L.h).

Yen and Kang (2010) used different starch concentrations (20, 40, and 60 g/L) in batch operation at 30ºC and observed that the batch with 20 g/L of initial starch provided the

maximum productivity and yield of 0.31 g/L.h and 98% respectively. The production of lactic acid is pH dependent as the production decreases if pH is not controlled

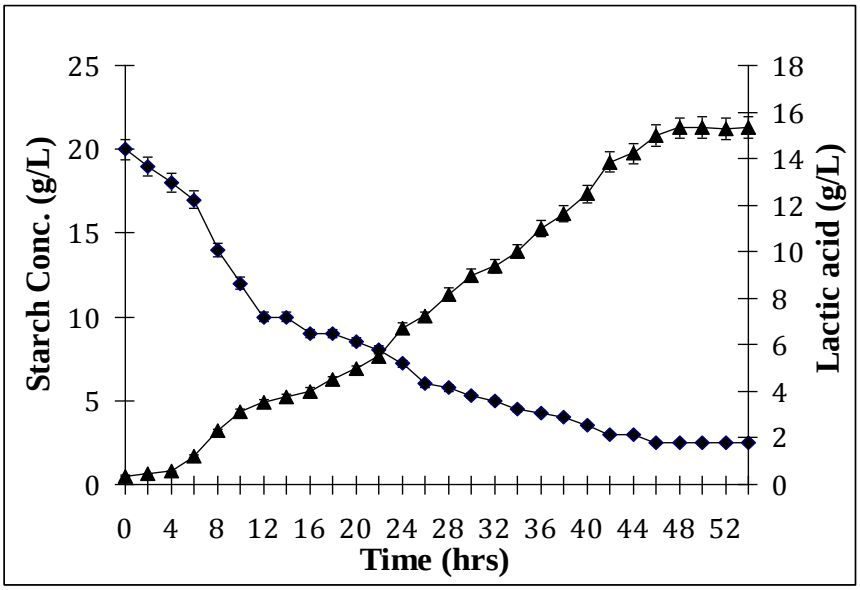


Fig. 4.23 Kinetics of lactic acid production with starch degradation in potato starch effluent. Lactic acid production is directly proportional to starch degradation at optimized temperature, pH and starch concentration of 30ºC, 5.5 and 20g/L respectively. The production of lactic acid is at equilibrium after 48 h and did not increase further. Results are mean of three independent replicates.

Table: 4.15 Kinetic parameters for lactic acid production by *Lactococcus lactis*

Kinetic parameter	Results
Product yield $Y_{P/S}$ (g/g)	0.78
Productivity (g/L.h)	0.325
Final concentration of lactate (g/L)	15.6
Residual starch (g/L)	1.2
Fermentation time (h)	48

In *Lactobacillus manihotivorans* LMG18010, without pH control, only 16.8 g of lactic acid was produced with 50 g of soluble starch whereas it increased 2.5 times and reached 40.7 g with a yield of 98.5% with a pH control at 5.0 (Ohkouchi and Inoune, 2006). A slightly lower productivity in the batch operation with 60 g/L of starch could be the

consequence of high lactic acid inhibition. These results were clearly different from those obtained from *L. amylophilus* (Yumoto and Ikeda, 1995), which found that the lactic acid concentration could be as high as 37.16 g/L from the batch operating with 60 g/L starch, which might be high enough to reduce productivity. To increase productivity and lactic acid concentration, a starch-controlled fed-batch operation with 20 g/L of initial starch was performed and controlled pH at 5.3. The maximum productivity of 0.75 g/L.h and the yield of 69% were obtained from the fed-batch operation with starch controlled at 8±1 g/L. (Yumoto and Ikeda, 1995).

Results from our study revealed that the isolate has potential for optically pure L-lactic acid production from potato starch. Extracellular amylolytic activity of the strain was found to be responsible for starch degradation during fermentation. Extracellular α-amylase had been purified and characterized from *Lactococcus lactis* IBB500 from fermentation medium containing potato starch as sole carbon source (Wasko *et al.*, 2010). The existence of one extracellular and one cell-bound α-amylase has also been reported for some starch-hydrolyzing streptococci (Lindgren and Refai, 1984).

4.15 Recovery of Lactic acid

As mentioned before, the lactic acid from the fermentation medium was recovered by the method of Benthin and Villadsen (1995). The colourless product (acid) obtained was further tested for the purity by inspecting for the presence or absence of organic acids other than lactic acid by TLC and HPLC.

4.15.1 Thin layer Chromatography (TLC)

It was desirable to examine the product following fermentation. TLC is a preliminary procedure for identification of organic acids produced in the media. The aliquots of

fermentation medium were examined by resolving on TLC plates along with reference standards of organic acids at a concentration of 10 % (w/v).

The chromatogram of lactic acid produced red spots. The chromatograms of acetic acid and butyric acid produced blue spots whereas that of succinic acid produced yellow spots and dark yellow for citric acid. Table 4.16 shows the Rf values of organic acids and the colour of the spots corresponding the acids. The sample produced a prominent red spot at Rf value of 0.51 indicative of lactic acid production.

In fermentation broth, organic acid profile (citric acid, lactic acid, formic acid, acetic acid, butyric acid, and propionic acid) that could be produced were determined using TLC (Lee *et al.*, 2001). Results confirmed the purity of lactic acid produced as no other organic acid production other than lactic acid was observed. The spot for lactic acid standard is observed at Rf values of 0.53. Lactic acid spots were visualized as red spots on the TLC plate at an Rf value of 0.51.

4.15.2 High Performance Liquid Chromatography (HPLC)

The authenticity of lactic acid was finally confirmed by HPLC. The chromatograms revealed the retention time of sample corresponding to the standards of organic acids (acetic acid, butyric acid, citric acid, lactic acid and succinic acid with peaks at 4, 6.3, 8.1, 10 and 12 min).

Table 4.16 Rf value and detection colour of standard organic acids and cultural broth of PSW on Thin layer chromatogram

Organic acids	Rf value	Daylight
		Methyl red + bromophenol blue
Acetic acid	0.44	Blue
Butyric acid	0.61	Blue
Citric acid	0.03	Dark yellow
Lactic acid	0.53	Red
Succinic acid	0.12	Yellow
Lactococcus lactis	0.51	Red

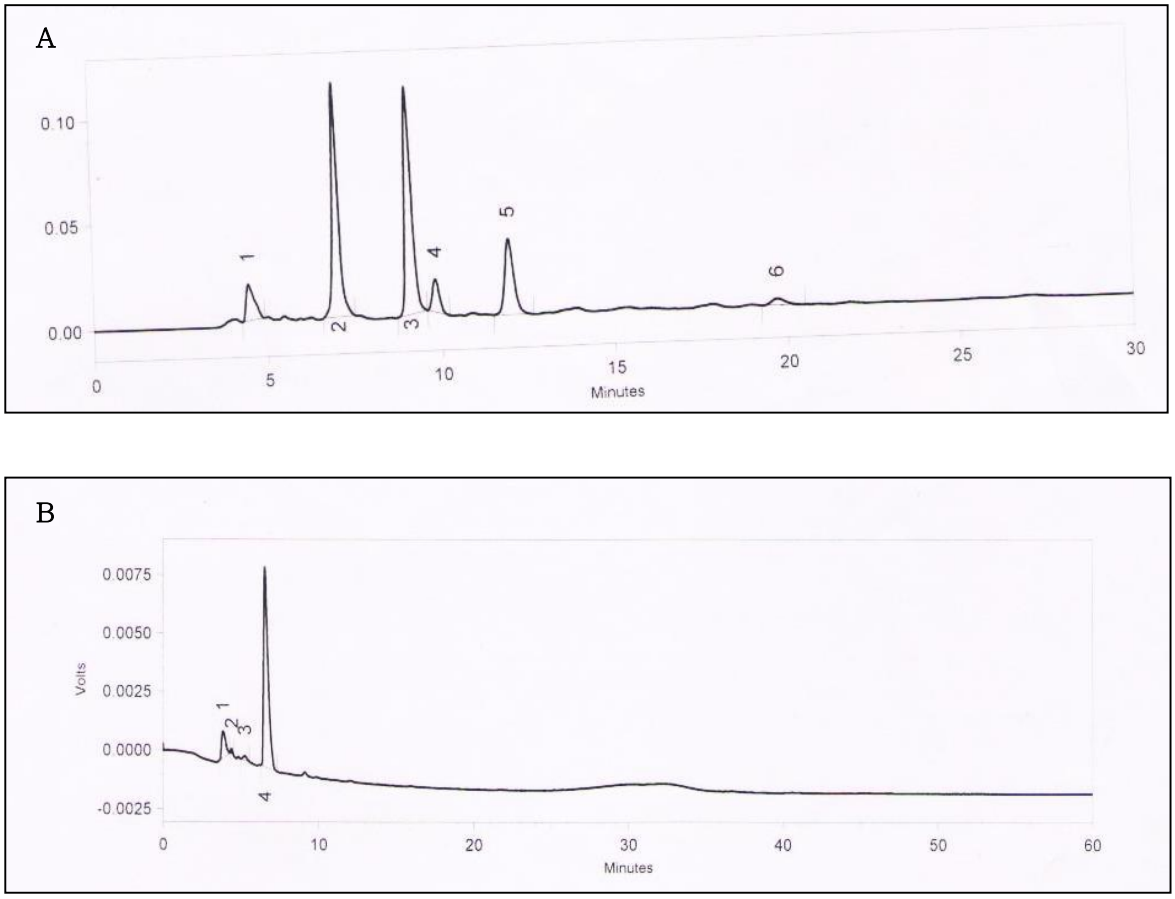


Fig. 4.24 A) HPLC chromatogram of standard organic acids. 1: succinic acid; 2: Lactic acid; 3: acetic acid; 4: Butyric acid; 5: Citric acid with peaks at 4.6, 6.8, 9.0, 9.8, 12.3 minutes respectively. B) HPLC chromatogram of Sample showing peak of Lactic acid at 6.1 minutes.

The production of lactic acid in the sample was evidenced from the appearance of a peak at a retention time of 6.1 min in Fig. 4.24B corresponding to lactic acid peak in standard at 6.3 min (Fig. 4.24A). Lactic acid concentration calculated from the peak area in sample corresponds to 15.2 g/L in the sample confirming the above results.

4.16 Enhanced production of lactic acid

4.16.1 Immobilization

Optimized parameters in minimal media with potato starch were applied for lactic acid production in potato starch effluent (containing 2% potato starch) with immobilized cells and compared with free cells. In order to efficiently immobilize bacterial cells, various parameters were optimized. Among the various preliminary experiments performed for the selection of process parameters effecting lactic acid production in PSW, alginate concentration, cell load and bead diameter were screened as the most significant parameters for maximal lactic acid production by immobilized *L. lactis*. Lactic acid production was compared with free cells and immobilized cells in PSW and found that the production increased from 15.2g/L to 17.2 g/L in PSW (Fig. 4.25) with cell load of 50 mg/mL, immobilized in agar and alginate respectively (Fig. 4.26). Production response with cell immobilized in agar (concentration of 2.5% and bead diameter of 2.0 mm) was lower i.e. 27% as compared to the cells immobilized in alginate (concentration of 3% and bead diameter of 2.5 mm) which showed a response of 42% in Potato starch effluent. Based on these results of conventional studies in minimal medium and modified MRS medium, alginate was preferred as a matrix for immobilizing *L. lactis* cells for lactic acid production in PSW.

Several reports indicate *Lactococcus lactis* to be a valuable strain involved in various fermentation processes, either batch or fed-batch or continuous, for production of metabolites and valuable other organic products from various expensive or inexpensive raw materials (Roukas and Kotzekidou, 1998; Ohashi *et al.*, 1999; Akerberg and Zacchi, 2000; Boonmee *et al.*, 2003). In these processes, the microorganism can be used either in a free form or immobilized (Roukas and Kotzekidou, 1991). It has been shown that, immobilized cell technology (ICT) with LAB could increase the biomass concentration maintained in the fermentation system (Champagne *et al.*, 1994; Norton *et al.*, 1994). Coupled with repeated-batch or continuous culture, ICT could largely increase process productivity and cell stability (Bertrand, 2002; Lamboley *et al.*, 1997).

Production of lactic acid with immobilized cells on alginates has been reported (Guoqiang *et al*., 1991; Yoo *et al.*, 1996; Roukas and Kotzekidou, 1998, Corton *et al.*, 2000), showing better results than with free cells. However, gels are considered to be inconvenient because they are chemically unstable and can easily be disrupted by lactate. The use of more stable supports such as porous foam glass particles (Bruno-Barcena et al, 1999), ceramic beads or porous glass (Gonclaves *et al.*, 1992), poraver beads (Senthuran *et al.*, 1999), and gluten pellets (Chronopoulos *et al.*, 2002) hardly offered a better alternative as they are relatively expensive materials. This can be attributed to the fact that industrialization needs a low cost, easily handled, food-grade purity support with high operational stability (Elezi *et al.*, 2003). Therefore, even though cell immobilization results give increased productivity compared to free cells, the viable industrial processes using immobilized cells have not been achieved.

4.16.2 Reusability and stability of immobilized cell

The reusability of the immobilized cells of *L. lactis* was compared with free cells (21 beads, equivalent to 50 mg/ml cells and free cells - 50 mg/ml cells) for the evaluation of lactic acid production under the optimized parameters of pH 5.5 and temperature 30°C. The immobilized bacterial cells and free cells were evaluated for their starch utilization potential in successive cycles.

After each cycle the alginate beads were filtered and the free cells were collected by centrifugation, washed with saline and phosphate buffer and were then reused for lactic acid production in the next set of PSW. All the reactions conditions were kept constant for every batch cycle. The reaction was carried out for next 24 h and the lactic acid during each cycle by immobilized and free cells is presented in Fig. 4.27. The results depict an accelerated production of lactic acid and reusability increased (till 3^{rd} cycle) of immobilized cells as compared to free cells (till 2^{nd} cycle) which is not significantly high. The use of immobilization is not beneficial and applicable on large scale due to its high processing cost and major disadvantage of beads being ruptured in adverse environmental conditions. Sirisansaneeyakul *et al.* (2007) reported that the immobilization produce enhanced lactic acid when paired with packed bed bioreactor to devise inexpensive process.

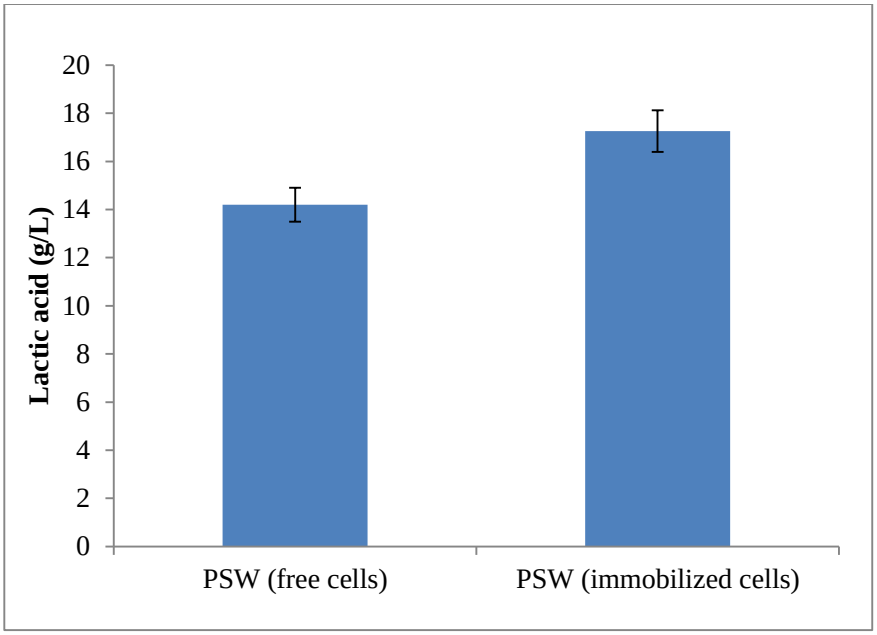


Fig.4.25 Comparative production of lactic acid with immobilized cells and free cells in Potato starch effluent

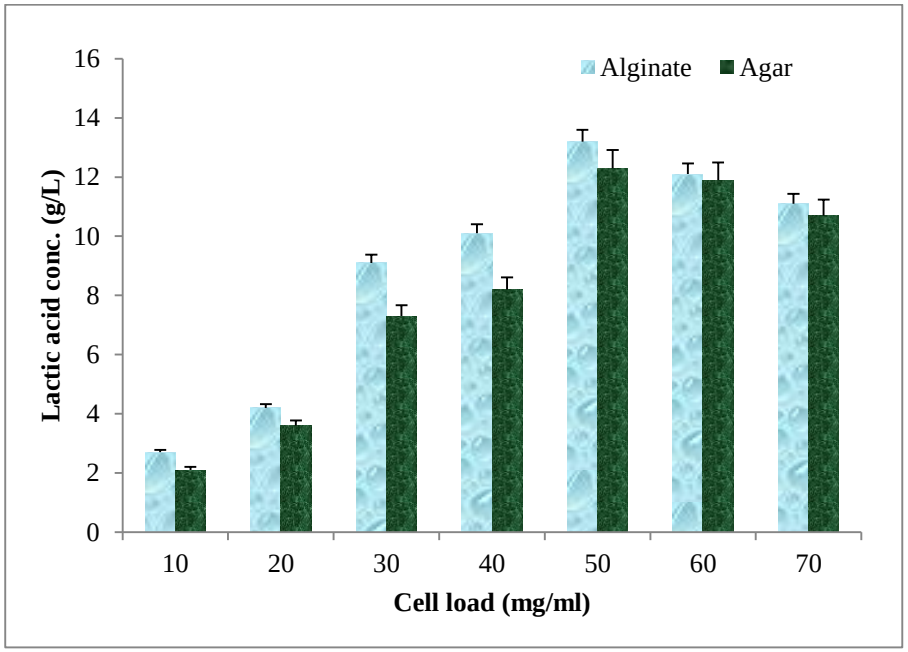


Fig. 4.26 Effect of cell load (w/v) in alginate beads and agar on lactic acid production

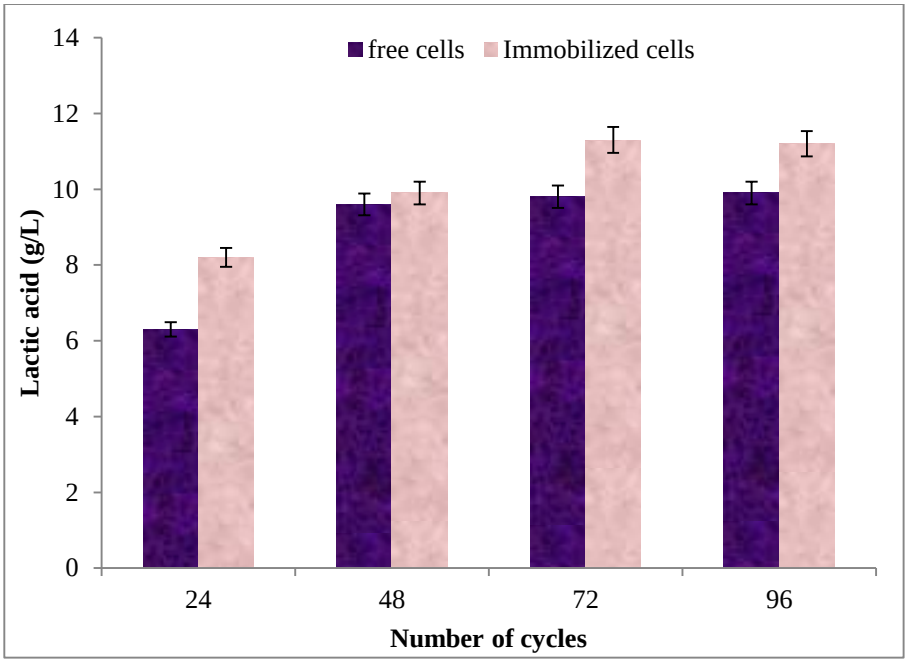


Fig. 4.27 Reusability and stability of free and immobilized cells of *L. lactis* for lactic acid production

4.16.3 Dialysis Sac Bioreactor

Homofermentative production of lactic acid is species specific and due to the end- product inhibition of lactic acid production in various fermentations, several attempts have been made to replace batch fermentation with continuous processes to improve lactate productivity. Improvements in productivity have been achieved using higher high cell density cultures, employing cell immobilization (Senthuran *et al.*, 1997), and by using cell-recycling modules (Ohleyer *et al.*, 1985; Bihal *et al.*, 1991; Xavier *et al.*, 1995), different fermentation feedstocks and different lactate-producing strains (Linko *et al.*, 1996; Moldes *et al.*, 1999).Additionally, some fermentation systems have incorporated lactate recovery units such as electrodialysis (Vonktaveesuk *et al.*, 1194; Boniardi *et al.*, 1997), extractive fermentation (Ye *et al.*, 1996) and ion exchange resins (Evangelista et aal., 1994) to increase both lactate production and productivity. Nevertheless, an efficient economically feasible and high density robust culture process for lactic acid fermentation

(LAF) for industrial purposes was required. The dialysis sac based bioreactor was developed for enhanced production of lactic acid sustainable in industrial environment. This process is also amenable to downstream processing as it reduces the cost.

The production of lactic acid was studied in an experimental set up shown in Fig. 4.28, where the cells of *L. lactis* were entrapped inside dialysis membrane. The production was compared in modified MRS medium, minimal medium and potato starch effluent. In dialysis sac bioreactor, carbon source (20 g/L) was completely consumed within 24 h. (Fig. 3b), whereas it took more than 48 h by free cells in the control shake flasks. The lactic acid productivity (0.79 g/L/h) and yield (0.94 g/g) in the dialysis bioreactor were 2.4 times and 1.2 times higher, respectively, than those 0.33 g/L/h and 0.78 g/g in the control, respectively.

The final lactic acid concentration achieved in dialysis sac bioreactor was 18.9 g/L (Fig. 4.30) compared to 15.6 g/L in shake flask (Fig. 4.29) in one cycle. To test the reusability of the cells, after each batch of fermentation, broth was withdrawn and equal volumes of fresh medium was added to the bioreactor. Fig. 4.29 demonstrates that the lactic acid productivity and yield dropped in shake flasks after the first cycle of fermentation and remained almost unchanged after second cycle of fermentation. The reduced lactic acid productivity and yield might be ascribed to the acidic environment (pH 2.5-3.0) of the fermentation broth which negatively affected the activity of the cells whose maximum activity is at pH 5.5. In dialysis bioreactor, the lactic acid productivity (0.79 g/L/h) and yield (0.94 g/g) was higher than control and increased for upto four cycles of fermentation (Fig. 4.30), thereafter the productivity decreased (Bhanwar et al., 2013c). The reusability of cells implied dialysis bioreactor to be an efficient alternative for treatment of Potato starch effluent using high density cultures of *L. lactis*.

Electrodialysis have been reported for production of lactic acid (Huang *et al.* 2007; Min-tian *et al.* 2005) but no report has been described using dialysis sac bioreactor for the production of lactic acid till date. In this study, the fabrication and use of dialysis sac bioreactor for production and recovery of lactic acid from an industrial waste, using probiotic bacteria is reported for the first time. This process also offers additional advantages in terms of recovery of enzymes especially α-amylase that is produced extracellularly & contained within dialysis sac. Another advantage is that the cells contained in the dialysis sac may be used for industrial use as cell mass can be directly used as animal feed or for human feed supplements (following clinical trials) or used for obtaining β-galactosidase, demonstrated in parallel studies with this culture (unpublished results).

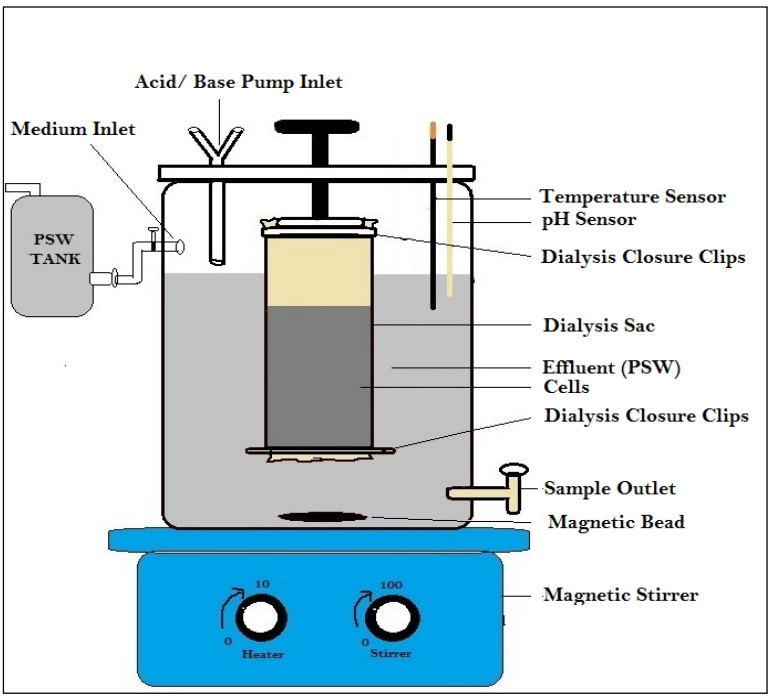


Fig. 4.28 Experimental set up for the study of lactic acid production by the dialysis membrane entrapped *L. lactis* cells

4.16.4 Viability and Reusability of cell entrapped in dialysis sac

The reusability of the *L. lactis* cells entrapped in dialysis sac was compared with free cells (equivalent to 10^8CFU/ml), for the evaluation of lactic acid production under the optimized parameters, at the original pH of PSW (pH 5.5) and at 30°C. The dialysis entrapped bacterial cells and free cells were evaluated for their starch utilization potential in successive cycles. After each cycle the cells were collected by centrifugation, washed with saline and phosphate buffer and were then reused for lactic acid production in the next set of PSW. All the reaction conditions were kept constant for every batch cycle. The reaction was carried out for 24 h and the lactic acid during each cycle is presented in Fig. 4.29. The results depict an accelerated production of lactic acid and high reusability (till 3rd cycle) of cells as compared to free cells (till 2nd cycle). Zymogram results indicated α-amylase to be functional for at least 3 cycles upto 72 h and no band in Lane 1 for 96 h. indicated that probably with the depletion of the growth factor and the age of the cells may have contributed to the loss of enzyme activity (Fig. 4.31).

The dialysis sac based bioreactor was effective in improving lactic acid production from PSW. High lactic acid productivity, yield and starch degradation were achieved compared to those of the shake flask fermentation (SFF; control). To our knowledge, dialysis sac bioreactor in comparison to electrodialysis is much easier and cost effective and has not been ireported earlier for direct starch conversion to L-Lactic acid production.

This study has demonstrated that this process not only increases the lactic acid productivity but it is also cost effective due to the use of inexpensive substrate comparative to media.

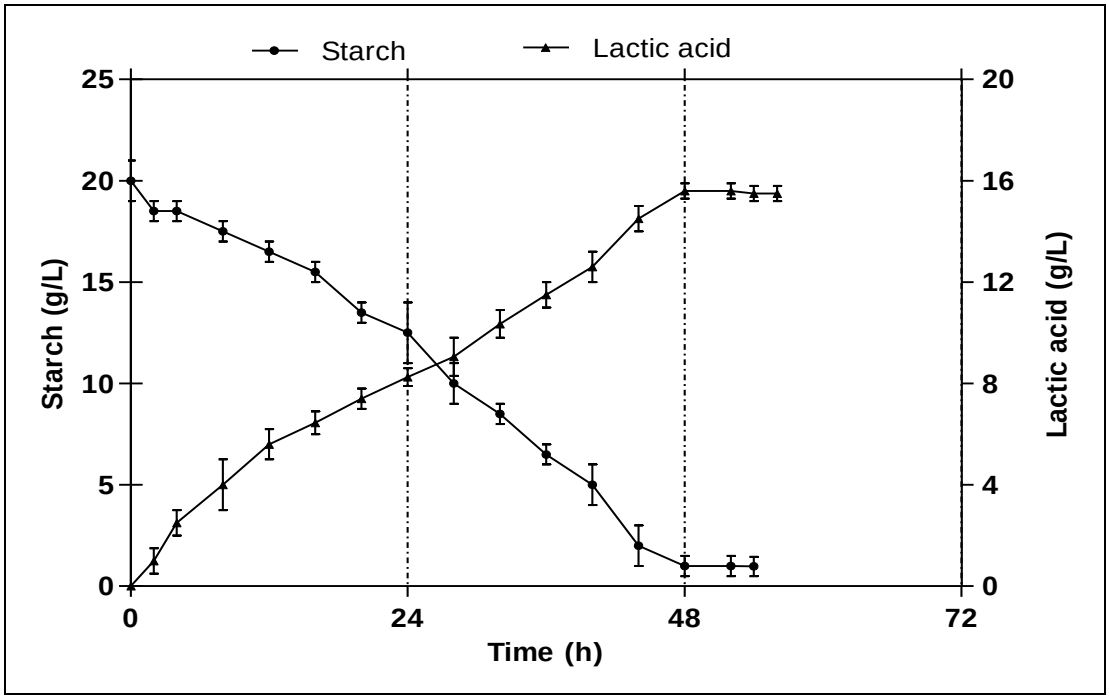


Fig. 4.29 Shake flask fermentation in PSW. Productivity of lactic acid: 1.2g/g starch

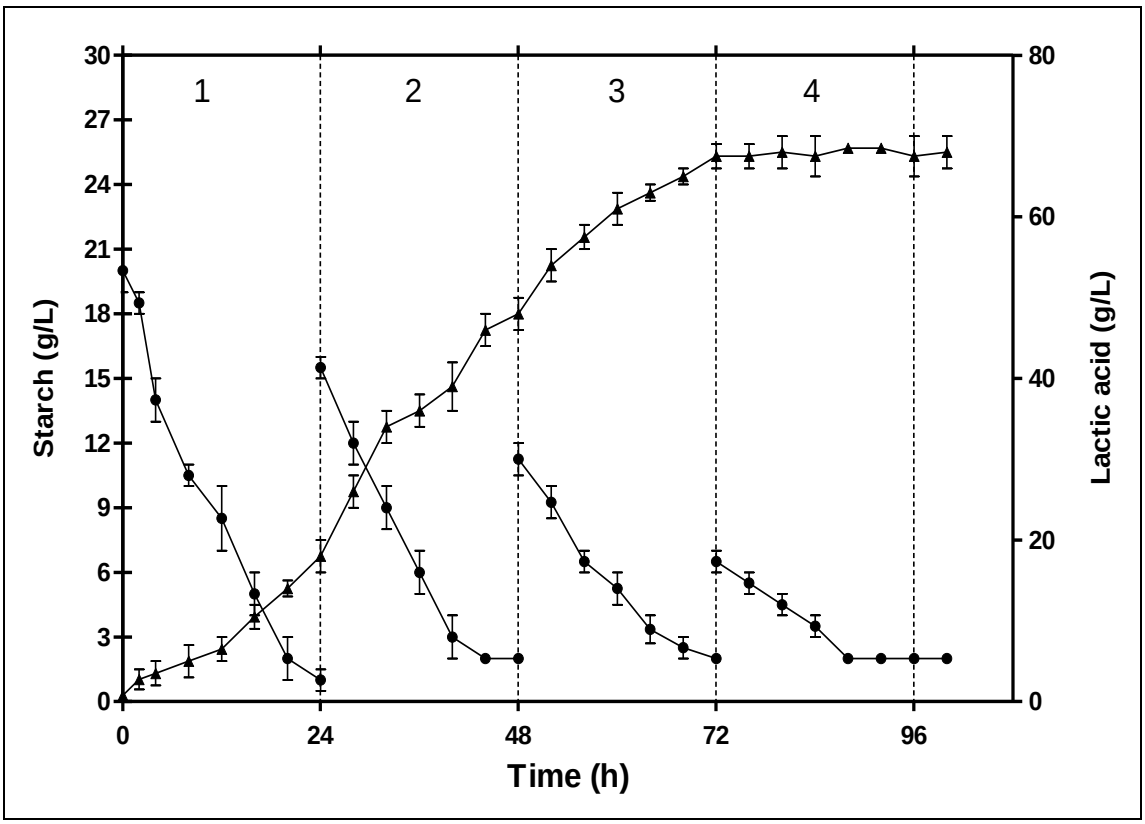


Fig. 4.30 Fed batch fermentation in dialysis sac bioreactor in PSW. Productivity of lactic acid:1.48 g/g starch

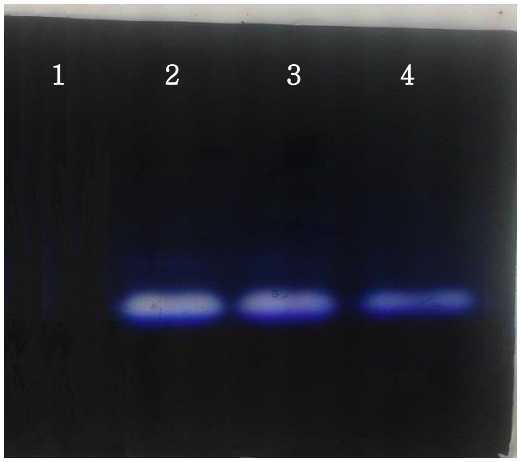


Fig.4.31 Native PAGE showing α-amylase activity over a period of four cycles in dialysis sac bioreactor. Lane 1: enzyme at 96 h.; Lane 2: enzyme at 24 h.; Lane 3: enzyme at 48 h.; Lane 4: enzyme at 72 h. No band at 96 h. depicts the loss of enzyme activity.

Commercially available pure L-Lactic acid costs approximately around Rs. 40,000/- per 100g (Sigma, MO, USA), whereas the L-lactic acid produced in this process costs around Rs. 4500/- for 100g Lactic acid that accounts to approximately 10 times reduction in the cost. It makes this process a significantly better process and commercially acceptable in market.

4.17 Lactic acid derivatives

A shift from a petroleum-based to a biomass-based economy warrants development, apart from biofuels, of biorenewable replacements for petroleum derived chemicals. In this regard, environmentally friendly biomass-derived esters may serve as alternatives to fossil-derived chemicals such as toxic halogenated solvents and glycol ethers.

Esterification is a well-known process and a number of industrially important chemicals such as methyl, ethyl and butyl esters, alkyl t-butyl ethers (MTBE and ETBE) are

produced using such reactions (Carey and Sundberg, 1990). Esterification of carboxylic acids with alcohols in presence of acid catalysts has been extensively investigated. Typical homogenous catalysts like H_2SO_4, HCl and $ClSO_3OH$ are used but due to their miscibility with the reaction medium, separation becomes a problem, hence ion exchange resins are preferred over homogeneous acids due to ease of product separation and catalyst recovery (Ayyappan *et al.,* 2009). Lactic acid and their esters derivatives are extensively used in food industry for preservation and flavoring purposes, as well as in the pharmaceutical and cosmetic industries (Gelbard, 2005).

As a last objective of this study, two esters of lactic acid, namely, methyl lactate and ethyl lactate have been produced by uncatalyzed esterification of lactic acid with alcohols and identification of both the esters was carried out by GC according to the method of Benedict *et al.* (2003).

4.17.1 Gas Chromatography

The produced lactic acid esters were analyzed by GC and the chromatograms obtained reveal typical chromatograms of standard ethyl lactate and methyl lactate in Fig. 4.31a and 4.32a respectively. The response was linear over the range 300–700 μg/ml with correlation coefficients between 0.990 and 0.999. The RSD (Relative Standard Deviation) for five injections of the 500 μg/ml standard for ethyl lactate and methyl lactate gave values of typically 4–5%. In standard chromatogram, the ethyl lactate total ion gave a single peak not attributable to the solvent with a retention time of approximately 4.1 min (Fig. 4.31a). The ethyl lactate in sample gave peak at 4.5 alongwith probably residual l-lactic acid, and water gave peaks at 2.0, 3.0 and 3.5 min (Fig. 4.31b).

The peak for methyl lactate in standard was observed at 30.3 min (Fig. 4.32a) whereas in

sample it is found at 30.65 min with three other peaks at 10.4, 10.9 and 20.6 min (Fig. 4.32b).

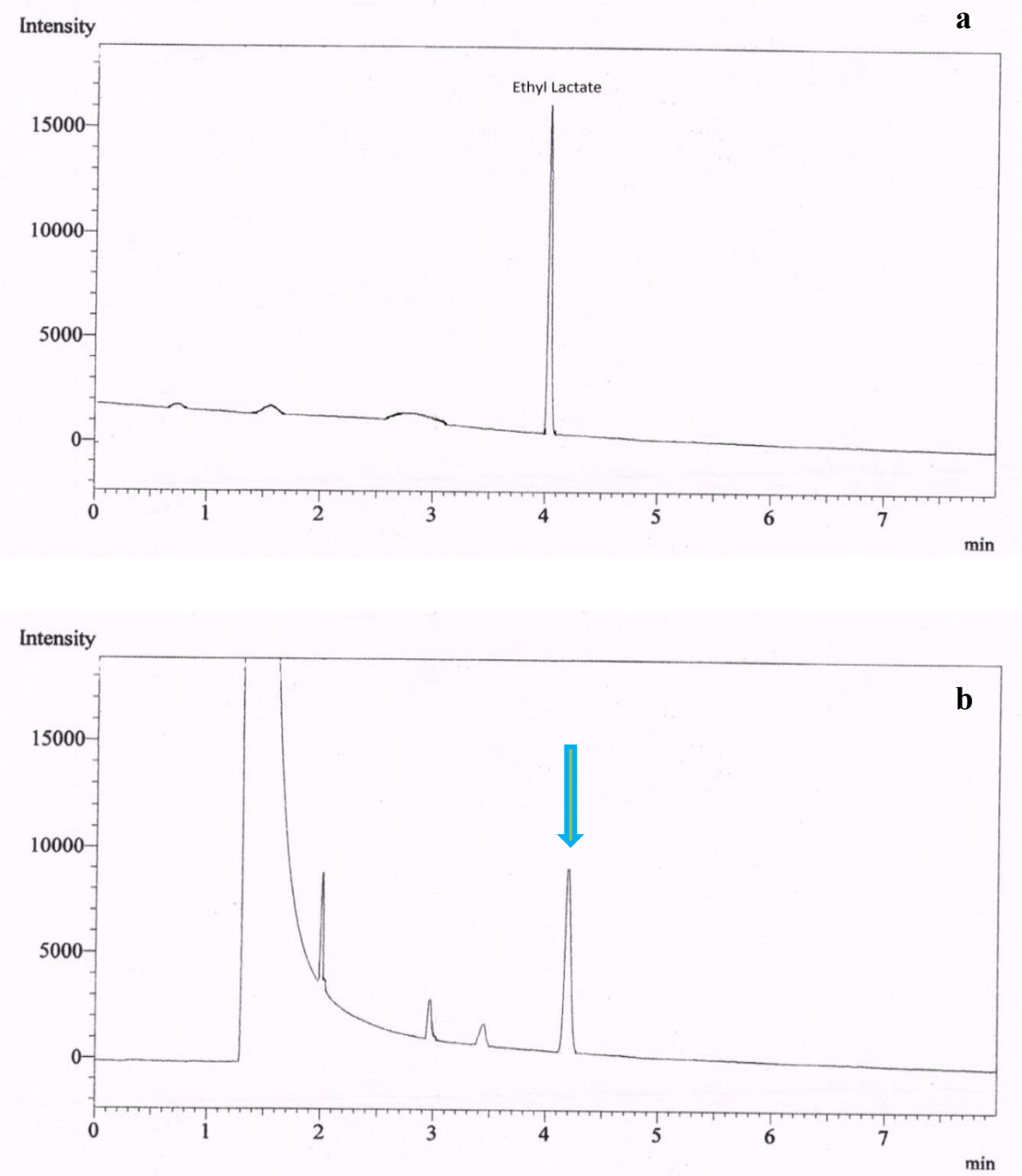


Fig 4.31 a) Standard Ethyl lactate Peak at 4.03 min.
Fig b) Sample: Ethyl lactate Peak at 4.15 min at a conc. of 12.42 g/L and productivity at 0.69 g/g

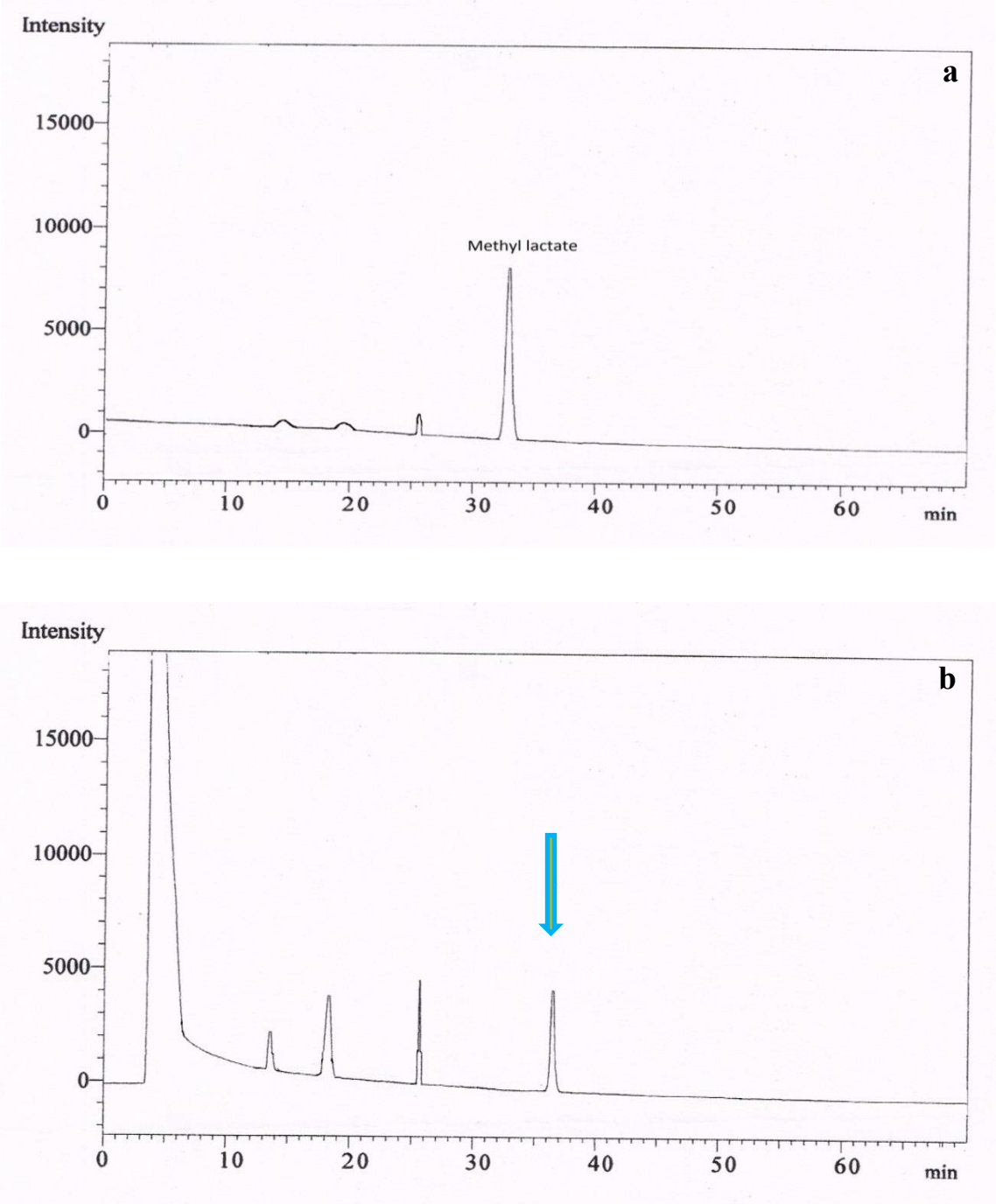


Fig 4.32a) Standard Methyl lactate Peak at 30.3 min.
b) Sample: Methyl lactate Peak at 30.6 min at a conc. of 13.69 g/L. and productivity at 0.76g/g

The lactic acid esters, namely, ethyl lactate and methyl lactate with a yield of 0.69 g/g starch and 0.79 g/g starch were prepared. There are various modes of action of lactic acid/lactate in consumer healthcare products and this determines the analytical

requirements of the method, such as, for example, the use of methyl/ethyl lactate in formulations. Lactic acid obtained can be of any grade (equal to or less than 99%). However, L-lactic acid produced in this study was approximately 89% pure. Peaks were identified in the sample by comparing with pure standards chromatograms.

Economically, the commercially available esters of lactic acid are expensive *viz.* Rs. 231/- per gram ethyl lactate and Rs. 231/- per gram of methyl lactate (Sigma, MO, USA) whereas, the cost reduces to 10 times in ethyl lactate at Rs. 23.4/- per gram and 2.4 times in methyl lactate at Rs. 94.9/- per gram methyl lactate using the process developed.

Chapter 5

Conclusion

Conclusions

This study was carried out in order to utilize potato starch waste for the production of lactic acid. The first experimental steps were to evaluate the waste to ensure the availability of nutrients and trace elements needed to support the growth and consequently the production of lactic acid and comparison between free cell and immobilized cell fermentation and then the fabrication of a dialysis sac bioreactor for enhanced lactic acid production leading to the formation of lactic acid esters: ethyl and methyl lactate respectively. The salient findings are noted below:

- Two hundred and ten strains of lactic acid bacteria were isolated from 180 fermented food samples of pickled mango, pickled yam, pickled garlic–chilly, pickled chilly, potato mixed hukuti mass and bhaati-jaanr mixed hukuti mass samples. Out of total 210 isolates, 174 isolates were Gram-positive rods and 36 isolates were Gram-positive cocci which occurred singly, in pairs, or in short chains. These isolates were screened and selected for starch hydrolyzing potential based on qualitative and quantitative starch hydrolysis tests on MRS media with potato starch as carbon source.
- A total 63 bacterial isolates were able to utilize potato starch in MRS-Starch agar plate, incubated at 37ºC for 48 h and three of these isolates, namely LA 56, LA 57 and LA 60 utilized potato starch at a comparatively higher percentage (50%, 55% and 85% respectively) which were finally identified by morphological, biochemical and phenotypic methods. The complete 16S rRNA sequence analysis revealed that the bacterial isolates had 97% to 99% similarity with the sequences of NCBI database. The isolate LA 56, isolated from potato leaves mixed hukuti mass, was identified as *Lactococcus lactis* strain HKT (GenBank accession number JN620210). On the basis of

phylogenetic data, isolate LA 57, isolated from Bhaati-jaanr mixed hukuti mass was identified as *Lactococcus lactis* strain HKBT-9 (GenBank accession number JN620216). Thus, based on morphological, physiological, biochemical properties and 16S rDNA sequence results, the isolated strain LA-60 was assigned as *Lactococcus lactis.*

- Due to highest starch hydrolyzing ability among all the isolates, LA 60 (*Lactococcus lactis*) was selected in the present study for the production of lactic acid from potato starch waste from agro industries. The biochemical mechanism behind starch hydrolysis primarily involved was α-amylase by *Lactococcus lactis* which was extracellular, inducible of starch since. The stability of the enzyme was checked by investigating the effect of pH (4-9) and temperature (25-75ºC) on α-amylase and maximum activity (2.54 U/mL) was observed at pH and temperature of 5.5 and 55ºC respectively.

- The growth kinetics of *Lactococcus lactis* with MRS medium and PSW depicted that no significant difference in both in the terms of specific growth rate and highest cell density which accounts for 0.0268 h^{-1} and 3.5 x 10^6 CFU/mL in MRS Starch medium respectively and 0.0216 h^{-1} and 3.8 x 10^6 CFU/mL respectively was observed. These results indicate the selected isolate to be a potential starch hydrolyzing strain.

- In the present study, different probiotic characteristics such as cell adhesion to solvents, gastrointestinal (pH 2.0) and bile salt (0.05%–0.3%) tolerance, antibiotic resistance and antimicrobial capacity of the isolated strains were determined to evaluate the probiotic potential of the strain. The results showed viable *L. lactis* cell count of 1.5 x 10^5 (CFU/ml) at 0 hours and 1.59x10^{10} (CFU/ml) at 24 h, being able to tolerate phenol, which indicate that they may reach the site of action, the small intestines unharmed. Because of

their metabolic properties, they would probably contribute to the reduction of cholesterol levels due to the presence of Bsh activity. In addition, it may also contribute to alleviation of lactose intolerance because of their β-galactosidase activity. These results indicate that there were no losses of viability of cell in simulated gastrointestinal condition. Overall, the above results emphasize the potential of this strain as a functional starter in probiotic formulations especially containing starch.

- The isolate was further analyzed for lactic acid production by optimizing the cultural conditions in minimal media and modified MRS media. Various cultural conditions including initial inoculum size, potato starch concentration, nitrogen source, initial pH of medium and incubation temperature were optimized for obtaining maximum lactic acid. The results indicated the optimum conditions of pH 5.0, temperature 30°C, potato starch concentration 20 g/L, with 1% inoculum (approximately 3.0 x 10^4CFU/mL), yeast extract as nitrogen source 0.5% at 48 h of incubation that yielded lactic acid 0.4 g/g and 0.5 g/g starch in minimal and modified MRS media respectively. Statistical optimization by Response surface methodology (RSM) increased the lactic acid production to 1.5 fold with a combination of pH 5.5, temperature 30°C with potato starch concentration at 20 g/L at 48 hrs.
- Using the optimized conditions, when PSW was inoculated with *Lactococcus lactis* in shake flask for 48 h, lactic acid was produced maximally at 15.6 g/L with a productivity and yield of 0.325g/L/h and 0.78 g/g of starch respectively. The acid was recovered from the fermentation broth and was administered to TLC and HPLC to confirm the purity and yield of the acid. The recovered acid was confirmed as L-lactic acid by HPLC and L-lactic acid and D-Lactic acid kits.

- The possibility of enhanced production of lactic acid was further investigated by techniques like immobilization with alginate or agar. Alginate was shown to be efficient in increasing lactic acid from 15.2g/L to 17.2 g/L with immobilized cells in PSW. Even though cell immobilization results in increased productivity compared to free cells, the viable industrial processes using immobilized cells have not been achieved due to stability purpose of immobilized beads. Therefore, a robust process was desired that can sustain in industries under environmentally harsh conditions which led to the development of a dialysis sac bioreactor.
- In dialysis sac bioreactor, final lactic acid concentration achieved after one cycle of 24 h was 18.9 g/L where 20 g/L starch was completely used. In fed batch fermentation, when PSWe was added after each cycle, the cells remained viable for 2 cycles and the yield of lactic acid at the end of the fermentation reaches to 1.48 g/g starch in comparison to shake flask fermentation at 1.2 g/g starch with cell viability upto one cycle i.e. for 48 hrs.
- As a last objective of this study, two esters of lactic acid, namely, methyl lactate and ethyl lactate have been produced by uncatalyzed esterification of lactic acid with alcohols and identification of both the esters was carried out by GC. The lactic acid esters, namely, ethyl lactate and methyl lactate with a yield of 0.69 g/g starch and 0.79 g/g starch were prepared in this study. Lactic acid and their esters derivatives are extensively used in food industry for preservation and flavoring purposes, as well as in the pharmaceutical and cosmetic industries.
- Economically, the commercially available esters of lactic acid are expensive *viz.* Rs. 231/- per gram ethyl lactate and Rs. 231/- per gram of methyl lactate (Sigma, MO, USA)

whereas, the cost reduces to 10 times in ethyl lactate at Rs. 23.4/- per gram and 2.4 times in methyl lactate at Rs. 94.9/- per gram methyl lactate using the process developed.

- Overall the results conclusively suggest a one-step microbially designed process for optically pure L-lactic acid and its derivatives production. A comparison of economies vis a vis similar or related process as well as commercially available products indicate this process to be prospective.

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APPENDIX

MEDIUM COMPOSITION

All culture media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

De Man, Rogosa and Sharpe broth (MRS broth)

Proteose peptone	10.00 g
Beef extract	8.00 g
Yeast extract	4.00 g
Tween 80 ((NH4)3C6H5O7)	1.00 g
tri-Ammonium citrate (CH3COONa.3H2O)	2.00 g
Sodium acetate trihydrate	5.00 g
MgSO4.7H2O	0.20 g
MnSO4.H2O	0.05 g
K2HPO4	2.00 g
Dextose	20.00 g
Distilled water added and brought up totalvolume to	1,000 ml
Final pH 6.2 ± 0.2 at 25°C	

De Man, Rogosa and Sharpe agar (MRS agar)

MRS medium was purchased from Himedia (Hi-Media Laboratories Pvt Ltd, India) and added with agar (15 g/l).

Modified MRS media

Modified MRS media contains potato starch at a concentration of 20 g/L instead of Dextrose in MRS medium

Composition of Minimal medium

Starch	2.0g
KCl	0.5g
$NaNO_3$	3.0g
$MgSO_4.7H_2O$	0.5g
$CaCl_2$	0.1g
KH_2PO_4	1.0g
$FeSO_4.7H_2O$	0.01g
Distilled water 1000ml	
pH 7.2	

Buffers and Solutions

1. TBE buffer (10X)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

2. Citrate buffer (0.1M), pH 5.5

Citric acid	(0.2 M) 116.25 mL

Sodium citrate	(0.2 M) 383.75 mL
Diluted to 1 L with distilled water and store 2°C-8°C	
3. 0.1M Phosphate buffer	
Monobasic sodium phosphate	(1M) 61.5 mL
Dibasic sodium phosphate	(1M) 38.5 mL
Dilute to 1 L with distilled water	
4. Agarose gel loading dye (6X)	
Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water 30.0%	

*Sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 min.

5. Ethidium Bromide	0.5µg mL-1
6. SSC 20X	
NaCl	3M
Sodium citrate	0.3 M (pH 7)
7. Wash buffer I	
SSC	2X
SDS	0.1%
8. Wash buffer II	
SSC	1X
SDS	0.1%
9. TE buffer 10X	
Tris-HCl	0.1 M (pH 8)
Na2EDTA	10 M (pH 8)
10. 4x Separating gel buffer (1.5M Tris, pH 8.8)	
Tris	3g
Distilled water	100ml
11. 4x Stacking gel buffer (0.5M Tris, pH 6.8)	
Tris	18.15g
Distilled water	100ml
12. 2x Sample buffer	
Tris	(0.5M, pH 6.8) 2.5ml
SDS	(10%) 4.0ml
Glycerol	(100%) 2.0ml
β-mercaptoethanol	0.8ml
Bromophenol blue	(0.1%) 300µl
Distilled water	(400µl) to 10ml
13. Tank buffer	
Tris	6.05g
Glycine	28.80g
SDS	1.0g
Distilled water	1000ml
14. Genomic DNA Extraction buffer	
Sodium acetate	100 mM
Na2EDTA	50 mM
NaCl	500 mM
SDS	1%
15. STET Buffer	
Sucrose	8.0% (w/v)

TritonX	100 - 0.5% (w/v)
EDTA	50.0 mM (pH 8.0)
Tris HCl	10.0 mM (pH 8.0)

16. E-buffer

Tris acetate	50mM (pH 8.0)

17. Lysis buffer

SDS	3%
Tris	50mM

pH was adjusted to 12.0 using 1M NaOH7

2. Reagents

Iodine solution (Gram's iodine)

Iodine	1.00 g
Potassium iodide	2.00 g
Distilled water added and brought up total volume to	300 ml

Tetramethyl-p-phenylenediamine dihydrochloride (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1.00 g
Distilled water added and brought up total volume to	100 ml

1. Phenol Sulfuric acid solution

Phenol	5%
Sulfuric acid (reagent grade)	96%
Sugar standards (reagent grade)	1 mg mL-1

2. Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na2EDTA	10mM

3. Plasmid extraction solution II

NaOH	5M
SDS	10%

4. Plasmid extraction solution III

K-acetate	5.0 M (pH 4.5)

5. Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

6. IPTG stock solution (0.1M)

IPTG	1.2 g

Add sterile distilled water to 50 ml final volume.
Filter sterilize and store at 4°C

7. X-Gal 2ml

100 mg 5-bromo-4-chloro-3-indolyl-Dgalactoside dissolve in 2ml N,N´-dimethylformamide. Cover with aluminum foil and store at 20°C

8. Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na2EDTA	10mM

9. Ribonuclease A

Stock solution	10mg/ mL

Working solution	10-15 g/mL

Ribonuclease A is prepared in a buffer containing 100mM Tris (pH 8.0) and 15 mM NaCl. To prepare DNAse free RNAse, the solution is boiled for 10 minutes, followed by the slow cooling, then dispensed into aliquots and is stored at -20oC for further use.

10. Lysozyme

Stock solution	10mg/mL
Working solution	300 - 400 mg/mL

Lysozyme was prepared freshly is water.

11. Acrylamide-bisacrylamide (30%)

Acrylamide	29.2g
Bisacrylamide 0.8g	
Distilled H2O	100ml

12. SDS (10%)

SDS	10g
Distilled water	100ml

13. Ammonium persulfate (10%)

Ammonium persulfate	0.1g
Distilled water	1.0ml

14. Bromophenol blue (0.1%)

Bromophenol blue	5mg
Distilled water	5ml

15. Staining solution

Coomassie blue (R-250)	0.3g
Methanol (AR)	80ml
Glacial acetic acid	20ml
Distilled water	100ml

16. Destaining solution

Acetic acid	100ml
Methanol	300ml
Distilled water to 1L	

17. Gel storing solution

Acetic acid	15ml
Distilled water to	200ml

18. Bromophenol blue 0.2% (w/v)

For β-amylase

Reagents

- 0.016 M Sodium acetate, pH 4.8
- 2 N Sodium hydroxide
- Dinitrosalicylic acid color reagent. Prepare by dissolving 1.0 gm of 3,5-dinitrosalicylic acid in 50 ml of reagent grade water. Add slowly 30.0 gms sodium potassium tartrate tetrahydrate. Add 20 ml of 2 N NaOH. Dilute to a final volume of 100 ml with reagent grade water. Protect from carbon dioxide and store no longer than 2 weeks.
- 1% Starch: Prepare by dissolving 1.0 gram of soluble starch (Merck) in 100 ml of 0.016 M sodium acetate buffer pH 4.8. Bring to a gentle boil to dissolve. Cool and, if necessary, dilute to 100 ml with reagent grade water. Incubate at 25°C for 4-5 minutes prior to assay.
- Maltose stock solution, 5 micromoles/ml. Prepare by dissolving 180 mg maltose (MW 360.3) in 100 ml reagent grade water. Incubate at 25°C for 4-5 minutes prior to assay.

Enzyme

Dilute to a concentraton of 1-10 micrograms per ml. A minimum of three different concentrations in this range should be run.

Using the maltose stock solution prepare a maltose standard curve as follows: In numbered tubes, prepare 10 maltose dilutions ranging from 0.3 to 5 micromoles per ml. Include two blank tubes with reagent grade water only

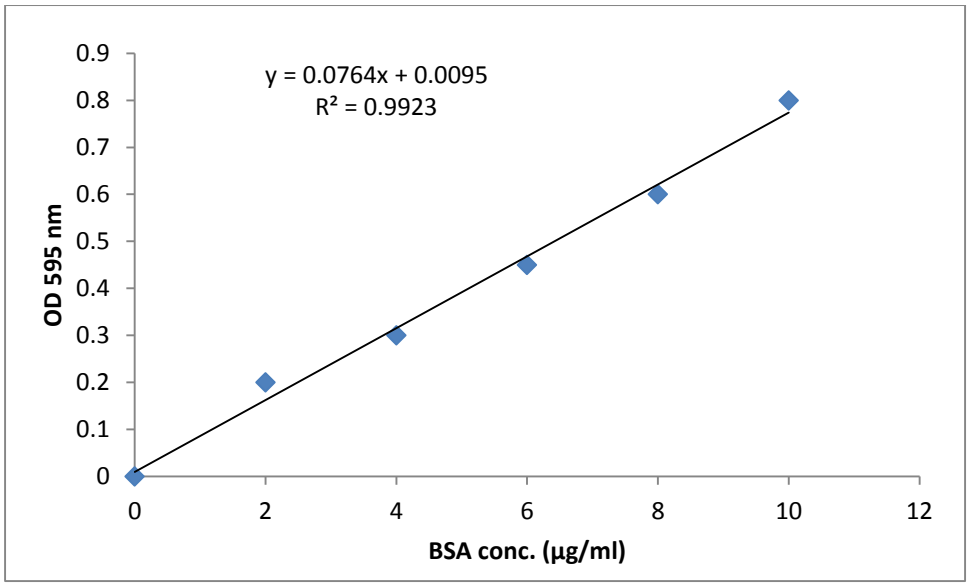
Bradford Protein Assay Reagents

Dye stock - Coomassie Blue G (C.I.# 42655) (100 mg) is dissolved in 50 mL of methanol. The solution is added to 100 mL of 85% H3PO4, and diluted to 200 mL with water. The solution should be dark red, and have a pH of -0.01. The final reagent concentrations are 0.5 mg/mL Coomassie Blue G, 25% methanol, and 42.5% H3PO4. The solution is stable indefinitely in a dark bottle at 4°C.

Assay reagent - The assay reagent is prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H2O. The solution should appear brown, and have a pH of 1.1. It is stable for weeks in a dark bottle at 4°C.

Protein Standards - Protein standards should be prepared in the same buffer as the samples to be assayed. A convenient standard curve can be made using bovine serum albumin (BSA) with concentrations of 0, 250, 500, 1000, 1500, 2000 µg/mL for the standard assay, and 0, 10, 20, 30, 40, 50 µg/mL for the microassay

Fig. 1 Standard curve of protein assay. Relationship between protein (µg/mL bovine serum albumin) and absorbance using Bradford assay.



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Use of *Lactococcus lactis* to enrich sourdough bread with γ-aminobutyric acid

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Abstract
Fried sourdough bread (bhatura) with an elevated amount of γ-aminobutyric acid (GABA) was produced using lactic acid bacteria (LAB). The LAB starter was screened and isolated from pickled yam showing highest GABA content and was identified as *Lactococcus lactis* subsp. *lactis*. The maximum GABA production in de Man Rogosa Sharpe (MRS) media supplemented with monosodium glutamate (MSG) was 110 mg/100 ml at pH 5, and 1–3% NaCl did not change the production of GABA significantly ($p > 0.05$). When MSG was replaced with *Vigna mungo* in sourdough, the amount of GABA for bhatura was 226.22 mg/100 g representing about 10-fold increase. A sensory evaluation resulted as the overall general acceptability of bhatura to be 4.91 ± 0.03 on a five-point hedonic scale. Thus, the results indicated the potential of *L. lactis* as a LAB starter for the production of GABA-enriched bhatura. Although other physiological effects can be expected in the product, animal and clinical studies are mandatory prior to application of this food.

Keywords: *γ-aminobutyric acid, sourdough, fried bread, lactic acid bacteria,* Lactococcus lactis, *bhatura*

Introduction

Sourdoughs are considered extremely complex ecosystems in which lactic acid bacteria (LAB) and yeasts represent the prevailing microflora (Corsetti et al. 1996). In this context, besides particular adaptative responses to the conditions prevailing during sourdough fermentation (Gobbetti and Corsetti 1997), the production of nutraceuticals by sourdough LAB could be particularly advantageous (Hammes and Ganzle 1997). Bhatura or puffed fried bread is a popular traditional sourdough bread commonly served as a midday meal or as breakfast in northern and eastern parts of the Indian subcontinent. γ-Aminobutyric acid (GABA), a non-protein amino acid, is primarily produced from irreversible α-decarboxylation of L-glutamic acid, catalysed by glutamic acid decarboxylase (GAD), which has been found in bacteria, plants and animals (Ueno 2000). GABA has multiple physiological functions such as tranquilizing effect, diurectic and hypotensive activity and as being an inhibitory neurotransmitter in sympathetic brain functions (Su et al. 2003; Komatsuzaki et al. 2005, 2007; Huang et al. 2007).

Earlier reports suggest that GABA-enriched foods have been effective in the regulation of sleeplessness, depression and autonomic disorders (Okada et al. 2000). To contribute to the development of traditional fermented food products with enhanced functional properties, this study aimed to screen GABA-producing microorganisms and to employ them for the consumption as a cheap source of this high value nutraceutical, i.e. GABA. In this study, LAB were isolated from commonly consumed pickle products, and the GABA-producing ability of the isolates was evaluated. Bhatura was prepared traditionally with minor modifications, i.e. a combination of wheat flour and *Vigna mungo* (added additionally for glutamate content) in the place of wheat flour (used in traditional recipe) was used. GABA directly from bhatura was desired because consuming foods with high GABA content as part of a customary diet may have the advantages of providing a regular GABA intake.

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Materials and methods

Microorganisms

Six commonly consumed pickles, i.e. mango, yam, mushroom, garlic, carrot and chilly, were screened for the presence of microorganisms with GABA-producing ability on de Man Rogosa Sharpe (MRS) containing either L-glutamic acid (50 mM glutamic acid) or monosodium glutamate (MSG). The isolate was identified by its colony morphology, Gram-staining and biochemical tests, as well as by 16S rRNA gene sequencing (Weisburg et al. 1991). Genomic DNA of the GABA-producing strain was isolated by lysozyme–proteinase K procedure (Smith et al. 1981).

GABA analysis

GABA in the supernatant was presumptively detected with some modifications by using thin-layer chromatography (TLC) as described by Cho et al. (2007), spectrophotometrically by the method of Zhang and Bown (1997) and confirmed by HPLC according to Rossetti and Lombard (1996) with minor modifications.

Optimization of culture conditions for GABA production

Effect of temperature, incubation time and initial pH and NaCl concentration on GABA production was studied. The isolated strain was cultivated in glutamic acid-supplemented MRS broth at temperatures (28–45°C) for 0–72 h at initial pH adjusted at 4–8 and NaCl concentration levels of 1%, 2% and 3% (w/w) for the results (Cho et al. 2007).

Production of fried sourdough bread 'Bhatura'

Bhatura was prepared using a traditional recipe (Singh 2004) with minor modifications in the composition. *V. mungo* flour (10 g) was added as a source of glutamate (approx. 0.4 g) to wheat flour (10 g).

Determination of GABA content in Bhatura

For estimating GABA content in the product, bhatura was extracted as per Zhao et al. (2011) and was analysed as suggested by Zhang and Bown (1997). Cell count followed by sensory evaluation was done using statistical software SAS version 89 (SAS Institute, Cary, NC, USA). The sensory quality of bhatura was evaluated by a panel of 10 trained judges by grading for sensory analysis and overall acceptability. The scores were given on a five-point hedonic scale where 1 represents worst and 5 represents best.

Results

Isolation and identification of high GABA-producing LAB and GABA content of pickle samples

LAB were isolated from *yam pickle* samples that showed the highest GABA content (99.0 mg/100 g of product) among the six different pickle products among those evaluated, the results of which are shown in Table I. The white colonies of LAB strains that were cultivated on MRS medium containing bromophenol blue were isolated, and their GABA-producing abilities were evaluated by TLC (data not shown). One strain with highest GABA-producing activity was subsequently identified by 16S rDNA sequencing analysis, after which the GABA-producing ability of the isolated strain was evaluated (Table II). The strain was identified as *Lactococcus lactis* subsp. *lactis* (Accession No. JN618456).

Table I. GABA content and pH value of six different pickle products.

Pickle types	Sample (*n*)	pH*	GABA content range (mg/100 g)
Yam	6	4.3 ± 0.5	79.5–99.0
Mango	8	3.6 ± 0.2	18.2–24.0
Mushroom	3	2.4 ± 0.5	15.7–28.8
Garlic	5	2.9 ± 0.1	19.9–27.8
Carrot	2	3.3 ± 0.3	17.1–19.5
Chilly	7	4.1 ± 0.4	19.2–26.9

Notes: Data are expressed as mean from three independent experiments; * Average of three mean values (SD).

Optimization of culture conditions for GABA production

The optimal temperature for GABA production was found to be 30°C at 48 h. High GABA production was obtained at the same temperature when MRS media supplemented with 5% MSG were used. GABA production rapidly decreased at temperatures 28 and 37°C (Figure 1), and there was negligible production

Table II. GABA production and sensory scores of control bhatura and bhatura prepared with *L. lactis* inoculated and fermented sourdough.

	Product*	
Attributes	Control†	*L. lactis* inoculated sourdough†
GABA production (mg/100 g)	0.13 (0.02)	226.22 (0.03)
Mouth feel	4.31 (0.05)	4.52 (0.05)
Taste	4.23 (0.03)	4.71 (0.02)
Sweetness	4.32 (0.03)	4.43 (0.03)
Saltiness	3.95 (0.03)	4.24 (0.05)
Off flavour	2.12 (0.03)	2.03 (0.02)
Consistency	4.61 (0.04)	4.71 (0.03)
Texture	4.03 (0.02)	4.24 (0.03)
Colour and appearance	4.41 (0.04)	4.51 (0.05)
General acceptability	4.80 (0.04)	4.91 (0.03)

Notes: Data are expressed as mean ± SD from three independent experiments; * scores were assigned numerical values from 1 (extremely weak) to 5 (extremely strong); values with different letters in the same column differ significantly ($p < 0.05$); † average of three mean values (SD); figures in parentheses represent standard deviation.

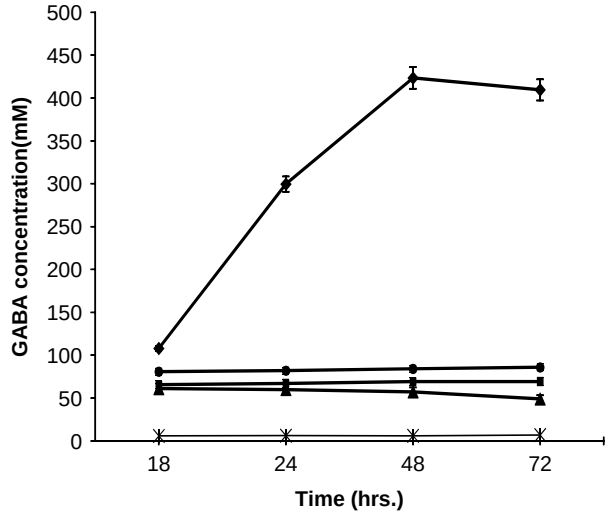

Figure 1. Effect of different temperatures: 30°C (◆), 34°C (■), 37°C(▲), 28°C (×), 48°C (+) and time on GABA production by *L. lactis*. *Values are mean of three replicates.

at 45°C. GABA biosynthesis in LAB is reported to be strictly pH regulated (Komatsuzaki et al. 2005), which has shown to have a significant effect on the production of GABA. A highest amount of GABA was obtained at pH 5.0 (Figure 2) that accorded with the previous reports about the optimal pH values for maintaining the activity of LAB, which were in the range of 4.0–5.0 (Cho et al. 2007). It has been suggested that a higher or lower pH may lead to partial loss of GAD activity and thus decrease the GABA production. The overall maximum GABA production in media by *L. lactis* obtained with optimal conditions was approximately 110 mg/100 ml. Thus, considering the average pH condition of sourdough which is around 4.2 (Messens et al. 2002) after 4 h of fermentation and prior to being processed, bhatura sourdough may have great potential for use as a GABA supplier. When varied concentration of NaCl (1–3%) was evaluated for GABA production by *L. lactis* to obtain the most appropriate salt conditions, it was found that the concentration of NaCl for up to 3% in the culture media did not have a significant ($p > 0.05$) effect on the amount of GABA produced.

GABA content and sensory properties of bhatura prepared from L. lactis-inoculated sourdough

GABA concentration in the bhatura prepared by *L. lactis* inoculated sourdough was 226.2 mg/100 g following 4 h of fermentation. The sensorial analysis resulted as the overall general acceptability of bhatura on a five-point hedonic scale to be 4.19 ± 0.03 (Table II). The cell count of *L. lactis* in the bhatura was negligible when compared with that in the sourdough (Figure 3). This can be supported by the fact that heating at high temperature (180°C) kills the bacteria.

Discussion

In summary, GABA-producing LAB strains that had different physiological characteristics, such as growth rate and acid-producing ability, were screened for use as starters of fermented food. As stated by Leroy and de Vuyst (2004), LAB contribute to the microbial safety or offer one or more organoleptic, technological, nutritional or health advantages, for example, some LAB produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins or

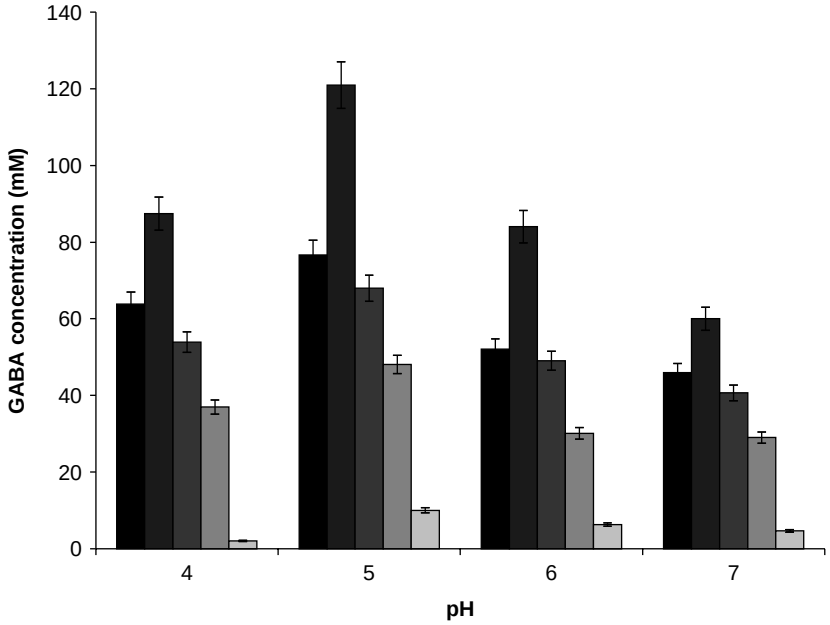

Figure 2. Effect of different temperatures: 28°C (■), 30°C (■) 34°C (■), 37°C(■), 48°C (■) and pH on GABA production by *L. lactis*. *Values are mean of three replicates.

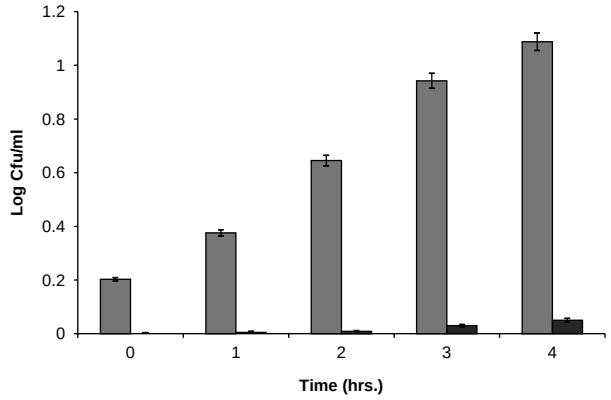

Figure 3. Cell count of *L. lactis* in bhatura sourdough fermented for 4 h (■) and bhatura (■). *Values are mean of three replicates.

useful enzymes, or that have probiotic properties. Acid-producing ability might be an important factor and it does not decrease in processing the food quality and taste of fermented foods. To develop fermented foods containing GABA with good taste, LAB strains with suitable acid and flavour production profiles should be chosen. Because of the high GABA-producing ability, *L. lactis* has great potential for use as a starter in the production of GABA-containing functional fermented foods.

There have been several reports on GABA production from many food products, for instance, Malaysian brown rice ((10.1–15.2 mg/g) Roohinejad et al. 2009), yoghurt ((424.67 μg/g) Park and Oh 2007), cheese ((177.0 mg/g) Nomura et al. 1998), Gaba tea ((>150 mg/100 g) Zhao et al. 2011) and kimchi ((251 mM) Cho et al. 2007) with LAB such as *Lactobacillus acidophilus*, *Lactobacillus buchneri*, *Lactobacillus delbrueckii* and *Streptomyces cinereus*. In this study, we utilized LAB and legume for preparing bhatura, a traditional fermented food with enhanced GABA levels. To the best of our knowledge, this is the first report in which high GABA (226.2 mg/100 g) production by *L. lactis* was achieved in a fermented food product.

Bhatura is a popular, economical traditional Indian food commonly eaten with curd or pickle or vegetables and is normally prepared from wheat flour fermented with a curd as inoculum. The principal LAB culture in sourdough has been identified as *L. lactis*. However, GABA production by LAB cannot be initiated in absence of L-glutamate in wheat flour, therefore, legume (*Vigna mungo*) addition was proposed which contains high levels of L-glutamate. This enabled the elimination of MSG from bhatura and provided moderate amount of protein (approx. 8.8 g/serving) for functional and nutritive properties. The *L. lactis* strain possessed excellent survival characteristics in the dough and produced high levels of GABA in bhatura.

Conclusion

In conclusion, this study is the first report in which high GABA production by *L. lactis* was achieved in a fermented food product, bhatura. The *L. lactis* strain possessed excellent survival characteristics in the sourdough and produced high levels of GABA. Furthermore, no differences in sensory properties of bhatura prepared from *L. lactis*-fermented sourdough and traditionally prepared bhatura were observed. These findings may contribute to enhancing the health benefits and increasing the commercial value of the traditional fermented food product, bhatura. Thus, the results indicated the potential of *L. lactis* as a LAB starter for the production of GABA-enriched bhatura.

Declarations of interest

The financial assistance from University Grants Commission, Govt. of India, New Delhi, for carrying out this work is gratefully acknowledged. We the authors state that the strain used and the work submitted in this journal is solely ours. The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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RESEARCH ARTICLE

Probiotic characterization of potential hydrolases producing *Lactococcus lactis* subsp. *lactis* isolated from pickled yam

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Abstract

The aim of this study was to characterize potential probiotic strain co-producing α-amylase and β-galactosidase. Sixty-three strains, isolated from pickle samples were screened for their hydrolase producing capacity by utilizing different starches as carbon source. One out of 63 strains, isolated from traditionally fermented pickled yam showing maximum hydrolase activity (α-amylase (36.9 U/ml) and β-galactosidase (42.6 U/ml)) within a period of 48 hours was identified as *Lactococcus lactis* subsp. *lactis*. Further, it was assessed for the probiotic characteristics under gastrointestinal conditions like acidic, alkaline, proteolytic enzymes, bile stress and found to exhibit tolerance to these stresses. The therapeutic potential of the isolate is implicated because of its antagonistic effect against enteric foodborne pathogens (*Salmonella typhimurium, Escherichia coli* 0157:H7, *Staphylococcus aureus, Yersinia enterocolitica* and *Aeromonas hydrophila*). The results of this study entail a potential applicability of the isolate in developing future probiotic foods besides the production of industrially significant hydrolases.

Keywords

α-amylase, β-galactosidase, co-production, hydrolase, *Lactococcus lactis*, pickled yam, probiotic

History

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Introduction

Traditional fermented foods prepared from the most common types of vegetables (such as yam, cabbage, cauliflower, turnip) are well known in many parts of the world. Some are utilized as colorants, spices, beverages and breakfast or light meal foods, while a few of them are used as main foods in the diet (Marshall & Danilo, 2011; Tamang, 2010). Pickled fruits, vegetable shoots as well as beverages prepared from cereals and edible flowers by fermentation generally form an essential constituent of the Indian population diets. These foods in the diet beyond meeting nutritional needs may modulate various physiological functions and play detrimental or beneficial roles in some diseases because of microorganisms associated with them (Kumar et al., 2012). Currently there are hundreds of traditionally fermented foods with different base materials and preparation technologies but only limited knowledge has been obtained regarding the microbiota associated with these products (Jeyaram et al., 2009).

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria that play a key role in the production of fermented foods and beverages with high relevance for human and animal health. These bacteria have also been recently exploited for other beneficial attributes like probiotic characteristics, bacteriocin and hydrolases production (Ruethaiwan et al., 2012).

The essential characteristics for LAB to be used as probiotics during manufacturing include the following: (i) recognition as safe (GRAS; generally recognized as safe); (ii) viability during processing and storage; (iii) antagonistic effect against pathogens; (iv) tolerance to bile acid challenge; and (v) adherence to the intestinal epithelium of the host among others (Begley et al., 2005; Lin et al., 2006; MacFarlane & Cummings, 2002; Vesterlund et al., 2005). It is generally considered that minimum numbers required for a probiotic to provide a health benefit are 10^7 CFU/ml (Jayamanne & Adams, 2006; Ross et al., 2005).

Many different microorganisms have been employed for the production of industrially important enzymes over decades. Among the group of hydrolases, two enzymes namely α-amylase and β-galactosidase are significantly important enzymes with numerous industrial applications (Konsoula & Liakopoulou-Kyriakides, 2007). α-Amylase, which catalyzes the hydrolysis of starch to low molecular weight products, is produced by a wide variety of microorganisms, but for commercial applications α-amylase is mainly derived from the microorganisms belonging to genus *Bacillus* (Pandey & Nigam, 2000; Violet & Meunier, 1989). For instance, α-amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus* and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries (Machius & Wiegand, 1995; Pandey & Nigam, 2000). β-Galactosidase (or lactase) which hydrolyzes the milk sugar, lactose, to its components glucose and galactose, is used to treat lactose intolerant patients, prevent lactose crystallization in frozen and condensed milk products, reduce water pollution caused by whey and also to increase the sweetening properties of lactose (Furlan & Schneider, 2000; Patel & Mackenzie, 1985). In view of the advantages offered by the application of LAB to different food substrates, there is always a requirement to isolate a potential strain(s) which can be industrially important and can impart health benefits to consumers.

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In previous studies, the co-production of α-amylase and β-galactosidase from *Bacillus subtilis* and its employment for the hydrolysis of various organic starches (Konsoula & Liakopoulou-Kyriakides, 2007) have been reported. This paper describes the screening of various isolates from pickled vegetables for co-production of α-amylase and β-galactosidase and further elucidates probiotic characteristics of the isolates for use as a potential probiotic strain and in other industrially important applications. The selected probiotic LAB was identified by 16S rDNA sequencing.

Materials and methods

Isolation of bacteria

Lactic acid bacterial strains were isolated from traditionally fermented pickles procured from local markets of Punjab and Orissa, India. All samples were collected in presterile poly-bags and screw capped bottles, kept in an icebox and transported to the laboratory for analysis. Samples were plated onto MRS agar to enumerate bacteria and predominant colonies were isolated. Plates were incubated for 48 hours at 37 °C.

Screening of hydrolase producing lactic acid bacteria

Two-stage enzymatic screening was done for α-amylase and β-galactosidase producing strains. All isolates were subjected to primary screening, while secondary screening was performed for the bacterial isolates showing enzymatic activities during the primary screening.

Primary screening

The starch utilizing strain was checked on modified MRS medium with RBB-soluble starch as the C-source and incubated at 37 °C for 24 hours. The starch utilization was monitored by the disappearance of the blue color of the medium based on amylase production. The screened isolates were further monitored for its β-galactosidase producing potential on modified MRS agar with 30 µg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and 1% soluble starch. The production of β-galactosidase was observed after incubating the LAB inoculated MRS agar containing X-gal plate for 24 hours at 37 °C.

Secondary screening

The screened isolates were further screened for maximum hydrolase producing potential on modified MRS agar having 1% variable starch sources, i.e. maize, potato, tapioca, fox nut and soluble starch. The enzymatic activity were determined (extracellulary and intracellularly) as explained by Giraud et al. (1993) and Karasova et al. (2002) for α-amylase and β-galactosidase, respectively.

Enzyme isolation

Cells were removed from the modified MRS medium containing 1% variable starch source (maize, potato, tapioca, fox nut and soluble starch) by centrifugation at $4500 \times g$ for 20 min at 4 °C. The cell pellet was washed with phosphate buffer saline (PBS, pH 7.0) twice and resuspended in PBS till further use. The cell-free culture supernatant was precipitated with $(NH_4)_2SO_4$ (80% saturation) followed by centrifugation (at $4500 \times g$ for 45 min). The precipitate was collected and resuspended in 0.1 M sodium phosphate buffer (pH 7.0) to obtain partially purified crude enzyme preparation. Protein was estimated according to the method of Bradford (1976) using crystalline bovine serum albumin as standard.

Enzyme activity assay

The characterization of the α-amylase producing strain was performed according to the methods of Giraud et al. (1993). The reduction in the color of the blue-colored starch–iodine complex was determined and the release of reducing sugar equivalents was estimated by the method of Miller (1959). Later, the isolates were subjected to the method of Miller (1998) to identify the strains producing β-galactosidase.

Phenotypic characterization

One isolate capable of hydrolyzing starch maximally was identified by its colony morphology, gram-staining and catalase test. The carbohydrate fermentation profile of the isolates was carried out by using API 50 CH strips and API 50 CHL medium according to the manufacturer's instruction (API System; BioMerieux, Marcy I'Etoile, France). Sediment from centrifuged culture broth was used to prepare the suspension at 10^5 CFU/ml. Following inoculation, cultures were incubated for 4 hours at 37 °C.

Genotypic characterization

Genomic DNA of the strain was isolated by using QIAamp DNA Mini kit as per the manufacturer's instructions (Qiagen, Valencia, CA). Bacteria-specific universal primers used for amplification of 16S rRNA gene were the forward primer 27F (5′-AGAGTTTGATCATGGCTC-3′) and the reverse primer 1327R (5′-CTAGCGATTCCGACTTCA-3′) (Weisburg et al., 1991). The 16S rRNA gene was amplified in 35 cycles with a Gene Amp PCR System 2400 (Perkin Elmer, Waltham, MA). The thermal program consisted of one cycle at 94 °C for 4 min, 35 cycles at 94 °C for 40 s, 46 °C for 40 s, 72 °C for 2 min, final one cycle of 72 °C for 15 s and stored at 4 °C. A 100-bp DNA ladder was used as the molecular marker (Fermentas, Germany). PCR products were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA) and sequenced from both ends with an ABI3700 DNA sequencer (Applied Biosystems, Foster City, CA) using the same oligonucleotide primers used for PCR. The sequenced 16S rDNA sequences for the bacterial isolate were analyzed to detect the presence of possible chimeric artefacts and compared with the similar gene sequences using the NCBI BLAST (National Library of Medicine). The phylogenetic tree of the sequence so obtained was constructed using MEGA 4.0 software (Tamura et al., 2007).

Probiotic characterization

Tolerance of artificial gastric juice

The ability of selected isolate to survive under gastric conditions was examined according to Casey et al. (2004) with little modifications. Overnight culture was washed with phosphate-buffered saline (PBS, pH 7.0), resuspended in synthetic gastric juice (pH 1.85 adjusted using HCl) and incubated at 37 °C for 3 hours. The artificial gastric juice consisted of (g/l): 3.5 D-glucose 1.28 NaCl, 0.6 KH_2PO_4, 0.11 $CaCl_2$, 0.239 KCl, 0.2 Ox bile, 0.1 lysozyme and 0.013 pepsin. Samples were withdrawn at regular intervals of 2 hours, serially diluted in PBS and enumerated on MRS agar to enumerate the viable cells.

Microbial adhesion to solvents

Microbial adhesion to solvents (MATS) was measured according to the method originally proposed by Rosenberg et al. (1980) and recently modified by Bellon-Fontaine et al. (1996). Briefly, bacteria were harvested in the stationary phase by centrifugation at $5000 \times g$ for 10 min, followed by washing in PBS (pH 6.2) twice and resuspending in 0.1 M PBS (pH 6.2) to an optical

density of 0.4 at 600 nm (A_0) (approximately 10^8 CFU/ml cell density). Further, 0.2 ml of solvent was added to 1.2 ml of cell suspension. Following pre-incubation at room temperature for 10 min, the two-phase system was mixed on a vortex for 2 min. To allow complete phase separation of the mixture, the aqueous phase was removed after 15 min and its optical density at 600 nm (A_1) was measured. The percentage of microbial adhesion to solvent was calculated as

$$[1 - (A_1/A_0)] * 100.$$

n-Hexadecane (polar), Chloroform (a monopolar and acidic) and ethyl acetate (a monopolar and basic) were used as solvents in this study.

Resistance to 0.4% (v/v) phenol

The ability of selected LAB strain to grow in the presence of phenol was tested by inoculating cultures (1% of overnight culture) in MRS broth with and without 0.4% phenol. Serial dilutions were spread-plated (100 µl aliquots) onto MRS agar at time 0 and after 24 hours of incubation at 37 °C to enumerate surviving bacteria (Xanthopoulos et al., 1999).

Bile salt hydrolase activity

Bile salt hydrolase (Bsh) activity of the culture was detected using the plate screening procedure described by Du Toit et al. (1998). Briefly, overnight culture was spotted onto MRS agar plates containing 0.5% (w/v) sodium taurodeoxycholate (Sigma, St. Louis, MO) and 0.37 g/l $CaCl_2$. Colonies with precipitation zones were considered Bsh-positive. *Lactobacillus acidophilus* ATCC 43121 and *E. faecium* E 179 were used as Bsh-positive and Bsh-negative control strains, respectively.

Determination of antibiotic resistance

The selected strains were investigated for their antibiotic resistance profile using the E-test (Viva Diagnostika, Cologne, Germany) using MRS agar and anaerobic incubation conditions and following the manufacturer's instructions. The minimum inhibitory concentration (MIC) values used to determine whether strains were susceptible or resistant were those as suggested by Scientific Committee for Animal Nutrition (SCAN) (Chesson et al., 2002).

Determination of antimicrobial potential of probiotic strains

Screening for antagonistic activity: The agar spot test as described by Schillinger & Lucke (1987) was used for screening for antagonistic activity of the selected strains against a variety of indicator strains: *Staphylococcus aureus* ATCC 9144, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterolitica* MTCC 840, *Salmonella typhimurium* ATCC 19585 and *Escherichia coli 057:H7* ATCC 43895. The agar spot test method of (Uhlman et al., 1992) was further used to test the activity of cell-free neutralized supernatants. The supernatants were tested against the same indicator strains used above.

Production of H_2O_2: Overnight cultures (10 µl) were spotted onto ABTS (2-2′-azino-di-3-ethylbenzthiazoline-6-sulfonic acid)-agar plates as described by Kostinek et al. (2005). The plates were incubated anaerobically at 37 °C for 72 hours and then were exposed to the atmosphere and H_2O_2 producing strains were considered positive when a violet halo surrounded the colony appeared (Marshall, 1979).

Statistical analysis

All the experiments were performed in triplicate. Error bars on graphs show the standard deviation. The data were analyzed by the analysis of variance (ANOVA) and the means of enzyme activity were compared by Tukey's test using GraphPad Prism version 5.0 (GraphPad Prism Software, Inc., La Jolla, CA).

Results

Isolation and primary screening of hydrolase producing LAB

Of the 63 indigenously isolated bacterial strains screened for α-amylase and β-galactosidase production, 20 showed positive result. Bacterial strains showed the formation of white colored colonies on RBB starch agar medium indicating amylolytic activity whereas blue colur colonies on X-gal agar medium indicating β-galactosidase activity (data not shown). Out of 20 isolates, only six isolates namely LA-1, LA-2, LA-3, LA-4, LA-5 and LA-6 showed high β-galactosidase and amylolytic activity (Table 1) and were subjected to further screening using different variable starch sources.

Secondary screening

The co-production of α-amylase and β-galactosidase by screened isolates was investigated in order to elucidate the source (intracellular/extracellular) of enzyme production. Maximum α-amylase activity was detected in the supernatant in comparison to whole cells, the isolate suggesting the enzyme to be extracellular whereas in case of β-galactosidase, maximum activity was exhibited by cell-free extracts in comparison to supernatant signifying an intracellular location of β-galactosidase. The isolate LA-6 exhibiting maximum enzyme activity (Figure 1) was selected to investigate the effect of different organic substrates on the co-production of α-amylase and β-galactosidase.

Effect of various organic substrates on α-amylase and β-galactosidase co-production

Initially bacterial growth was carried out at 37 °C with agitation at 120 rpm and pH was maintained at 6.5. A modification of MRS medium was adopted with 20 g/l of starches (maize, potato, tapioca, fox nut and soluble starch) replacing glucose. Samples were withdrawn at regular intervals of 2 h and analyzed for enzyme activity. Figure 2 shows the production of α-amylase and β-galactosidase in the presence of various starches as carbon sources at 2% (w/v) concentration. Although all carbon sources supported good growth of the microorganism, soluble sugars were easily metabolized carbon sources giving relatively higher growth in comparison to starch.

Enzyme production

Maximum α-amylase activity was detected in the supernatant in comparison to whole cells (Figure 1), suggesting the enzyme to be extracellular whereas in case of β-galactosidase, maximum activity was exhibited by cell-free extracts in comparison to supernatant (Figure 1) signifying an intracellular location of β-galactosidase.

Table 1. Screening of lactic acid bacteria for α-amylase and β-galactosidase activity.

Bacteria	α-Amylase activity (U/ml)	β-Galactosidase activity (U/ml)
LA-1	22.1	21.7
LA-2	24.6	23.3
LA-3	19.4	20.5
LA-4	27.3	22.8
LA-5	19.9	27.1
LA-6	36.9	42.6

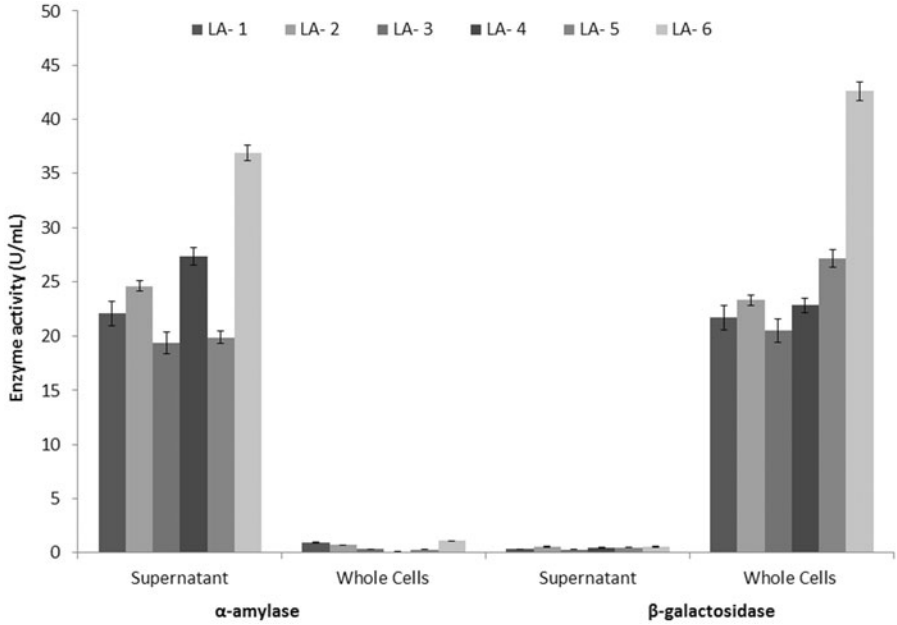

Figure 1. Comparison of both α-amylase and β-galactosidase activity of supernatant and whole cell of LA 1–6 culture isolates in MRS. Error bar denotes average of three replicates. The graph represents α-amylase and β-galactosidase activity in supernatant and whole cells of six culture isolates. It shows the maximum production of both the enzymes by one isolate (LA-6).

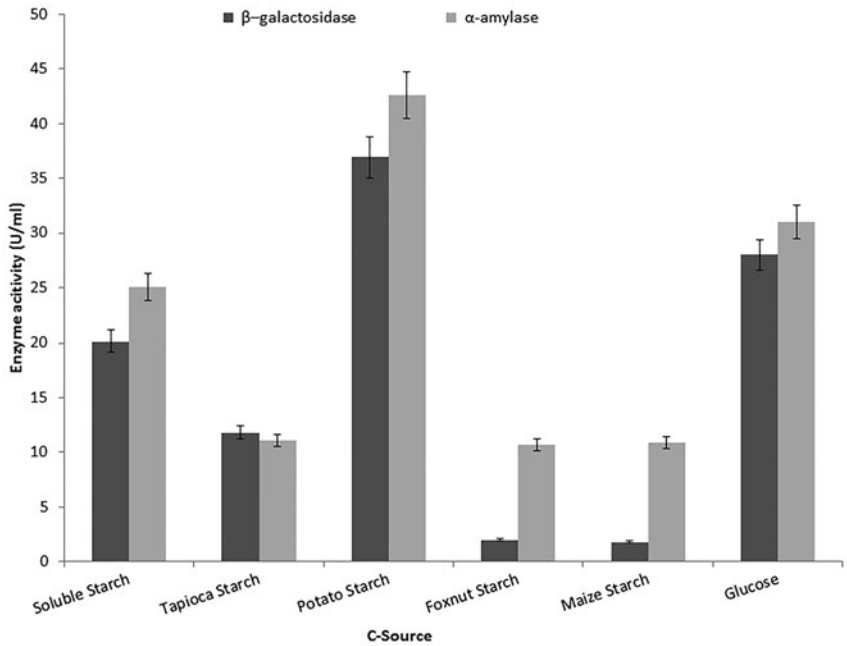

Figure 2. Comparison of α-amylase activity (■) and β-galactosidase activity (■) of the isolate with different substrate in MRS. Error bar denotes average of three replicates. The isolate selected for maximum production of enzyme activity in medium is supplemented with various starches as carbon source; potato starch proved to be the best among the selected ones for maximum production of enzymes.

Kinetics of α-amylase and β-galactosidase production

The isolate was grown in potato starch exhibited β-galactosidase activity after 4 hours of incubation. The activity increased gradually with incubation time and reached highest at 14–16 hours of cellular growth (Figure 3). The total α-amylase activity initially increased and was maximum following 12–14 hours of incubation. This initial part where the increase in activity was observed comprised the exponential phase and early stationary phase. However, as incubation continued, total α-amylase activity became stable from 40–48 hours and decreased further. In general, a decline in total enzyme activity in both cases was observed after the stationary phase during the growth experiments. The decline in total enzyme activity could be attributed to inhibition of cellular functions due to lowering of pH, depletion of nutritional factors from the growth medium, deactivation of the enzyme due to low pH catabolite repression, or/and inducer exclusion. LAB maintains a cytoplasm that is more alkaline than the medium, but the medium is acidified during growth by secretion of lactic acid. However, if the cytoplasmic pH decreases below a threshold pH, cellular functions are inhibited and the intracellular enzymes can be deactivated (Kashket, 1987).

Phenotypic characterization

The isolate LA-6 capable of hydrolyzing starch maximally was investigated for its phenotypic characteristics like colony morphology, gram-staining and catalase test. The carbohydrate fermentation profile and enzyme activity of the isolate was also carried out (Table 2).

Genotypic characterization

The best screened strain was finally characterized by sequencing of its PCR-amplified 16S rDNA gene. Comparison of the 16S rDNA gene sequence of the strain with those in the database

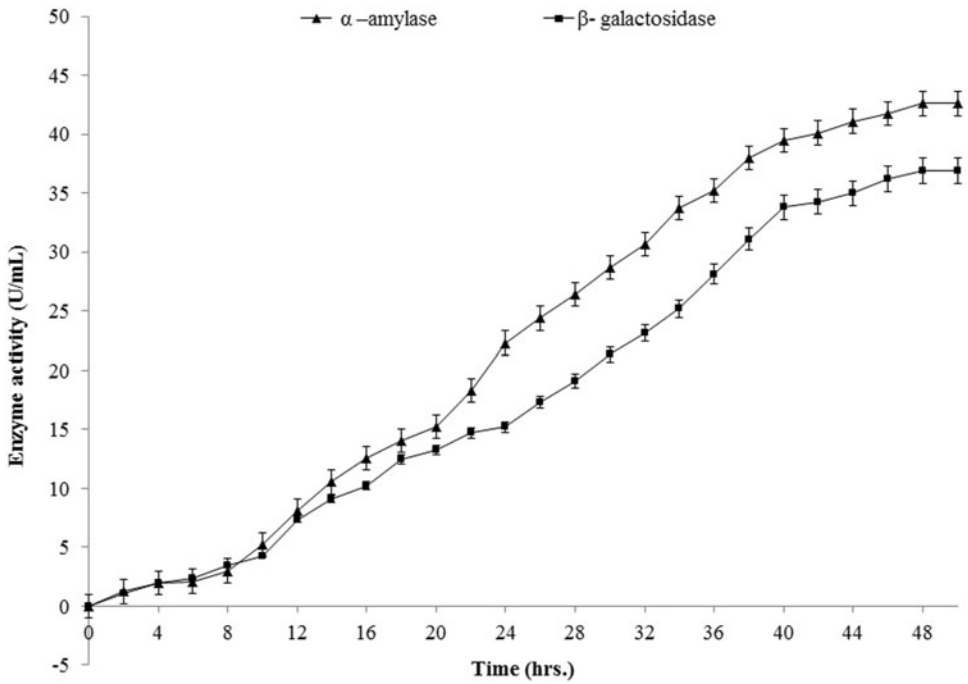

Figure 3. Kinetics of α-amylase (▲) and β-galactosidase (■) production.

Table 2. Phenotypic characterization of the isolate.

S. No.	Characteristics	Isolate
1	Cell Shape	Spherical
2	Cell Arrangement	Singly/paired/short chains
3	Cell Size (μm)	0.38–0.51 μm
4	Gram Staining	+
5	Spore forming	No
6	Aerobic growth	+
7	Anaerobic Growth	+
8	Catalase Test	–
9	Oxidase Test	–
10	Motility	Non motile
11	Gas from D-Glucose	–
12	Growth 15/45 (°C)	+/–
13	Growth at 0% NaCl	+
	3.0% NaCl	+
	4.0% NaCl	+
	6.5% NaCl	–
	8.0% NaCl	–
14	Hydrolysis of	
	Starch	+
	Skim Milk	+
	Gelatin	–
	Arginine	+
15	Lactic acid configuration	L-form
16	Sugar fermentation profile	
	Fructose	+
	Gentibiose	–
	Galactose	+
	Lactose	+
	Maltose	+
	Mannitol	–
	Mannose	–
	Salicine	+
	Sucrose	+
	Arabinose	–
	D-Xylose	–
	Trehalose	–
	Raffinose	+
17	Vogues Praskeur	+
18	Nitrate reduction	–
19	H_2S	–

showed 99% homology to those of *L. lactis* ssp. *lactis* (Figure 4). Therefore, the strain was identified as *Lactococcus lactis* ssp. *lactis* (GeneBank Accession No. JN618456).

Probiotic characterization

Tolerance to gastric juice

Sixty-three isolates from pickles were screened for co-producing α-amylase and β-galactosidase and their survival under simulated gastrointestinal conditions. One strain LA-6, co-producing α-amylase and β-galactosidase was assessed for its survival in gastric juice and was therefore selected for further studies of probiotic features. The effect of the successive passages through artificial juices on the viability of the *L. lactis* strain is shown in Figure 5. *Lactococcus lactis* showed notable survival (99%) in simulated acid gastric juice. The slight decline in the live counts of *L. lactis* seemed to be due to the effect of gastric juice (determined after 1 hour incubation with simulated acid gastric juice), because in the following stages of incubation, cell counts increases approximately 0.9 log units. Results show tolerance of *L. lactis* toward simulated gastric juice.

Microbial adhesion to solvents

Only microbial adhesion to *n*-hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent, as noted above. The values of MATS obtained with chloroform and ethyl acetate were recorded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacteria, respectively (Bellon-Fontaine et al., 1996).

Three different solvents were employed by using the MATS method to evaluate the hydrophobic/hydrophilic cell surface properties of *L. lactis* and its Lewis acid–base (electron donor and acceptor) characteristics. First, direct measurements of the cell surface hydrophobicity and hydrophilicity were carried out by the partitioning of cells between aqueous and *n*-hexadecane at a high ionic strength of 0.1 M (pH 6.2). The results indicated that a comparatively less percentage (36.7%) of bacteria which adhered to this apolar solvent, demonstrated a hydrophilic surface

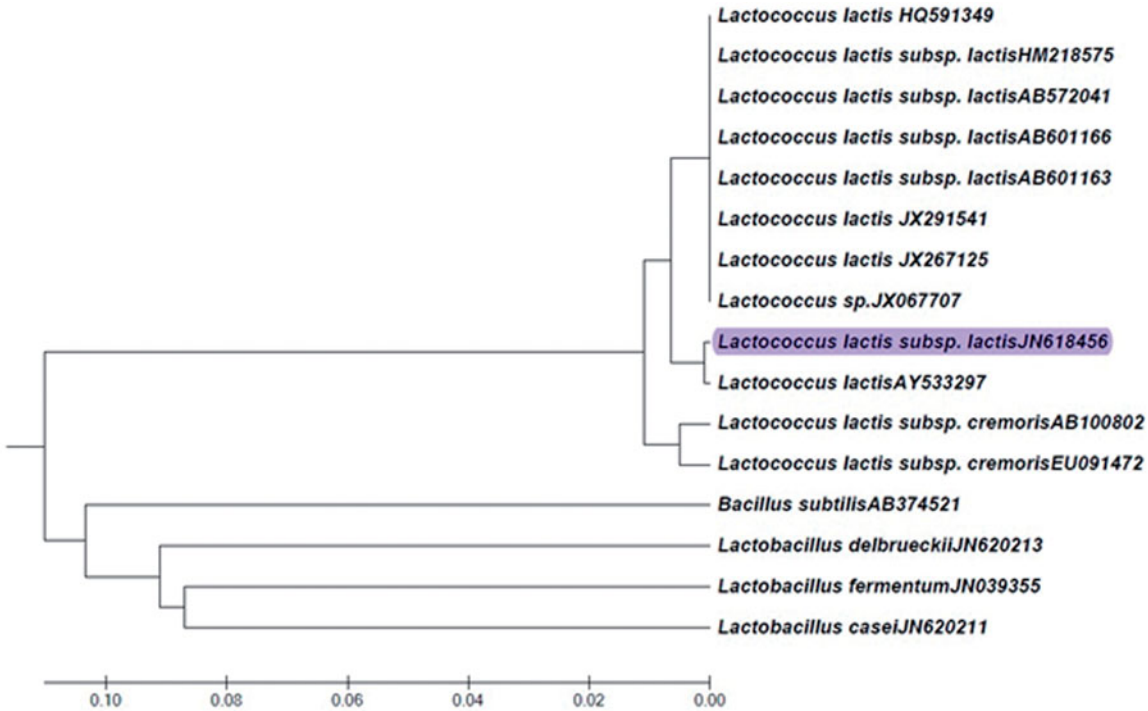

Figure 4. Neighbor-joining tree of *L. lactis* subsp. *lactis* based on bacterial 16S rRNA sequence data from different isolates of current study along with sequences available in GenBank database. *B. subtilis* was used as outgroup taxa.

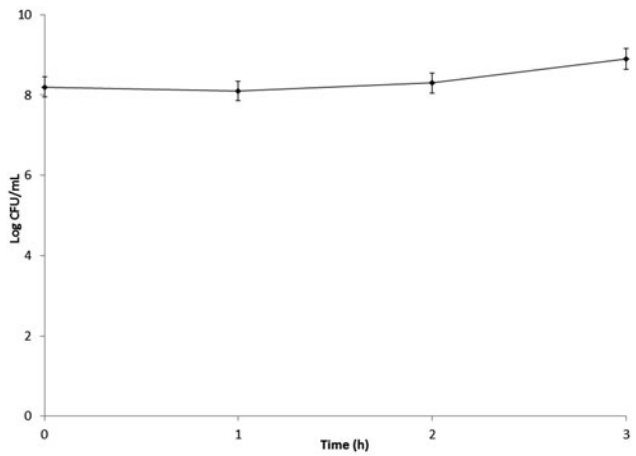

Figure 5. Effect of simulated gastric juice on viability of *L. lactis*. The values shown are mean of three experiments. Bar represents the standard error.

Table 3. MATS for *Lactococcus lactis*.

Solvent	Nature of solvent	% of adhesion (±SD)
n-hexadecane	Apolar	36.7 ± 2.1
Chloroform	Monopolar, acidic	72.3 ± 2.8
Ethyl acetate	Monopolar, basic	24.5 ± 1.5

Means ± standard deviations of two measures of at least three separate experiments.

(Table 3). Microbial adhesion to chloroform showed an overall strong affinity (72.3%) of *L. lactis* to this acidic solvent and electron acceptor. These higher values of adhesion were compared with those obtained for *n*-hexadecane because both solvents possess the same Vander Waals properties. The important difference observed was due to the implication of Lewis acid–base interactions resulting from the electron donor and basic character of bacterial strains. The data obtained for ethyl acetate, which is a strongly basic solvent and electron donor, produced results contrary to those encountered with chloroform: the bacterial adhesion to this third solvent was low (24.5%). It confirmed the non-acidic character of the bacterial strains studied.

Table 4. Ability of *L. lactis* to grow in the presence of phenol 0.4%.

Viable count[a] ($\log_{10}$ CFU/ml)					
MRS Broth			MRS Broth + phenol 0.4%		
0 hours	24 hours	Increase[b]	0 hours	24 hours	Increase[b]
8.09 ± 0.06	9.96 ± 0.11	1.87	8.05 ± 0.43	8.63 ± 0.09	0.58

[a]Log mean counts of two trials (average ± S.D.); Increase[b] = $\log_{10}$(final population)-$\log_{10}$(initial population); $n = 3$; observations comes from three replicate assays; data are represented as mean ± SD.

Phenol resistance

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols (Suskovic et al., 1997). Resistance to phenol was tested as an additional indicator for survival under intestinal conditions (Xanthopoulos et al., 1999) for the same screened probiotic candidate strain which survived *in vitro* intestinal passage as described above. *Lactococcus lactis* strain was less sensitive to phenol and tolerated phenol at 0.4% for 24 hours as its numbers did not decrease from initial inoculums of approximately log 8.1–8.5 (Table 4).

Bile salt hydrolase activity

To test the ability of the selected strain to hydrolyse the sodium salt of taurodeoxycholic acid, Bsh activity was evaluated. The screened isolate displayed Bsh activity by providing the white

precipitation zone around the colonies on plate assay when compared with *Lactobacillus acidophilus* ATCC 43121 and *E. faecium* E179 (controls). The isolate demonstrated high Bsh activity by expressing precipitation zone diameter greater than 15 mm and it was noted that the strain also exhibited high resistance to duodenum juice containing 0.5% bile salts (data not shown), which may be related with its Bsh activity as suggested by De Boever et al. (2000).

Antibiotic resistance

Antibiotic susceptibility of isolate is one of the crucial criteria for the safety point of view of potential probiotics since bacteria used as probiotics may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria. The *L. lactis* strain was resistant to gentamycin, vancomycin as well as to chloramphenicol and ciprofloxacin (Table 5). In contrast, it was susceptible to the antibiotics erythromycin, ampicillin, penicillin, tetracycline and clindamycin (Table 5), from the MIC breakpoint values as suggested by SCAN (Chesson et al., 2002).

Antagonistic activity

In the agar spot test, the *A. hydrophila* ATCC 35654, *S. typhimurium* ATCC 19585, *E. coli* 0157:H7 ATCC 43895, *S. aureus* ATCC 9144 and *Y. enterolitica* MTCC 840 indicator strains showed weak to strong inhibition (zone of inhibition of more than 1 mm from edge of producer colony up to 10 mm, Table 6). The inhibitory activity of *L. lactis* might be due to the production of a heat-stable bacteriocin, because the heated and neutralized cell-free supernatant of the producer culture also exhibited antimicrobial activity upon comparison to live cells in the agar spot test. Therefore, the inhibitory activity observed could be explained by the production of bacteriocin and organic acids. Hydrogen peroxide could hypothetically also act as an inhibitory substance, but the incubation of the plates under anaerobic conditions discards this as cause for the observed inhibition. Further, production of H_2O_2 under aerobic conditions was investigated and the strain was found to produce H_2O_2.

Table 5. Antibiotic susceptibilities of *L. lactis.*

Antibiotics	MIC (μg/ml)	SCAN breakpoints (μg/ml)
Vancomycin	≤1.2	4.0
Gentamycin	≤5.5	32.0
Ampicillin	≤0.12–0.24	2.0
Penicillin	≤0.5–1.0	2.0
Chloramphenicol	≤2.0–4.0	8.0
Clindamycin	≤0.15	4.0
Erythromycin	≤0.50	2.0
Ciprofloxacin	≤1.0–3.0	4.0
Tetracycline	≤1.5	4.0

Table 6. Antagonistic activity of *L. lactis.*

Organism	Isolate
Aeromonas hydrophila, MTCC 646	+++
Salmonella typhimurium ATCC 19585	+++
Escherichia coli 0157:H7 ATCC 43895	++
Staphylococcus aureus ATCC 9144	+++
Yersinia enterolitica MTCC840	+

ATCC American type Culture Collection, MTCC Microbial Type Culture Collection

+, inhibition zone <10 mm; ++, inhibition zone >11–19 mm; +++, inhibition zone >20 mm.

Discussion

Lactic acid bacteria are the most abundant bacteria in gastrointestinal tract (GIT). They are the most important food fermentors and used as starters. These also have a key role in texture and flavor enhancing properties of various dairy products. In this study, production of α-amylase and β-galactosidase by potential probiotic culture was investigated.

In the presence of starch as carbon source at 37 °C, *L. lactis* grew and released amylolytic activity in the culture medium. The maximum extracellular α-amylase production was obtained from LA-6 isolate (Figure 1) during the exponential phase of growth. The α-amylase production pattern indicates that the induction of α-amylase occurred during the lag phase in the presence of starch. Cell growth and amylase production reached maxima values at the same time (48 hours) (Figure 3). This phenomenon is generally attributed to the nutritional activity of the bacterium, which needs glucose for its growth. Since the extracellular medium contains starch which cannot cross the cell membrane for bacterium nutrition, cells secrete α-amylase extracellularly for breaking down starch molecules and releasing oligosaccharides such as glucose which can then enter into the cell for the bacterium nutrition (Fossi et al., 2011)

β-Galactosidase is an enzyme that is produced by some of bacteria, especially lactobacilli in dairy products like yoghurt, cheese and milk. Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world (Heyman, 2006). Favier et al. (1996) reported a method to detect bacteria with β-galactosidase activity by X-gal. Colonies growing on X-gal medium with green color were regarded as bacteria containing β-galactosidase enzyme (Favier et al., 1996). In this study, when the method of Favier et al. (1996) was applied to 63 strains, only six isolated strains containing β-galactosidase enzyme had green colonies (some producing strong and rapid while other producing low and slow enzyme). As indicated in the results (Figure 1), β-galactosidase production was intracellular and was maximum in isolate LA-6 in comparison to five isolates namely LA 1–5.

Different probiotic characteristics such as cell adhesion to solvents, gastrointestinal and bile salt tolerance, antibiotic resistance and antimicrobial capacity of the isolated strains were also determined in this study. Antagonistic properties of probiotic strains are essential in order to prevent the infection and/or invasion of pathogenic bacteria, whereas gastrointestinal and bile salt tolerance is essential for the potential use as probiotic in different formulations. In this preliminary screening of the antimicrobial capacity of the strains, five different strains of the gastrointestinal pathogens namely, *S. typhimurium*, *E. coli*, *Aeromonas hydrophila*, *Yersinia enterolitica* and *Staphylococcus aureus* were selected as indicator strains. Interestingly, *L. lactis* (LA-6) isolated from pickled yam have strong antagonistic activity against *Salmonella*, *Staphylococcus* and *Aeromonas* sp. (Table 6).

In this experimental design, 0.05%–0.3% bile concentration were used, as it corresponded to that found in the human intestinal tract and 0.3% bile is the maximum concentration that is present in healthy men (Graciela et al., 2001). Therefore, prior to the selection of probiotic bacteria for human consumption it must be endurable to 0.3% bile concentration (Gilliland et al., 1984). *L. lactis* was able to tolerate up to 0.3% of bile concentrations. The viable cell count of *L. lactis* found on MRS agar media were 1.5×10^5 (CFU/ml) at 0 hours and 1.59×10^{10} (CFU/ml) at 24 hours (cells were plated from artificial gastric juice at concentration (Gilliland et al., 1984)). These results indicate that there were no losses of viability of cell in simulated gastrointestinal condition. From this experiment, the results indicate that in

GIT environment the gastric juice will have minor or possibly no adverse effect on our isolated probiotic bacteria. Bacterial culture plated on MRS agar medium from simulated gastric juice (pH 2.2) showed two morphologically different colonies at 0 and 24 hours.

Overall, the current study emphasize on the potential of the strain as a functional starter in probiotic formulations. Moreover, the strain may be exploited for commercial production of hydrolases from different starches. However, further studies involving its survival and behavior in food matrices as well as its efficacy through clinical trials are mandatory prior to human applications.

Conclusion

The current study focuses on a potential applicability of the isolate for production of both hydrolases from a cheap agro industrial source such as potato starch. Results of this study also implicate the development of probiotic selection and its use in functional food. *Lactococcus lactis* ssp. *lactis*, isolated from indigenous pickled yam was selected for the co-production of α-amylase and β-galactosidase and its potential for use as probiotics. LAB utilized potato starch, an agro processing industry waste, in comparison to the commonly consumed starches (maize, tapioca, fox nut and soluble starch) and exhibited maximum α-amylase production. Further, the strain may be useful for the processing of pickled vegetables and fermented vegetable juice, which has been reported as a healthy beverage for vegetarians and lactose allergic consumers.

Declaration of interest

The authors do not have any conflict of interest for the publication of this work.

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SHORT COMMUNICATION

Effective conversion of industrial starch waste to L-Lactic acid by *Lactococcus lactis* in a dialysis sac bioreactor

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Abstract We describe here a simple technological process based on the direct fermentation of potato starch waste (PSW), an inexpensive agro-processing industrial waste, by a potential probiotic strain, *Lactococcus lactis* subsp. *lactis*, for enhancing L-lactic acid production. To maximize bioconversion and increase cell stability, we designed and tested a novel dialysis sac-based bioreactor. Shake flask fermentation (SFF) and fed batch fermentation in the dialysis sac bioreactor were compared for L-lactic acid production efficiency. The results showed that the starch (20 g/L) in the PSW-containing medium was completely consumed within 24 h in the dialysis sac bioreactor, compared with 48 h in the SFF. The maximum lactic acid concentration (18.9 g/L) and lactic acid productivity (0.79 g/L·h) obtained was 1.2- and 2.4-fold higher in the bioreactor than by SFF, respectively. Simultaneous saccharification and fermentation was effected at pH 5.5 and 30 °C. *L. lactis* cells were viable for up to four cycles in the fed batch fermentation compared to only one cycle in the SFF.

Keywords Dialysis sac bioreactor · *Lactococcus lactis* · Fermentation · L-Lactic acid · Potato starch waste

Many food and agro-based industries throughout the world produce large volumes of waste which are often discharged into the environment, possibly with harmful ecological effects. Effective utilization, recycling and reprocessing of these wastes would therefore be beneficial to the environment (Abdel and Sonomoto 2010) and potentially economically advantageous. For example, potato processing plants release an appreciable amount of starch into wastewater streams which could be utilized as a cheap substrate by microorganisms to produce economically valuable organic acids, such as lactic acid, acetic acid, among others. The wide application of lactic acid (2-hydroxypropionic acid, $CH_3CHOHCOOH$) in the food, cosmetic, pharmaceutical and chemical industries (Dumbrepatil et al. 2008; Gegios et al. 2010; Walton et al. 2010; Wanga et al. 2010; Zhao et al. 2010) has significantly increased interest in lactic acid production on a large scale (Palaniraj and Nagarajan 2012). Although lactic acid can be manufactured either via chemical synthesis or by a biological approach, the former is associated with several major disadvantages, including environmental issues and the depletion of petrochemical resources. Consequently, in recent years, focus has been directed towards the development of economically effective and sustainable biological/biotechnological approaches to produce lactic acid on an industrial scale (Wee et al. 2006).

Microorganisms in general play an important role in waste utilization through their ability to degrade/convert organic waste, such as wastes from various food-processing industries, and lactic acid bacteria (LAB) can produce lactic acid using such wastes as substrates (Datta et al. 1995). Historically, refined sucrose was the most commonly used substrate for the industrial production of lactic acid. More recently, corn refiners have started to manufacture lactic acid from corn starch, which is produced as a by-product from the wet milling of corn: the starch is liquefied and saccharified by enzymes to glucose, which is then fermented to lactic acid (Shen and Xia 2006). However, the industrial application of an microorganism which could produce lactic acid directly from starch-containing wastes would obviate the need for saccharification and/or liquefaction, resulting in large savings in production costs. As a first step in this direction, various LAB, including *Lactococcus lactis* ssp. *lactis* ATCC 19435, *L. lactis* and *Lactobacillus delbrueckii*, *L. casei* NRRL B-441 and *L. amylovorus* ATCC 33620, have been shown to have the

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ability to produce lactic acid directly from starchy substrates (John et al. 2009).

The aim of our study was to develop a process in which L-lactic acid can be produced from a cheap agro-processing industry waste, namely, potato starch waste, by direct fermentation. We also attempted to increase lactic acid production by setting up a novel dialysis sac-based bioreactor to facilitate the recycling and reuse of the *L. lactis* cells for an extended period.

Lactococcus lactis subsp. *lactis* used in this study is a lactic acid-producing strain isolated from pickled yam (Bhanwar et al. 2013). Three types of media were used in this study (1) de Man–Rogosa–Sharpe (MRS medium; Sigma, St. Louis, MO), (2) modified MRS medium (containing potato starch instead of D-glucose), (3) potato starch waste (PSW; a kind gift from PepsiCo., Patiala, India; stored at 4 °C until used). The strain was maintained on MRS agar plates at 4 °C and sub-cultured weekly. All other chemicals and reagents used were of highest grade available commercially.

To test the feasibility of improving lactic acid production by free cells, various experiments were conducted in MRS medium, modified MRS medium and PSW by inoculating approximately 10^8 CFU/mL seed culture into flasks containing 100 mL medium. The flasks were incubated with shaking at 120 rpm, 30 °C for 96 h. The concentration of carbon source in the media was varied (0.5, 1, 2 %). PSW was diluted to achieve the same final concentration of starch as the carbon source in the other two media. Aliquots (5 mL) of each culture were withdrawn at 8-h intervals and centrifuged at 10,000 rpm for 10 min; residual starch and lactic acid were estimated in the supernatant. In a parallel experiment, the pellet was washed and resuspended in 0.85 % saline before absorbance was read at 600 nm to obtain the cell density.

To facilitate the recycling and reuse of the *L. lactis* cells, we used a novel bioreactor equipped with a dialysis membrane (Ganguli and Tripathi 2002) for improved lactic acid production (Fig. 1). Briefly, dialysis tubing (Cellulose; Sigma-Aldrich) was thoroughly washed with sterile triple-distilled water and clipped at one end to produce a sac. The wet weight of log phase *L. lactis* cells (10 mg) corresponding to 5.5×10^8 CFU/mL was packed into the dialysis sac. Each sac was then tied at the upper end with clips and submerged completely in a vessel containing one of the culture media and incubated with stirring at 30 °C for 96 h. Aliquots (5 mL) of medium were withdrawn at various intervals and used to estimate residual starch and lactic acid.

During fermentation, the pH was maintained at 5.5 by adding 2 mol/L NaOH or 1 mol/L HCl, and the temperature

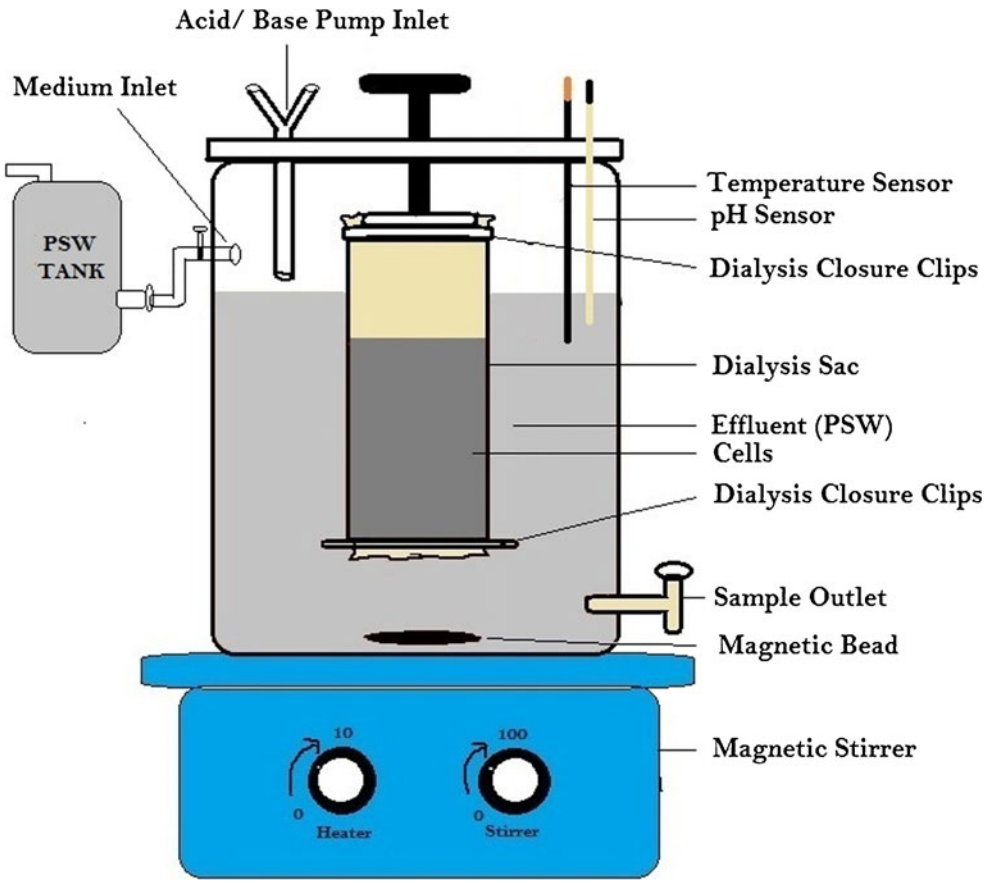

Fig. 1 Experimental setup for the study of starch utilization in fed batch fermentation by cells of *Lactococcus lactis* in the potato starch waste (*PSW*) medium. The volume in the vessel was maintained at 500 mL and the temperature was monitored using a temperature sensor

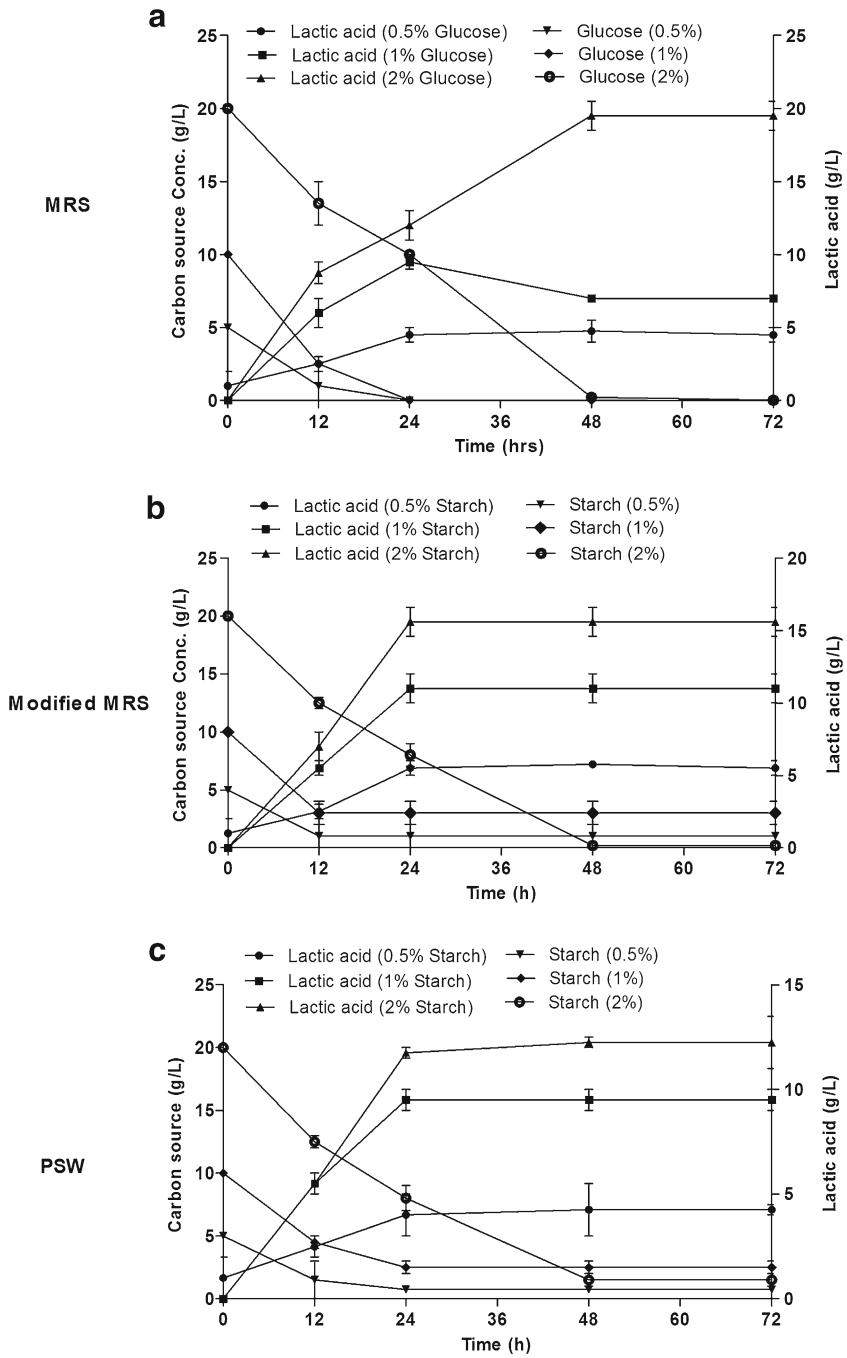

Fig. 2 Kinetic study of L-lactic acid production in shake flask fermentation (SFF) using modified de Man–Rogosa–Sharpe (*MRS*) medium (**a**), MRS medium (**b**) and PSW-containing medium (**c**)

was kept at 30 °C, which had been determined earlier to be the optimal culture temperature (data not shown). Liquid samples (5 mL) were taken at predetermined time intervals and centrifuged at 10,000 rpm for 10 min. The supernatants were collected and properly diluted for high-performance liquid chromatography (HPLC) analysis. Cell growth was monitored by measuring the optical density at 600 nm using a UV-spectrophotometer.

Due to starch depletion from the media over time, during the course of the experiment we withdrew medium from the dialysis bioreactor and replaced it with fresh medium. The allowed us to check the reusability of the dialysis sac-entrapped cells for lactic acid production. The viability and stability of the cells entrapped in the dialysis sac was checked by diluting them in phosphate buffered saline (pH 7.2) and plating them on MRS agar plates under sterile conditions. Lactic acid production was estimated as described below.

Lactic acid was analyzed on an HPLC system (1200 Series; Agilent Technologies, Santa Clara, CA) coupled with an UV–VIS detector and an HPLC column (Acclaim Organic

acid Column; 5 μm, 4×250 mm; Thermo Scientific, Waltham, MA). The mobile phase was a sodium sulfate (100 mM) solution (pH 2.65 adjusted with MSA), and an isocratic elution was used at a flow rate of 0.6 mL/min. The detection of lactic acid was set at λ=210 nm (Naveena et al. 2005). The calculation of lactic acid content was based on the peak area registered at the specific retention time for lactic acid and by taking the regression curve factor into account. Residual starch was detected as described by Giraud et al. (1993).

All analyses were done in triplicate, and the statistical comparison of data was performed by analysis of variance to reveal significant differences for each parameter among species. The correlation coefficient (R^2) and P value were used to show correlations and their significance. A probability value of $P< 0.05$ was adopted as the criterion for a significant difference.

Lactic acid production was initially quantified in shake flasks (control) under previously optimized conditions of temperature (30 °C) and pH (5.5) (unpublished data). Lactic acid productivity increased with increasing cell number which led in turn to a decreasing starch concentration (Zhou et al. 1999). There was a gradual increase in lactic acid production with increasing initial starch concentration from 0.5 to 2 %, but there was no significant increase at 3 % starch concentration (data not provided). One possible explanation for this findings is decreased free availability. It is possibly that at lower concentrations (0.5–2 % starch), the starch was readily available to *L. lactis* cells, but as the concentration increased to >2 %, the availability of free starch increased to such a level that it did not dissolve completely in water to become available to the cells, thereby decreasing the production of lactic acid.

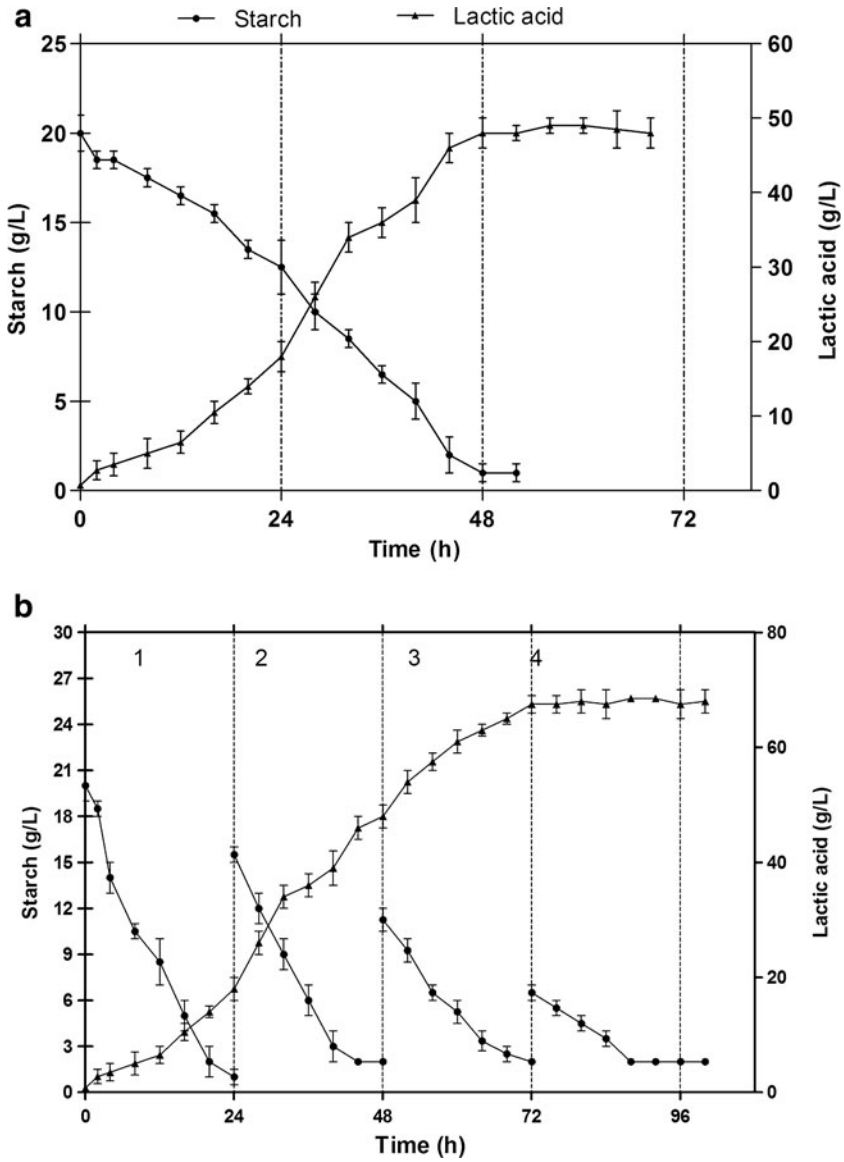

Fig. 3 Kinetics of lactic acid production and starch degradation in PSW-containing medium in SFF (**a**) and fed batch fermentation (**b**) in a dialysis sac bioreactor

The maximal lactic acid concentration in MRS medium (19.5 g/L) (Fig. 2a) was 1.11-fold higher than that in modified MRS medium (15.6 g/L) (Fig. 2b) and 1.59-fold higher than that in PSW-containing medium(12.25 g/L) (Fig. 2c). These results indicate that the glucose in the MRS media is a more readily available carbon source for the production of lactic acid (Cock and de Stouvenel 2006) than starch granules.

When *L. lactis* cells were directly used in the shake flask fermentation (SFF) containing either MRS broth or modified MRS broth or PSW, the cells remained viable for 2 days. Complete starch degradation was not achieved due to the loss in cell viability caused by the acidic environment that developed in the culture flasks. The production of lactic acid did not increase significantly even after starch supplementation (Fig. 3a). Therefore, to overcome these problems and facilitate recycling and reuse of the cells, we tested the media and *L. lactis* cells in a two-in-one dialysis sac-based bioreactor (Fig. 1).

Starch/glucose (20 g/L) was completely consumed within 24 h in the dialysis sac bioreactor (Fig. 3b) in comparison to >48 h by free cells in the control shake flasks. Lactic acid productivity (0.79 g/L·h) and yield (0.94 g/g) in the dialysis bioreactor were 2.4- and 1.2-fold higher, respectively, than those (0.33 g/L·h and 0.78 g/g) in the control flasks. The final lactic acid concentration (18.9 g/L) after 24 h of culture in the dialysis bioreactor with 20 g/L starch as substrate was 1.2-fold higher than that in the control (15.6 g/L) after 48 h. When the initial glucose/starch concentration was increased to 30 g/L (data not shown), glucose/starch was not completely mixed within the medium and became unavailable to the cells, thereby decreasing lactic acid productivity and yield (0.55 g/L·h and 0.51 g/g, respectively).

To test the reusability of the cells, after each batch of fermentation, the broth was withdrawn and an equal volume of fresh medium was added to the bioreactor. Figure 3a shows that lactic acid productivity and yield dropped in the shake flasks after the first cycle of fermentation and remained almost unchanged after the second cycle of fermentation. The reduced lactic acid productivity and yield might be ascribed to the acidic environment (pH 2.5–3.0) of the fermentation broth which negatively affected the activity of the cells whose maximum activity is at pH 5.5–6.2. Lactic acid productivity (0.79 g/L·h) and yield (0.94 g/g) were higher in the dialysis bioreactor than in the control and increased for up to four cycles of fermentation (Fig. 3b), following which the productivity remained unchanged. The reusability of cells implies that the dialysis bioreactor could be an efficient alternative for the treatment of PSW.

There have been several reports on the production of lactic acid using electrodialysis (Min-tian et al. 2005; Huang et al. 2007), but to our knowledge there has been no report on the use of a dialysis sac bioreactor for lactic acid production. More specifically, we believe that our study is the first to examine the recovery of lactic acid from an industrial waste using probiotic bacteria in the setting of a dialysis sac bioreactor. One additional interesting finding is that the cells contained in the dialysis sac and the enzymes produced can be recovered and may be used for industrial use.

The dialysis sac-based bioreactor used in our study was effective in improving lactic acid production from PSW. Higher levels of lactic acid productivity, yield and starch degradation were achieved in the dialysis sac bioreactor than in the SFF (control). Based on our results, we suggest that the dialysis sac bioreactor represents an easier setup and is more cost effective than electrodialysis, which has been used for years to produce lactic acid. This is the first report of such a reactor being adapted for direct starch conversion to L-Lactic acid production. Rigorous large-scale studies are warranted before this technology can be used in industrial-scale wastewater treatment plants. However, the technology may lead to a new process for the treatment of starch-enriched agro-waste and the production of a non-toxic by-product (lactic acid) using indigenous LAB isolated from pickled yam. We have demonstrated that our novel dialysis bioreactor not only increases lactic acid productivity but that it is also cost effective due to the use of a relatively inexpensive substrate.

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Conflict of interest None.

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α-amylase and β-galactosidase production on Potato starch waste by *Lactococcus lactis* subsp *lactis* isolated from pickled yam

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Potato starch waste, a chips industry effluent, was used for the production of industrially important enzymes by an amylolytic strain of lactic acid bacteria, isolated from pickled yam & identified as *Lactococcus lactis.* The strain was observed to co-produce α-amylase and β-galactosidase. Potato starch waste was efficiently utilized (91.6%) along with high co-production of α-amylase and β-galactosidase. Optimum culture conditions favouring maximum production of these two hydrolases in MRS medium containing 2% potato starch were temperature 55°C, pH 7 and temperature 35°C, pH 5 for α-amylase (2.54 U/mL) and β-galactosidase (2.67 U/mL) respectively. In potato starch waste, *Lactococcus lactis* retained viability and could co-produce α-amylase (17.54 U/mL) and β-galactosidase (25.35 U/mL) at a temperature of 45ºC and pH 6.5 within a period of 48 hrs. Thus, the study suggests a potential applicability of the isolate for the production of industrially significant hydrolases from a cheap agro industrial source.

Keywords: β-galactosidase, α-amylase, *Lactococcus lactis*, potato starch waste, co-production

Introduction

Microorganisms have been employed for the production of industrially important enzymes over decades. Among the group of hydrolases, two enzymes namely α-amylase and β-galactosidase are significantly important enzymes with industrial applications[13]. *Lactobacillus manihotivorans* LMG18011using soluble starch and food wastes as substrates[19] and *L. amylovorus* utilizing raw corn starch, rice starch and wheat starch medium[35] are few examples of microorganisms utilizing cheaper substrates for enzyme production. Complete starch hydrolysis requires two kinds of enzyme activities: against α-1,4 and α-1,6 glycosidic bonds. These two types of activities can be shown by one enzyme (amylopullulanase) or by two enzymes, such as α-amylase and pullulanase type I[27]. α-Amylase (E.C.3.2.1.1) catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch and have significant application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries[9,11,26]. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries[5].

Currently, various microbial amylases available commercially holds a broad spectrum of applications as compared to chemical hydrolysis of starch in starch processing industry; plant and animal α-amylases[30]. A most recently isolated ALAB strain *L. paracasei* B41 was the first amylolytic representative of *L. casei* group[23] among other amylase producing bacteria such as *Lb. plantarum* and *Lb. manihotivorans*[15,18], isolated from cassava-based fermented products and *Lb. cellobiosus*[29], *Lb. amylovorus*[16], and *Lb. amylolyticus*[4]. The amylolytic lactic acid bacteria (genera of *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Carnobacterium,* and *Weissella)* producing starch-modifying enzymes have been summarized[24].

β-galactosidase (EC.3.2.1.23), most commonly known as lactase, hydrolyses lactose into its monomers glucose and galactose. It has potential applications in food processing industry and is produced in a variety of sources, including plants, animals and microorganisms[17,1,21]. The dairy industry employs β-galactosidase enzyme produced by the microorganisms belonging to genera of *Lactobacillus* and *Bifidobacterium*[7,10,34]. However, different

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microorganisms differ in their respective physiological conditions for the optimal production of enzyme. Though β-galactosidase has been identified in a wide variety of fungal, yeast and bacterial cultures, Streptococcus thermophilus and Bacillus stearothermophilus can be considered as potential bacterial sources[21]. For instance, β-galactosidase based medical and industrial applications include cleavage of blood group A and B glycotopes, biosensor for specific lactose determination in milk and disease diagnosis, treatment of lactose malsorption, production of lactose hydrolysed milk[3]. Lactic acid bacteria have been exploited for production of both these enzymes separately, but very few studies have investigated the production of both these enzymes simultaneously[13].

Thus, the main focus of the study was to optimize the culture conditions such as temperature and pH for co-production of industrially important α-amylase and β-galactosidase from economically viable sources like potato starch waste of potato based snack food industry.

Materials and Methods

Chemicals

Potato starch was purchased from HiMedia, Mumbai, India and potato starch waste water was a kind of a gift from local potato chips industry (Patiala, Punjab, India). All other reagents and chemicals were purchased from HiMedia (India) or Sigma (USA). Modified MRS was prepared containing starch in place of carbon source at 2% concentration.

Potato Starch Waste water

The compositional analysis of potato starch waste water including reducing sugar[13], starch[8], pH, total solids, and chemical oxygen demand (COD), Biochemical oxygen demand (BOD) and total nitrogen was done as per APHA standard methods (2005) for water and wastewater[2] before and after analysis.

Screening of Bacterial isolates

Six strains of lactic acid bacteria, isolated from pickled yam and other fermented foods were screened[4] based on their starch utilizing potential. Further, screening was based on the selection of α-amylase and β-galactosidase producing lactic acid bacteria. The starch utilizing strains were screened on modified MRS medium by using Remazol Brilliant Blue **(**RBB) R salt (Acros Organics, New Jersey, USA) and potato starch (HiMedia, Mumbai, India) for α-amylase production. Remazol Brilliant blue (RBB)-starch agar medium was prepared by the method of Omemu *et al.* (2005). The isolates were plated on MRS-RBB starch agar plate and incubated at 37^{o}C for 24hrs. The starch utilization was monitored by the disappearance of the blue color of the medium based on the intensity of amylase production. The screened isolates were further monitored for their β-galactosidase production on MRS agar supplemented with 30μg ml^{-1} of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and incubated at 37°C for 24 hrs.

Biochemical analysis

One isolate capable of hydrolyzing starch maximally was identified by its colony morphology, gram-staining, and biochemical tests (catalase test, carbohydrate fermentation etc.), as well as by 16S rRNA gene sequencing[33].

Genetic Identification

Genomic DNA of the strain was isolated by usimg QIAamp DNA Mini kit as explained **(**Qiagen, Valencia, CA, USA). Bacteria-specific universal primers used for amplification of 16S rRNA gene were the forward primer 27F (5'-AGAGTTTGATCATGGCTC-3') and the reverse primer 1327R (5'-CTAGCGATTCCG ACTTCA-3') 33. The 16S rRNA gene was amplified in 35 cycles with a Gene Amp PCR System 2400 (Perkin Elmer, Waltham, MA, USA). The thermal program consisted of one cycle at 94ºC 4 min, 35 cycles of 94ºC 40s, 46ºC 40s, 72ºC 2 min, final one cycle of 72ºC 15s and stored at 4ºC. A 100-bp DNA ladder was used as the molecular marker (Fermentas). PCR products were purified using QIA quick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced from both ends with an ABI3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the same oligonucleotide primers used for PCR. The sequenced 16S rDNA sequences for the bacterial isolates were analyzed to detect the presence of possible chimeric artefacts and compared with the similar gene sequences.

Enzyme production

The identified strain was grown in MRS medium and inoculated with 1% (v/v) (10^5 CFU/mL) overnight grown culture and incubated at 30ºC for 24 h. The carbon source in the medium was substituted with 0.5, 1, 2 and 3% (w/v) potato starch. Aliquots were removed at regular intervals for biomass

and enzyme activity determination. The enzyme co-production was estimated in Potato starch waste thereafter.

Enzyme assay

α-Amylase production was studied on modified medium of MRS containing 20 g/l of potato starch source. 1% bacterial isolate was inoculated in respective modified MRS medium at pH 7.0 for 24 hrs. Aliquots were withdrawn periodically and centrifuged at 8000 rpm for 5 min and supernatant was analyzed for enzyme activity. The extracellular Amylase activity was assayed by measurement of the iodine complexing ability of starch as previously described (Giraud et al. 1993). The enzymatic activity was determined at different pH values (3.5-6-5; 0.1 mol/1 citrate-phosphate buffer) and temperatures (30-60ºC). One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described above.

β-galactosidase was estimated as described by Miller (1959). Briefly, the crude enzyme solution was diluted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer (pH 6.0), and was added to 0.5 ml of 6 mM o-nitrophenol-galactopyranoside (ONPG) in the same buffer. The reaction mixture was incubated at 40ºC for 30 min. The reaction was ended by adding 0.5 ml of 1 M Na2CO3 and the concentration of o-nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm, using a standard calibration curve. The enzyme activity was expressed as specific activity (U/ml soluble protein) and one unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 nmol ONP per minute.

Effect of pH on enzyme production

The effect of pH on enzyme production was investigated by adjusting the pH of media to 4, 5, 7 and 9. The media was inoculated by the isolate and incubated at 37°C for 24 hrs. Samples were withdrawn and the enzyme activity was determined as described previously.

Effect of temperature on enzyme production

The effect of temperature was investigated by incubating the media at different temperatures (25–75°C). The samples were withdrawn and promptly chilled in ice at the times indicated and the remaining β-galactosidase activity was determined as described in the enzyme assay.

Statistical analysis

All the experiments were performed in triplicate. Error bars on graphs show the standard deviation. The data were analyzed by analysis of variance (ANOVA).

Results

Characterization & Composition of Potato starch waste

The potato wastewater, with characteristics as shown in Table 1, used in this investigation was collected from Potato Chips Industry, Patiala, Punjab, India, The maximum starch reduction was 91.6% after 48 hrs and it did not decrease further on incubating for more time.

Selection of α-amylase & β-galactosidase producing lactic acid bacteria

One of the six of lactic acid bacterial strains showed the formation of white colour colonies on RBB starch agar medium indicating amylolytic activity, whereas blue colour colonies on X-gal agar medium indicated β-galactosidase activity (data not shown). The strain turned blue on X-gal agar depicts the inhibition of β-galactosidase enzyme and was selected for further analysis.

Identification of the strain

The selected strain isolated from the pickled yam was identified as *L. lactis* based on its physiological and biochemical characteristics. The isolate was gram-positive, catalase-negative, nonmotile, coccus, creamy, little sticky and smooth. The preliminary result obtained with API 50 CH test kit allowed the identification of isolate as *Lactococcus* spp. with good correlation at the genus level (98.2%). Further the complete sequence of the 16S rRNA of the strain with those in the database showed 99% homology to those of *L. lactis* subsp. *lactis*. Thus the strain belonged to *L. lactis* subsp. *lactis* species and was designated

Table 1—Composition and Characteristics of potato wastewater

Parameter	Before treatment (g/l)	After treatment (g/l)
Total Solids	45 ± 2.0	32 ± 2.0
Total insoluble solids	5.9 ± 0.5	2.6 ± 0.5
Total soluble solids	30.5 ± 1.0	28 ± 1.5
Starch	18 ± 1.5	1.5 ± 0.5
Reducing Sugars	1.3 ± 0.8	0.1 ± 0.01
COD	26 ± 1.0	6.0 ± 0.5
BOD	40 ± 2.0	15 ± 1.0
Total Kjeldhal Nitrogen	1.5 ± 0.2	0.3 ± 0.05

L. lactis subsp. *lactis* (Figure 1) (GeneBank accession number JN618456).

Enzyme kinetics of α–amylase and β-galactosidase

The identified strain was found to be the best co-enzyme producer & specific activity of α-amylase and β-galactosidase produced by this strain was 2.54 U/ml and 2.67 U/ml respectively with a biomass of 0.31 mg/mlwhen 2% potato starch as C-source was used in comparison to 0.5, 1 and 3% starch (Figure 2). In potato starch waste (PSW), containing approx. 2% potato starch, the production of α-amylase and β-galactosidase increased exponentially to a value of 17.54 U/ml and 25.35 U/ml respectively after 24 hrs of incubation with biomass of 0.42 mg/ml and productivity at 0.87 U/mg of starch and 1.21 U/mg of starch for α-amylase and β-galactosidase respectively.

Effect of pH on enzyme production

L. lactis was inoculated into MRS media with different pH (4, 5, 7, 9) and incubated at 37ºC for 2 days. The enzymes were extracted and the specific activity of enzymes produced was recorded. A differential enzyme production (α-amylase and β-galactosidase) was observed at a pH range of 4-9 (Figure 3), which depicts an optimum pH of 6.5 for the highest production of enzymes. Wallenfels and Weil (1972) suggested that decline in enzyme activity above and below of optimum pH may be possible due to the formation of an improper ionic form of the substrate or enzyme (or both), inactivation of the enzyme or from a combination of these effects. In this study, the effect of pH on enzyme stability was not determined, only the activity of the enzyme was determined. Therefore an explanation to the decrease in activity above and below optimum pH cannot be put forth. However, as summarized, data from earlier studies on the influence of pH on the enzymatic hydrolysis of ONPG (ortho-Nitrophenyl-β-galactoside) were fitted to a bell-shaped curve with maximal enzymatic activity between pH 7.0 and 7.4[31].

Effect of temperature on enzyme production

L. lactis was inoculated in MRS medium containing potato starch and incubated at different temperatures (30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC, 65ºC and 75ºC). Figure 4 depicts the optimum temperature of 35°C, at which specific β-galactosidase activity was maximum (2.67 U/ml) whereas the specific activity of β-galactosidase substantially declined when *L. lactis* was incubated above 35°C. For α-amylase, the specific activity increased up to 55°C, maximum specific activity was calculated as 2.54 U/ml, and decrease in specific activity was observed above this temperature.

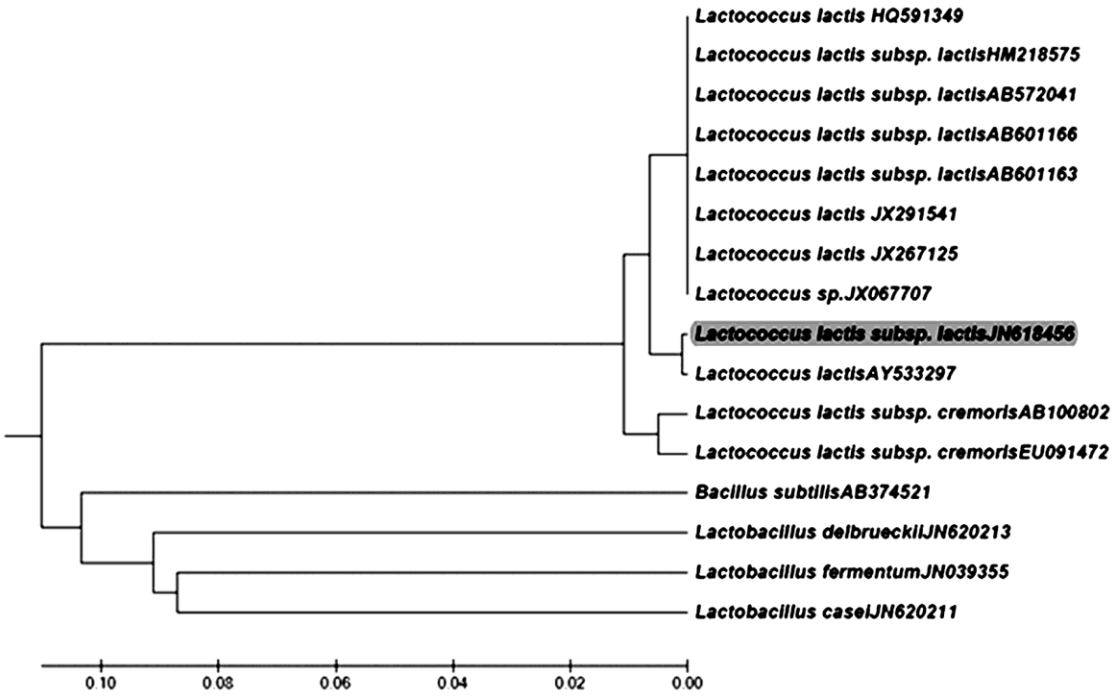

Fig. 1—Phylogenetic analysis of *Lactococcus lactis*subsp. *lactis*

Enzyme Co-production

When co-production of both the enzymes was studied in MRS media and PSW, α-amylase activity observed was as low as 1.48 U/ml and β-galactosidase activity was 2.2 U/ml in MRS medium but in studies with potato starch containing effluent α-amylase activity observed was 17.54 U/ml whereas β-galactosidase activity was 25.35 U/ml at temperature 45ºC and pH 6.5 (Figure 5). The resulting profile of *Lactococcus lactis* differs from *Lactococcus lactis* IBB500 that showed optimum amylolytic activity[32] at a lower pH (4.5) and temperature (35°C).

This can be explained by the fact that as the starch in the effluent gets consumed the production of enzymes increases and consequently following a decrease in starch content in the media, the enzyme activity decreases (Figure 6).

During the growth experiments, it was observed that there was a decline in total enzyme activity in both cases after the stationary phase. This decline was attributed to inhibition of cellular functions due to lowering of pH, depletion of nutritional factors from the growth medium, deactivation of the enzyme due to low pH catabolite repression, or/and inducer exclusion. This is due to the reason that during fermentation, the medium is acidified during growth by secretion of lactic acid by lactic acid bacteria. As reported earlier, if the cytoplasmic pH decreases below a threshold pH, cellular functions are inhibited and the intracellular enzymes can be deactivated[12].

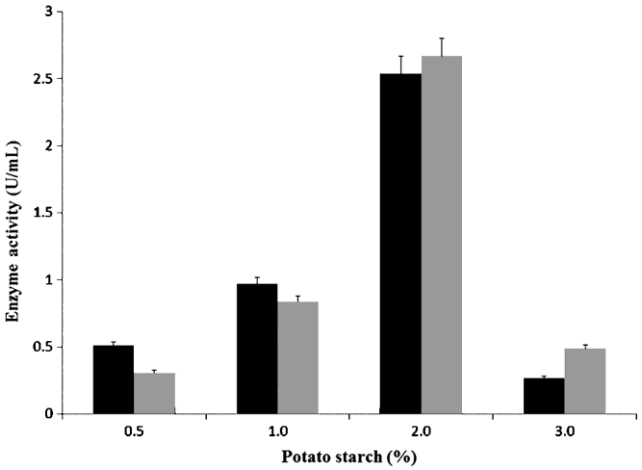

Fig. 2—Production of α-amylase (■) and β-galactosidase (■) by *L. lactis* on different concentrations of Potato starch in MRS media

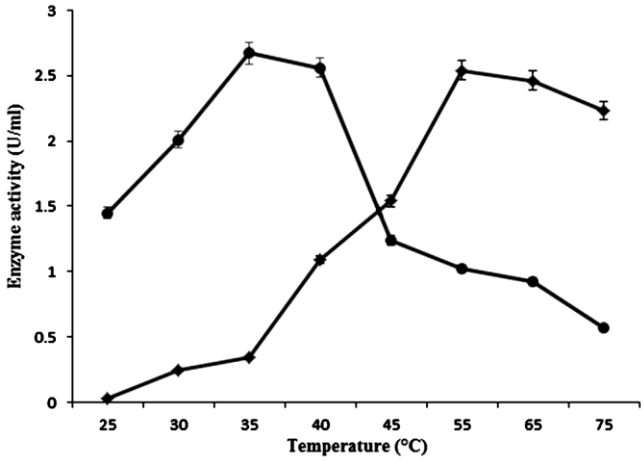

Fig. 4—Effect of temperature (25-75ºC) on α–amylase (●) and β-galactosidase (●) activity

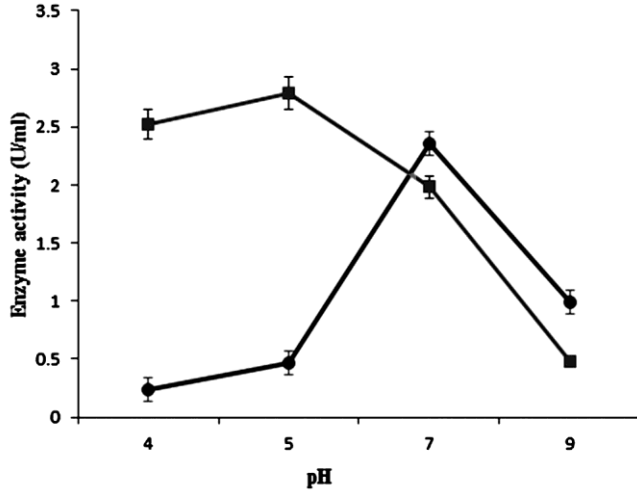

Fig. 3—Effect of pH (4-9) on α–amylase (■) and β-galactosidase (●) activity

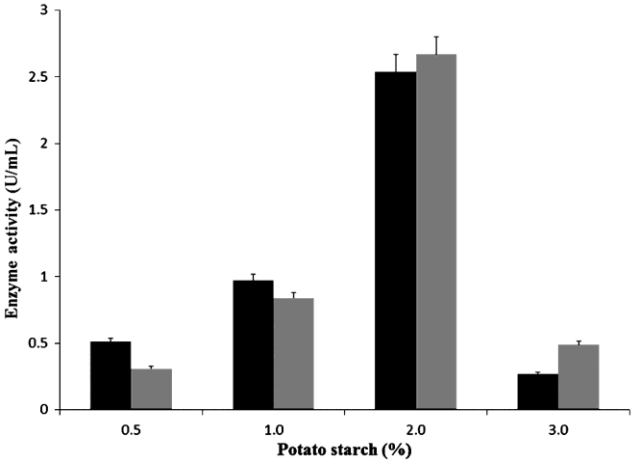

Fig. 5—Comparison of co-production of α-amylase and β-galactosidase activity in Potato starch waste (■) and MRS media (■).

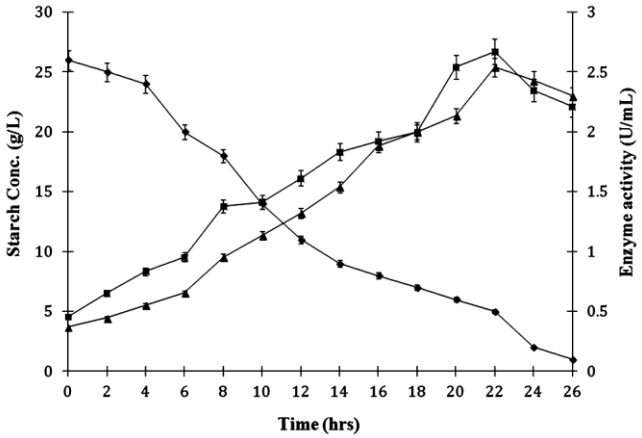

Fig. 6—Effect of starch (♦) utilization on enzyme kinetics of α–amylase (▲) and β-galactosidase (■) produced by *Lactococcus lactis* grown over a duration of 26 h)

When compared to modified MRS medium, the maximum activity of both enzymes was observed in potato starch waste than in modified MRS medium and the kinetics of co-production showed that α-amylase shows relatively lower activity as compared to β-galactosidase. It is interesting to note that production of these enzymes have been exploited by various microorganisms; *Lactobacillus amylovorus, Lactobacillus plantarum, Lactobacillus manihotivorans, and Lactobacillus fermentum*[25,28].

Cheaper sources containing both carbon and nitrogen sources are now mainly used as substrate for commercialized production processes and the ability of the microbes to grow and produce enzymes using these sources has been a point of interest[22]. Several studies have indicated the feasibility of cheaper media components, for instance, high β-galactosidase production in the presence of corn flour along with the corn steep liquor[13]. In this context, agroprocessing industries are interesting targets for providing their waste product which may be subsequently utilized using appropriate biotechnological interventions for generating value added products. The results of our study suggest a possibility of improvising a process for simultaneously producing α amylase and βgalactosidase from potato industry waste without addition of any growth enhancing components.

Conclusion

Based on the present study, it is concluded that *L. lactis* grown in MRS medium containing potato starch exhibited maximum enzyme activities. In view of this, a cheaper source containing potato starch i.e. potato starch waste from potato snack food industry was explored as the substrate for the production of these enzymes, the culture conditions for optimal co-production was subsequently standardized. The notably high production of extracellular α-amylase and intracelular β-galactosidase from a cheap source i.e. potato starch waste, without addition of any growth promoters, suggests the potential applicability of *L. lactis* for commercial production of these enzymes. Besides optimizing a fermentation process by developing an economically viable process for the production of enzymes from a cheap agrowaste, there has been a significant reduction in the COD, BOD, suspended solids and other parameters which leads to discharge of this effluent environmentally safe. Finally, the low cost of production of these enzymes alongwith their significant activity and stability constitute two important advantages for their possible industrial use To the best of our knowledge, this is the first report of a lactic acid strain co-producing α-amylase and β-galactosidase from potato starch waste.

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