ISOLATION AND CHARACTERIZATION OF STARCH DEGRADING LACTIC ACID BACTERIA

A
Dissertation
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In
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UNDER THE GUIDANCE OF
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Dated: June, 2006. ` Shalini Aggarwal
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CERTIFICATE

This is to certify that the thesis entitled "ISOLATION AND CHARACTERIZATION OF STARCH DEGRADING LACTIC ACID BACTERIA" submitted by Ms. Shalini Aggarwal (3040024) in partial fulfillment of the requirements for the award of Degree of Master of Sciences in Biotechnology to Thapar Institute of Engineering and Technology (Deemed university), Patiala, is a record of student's own work carried out by her, under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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OBJECTIVES

This dissertation aims at making a work plan to isolate potential strain of amylolytic lactic acid bacteria which is a potential lactic acid producer and to characterize it biochemically and to optimize culture conditions for the process. The ultimate objective of this research is to optimize the process for its application an industrial scale for the treatment of waste stream from snack food industry and to obtain value added products from it.

The objective can be achieved by following the described plan as seen below:

- Isolation of starch hydrolyzing and lactic acid producing strains of bacteria from fermented foods.
- Establishing the growth profile of he selected strain.
- Finding out he starch hydrolysability (qualitatively and quantitatively).
- Determine the ∞-amylase activity by the organism.
- To immobilize the culture and to study its effect on the overall process conditions.

CONTENTS

INTRODUCTION

Judicious recycling, reprocessing and finally utilization of food processing residues offer potential of returning these by-products of agro based industries, to beneficial uses rather then their discharge to the environment which might cause detrimental environment effects. Effective utilization of food residues can occur if through biotechnological interventions these wastes can be made utilizable by a minor industry. Food industry produces large volumes of wastes, both solids and liquids; this waste pose increasing disposal and pollution (High BOD or COD) problems and represents a loss of valuable biomass and nutrients. However, despite of their pollution and hazard aspects, in many cases, food processing wastes have a good potential for conversation into useful products of higher value as by-product, or even as raw material for other industries. Organic acids are examples of such valuable by-product of the fermentation of high carbohydrate containing industrial substrates. For instance, potato-processing plants release an appreciable amount of starch in wastewater streams, additionally; potatoes, which do not fit the standard quality criterion, are discarded. They therefore could be utilized cheaply as substrate for microorganisms producing intermediate volume high value organic acids like lactic acid.

Lactic acid, an intermediate-volume especially chemical is under increasing demand in Food, Pharmaceutical and Chemical Industries and for production of Poly lactic acid polymers, which possess excellent biomedical applications. The global production of this organic acid is estimated to be 100 million pounds/yr and is expected to grow by 8.6% annually (Narayanan et. al, 2004.) Lactic acid is currently manufactured either through chemical or microbial route via fermentative mode. In India, the annual production

capacity of lactic acid is 6000 T and an estimate gap of 2300 T in supply by the year 2015 have been predicted, if the present level of production is not increased (TIFAC 2001). Wastes containing starch generated from food processing plants may be regarded as a viable option for meeting this growing demand for lactic acid, if appropriate biotechnological interventions are used. Specific sectors amongst the Indian food processing industry need to be targeted. The snack food sector have expanded significantly in the recent years, for instance the Indian snack market is currently one of the largest snack markets in the Asia – Pacific Region (valued @ $307.7 million in 2001) (http:www.stat-usa.gov/). Potato chips are by far the largest product category within snacks, and generate 85% of the total market revenue. Additionally, the market for branded potato chips has been growing rapidly at approximately 20% annually. Consequently there has been a tremendous increase in potato consumption by this sector with a current average of approximately 450 MT/day.

Discarded, off-grade potatoes account for as much as 6.7 MT/day in addition to starch (50Gms/ltr) containing effluents (up to 6000ltr/day) (World bank, 2004) in potato processing industries. Both off-grade potatoes and processing effluents can utilize appropriately as a medium for fermentative production of Lactic acid using strains of amylolytic lactic acid bacteria. Considering an approximate content of 300 kgs of starch contained in the effluent, 75Kg per day of Lactic acid may be obtain from such fermentations. The fermentative yield mentioned is noteworthy in proportion to meet the total demand of LA. No studies have however addressed the reutilization of potato waste in terms of both treatment and value addition in the above lines. A sustainable approach

for production of lactic acid would encompass utilization of starch containing agro wastes through a fermentative mode by Lactic acid bacteria. Lactic acid bacteria are among the best-studied microorganisms for human health beneficial effects and for fermentation. Important new developments have been made in the research of lactic acid bacteria in the areas of multidrug resistance, bacteriocins, quorum sensing, osmoregulation, autolysins and bacteriophages. Progress has also been made in the construction of food grade genetically modified Lactic acid bacteria. These have opened new potential applications for these microorganisms in various industries (Konings et al. 2000). The desirable characteristics of these microorganisms for industrial use are their ability to rapidly and completely ferment cheap raw materials, requiring minimal amount of nitrogenous substances. In turn providing high yields of preferred stereo specific lactic acid under conditions of low pH and high temperature, production of low amounts of cell mass and negligible amounts of other byproducts. Therefore non-dairy isolates of lactic acid bacteria capable of degrading raw starch and converting them to Lactic acid could be key to the development of a viable biotechnology based value addition process for agro wastes containing starch.

Introduction to Lactic acid

Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or hydrocarbon group; and a central carbon atom having an alcohol carbon group. Lactic acid exists in two optically active isomeric forms. Lactic acid is soluble in water and water miscible organic
solvents but insoluble in other organic solvents. It exhibits low volatility. The various reactions characteristic of an alcohol which lactic acid (or it esters or amides) may undergo are xanthation with carbon bisulphide, esterification with organic acids and dehdrogenation or oxygenation to form pyruvic acid or its derivatives.

Lactic acid is used as emulsifying agent in baking foods (stearoyl-2-lactylate, glyceryl lactostearate, glyceryl lactopalmitate). The manufacture of these emulsifiers requires heat stable lactic acid, hence only the synthetic or the heat stable fermentation grades can be used for this application (Datta, 1995; Sodegard, 1998). Technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Various textile finishing operant and acid dying of food require low cost technical grade lactic acid to compete with cheaper inorganic acid. Lactic acid is being used in many small scale applications like pH adjustment hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyd resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electropolishing baths, detergent builders. Lactic acid has many pharmaceutical and cosmetic applications and formulations in topical ointments, lotions, anti acne solutions, humectants, parenteral solutions and dialysis applications, for anti carries agent. Calcium lactate can be used for calcium deficiency therapy and as anti caries agent. Its biodegradable polymer has medical applications as sutures, orthopaedic implants, controlled drug release etc. Polymers of lactic acids are biodegradable thermoplastics. These polymers are transparent and adjusting the composition and the molecular weight can control their degradation. Their properties approach those of petroleum derived plastics. Lactic acid esters like ethyl/butyl lactate can be used as green solvents. They are high boiling, non-toxic and degradable components. Poly L-lactic acid with low degree of polymerization can help in controlled release or degradable mulch films for large-scale agricultural applications (Datta, 1995).

Synthesis of Lactic acid:

There are two methods for the syntheses of Lactic acid are chemical synthesis and fermentation of the solution containing carbohydrate. The problem with chemically synthesized acids is the racemic properties. Fermented acids can produce desired isomers like L (+) and D (-) Lactic acids (Jin et. al, 2003), which are more important in modern applications for the acids uses such as biodegradable plastics. The properties that are derived from the different forms of the isomer are very different. For instance higher optical purity of the L (+) lactate polymer leads to higher melting point and crysatllinity.

Chemical synthesis:

The commercial process of chemical synthesis is based on lactonitrile (Narayanan et. al, 2004). Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This crude lactonitrile is recovered and purified by distillation. It is then hydrolyzed to lactic acid, either by conc. HCl or by H_2SO_4 to produce corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate, which is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce Lactic acid and methanol, which is recycled.

(a)Addition of Hydrogen Cyanide

$$\underset{\text{acetaldehyde}}{CH_3CHO} + \underset{\text{hydrogen cyanide}}{HCN} \xrightarrow{\text{catalyst}} \underset{\text{lactonitrile}}{CH_3CHOHCN}$$

(b) Hydrolysis by H_2SO_4

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

Lactonitrile sulphuric acid lactic acid ammonium salt.

(c) Esterification

$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H2O

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate Lactic acid Methanol

Microbial production of lactic acid:

Though chemical synthesis produces a racemic mixture, stereo specific acid can be made by carbohydrate fermentation depending on the strain being used. It can be described by 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.

(a) Fermentation and neutralization

$C_6H_{12}O_6 + Ca(OH)_2 \xrightarrow{\text{fermentation}} (2CH_3\ CHOHCOO^-)\ Ca_2+ + 2H_2O$

Carbohydrate Calcium hydroxide Calcium lactate

(b) Hydrolysis by H2SO4

$2(CH_3CHOHCOO^-)\ Ca_2^{+} + H_2SO_4 \longrightarrow 2\ CH3CHOHCOOH + CaSO_4$

Calcium lactate Sulphuric acid Lactic acid Calcium sulphate

(c) Esterification

$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H2O

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate Lactic acid Methanol

The broth containing calcium lactate is filtered to remove cells, carbon treated, evaporated and acidified with sulfuric acid to get lactic acid and calcium sulfate. The

insoluble calcium sulfate is removed by filtration; hydrolysis, esterification, distillation and hydrolysis obtain lactic acid. 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 depending upon the microorganisms (Shuler, 2003).

Homofermentative lactate fermentation:

Homofermentative lactic acid bacteria 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.

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

Examples are *Lactobacillus lactis, Streptococci, Enterococcus faecalis.*

Heterofermentative lactate fermentation:

Heterofermentative lactic acid bacteria produce 1 molecule of lactate along with 1 molecule of ethanol and 1 molecule of carbon dioxide (or acetic acid)

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

Examples are *Leuconostoc sp., Lactobacillus brevis, and Lactobacillus fermentum*

Starch, lactic acid and Lactic acid bacteria:

Lactic acid is produced ultimately by the fermentation of glucose, however instead of glucose most plants have high contents of linearly linked glucose molecule called starch. This starch is kept in the plant organelle chloroplast and amyloplasts. Starch is a polysaccharide made of individual monosaccharide (glucose units) and is linked by $\propto$ (1-6) and $\propto$ (1-4) bonds. The polysaccharide consists of a mixture of two types of polymers namely amylose and amylopectin. Amylopectin is a highly branched chain of D (-) glucose residues, while amylose is a much more linear polymer(Hizukuri, 1996).

The Lactic acid bacteria are collectively assigned to the family Lactobacteriaceae. This group exhibits heterogeneous morphology including long and short rods as well as cocci of the streptococcus type. They have been very well characterized physiologically. Some of the features of Lactobacteriaceae are listed below. (Schlegel, 1997)

- All its members are gram positive and do not form spores (with the solitary exception of *Sporolactobacillus inulinus*)
- Most of them are non-motile.
- They all are dependent on carbohydrate for their energy supply and excrete lactic acid.
- They do not contain haemins (cytochrome, catalase)
- In spite of the absence of the haemins, the Lactobacteriaceae especially the streptococci can grow in the presence of oxygen. Thus they are anaerobes but aerotolerant.
- They require complex media containing several growth factors such as vitamins, lactoflavin, thiamine, nicotinic acid, folic acid, biotin, pantothenic acid etc.

Thus lactic acid bacteria may be regarded as metabolic cripples that have lost their ability to synthesize a no. of metabolites as a consequence of their specialization for growth on milk and other hand they have an ability that most microorganisms lack; they can utilize lactose. Lactose apparently dose not occur in the plant kingdom; it is produced, excreted and ingested in milk by mammals. The utilization of lactose by microorganisms can thus be regarded as an adaptation to ecological conditions in the mammalian digestive tract. Lactose is a disaccharide that must be hydrolyzed before it can enter the catabolic pathway for hexoses (Schlegel, 1997).

$$\text{Lactose} + H_2O \longrightarrow \text{D-Glucose} + \text{D- Galactose}$$

The hydrolyzed product, galactose after the phosphorylation by a specific galactokinase is isomerised to glucose – 1 – phosphate.

Occurrence and habitat :

The distribution of Lactic acid bacteria in nature is related to their high demand of nutrients and their type of energy generation (Schlegal, 1997).They are hardly found in air or soil. Their natural habitats are

- Milk and the places where milk is produced or processed (*Lactobacillus Lactis*, *L. bulgarics*, *L. helveticus*. *L.casei, L.fermentum. L. brevis, L. diacetalactis*)
- Intact and rotting plants (*Lactobacillus plantarum, L. delbrueckii, L. fermentum, L. brevis, Lactococcus lactis, Leuconstoc mesenteroides*)
- Intestinal tracts of mucos membrances of animals and humans (Lactobacillus acidophilus, Bifidobacterium *sp.*, *Enterococcus faecalis*, *Streptococcus salivarius*, S. *pyogenes*, *S. pneumoniae*)

Amylolotic Lactic acid bacteria

Lactic acid bacteria possessing amylases can utilize starch fermentatively for lactic acid production. Amylolytic lactic acid bacteria (ALAB) account for a substantial portion in different types of foods and are widespread among the non-dairy food environments and different geographical areas.Amylolytic lactic acid bacteria have been isolated from swine and cattle waste-corn fermentations in the USA (*Lactobacillus amylophilus* and *Lact. amylovorus*) (Nakamura and Crowell 1979; Nakamura 1981), fermented cassava roots in Congo and Niger (*Lact. plantarum* strains) (Nwankwo et al. 1989;Giraud et al. 1991), chicken crops in France (*Lactobacillus* strains) (Champ et al. 1983), fish silage in Sweden (*Leuconostoc* strains) (Lindgren and Refai 1984), fermented fish and rice food in Japan (*Lact. plantarum*) (Olympia et al. 1995), maize sourdough (*Lact. fermentum*)(Agati et al.1998) and from cassava sourdough(*Lact. manihotivorans)*(Morlon-Guyot et al. 1998). Lactic acid production from different types of starches by *Lact. amylophilus* and *Lact. amylovorus* (Zhang and Cheyran 1991; Mercier et al. 1992; Yumoto and Ikeda 1995; Xiaodong et al. 1997) have been reported. Most of the known ALAB are (*Lact. Amylophilus, Lact. Manihotivorans* and *Lact.amylovorous)* capable of producing L (+) lactic acid have been reported (Nakamura 1981, Morlon-Guyotet al. 1998, Naveena et al, 2004). A substantial number of strains of ALAB have been isolated recently, from Nigerian traditional fermented foods (fufu, burukutu, ogi-baba and kunu-zakki) with the aim of selecting efficient amylase-producing strains. Nine isolates have been characterized on the basis of their phenotypic and taxo-molecular characteristics. *Lactobacillus fermentum*, was found to be the most efficient amylase producer. The strain differed with lactic caid production with respect to *L. fermentum* OgiE1 and Mw2 earlier isolated from Benin maize sourdough and reported as efficient ALAB's.

Laboratory scale studies with currently isolated ALAB strains (Mueller etal, 1990) have shown that saccharification takes place during the fermentation process eliminating the need for complete hydrolysis of the starch to glucose prior to fermentation. The cost savings of this alternative are substantial since it eliminates the energy input, separate reactor tank, time, and enzyme associated with the typical pre-fermentation saccharification step. The only pre-treatments that may be required are gelatinization and enzyme-thinning of the starch to overcome viscosity problems associated with high starch concentrations and to make the starch more rapidly degradable. The fermentation process could then be optimized for temperature, substrate level, nitrogen source and level, mineral level, B-vitamins, volatile fatty acids, pH, and buffer source. Furthermore, both the rate of reaction and the final level of lactic acid obtained in the optimized liquefied starch processes have been shown to be similar to that obtained with standard lactic acid producing *L. delbrueckii* (using glucose as the substrate).Few studies in India have attempted to isolate and evaluate the starch degrading potential of amylolotic lactic acid bacteria. This work emphasizes on the isolation of ALAB's and on evaluation of their ability to degrade starch with consequent production of lactic acid.

MATERIALS AND METHODS

ISOLATION OF AMYLOLTIC LACTIC ACID BACTERIA (ALAB):

Fermented dough, Fermented batters and Green chutney's were used for isolation of amylotic lactic acid bacteria. From each sample 5-10 g amounts were taken and were inoculated in 100 ml of MRS broth which was incubated at 28^0c for 24 hrs. Aliquots were diluted in 0.85% sterile saline and spread on MRS agar plates, which were incubated for 24-48 hr at 28^0C, in order to isolate the lactobacillus species.

- ✓ Then colonies with different morphologies were streaked on to separate plates of MRS agar and starch agar to identify their starch utilizing potential.
- ✓ The colonies, which were showing zone of clearance in starch agar plates, were maintained on to MRS agar plate s as well as on to MRS agar slants.
- ✓ Morphological characteristics of the colonies that exhibited starch degradation were done via Gram's staining.

Standard strains:

In addition to above isolates a freeze-dried culture of *Lactobacillus plantarum* MTCC 2621 was procured from MTCC at institute of microbial technology (IMT), Chandigarh. All isolates were preserved at-80^{0}C in 40% glycerol throughout the course of the study.

QUALITATIVE STARCH HYDROLYSIS TEST FOR MICROORGANISM:

Different isolates were streaked on to individual MRS agar plates and were incubated at 300C. Then from each plate isolated colonies were picked up and streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 30^{0}C for 24-48 hrs., individual plates were flooded with Gram's iodine (Gram's iodine- 0.15% iodine crystals added to 1.5% potassium iodide solution. Stored at room temperature.) to produce a deep blue colored starch-iodine complex. If a strain is amylolytic then it starts hydrolyzing the starch present in the plate nearby its growth and in the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain. The zone of decolorization becomes visible within few seconds of addition of I_2-KI solution and removing excess of the solution. Along with these isolate the standard strain was also subjected to this test.

QUANTITATIVE STARCH HYDROLYSIS TEST FOR ISOLATED MICROORANISMS:

Only those isolates (a total of 4) that give a positive result in qualitative starch hydrolysis test were subjected to this quantitative test. For this, individual isolates were grown on the respective media broth for 24 hrs and then an aliquot of 2 ml was withdrawn at regular intervals of 4 hrs and starch degradation profile was established. For this 1ml of broth was centrifuged to pellet out bacterial growth. Then the supernatant in each case was diluted 100 times with distilled water. To 10 ml of this 1 ml of Gram's iodine was added in a test tube. The mixture was vortexed and the absorbance of the resultant blue colored complex was measure d at 585 nm with a spectrometer. The concentration of the residual starch in each case was worked out from a standard curve.

LACTIC ACID PRODUCTION AND ESTIMATION:

Then the supernatant was transferred the isolates that hydrolyzed starch as was evident from qualitative starch hydrolysis test were tested for production of lactic acid. The lactic acid produced was estimated by titration and via a standard kit, which is specific to the presence of L-Lactic acid.

For estimation of lactic acid fermented broth was taken in falcon tubes and centrifuged at 10000 rpm for 10 minutes to pellet out the bacterial growth. Then the supernatant was transferred to a beaker and the solution was heated to 80^{0}C. To it 5% $Ca(OH)_2$ was added drop wise till the pH becomes 7. Next the broth was filtered using a filter paper and the filtrate was discarded. The precipitates on filter paper were dissolved in a conical flask using minimal volume of 0.1 N HCl. After dissolution of the precipitate, the solution is titrated against 0.1N NaOH using phenolphthalein as indicator.

Standardization of NaOH was done using 0.1N oxalic acid, for which 10 l of 0.1N NaOH was taken in a conical flask and titrated against 0.1n standard oxalic acid using phenolphthalein as indicator.

[calculation: let us suppose that the strength of the NaOH that was used for titration was xN and that y ml of it was consumed by 5 ml of the lactic acid that was produced after 2folds of dilution. then the gram equivalent of NaOH that was used was (x*y)/1000. since reaction takes place in equivalent amounts the gram equivalent wt. of lactic acid is 90gms., the amount of lactic acid present in the titrand was (x*y)/1000 grams. This amount of lactic acid comes from z ml of fermentation broth so the strength of lactic acid produced was (x*y*90*1000/ (1000*z) g/l.]

Identification of predominant bacterial isolate(s)

Gram staining: **Gram staining was performed to study the Gram character of the isolated strains from total aerobic count.**

- Cell morphology: **The morphology of the cells was observed under compound microscope in 40x.**
- Spore formation: **The cells were tested for the formation of spores under microscope only.**
- Catalase activity: **The growth from TSA slant was used for Catalase test on glass slide and illuminated properly to observed production of gas bubbles.**
- Glucose fermentation: **The cells were tested for the fermentation of glucose by adding a small suspension of O/N grown culture of the cells from TSA slant.**
- Growth at different temperatures: **The growth of the cells from TSA slant was observed at four temperatures viz. 15, 30 45, 50^{0}C.**
- Growth in a medium with NaCl (%): **The growth of the cells from TSA slant was observed at NaCl concentration of 4 & 6.5.**
- Gelatin Liquefaction: **For this agar medium plate supplemented with 0.4% gelatin was prepared. Cells were streaked on it and cultured overnight. Then it was poured with 15% $HgCl_2$ in 20% vol/vol conc. HCl.**
- Milk curdle: **100µl of O/N grown culture of cells from TSA slant was inoculated in 10 ml of milk and kept at ambient temperature to check curdling of milk.**

- pH optimum: **The growth of cells at different pH, was observed. The pH, range was 6.5, 5.8-6.5**

The results so obtained were used to identify the isolates using Bergeys manual of determinative bacteriology.

GROWTH KINETICS of STARCH DEGRADING, LACTIC ACID PRODUCING ISOLATE(S) IN NUTRIENT MEDIUM:

The strain(s) of *Lactobacillus* 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 isolated colony was picked up from the plate and was inoculated in 10ml of sterile nutrient broth in a test tube to grow it overnight (20hrs) in order to generate a growing and activated inoculum. Form this 1 ml of the inoculum i.e. 1% culture was transferred to 100 ml of sterile nutrient medium and it was incubated at 30^0C at 120 rpm for 96 hrs. To work out the growth profile of the organism, 2ml of aliquots were withdrawn from the flask at a regular interval of 4 hrs. in sterile eppendorf's . These eppendorf's were vortexed and the absorbance was taken against sterile medium at 590 nm using a spectrophotometer.

RESIDUAL REDUCING STARCH ESTIMATION (Miller, 1959):

The residual reducing sugar content was also estimated at regular intervals of 4 hr spectrophotometrically using DNS method. The 0-hr reading corresponds to the uninoculated medium.

This method tests for the presence of the free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde or ketone group present in the sugar. Simultaneously, 3, 5-di nitro salicylic acid is reduced to 3-amino, 5- nitro salicylic acid under alkaline conditions:

Aldehyde group $\longrightarrow$ carboxyl group

3, 5- dinitrosalicylic acid reduction 3-amino, 5- nitro salicylic acid

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

Materials required: 1% DNS Reagent-

Took 10g of dinitrosalicylic acid, 0.5g of sodium sulfite, 10g of sodium hydroxide, and 2g of phenol and made up the volume to 1 liter.40% sodium potassium tartarate solution. Standards – glucose 10g/lt stock solution, for standard curve 0.5, 1, 2, 3, 4, 5 g/l solutions of glucose were made. Sample –10 times diluted fermentation broth.

Method: 3 ml of DNS reagent was added to 3 ml of the samples taken in capped test tubes. The mixture was heated at 90^0C for 15 min. to develop the red brown color. 1 ml of a 40% potassium sodium tartarate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded with a spectrometer at 575nm.

<u>Optimization of temperature and pH for the production of lactic acid:</u>

The optimization was carried out for duration of 96 hrs.Both for temperature and pH optimization the experiments were carried out in triplicates. For temperature optimization the three standards temperatures used were 28^0C, 34^0C and 37^0C while for pH optimization, the three standards used were 4, 6 and 8. 100 ml of sterile MRS medium was used for the experiment. The respective pH's were adjusted with conc. NaOH (10N) and conc. HCl (16N). For temperature optimization, the initial pH was kept at 6 while for pH optimization the temperature was kept constant at 28^0C (incubation temperature). Mediums were inoculated with 1 ml of overnight grown *Lactobacillus* isolate (1% inoculum level) in each case. Then from each culture lactic acid was estimated titrimetrically at a regular interval of 24 hr. During the course of this experiment pH control was not automated.

Yield coefficient for starch hydrolysis:

Based on the hydrolysis of starch, yield coefficient of each of the microorganisms was calculated. For this weight of each of these eppendorf's which contained the bacterial pellets was taken after incubating the eppendorf's at 100^0C for 12 hrs (dry wt.) When the wt. of the empty eppendorf's was subtracted from this wt. respectively, the amount of biomass produced for 2 ml was calculated. Consequently the biomass produced per liter was calculated. From the amount of residual starch the amount of starch that has hydrolyzed after 12 hrs of growth was found out by subtracting residua starch content from the initial starch content (obtained from the uninoculated medium). The amount of starch hydrolyzed per liter of the nutrient broth when divided by the amount of biomass produced per liter of the broth gives the yield coefficient with respect to starch hydrolysis (Shular , 2003).

DETERMINATION OF AMYLASE ACTIVITY EXHIBITED BY THE ORGANISM:

An aliquot of 2ml of the culture was withdrawn at an interval of 4 hrs for 96 hrs. from this 1.5 ml of culture was taken aseptically in sterilized eppendorf's and was pelleted down to get the supernatant and this supernatant was proceeded with for the estimation of amylase activity.

Method: For determining activity 1ml of crude enzyme was taken and was added in a mixture of 1ml of standard 1% starch solution (1% w/v starch solution- added 1g of starch in 100ml of distilled water and to be stored at 4^0C.) and 0.1 ml of citrate buffer (Citrate buffer-Took 50ml of 0.05 M/liter citric acid in a volumetric flask and made up the volume by using 0.05 M/ liter tri-sodium citrate to get citrate buffer with pH4.5..Prepared citrate buffer was stored at 4^0C.). This mixture was vortexed and kept in a water bath at 60^0C for 60 minutes. After incubation the stand was removed and reaction was stopped by keeping the reaction tubes in boiling water bath at 100^0C for 2 minutes. The mixture was brought to the room temperature and 3 ml of DNS reagent was added to it and the mixture was vortexed and capped and kept in a pre-heated water bath at 90^0C for 15 minutes. This mixture was cooled to room temperature and absorbance was taken at 575nm.

L-Lactic acid content determination by analytical kit:

An analytical kit (Randox laboratories, UK) was used for the estimation of the L-Lactate content produced in the fermentation broth by *Lactobacillus* isolate. The kit relies on UV spectrophotometery and uses NAD+ linked l-Lactate dehydrogenase assay. The assay was carried out after 96 hrs of incubation of the potato meal with increasing concentration of potato in medium and for the rice meal used for production of lactic acid. The fermentation broth used here was also used to determine the lactic acid by conventional titrimetric method. Comparison of both the values will give us the insight to what was the actual percentage of L-Lactic acid in the broth along with other acids which was detected in during titrimetric method, since the kit is sensitive to only L form of the lactic acid.

Method: 2ml aliquots were withdrawn from the fermentation broth at regular intervals in sterile eppendorf's. These eppendorf's were centrifuged to pellet down the suspended cells in the culture and the clear supernatant was taken in autoclaved eppendorf's. This supernatant was than used to find out the concentration of L-Lactic acid in the culture by following the standard protocol as described by the manufacturer.

USAGE OF ENTRAPPED CELLS FOR STUDYING THE VARIOUS ASPECTS OF STARCH DEGRADATION:

From an overnight growing cell culture of *Lactobacillus* isolate, 10ml of culture was transferred to sterile falcon tube and centrifuged to pellet down the cells. Then these pelletized cells were re-suspended in 4ml of fresh sterile nutrient medium and this culture was used as the entrapped cell culture in the dialysis sac.

Method: A 5cms long dialysis sac was autoclaved and filled with the re-suspended culture, the sac was closed with sterile clips on both sides and dipped partially in the media, sealed from top and incubated at 30^0C at 80 rpm, so as to ensure the medium entry in and out of the dialysis sac. Aliquots were withdrawn from this assembly, and from outside medium at 24 h and 48 h of incubation to check for the amount of starch degradation, amylase activity and L-lactic acid concentration. The whole set up of assembly and withdrawing of samples was done in laminar air flow so as to avoid any contamination. The viability of cells was checked by diluting them and plating them on MRS agar plates.

Starch hydrolysability:

For this the nutrient medium used for the purpose was supplemented with 1% soluble starch, an aliquot was taken from uninoculated medium which give us the initial value of the starch present and then the aliquots withdrawn were tested for the concentration of the residual starch after 24 and 48 hours of incubation.

Amylase activity:

The amylase activity in the medium was estimated by following protocol described earlier. An aliquot of 1 ml of the supernatant of the cell culture was taken and the activity was estimated. Later the activity was also estimated inside the sac by taking an aliquot of 2 ml from the sac and centrifuged so as to obtain 1 ml of supernatant which was used for estimation after 48 hrs.

L-lactic acid production:

The lactic acid productivity was estimated in the medium at an interval of 24 and 48 hrs. following incubation by using the analytical kit. For this, 1 ml supernatant was taken and was subjected to estimation as described by the manufacturer.

Table 1: IDENTIFICATION and CHARACTERISIZATION of *Lactobacillus plantarum*:

Characteristics	*Lactobacillus plantarum* ATCC	*Lactobacillus plantarum* MTCC2621
I. Morphological and culture characteristics		
• Color	Cream-colored – beige	White, Cream-color – beige
• Shape	Isolated little sticks	Isolated little sticks
• Gram's reaction	Yes	Yes
• Development on solid medium	Smooth round colonies	Smooth round colonies
• Development on liquid medium	Uniform turbidity, sediment formation	Uniform turbidity, sediment formation
II. Physiological characteristics		
• pH	5-7	4.5-6.5
• temperature(^{0}C)	30-37	28-30
III. Biochemical Characteristics		
Acid from:		
• Ribose	Yes	Yes
• Galactose	Yes	Yes
• D-glucose	Yes	Yes
• D-fructose	Yes	Yes
• D-manose	No	No
• Ramnose	Yes	Yes
• Manitol	Yes	Yes
• Sorbitol	Yes	Yes
• Maltose	Yes	Yes
• Lactose	Yes	Yes
• Sucrose	Yes	Yes
Fermenting type	Homofermentative	Homofermentative
Enzyme production:		
• Catalase	No	No
• Amylase	No	No

Lactic acid production of the isolated micro-organisms and the standard strain:

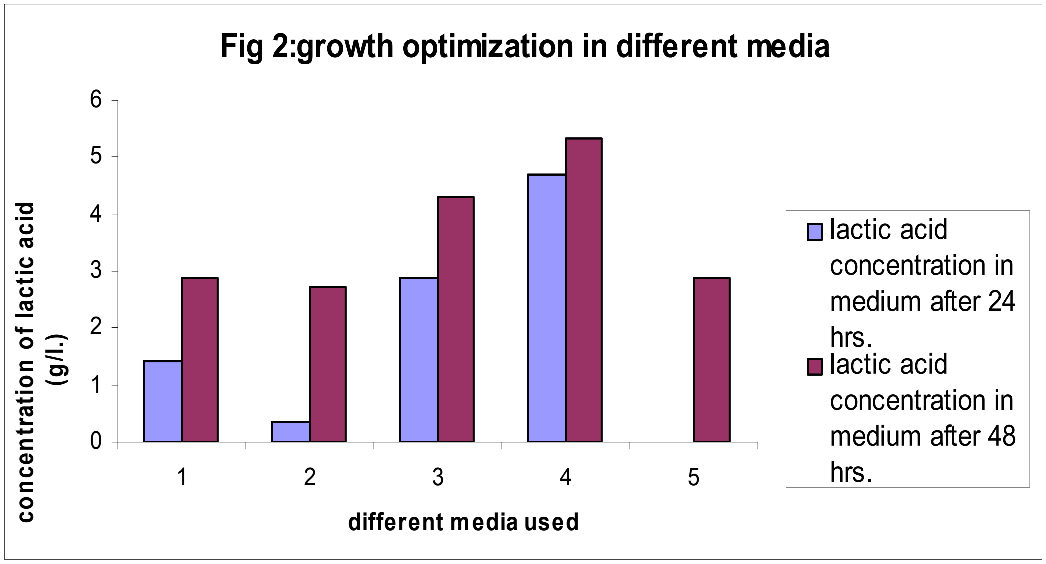
Table: 2

Name	Type of organism	Morphology of colony	Starch hydrolysability	Lactic acid productivity
Isolate A	Bacteria	White, round, smooth	+	-
Isolate B	Bacteria	Cream, round, rough	+	-
Isolate C	Bacteria	Small round and white colored	+	-
Isolate S	Bacteria	Yellowish, slimy, round	+	-
Lactobacillus plantarum MTCC2621	Bacteria	White, Cream-color – beige	+	+

(+ relates to positive & - relates to negative)

Only one isolate conformed to the desired attributes were identified as *Lactobacillus plantarum* were used for all experiments carried out further.

Growth optimization in different media:



(Here 1=nutrient medium, 2=MRS, 3= modified MRS, 4=Potato meal, 5= Rice meal)

Fig 2, shows that potato meal has the potential to furnish micro organisms with carbon source in the form of starch and sugar. The advantage of such a feedstock, in comparison to other media is that it contains all the necessary nutrients for the organism's survival and metabolite production and is much cheaper in comparison to MRS for instance. It can also be seen that nutrient media also act as a promising media since being minimal media still it provides a substantial growth of organisms. Thus, the discarded potato-waste materials as substrate offers advantages of minimal processing and supplementation besides being economical they are quite promising for production of lactic acid, in addition to being both environmentally and economically favorable.

GROWTH KINETICS IN NUTRIENT BROTH SUPPLEMENTED WITH 1% STARCH:

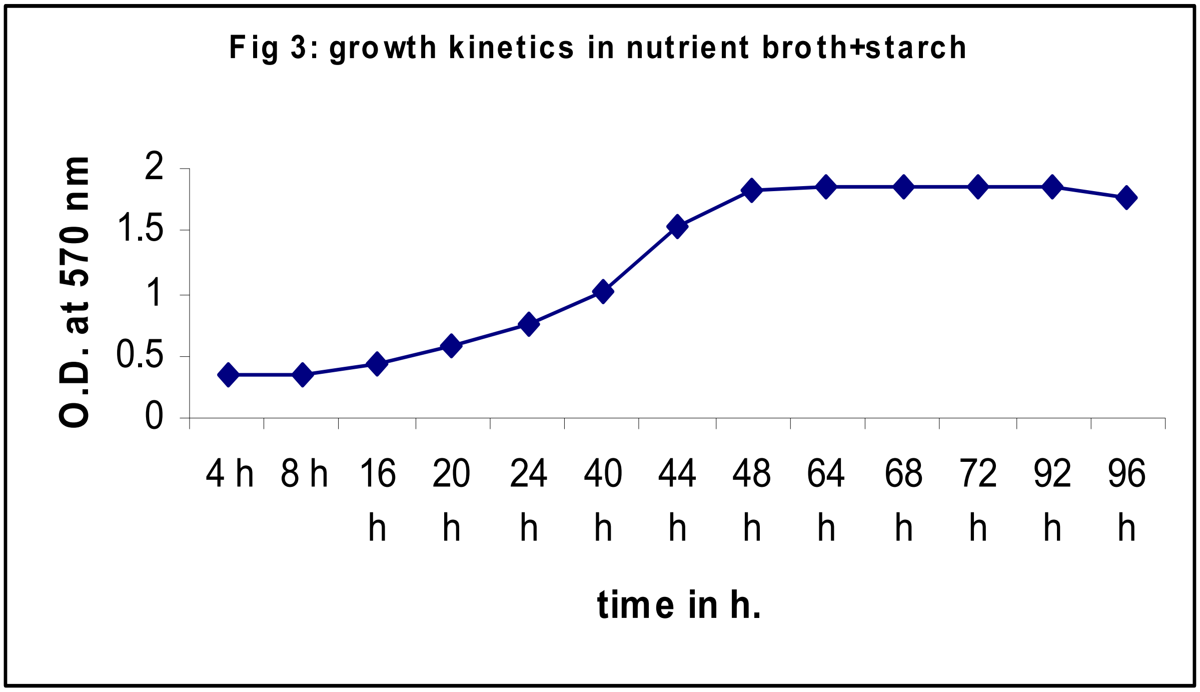


Fig 3 shows the growth kinetics of *Lactobacillus plantarum* The lag phase starts right after the inoculation and rests for 12 hrs. In this phase the bacteria adapts to the nutrient medium. After the lag phase is over the maximum division phase is observed in exponential or logarithmic phase of growth. The stationary phase, which continues till 42 hrs begins after the log phase; here the cells in the culture no longer reproduces because the concentrations of the nutrients vastly decreases during the exponential growth phase. The transition of the stationary phase from the exponential phase is gradual because the growth rate of the cells in the culture declines even before the substrate has been consumed significantly. After sufficient time has elapsed, the culture shows a decline in growth in the death phase, where the bacterial cells may be because of scarcity of the nutrients, accumulation of acid and other metabolic waste products. Approximately the phase starts at 92 h after the incubation and continues thereafter.

STARCH DEGRADATION PROFILE ON NUTRINT MEDIUM:

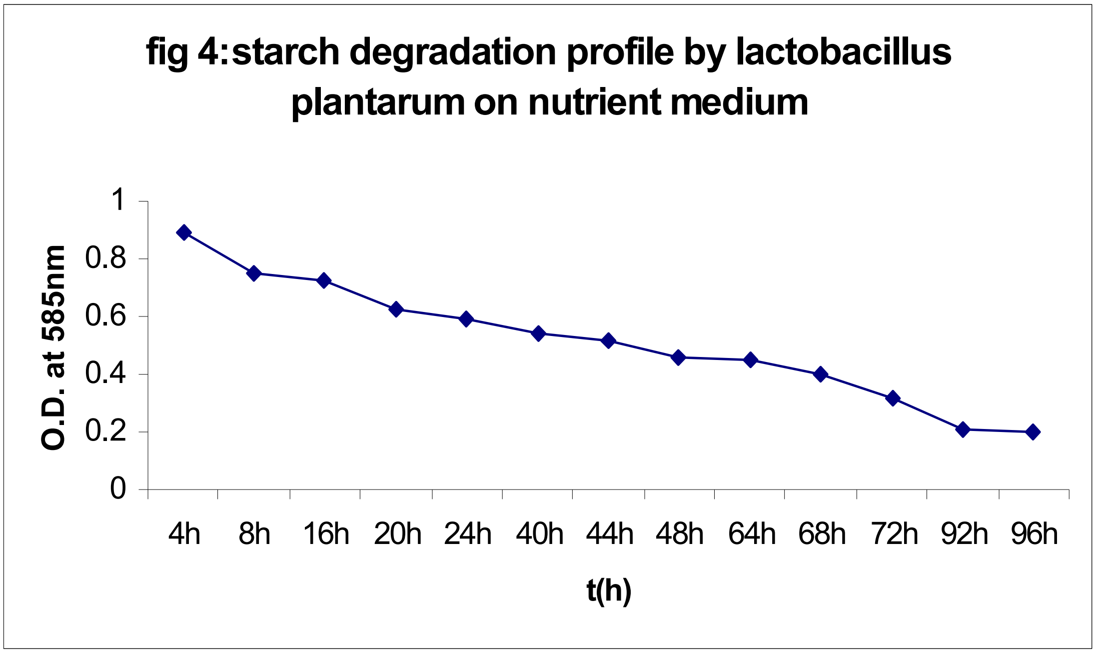


Fig 4 shows the pattern of the degradation of starch in the medium; the rate of degradation is faster in the initial stages and slows down as the culture proceeds to the stationary phase. The rate of degradation is very high initially and subsequently slows down and becomes constant. This could be due to the rapid uptake of starch by the cells during the initial cell growth period during which the biomass increases considerably but in later stages the biomass tends to remain almost constant whereas the concentration of lactic acid increased with time with slow decline in the cell biomass may be due to the cell lysis occurring in the culture.

QUATIFICATION OF THE AMYLASE ACTIVITY BY *Lactobacillus plantarum* IN NUTRIENT MEDIUM:

Fig :5

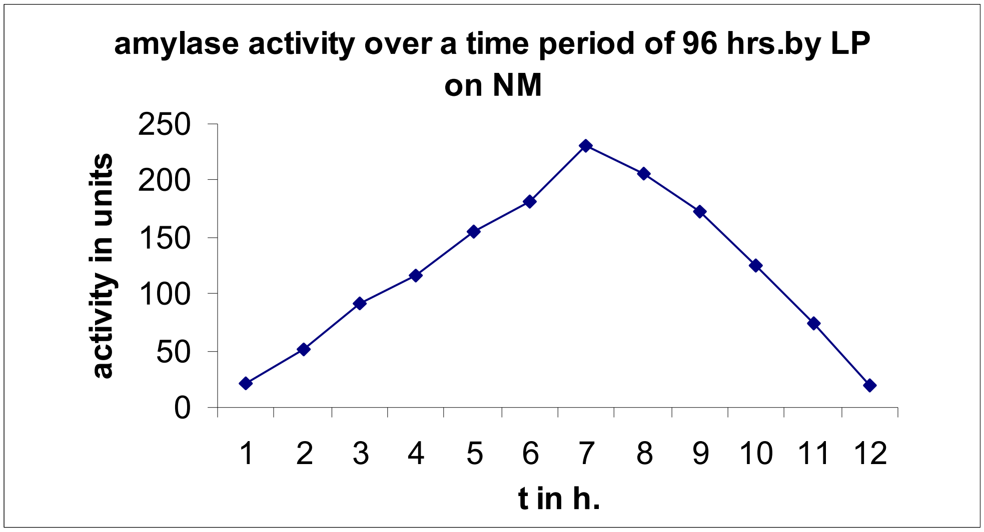


Table 3: Amylase activity in different media at the time interval of 24 hrs. and 48 hrs.

Media T (h.)	Amylase activity in : Nutrient media	Amylase activity in MRS	Amylase activity in Modified MRS	Amylase activity in Potato meal	Amylase activity in Rice meal
24h	27.7	8.6	17.9	29.2	8.6
48h	9.6	6.2	6.21	9.6	6.2

(One enzyme unit is defined as the amount of enzyme that liberates1µmole of reducing sugars per minute)

From Table 3, it is clear that the amylase activity of the culture in nutrient broth shows a sharp linear increase after 4 hrs of incubation till it reaches to late log phase of the growth whereas as soon as the culture enters the stationary phase the cells show a linear decrease in the amylase activity where as this phase exhibits the maximum production of lactic acid, which could be understood as, that, the starch degradation and amylase activity is directly proportional to each other but as soon as there is accumulation of enough free glucose/reducing sugar molecules the lactic acid production picks up and by this time the culture reaches to late log phase where the biomass remains almost constant and in later phase the cell lyses occur, dropping the amylase activity to lower levels

TEMPERATURE OPTIMIZATION FOR LACTIC ACID PRODUCTION BY *Lactobacillus plantarum* AT pH 6

Table 4:

Temperature	Lactic acid* produced after- **24 hours**	Lactic acid* produced after- **48 hours**	Lactic acid* produced after- **72 hours**	Lactic acid* produced after- **96 hours**
28^0C	1.58	1.46	1.42	1.48
34^0C	0.98	1.12	0.9	0.9
37^0C	0.8	0.85	0.8	0.85

*Lactic acid production in g/l

pH OPTIMIZATION FOR LACTIC ACID PRODUCTION BY *Lactobacillus plantarum* AT TEMPERATURE 28^0C

Table 5:

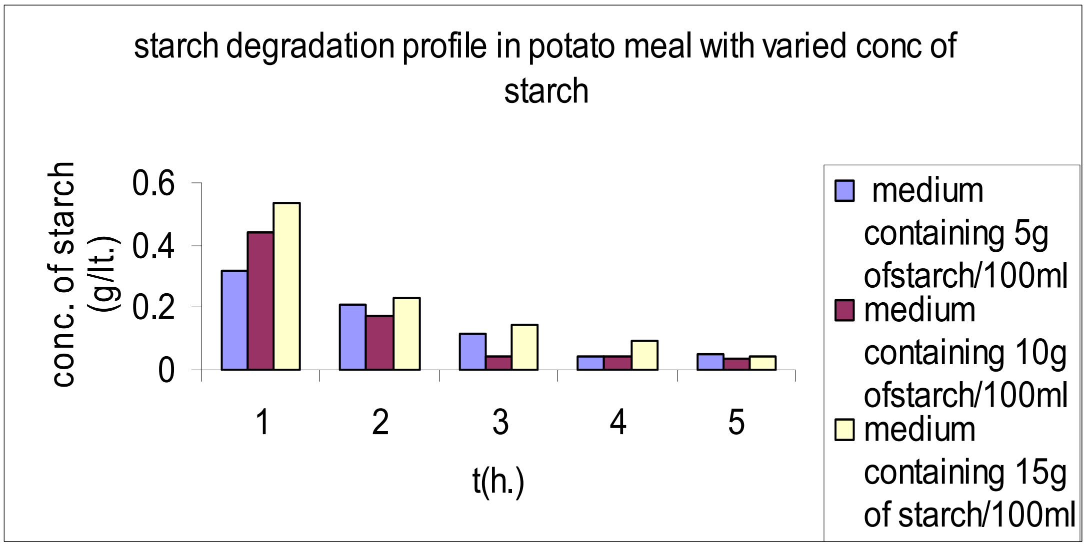
pH	Lactic acid* produced after- **24 hours**	Lactic acid* produced after- **48 hours**	Lactic acid* produced after **72 hours**	Lactic acid* produced after- **96 hours**
4	0.56	0.48	0.48	0.46
6	1.58	1.46	1.42	1.48
8	0.29	0.42	0.9	0.98

*Lactic acid production in g/l.

As evident from the above tables, the suitable temperature and pH for lactic acid production was 28^0C and 6 respectively. For example lactic acid concentration at 28^0C (1.58 g/l) after 24 hrs is over 60% more than the same at 34^0C (0.98g/l). Again lactic acid concentration at pH 6 after 24 hrs (.58 g/l) is almost thrice the amount of the same at pH 4(0.56g/l). The general trend of lactic acid production except in the case of pH 8 was that the production of the acid is completed with in first 24 hrs. After 24 h there is negligible change in the extra cellular concentration of lactic acid. This may be due to the completion of the exponential phase as well as stationary phase and starting of the death phase (as in inferred from the growth kinetics of Lactobacillus *plantarum*) in which the bacteria shows no metabolic activity in general. The case for the pH 8 is significantly different from all the other cases and the lactic acid concentration increases from as little a value of 0.28 g/l at 24 h to a value of 0.9 g/l at 72 h. the possible case may be the decreases in the pH due to acid production; the initial pH in this case was 8, which is not at all a favorable ph for the strain. Thus the bacteria were sluggish on the production of lactic acid. When the pH started decreasing and started coming to a value close to more favorable 6 the rate of metabolic activity increases markedly and the lactic acid concentration shoots up.

EFFECT OF INTIAL STARCH CONCENTRATION ON STARCH DEGRADATION PROFILE:

Fig: 6



(1= 0hr, 2=24hr, 2=48hr, 4=72hr, 5= 96hr)

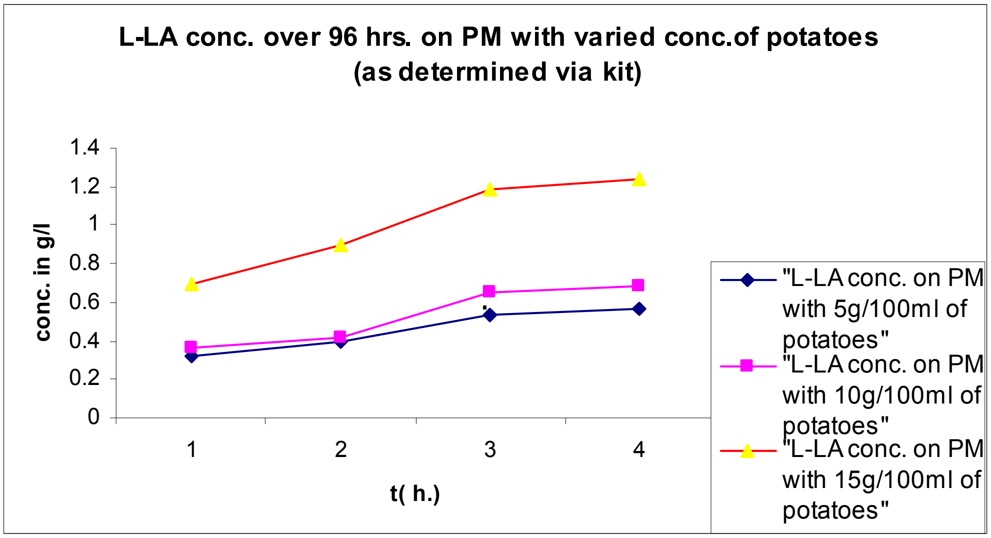
As evident from Fig 6, the starch degradation rate is significantly high in the first 24 hrs and we found out that the increasing concentration of nutrients in the medium does not inhibit the starch hydrolysability and amylase production in any way and shows a linear pattern of degradation. It can be said that there is a constant trend in starch degradation profile by organism in all concentrations where as it can be seen that the residual starch conc./amount is almost same for all the three concentrations.

LACTIC ACID PRODUCTION BY *Lactobacillus plantarum* IN POTATO MEAL:

The effect of varying the starch concentration in the medium between 50-150 g/lt. (dry wt.) on the production of lactic acid production has been shown in the figure above. Lactic acid concentration seems to be high in initial few hours with high concentration of nutrients but as the process proceeds it can be seen that there is maximum production of lactic acid in low concentrations which clearly indicates that the high concentrations of nutrient leads to formation of certain metabolites which act as inhibitory compounds or it could be quite possible that the substrate concentration itself act as inhibitory to the production of desired metabolite.

L-LACTIC ACID CONCENTRATION IN POTATO MEAL:

Fig : 7



(1=24hr, 2=48hr, 3=72hr, 4= 96hr)

As evident from above the Figure, concentration of the L- lactic acid as determined by using the analytical kit shows a considerable increase in L-lactic acid concentration with the increase of the nutrient level in the medium from 5-15g/100ml in the medium which is quite contradictory to the conclusion we can make out from the previous result. But as the metabolite to our interest is the L-lactic acid, it can be said that with the increase in the nutrients in the medium there is no inhibitory effect to the cells for the production of amylase but the other metabolites i.e. other organic acid's production is inhibited which is detected in titrimetric estimation which is non specific to L-lactic acid. It can also be observed that the production of lactic acid increases from late log to next 24 hrs during the stationary phase. This fact is of great interest to us from the commercial application point of view of this process.

STARCH DEGRADATION PROFILE IN NUTRIENT MEDIUM USING A DIALYSIS SAC:

Fig : 8(a)

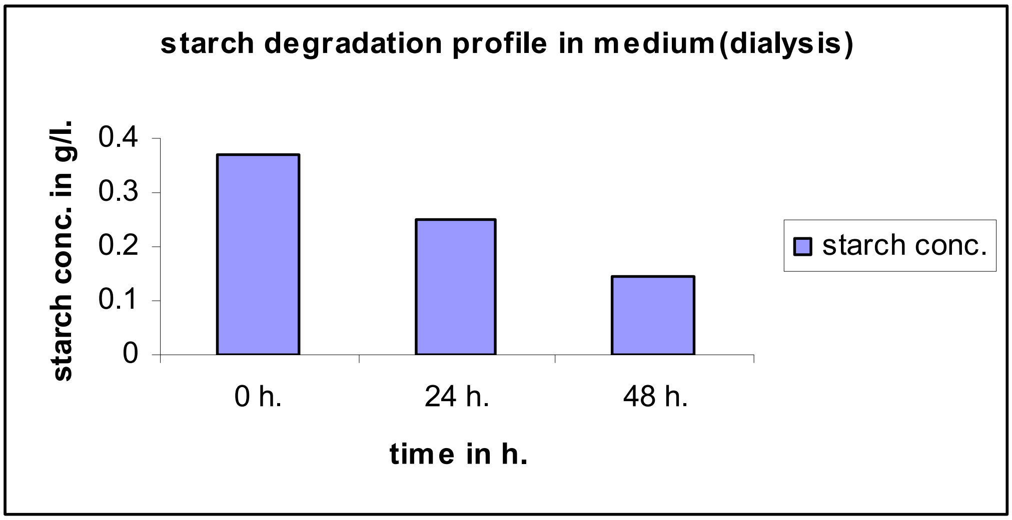
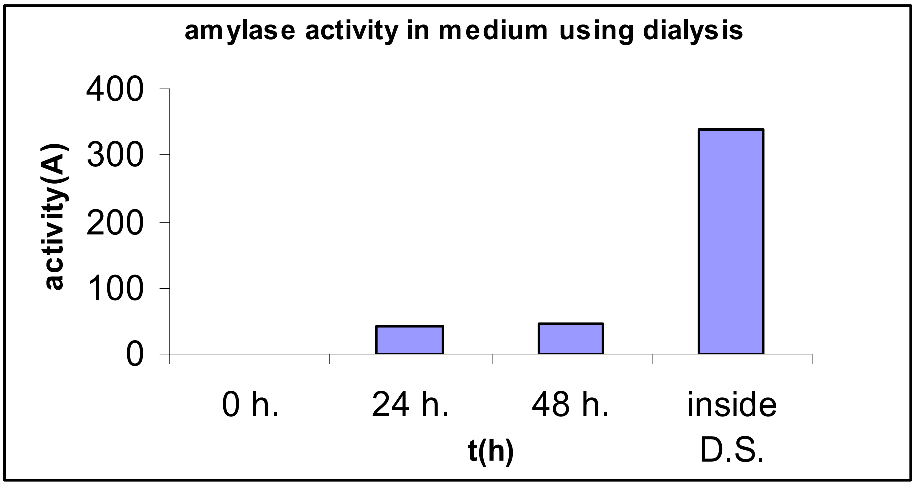


Fig 8 shows the pattern of starch hydrolysis outside the dialysis sac by the culture in nutrient media, the trend is almost similar as shown by the free cell culture; in fact it was observed that the rate of removal of starch by the entrapped cells is faster and more efficient than free cell culture. Further, the cells in the culture sustain for a longer duration (than free cells) inside the sac. This process has an additional advantage of providing an efficient and lowercost intensive process for down-streaming. This process could prove to be a promising modification in the whole process and would improve the applicability of the process.

COMPARISON OF AMYLASE ACTIVITY INSIDE AND OUTSIDE THE DIALYSIS SAC:

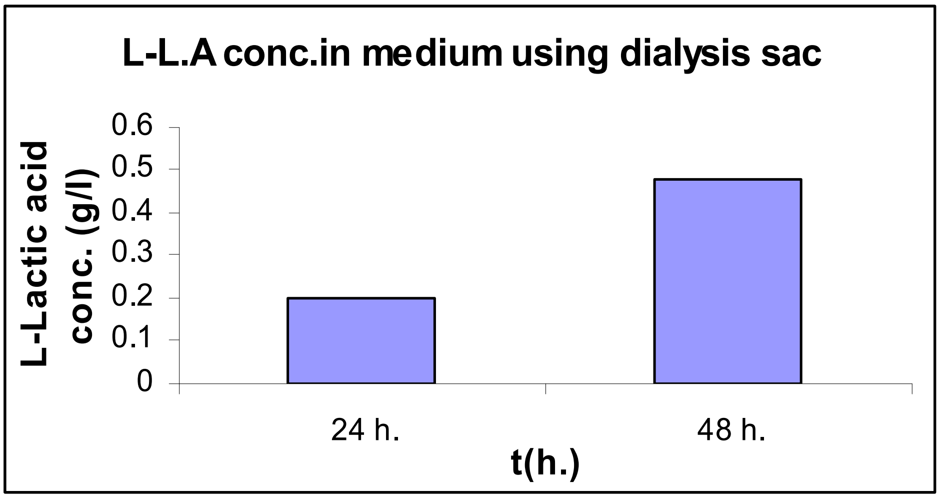
Fig 8 (b)



The extra cellular amylase activity was determined inside the dialysis sac was found to be 6 folds higher(Fig 8b), than what it was present outside the dialysis sac, which indicated that the majority of the enzyme remains entrapped in the sac and is of size greater that 7 kDa. This result also indicates towards the increased efficiency of the cells when entrapped it can thus be concluded that this process will ease the problems associated with the down streaming process which accounts for one of the major percentage of the cost intensive tasks. It was also seen that this culture maintain high amylase activity even after 48 hrs inside the sac which ensures its reusability.

L-LACTIC ACID CONCENTATION IN MEDIUM WITH CELLS ENTRAPPED IN DIALYSIS SAC:

Fig 8(c)



A constant increase in the L-Lactic acid concentration was observed in the media (Fig 8c) outside the sac where the cells as well as the enzyme are entrapped inside the dialysis sac which ensures the promising applicability of the process and indicates a useful modification in the process to enhance the productivity and suitability of the overall process.

In conclusion, our results indicate that the *Lactobacillus plantarum* isolated from fermented batter possess starch degradation capability through production of extracellular amylase, with consequent production of Lactic acid. Although the yield of Lactic acid is not substantial, with free cells. This is in concurrence with observations of other workers elsewhere, who isolated *L.plantarum* and evaluated for lactic acid production. The strain shows maximum production and starch degradation at temperatures of 28^0C and pH of 6. Dialysis entrapped cultures could aid in easy recovery as well as longer use of free cells. From initial observations the isolated strain shows promise in production of Lactic acid from starch and therefore may be used in conversion of starch to Lactic acid from agro processing waste containing starch. However in order to achieve industrial scale value addition process further experiments are warranted.

1 Composition of MRS medium

• Peptone	10.0g
• Beef extract	10.0g
• Yeast extracts	5.0g
• Glucose	20.0g
• Tween $_{80}$	1.0ml
• Na_2HPO_4	2.0g
• Sodium acetate	5.0g
• Triammonium citrate	2.0g
• $MgSO_4.7H_2O$	0.2g
• $MnSO_4.4H_2O$	0.2g
• Agar	15.0g
• Distilled water	1000ml
• pH	6.2-6.6

2 Composition of Nutrient medium

• Soluble starch	10.0g
• NaCl	5.0g
• Bactotrytone	2.0g
• Distilled water	1000ml
• pH	7.2

3 Composition of Minimal medium

• Starch	5.0g
• 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

4 Composition of Potato meal

• Pre –boiled and mashed potatoes	5-15g
• Distilled water	100ml
• pH	6.5-7

5 Composition of Modified MRS medium

• Peptone	10.0g
• Beef extract	10.0g
• Yeast extracts	5.0g
• Starch	20.0g
• Tween $_{80}$	1.0ml
• Na_2HPO_4	2.0g
• Sodium acetate	5.0g
• Triammonium citrate	2.0g
• $MgSO_4.7H_2O$	0.2g
• $MnSO_4.4H_2O$	0.2g
• Agar	15.0g
• Distilled water	1000ml
• pH	6.2-6.6

6 Composition of Rice meal

• Pre boiled rice	10.0g
• Distilled water	100ml
• pH	6.5-7

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