Lactic acid production from food processing wastes

A
DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree of

Masters of Science
(Biotechnology)

BY
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CERTIFICATE

This is to certify that the dissertation entitled **'Lactic acid production from food processing wastes'** submitted by Rajarshi Biswas (Reg. no 3030118) 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 an authentic record of Student's own work carried out by him 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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Contents

Abstract

A *Lactobacillus plantarum* culture (*Lactobacillus plantarum* MTCC2621) was characterized for its ability to degrade starch and consecutively produce lactic acid. The optimum starting pH and temperature were found to be pH 6 and 28^0C respectively. Under these conditions, the bacteria produced 1.58g/l of lactic acid after one day of growth. This lactic acid comes from the hydrolysis of starch, which was mediated by extracellular amylases. When the pH was controlled the lactic acid production was significantly increased yielding a value of 1.85g/l at a constantly maintained pH 6 and incubation temperature of 28^0C after a day of growth. L-lactate content of the produced lactic was quantified using a biochemical kit and it was found that the strain was actually producing a DL mixture of lactic acid with D:L ratio closely equals to 4:1.

In the next phase of the experiments feasibility studies was carried out with a food processing effluent (a potato wash effluent, obtained from a snack food company). This study indicated that the strain could survive and grow, break down starch and produce lactic acid from the effluent.

Introduction

Food processing wastes may be regarded non-product flows of raw materials whose economic values are less than the cost of collection and recovery for reuse; and are therefore discarded. These wastes could be considered valuable by-products if appropriate technical means are used to increase the value of the subsequent products to exceed the cost of reprocessing. Recycling, reprocessing and eventual utilization of food processing residues offer potential of returning these by-products to beneficial uses rather than their discharge to the environment which might cause detrimental environmental effects. Effective utilization of food residues can occur if through biotechnological interventions these wastes can be made utilizable by a secondary industry. Food industry produces large volumes of wastes, both solids and liquids; these wastes pose increasing disposal and pollution (High BOD or COD) problems and represent a loss of valuable biomass and nutrients. However, inspite of their pollution and hazard aspects, in many cases, food processing wastes have a good potential for conversion 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. 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 specialty chemical is under increasing demand in Food, Pharmaceutical and Chemical Industries and for production of Polylactic 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 estimated 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 and specific sectors amongst the Indian food processing industry are 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 450MT/day.

Discarded, off-grade potatoes account for as much as 6.75 MT/day in addition to starch (approximately 50g/L) containing effluents (up to 6000L/day) (World bank, 2004). Both off grade potatoes and processing effluents can be utilized conveniently as a medium for fermentative production of Lactic acid using appropriate strains of amylolytic lactic acid bacteria. Considering an approximate content of 300Kg of starch contained in the effluent, 75Kg (/day) of Lactic acid may be obtained through such fermentations. The fermentation yield mentioned is noteworthy in proportion to meet the total demand of lactic acid. No studies have however addressed the reutilization of potato wastes in terms of both treatment and ‘value addition’ in the above lines.

Scope of the dissertation:

This dissertation encompasses a systematic study for isolation and characterization of potential amylolytic lactic acid bacteria, optimization of their lactic acid production by them and feasibility of the same in potato starch containing industrial effluents. The consequent results could be used by potato processing industries for subsequent value addition to the waste.

This scope can be achieved under the framework of the following **objectives:**

1. Isolation and characterization of starch degrading microorganisms.
2. Examination and quantification of Lactic acid conversion from starch into Lactic acid by microbial isolates.
3. Comparison of relative productivity of starch degrading isolates for lactic acid production.
4. Optimization of cultural parameters (pH, temperature, nutritional properties, etc) for maximizing conversion to lactic acid by screened isolates.
5. Simulation studies using starch containing industrial effluents for conversion to lactic acid by screened microbes.

Literature review

The literature review focuses on the history, uses and synthesis of Lactic acid and microbes that are related to lactic acid formation. This section also looks briefly at the biochemistry behind the production of Lactic acid and physiology of lactic acid bacteria. Finally in this section, we conclude with the choice of microorganism for fermentation.

1. History of lactic acid:

The first isolation of Lactic acid is documented to have occurred as early as 1789, when Scheele separated the acid from sour milk. The name Lactic acid was derived from the sugar found in milk, lactose and the microbe first used to ferment the acid, was *Sreptococcus lactis*. In 1980, lactic acid was the first acid to be fermented at an industrial level (Zadow, 1992). It is documented that commercial quantities of the acid have been produced since the earlier part of the twentieth century.

2. Chemical properties of Starch and Glucose:

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 organelles chloroplasts and amyloplasts. Starch is a polysaccharide made of individual monosaccharide (glucose units) and is linked by α (1-6) and α (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).

3. Use and properties of Lactic acid:

Lactic acid is the most widely occurring organic acid. Traditionally, the acid has numerous applications in the food and beverage, pharmaceutical and chemical industries. However more modern applications include avenues like biodegradable plastics and medical care. The demand on production of Lactic acid is increasing almost daily. Some of the most important uses of Lactic acid are listed below (Narayanan et. al 2004), (Naveena et. al 2004).

- **Food industries**:

FDA has classified lactic acid as GRAS for use in food industries as general-purpose food additive in US. L (+) isomer, one of the stereoisomers of Lactic acid is preferred in food and dairy industries due to the presence of L (+) Lactate dehydrogenase in human being.

1) Lactic acid is added to margarine, butter, yogurts etc. for its pleasant taste (taste enhancer)
2) Lactic acid is used as pickling agent for olives and pickled vegetables.
3) It is also used as jelling agent for jams and jellies.
4) Ca-lactate is added to milk and other sports drink as mineral supplement
5) Lactic acid and its salt can increase the shelf life of food products like sausages, hams, poultry, fish, etc. by 30 to 50 %
6) A large mass fraction of (>50%) fermentation grade Lactic acid is used to produce emulsifying agents such as sodium and calcium stearoyl lactate in bakery goods.
7) Calcium salt of this acid is a good dough conditioner and the sodium salt is both conditioner and emulsifier for Yeast leavened bakery products.

- **Pharmaceutical Industries:**

Poly lactic acid polymers are biocompatible, biodegradable and restorable materials used in medical application as sutures, orthopedic implants, controlled drug release etc. Polymers of Lactic acid, after adjusting the composition and the molecular wt., can control the degradation of biodegradable transparent thermoplastics. Other applications in this industry are formulation of ointments, lotions, anti-acne solutions and dialysis applications. Ca-lactate can be used for calcium deficiency therapy and as anti carries agents.

- **Chemical Industries:**

Lactic acid is used as acidulant in Leather tanning industries and in small scale operations like pH adjustments, hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifiers, solder flux, lithographic and textile printing developers, adhesive formulations, in electroplating and electro polishing baths, detergent builders etc. Lactic acid esters like ethyl/butyl lactates can be used as green solvents. They are high boiling, non-toxic and degradable components.

4. Synthesis of lactic acid:

Two possibilities for the synthesis of Lactic acid are chemical synthesis and fermentation of solution that contains carbohydrate. The problem with chemically synthesized acids is their 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 crystallinity.

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 process occurs in liquid phase at high pressures. The 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.

$CH_3CHO + HCN \rightarrow CH_3CHOHCN$

Lactonitrile

$2CH_3CHOHCN + 2H_2O + H_2SO_4 \rightarrow CH_3CHOHCOOH + (NH_4)_2SO4$

ammonium sulphate

$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$

Microbial production *via* Fermentation:

Stereo specific lactic acid can be 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

$2(CH_3CHOHCOO^-)\ Ca2^+ + H_2SO_4 \rightarrow 2CH_3CHOH + CaSO4$

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).

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.

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

Examples are: *Lactococcus lactis, Streptococci, Enterococcus faecclis.*

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)

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

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

Fermentation Vs Chemical synthesis:

Chemical synthesis suffers from some drawbacks, which are

1) Chemical synthesis is less efficient in terms of % conversion when compared to fermentation.
2) Chemical synthesis always produces a racemic (a DL mixture) mixture instead of a particular stereoisomer**.**
3) It employs harsh conditions such as high pressure, which is often unattainable at lab scale.

5. Microorganisms in lactic acid fermentation:

It is very much feasible to obtain strains of lactic acid producing microorganisms that produce only L (+) or D (-) Lactic acid (Gonzalez – Varay 1996). L (+) Lactic acid is commercially produced by fermentation processes using lactic acid bacteria or fungi such as *Rhizopus* in submerged cultures (Yin et. al 1997). *Rhizopus arrhizus* and *Rhizopus oryzae* are widely accepted producers of lactic acid (Jin et. al 2003) for the high degree of optical purity associated with the Lactic acid that they produced, However, the production of L (+) Lactic acid based on the total carbohydrate consumed is greater for lactic acid bacteria, which often makes it a choice of preference.

The processing of fruits and vegetables leads to large amounts of organic residues. In general most of these waste is used as cattle feed or compost. However greater environmental and economic benefits could result from conversion of these wastes to by products of higher value. Carbohydrate (especially starch) containing waste streams is the topic of much discussion in industry today for their disposal and pollution (High BOD or COD) problems. The potential of gaining usable and valuable by-products is also gaining significant attention. Specifically targeting high chemical and biochemical oxygen demand contained by these waste streams (which suffocate the life of marine organisms and degrade the potential of the streams to be used for agricultural purposes) will lead to some advances in near future. The ultimate aim of the industries is to lower the BOD and COD of liquid waste releasing from a plant and producing valuable by- products instead of paying for the disposal of the waste stream.

Lactic acid bacteria possessing amylases can utilize starch fermentatively for lactic acid production. Such amylolytic lactic acid bacteria (ALAB) account for 65% of isolates from 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, 1981), fermented cassava roots in Congo and Niger (*Lact. plantarum* strains) (Nwankwo et. al 1989;), chicken crops in France (*Lactobacillus* strains) (Champ et. al 1983), 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. Although most of the known ALAB are DL lactic acid producers from starch, strains of ALAB (*Lact. Amylophilus, Lact. Manihotivorans* and *Lact. amylovorous)* capable of producing L (+) lactic acid have been reported (Nakamura 1981, Morlon-Guyotet. et. al 1998, Naveena et. al 2004). In contrast to the increasing efforts of isolation of novel strains of ALAB elsewhere, few studies (Naveena et. al 2004) have focused on characterization of ALAB cultures from Indian foods or agricultural wastes. The potential importance of starch fermentation by lactic acid bacteria, have opened up avenues to value add starch containing food-industry effluents. Characterization of appropriate strains of ALAB, insights on the physiology of ALAB and optimization of cultural parameters to enhance lactic acid production is therefore necessary to develop new processes and improve existing techniques on a more rational basis for producing Lactic acid from starch wastes.

6. Physiological characteristics of Lactic acid bacteria:

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)

1) All its members are gram positive and do not form spores (with the solitary exception of *Sporolactobacillus inulinus*)

2) Most of them are non motile

3) They all are dependent on carbohydrate for their energy supply and excrete lactic acid

4) They do not contain haemins (cytochrome, catalase)

5) 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.

6) 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 media that are rich in nutrients and other growth factors. On the other hand they have an ability that most microorganisms lack; they can utilize lactose. Lactose apparently does 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).

(Physiological characteristics… continued.)

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{D-Glucose} + \text{D-Galactose}$$

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 (Schlegel, 1997). They are hardly found in air or soil. Their natural habitats are

1) Milk and the places where milk is produced or processed (*Lactobacillus lactis, L. bulgaricus, L. helveticus, L.casei, L.fermentum, L. brevis, L. diacetalactis*)
2) Intact and rotting plants
   (*Lactobacillus plantarum, L. delbrueckii, L. fermentum,, L. brevis, Lactococcus lactis, Leuconostoc mesenteroides*)
3) Intestinal tracts of mucous membranes of animals and humans
   (*Lactobacillus acidophilus, Bifidobacterium, Enterococcus faecclis, Streptococcus salivarius, S. bovis, S.pyogenes, S. pneumoniae).*

7. Choice of microorganism:

The desirable characteristics of microorganisms are their ability to rapidly and completely ferment cheap raw materials, requiring minimal amount of nitrogenous substances, 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 amount of other byproducts. Most importantly the choice depends upon carbohydrate to be fermented (Nrayanan et. al 2004)

A summary of substrate utilization can be represented (Schlegel, 1997).

Lactobacillus delbrueckii subsp. ***delbreuckii***	Sucrose
Lactobacillus delbrueckii subsp. ***bulgaricus***	Lactose
Lactobacillus helveticus	Lactose and galactose
Lactobacillus amylophylus	Starch
Lactobacillus amylovirus	Starch
Lactobacillus lactis	Glucose, sucrose and galactose
Lactobacillus pentosus	Sulphite waste liquor

Materials and methods

1. Isolation of amylolytic lactic acid bacteria (ALAB):

The search for amylolytic lactic acid bacteria was done using a) potato cultivation soil from Thapar Institute of Engineering and Technology, Patiala, Punjab b) a sewage treatment effluent obtained from a local vegetable processing factory at Rajpura, Punjab c) a food processing effluent (which is a potato wash effluent) obtained from Frito-Lay food incorporates, village Channo, Sangrur, Punjab.

a) Isolation from potato cultivation soil: For this 1g freshly procured soil was added into 100 ml nutrient broth supplemented with 0.5% soluble starch and was incubated overnight at 37^0C in shaking condition. Next day, the microbial consortium was serially diluted using sterile saline (0.85% NaCl solution) up to a concentration of 10^{-8}. The dilutions 10^{-8}, 10^{-7} and 10^{-6} were taken and 100µl of each of these dilutions were spread in nutrient agar plates supplemented with 0.5% soluble starch. All the plates were incubated at 37^0Cfor 24-48 h for bacterial growth and the morphological characteristics of the colonies that started forming in these plates were observed over next two days.

b) Isolation from sewage treatment and food processing effluents: For this, 1ml of each of the effluents were added separately into100 ml nutrient broths supplemented with 0.5% soluble starch and was incubated overnight at 37^0C in shaking condition. Following the same method as above the microbial consortiums were serially diluted, grown in plates and the morphological characteristics of the colonies that formed were observed.

Standard strains:

In addition to the above isolates a freeze-dried culture of *Lactobacillus plantarum* MTCC 2621 was procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMT), Chandigarh. The strain has yet not been reported to produce Lactic acid from carbohydrate containing

waste streams. After completion of the experiments, its culture was preserved at -80^{0}C in 40% glycerol.

2. Qualitative starch hydrolysis test for isolated microorganisms:

Different isolates were grown individually in nutrient agar plates (+ 0.5% starch) at suitable temperature. Then from each plate isolated colonies were picked up and streaked in straight lines in separate nutrient agar plates supplemented with 0.5% soluble starch. After one or two day's growth, individual plates were flooded with 0.15% Iodine in 1.5% potassium iodide solution 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 this zone of degradation no blue color forms, which is the basis of detection and screening of an amylolytic species. 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 isolates the two standard strains were also subjected to this test.

3. Quantitative starch hydrolysis test for isolated microorganisms:

Only those isolates (a total of five) that gave a positive result in qualitative starch hydrolysis test were subjected to this quantitative test. For this, individual isolate were grown on their respective media broth for 12 h and then 2ml of each of the broths was centrifuged to pellet out the bacterial growth. Then the supernatant in each case was diluted 100 times with distilled water. To 10 ml of this 1ml of 0.15% Iodine in 1.5% potassium iodide solution was added in a test tube. The mixture was vortexed and the absorbance of the resultant blue colored complex was measured at 585 nm with a spectrophotometer. The residual starch content of the uninoculated broth in each case was also calculated. The concentration of the residual starch in each of the supernatant was worked out from a standard curve.

4. 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 the eppendorfs which contained the bacterial pellets was taken after incubating the eppendorfs at 100^0C for 12 h (dry wt.) when the wt. of the empty eppendorfs was subtracted from this wt. respectively, the amount of biomass produced for 2ml was obtained. Consequently the biomass produced per liter was calculated. From the amount of residual starch the amount of starch that has hydrolyzed after 12 h of growth was found out by subtracting residual starch content from the initial starch content (obtained from the uninocculated broth). The amount of starch hydrolyzed per liter of the nutrient broth when divided by the amount of biomass produced per liter of the nutrient broth gives the Yield coefficient w.r.t. starch hydrolysis (Shuler, 2003).

5. Lactic acid production and estimation:

The isolates that hydrolyzed starch as was evident from qualitative starch hydrolysis test were tested for production of lactic acid. The lactic acid produced (if any) was estimated by titration. Medium used was MRS + 0.5% starch.

For estimation of lactic acid 50ml of fermented broth was taken in Falcon tubes and centrifuged at 10000 rpm for 5 minutes to pellet out the bacterial growth. Then the supernatant was transferred to a beaker and the solution was heated to 80^0C. To it 5% $Ca(OH)_2$ was added drop wise till the pH becomes 7. Next the broth was filtered using Whatmann 42 filter paper and the filtrate was discarded. The precipitate in the filter paper was dissolved in a conical flask carefully using minimal volume of 0.1N HCl. After dissolution of the precipitate, the solution was diluted using distilled water for titration against 0.1N NaOH (standardized) using phenolphthalein as indicator. Standardization of NaOH was done with 0.1N standard oxalic acid; for which 10 ml 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 NaOH that was used for titration of lactic acid was S N and that x ml of it was consumed by 5ml of the lactic acid that was produced after 2 folds dilution. Then the gram equivalent of NaOH that was used was (S*x)/1000. Since reaction takes place in equivalent amounts the gram equivalent of lactic acid that was produced was also (S*x)/1000. Again, since the gram equivalent wt. of lactic acid is 90gram, the amount of lactic acid present in the titrand was (S*x)*90/1000 gram. This amount of lactic acid comes from 50 ml of fermentation broth so the strength of lactic acid that is actually produced was (S*x)*90*1000/(1000*50) g/l which simplifies to (S*x)*1.8 g/l.]

6. Growth kinetics of *Lactobacillus plantarum* MTCC2621 in MRS medium:

The strain of *Lactobacillus plantarum* was grown on MRS plates by the method of four quadrant streaking using a sterile inoculation loop. For growth kinetics, a single isolated colony was picked up from its plate with the help of a sterile inoculation loop and added to 5 ml of sterile MRS broth in a test-tube to grow overnight (18 h) in order to activate the colony taken from the plate. From this suspension of overnight grown bacterial culture, 1ml suspension was transferred in 100 ml of sterile MRS medium (1% inoculum). This served as the culture from which the growth kinetics was worked out. For this, at regular intervals (2, 4, 6, 8 …24 h) 4 ml (2 ml * 2 times) of suspension was taken out aseptically in eppendorfs using micropipette and centrifuged at 10000 rpm for 5 minutes to palletize the bacterial growth. Then the pellet was washed two times using sterile saline (0.85% NaCl solution), pelletized and redissolved in sterile saline. Finally the absorbance of this suspension was taken against sterile saline at 590nm using a spectrophotometer.

7. Estimation of different constituent utilization by *Lactobacillus plantarum* MTCC2621 from MRS medium:

During its growth in the medium, *Lactobacillus plantarum* uses different medium constituent such as starch, total, reducing sugar etc. To get an insight of the utilization pattern of these constituents, time profiles of residual concentrations of these quantities is necessary. For all these three quantities MRS medium supplemented with 0.5% starch was used and time profile over 24 h was done, however, for total and reducing sugars this was extended to 44 h. The 0 h reading corresponds to the reading of uninoculated media.

Residual starch estimation:

The residual starch content was estimated at a regular time interval of 4 h (0, 4, 8…) spectrophotometrically using the same method as mentioned in Quantitative starch degradability test for isolated microorganisms.

Residual total sugar estimation (Anal. Chem. 1956):

The residual total sugar content was estimated at a regular time interval of 4h (0, 4, 8…) spectrophotometrically using *phenol sulphuric acid carbohydrate assay*.

Materials required: Standards- glucose 1 g/l stock solution. For standard curve 20, 40, 80, 120, 160 µl of glucose stock was used and volume of each sample was made up with dH_2O to a final volume of 200 µl, to produce a concentration range 0.1, 0.2…0.8 g/l. Blank used was 200 µl water. Samples-100 µl of 10 times diluted fermentation broth. The volume was made up to 200 µl. with dH_2O

Method: 200 µl of 80% Phenol solution (80% Phenol by weight) was added to each of the Test tubes containing samples for standard curve and residual sugar estimation. Then the test tubes were vortexed. Next 4.0 ml concentrated Sulphuric Acid was added in a stream and the test tubes were kept for 10 min. at room temperature. Finally the absorbance was read at 490nm.

Residual reducing sugar estimation (Miller, 1959):

The residual reducing sugar content was also estimated at a regular time interval of 4 h (0, 4, 8…) spectrophotometrically using *DNSA* (dinitrosalicylic acid) method. The 0 h reading corresponds to the reading of uninoculated media.

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

Oxidation

aldehyde group ----------> carboxyl group

Reduction

3,5-dinitrosalicylic acid ----------> 3-amino, 5-nitrosalicylic 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% dinitrosalicylic Acid Reagent Solution, (dinitrosalicylic acid 10 g, Sodium sulfite 0.5 g, Sodium hydroxide 10 g and water to make up the volume up to 1 liter) 40%Potassium sodium tartarate solution, Standards-glucose 10 g/l 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 samples taken in capped test tubes. The mixture was heated at 90º C for 5-15 minutes to develop the red-brown color. 1 ml of a 40% potassium sodium tartrate (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 spectrophotometer at 575 nm.

8. Determination of starch hydrolyzing principles:

The starch hydrolyzing enzymes are proteins by nature. So an estimation of the increase in protein content in the fermentation broth during the growth of the bacteria will be indicative of the production of extracellular amylases, which are the main starch hydrolyzing enzymes. If at regular intervals starch hydrolysis is estimated along with protein production and the former is divided by the later, then it will give an estimation of the amylase activity. This is the basis of the present work. Here the medium used was again MRS supplemented with 0.5% soluble starch. The protein and the residual starch content of the fermentation broth was estimated at regular intervals of four hours and extended up to 44 h (0, 4, 8…). The protein content was estimated using *Hartree-Lowry* method and residual starch was estimated using the same protocol as mentioned in Quantitative starch hydrolysablity test for isolated microorganisms. The amount of starch hydrolyzed was found by subtracting the residual starch amount from the initial amount of starch (0 hr reading). And the amount of protein produced for each hr was found by subtracting each hr's protein content from initial protein amount (0 hr reading). Since both of them have the unit g/l, the resultant fraction (enzyme activity) as evident is dimensionless (does not have any unit).

***Hartree-Lowry* Protein Assay (Hartree, 1972):**

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten-blue.

Materials required*:* Reagent-A which Consists of 2 gm sodium potassium tartrate,$4H_2O$, 100 gm sodium carbonate, 500 ml 1N NaOH, water to make up the volume to one liter (that is, 7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration), reagent-B which Consists of 2 gm sodium potassium tartrate,$4H_2O$, 1 gm copper sulfate ($CuSO_4$,$5H_2O$), 90 ml H_2O, 10 ml 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulfate),

reagent-C which Consists of 1 volume Folin-Ciocalteau reagent diluted with 15 volumes of water. For standard curve, a series of dilutions of 3 g/l bovine serum albumin stock in sterile distilled water (to give concentrations of 0.1 to 0.6 g/l) was made. For analyzing the fermentation broth's protein content, the broth was diluted 10 times with distilled water.

Method: 1.0 ml of each dilution for standard curve as well as protein containing broth was added to 0.90 ml reagent-A in separate test tubes and was mixed. The tubes were incubated for 10 min in a 50^0C bath and then were cooled to room temperature. Next 0.1 ml reagent-B was added to each tube, mixed and the test tubes were incubated for 10 min at room temperature. Then rapidly 3 ml reagent-C was added to each tube and mixed. Again test tubes were then incubated for 10 min in the 50^0C bath and were cooled to room temperature. Finally the absorbance was taken at 650 nm.

9. Optimization of temperature and pH for the production of lactic acid:

The optimizations were carried out for a four-day period (96 h).

Both for temperature and pH optimization the experiments were carried out in triplicates. For temperature optimization the three standard temperatures used were 28^0C, 34^0C and 37^0C while for pH optimization, the three standard pH used were pH 4, pH 6 and pH 8. 250 ml of sterile MRS medium supplemented with 0.5% soluble starch was used for the experiment. The respective pHs were adjusted with conc. NaOH (10 N) 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 2.5ml of overnight -activated *Lactobacillus* culture (1% inoculum level) for each case. Then from each culture lactic acid was estimated titrimetrically at a regular interval of 24 h. During the course of this experiment pH was not controlled.

10. Simulation with Food processing effluent (FPE):

Different combination of filter sterilized food processing effluent (FPE) with sterile MRS medium was made ranging from 5% MRS (95 ml FPE + 5 ml MRS) to 80% MRS (20 ml FPE + 80 ml MRS). Each of such combinations was then inoculated with overnight-activated *Lactobacillus* culture (at 1% inoculum level) and incubated at 28^0C. After 18 h of growth, individual suspension was taken out from the test tubes containing the different combinations of nutrients in eppendorfs. 2 ml of the suspensions were taken in eppendorfs and centrifuged at 10000 rpm for 5 minutes to palletize the bacterial growth. Then the pellets were washed two times using sterile saline (0.85% NaCl solution). Finally the wt. Of the pellets were taken after incubating the eppendorfs in 100^0C for 12 h (dry wt.). Consequently the growth for one liter was calculated. On the other hand the viability of the cultures were checked separately by plating method after pelletization and serial dilution. Since even 5% MRS (95 ml FPE + 5 ml MRS) showed considerable growth, this combination was used for the further experiments. Based on growth kinetics, starch hydrolysablity and lactic acid productivity of a comparative study with pure MRS medium was carried out using this simulation medium.

Growth kinetics:

From a suspension of overnight grown *Lactobacillus plantarum* MTCC 2621 culture, 100µl was transferred in 100 ml of simulation medium (1% inoculum). This served as the culture from which the growth kinetics was worked out. For this, at regular intervals (2, 4, 6, 8 …24 h) 4 ml (2 ml * 2 times) of suspension was taken out in eppendorfs using a micropipette and centrifuged at 10000 rpm for 5 minutes to palletize the bacterial growth. Then the pellet was washed two times using sterile saline (0.85% NaCl solution) and redissolved in sterile saline. Finally the absorbance of this suspension was taken against sterile saline at 590nm.

Starch hydrolysablity:

For this the simulation medium was supplemented with an autoclaved solution of soluble starch in volume such that the final conc. of starch in the broth becomes 0.5%. Then from a suspension of overnight grown *Lactobacillus plantarum* MTCC 2621 culture, 100µl was transferred in 100 ml of simulation medium (1% inoculum). The residual starch content was estimated at a regular time intervals of 4 h (0, 4, 8 …) spectrophotometrically using the same method as mentioned in Quantitative starch hydrolysablity test for isolated microorganisms. The 0 h reading corresponds to the reading of uninoculated media.

Lactic acid productivity:

The Lactic acid productivity in the simulation medium was estimated over a 24 hr period. Here again, from a suspension of overnight grown *Lactobacillus plantarum* MTCC 2621 culture, 100µl was transferred in 100 ml of simulation medium (1% inoculum). Then Lactic acid was estimated titrimetrically at a regular time intervals of 4 h (0, 4, 8 …).

11. Lactic acid production under controlled pH condition:

In order to check whether controlling the pH does produce any significant change in the level of production of lactic acid, an ex-citu 2 liter steel bioreactor (made by *Bioage instruments*, India) was used equipped with pH probe and controlling adjustments. Systemically activated Lactobacillus culture was inoculated in the bioreactor (containing previously autoclaved medium) and lactic acid was estimated from it after a period of 24 h titrimetrically.

12. L-lactate content determination by analytical kit:

An analytical kit (made by *Randox laboratories*, UK) was used for the estimation of the L-lactate content produced in the fermentation broth by *Lactobacillus plantarum*. The kit relies on UV spectrophotometry and uses NAD^+ linked L-lactate dehydrogenase assay. The experiment was carried after 24 h period of incubation. The fermentation broth that was used for controlled pH condition was used here also. Comparison of both the values will give us an insight to whether any particular isomeric form or a DL mixture is actually produced by *Lactobacillus plantarum.*

Result and discussion

1. Starch hydrolysablity test (qualitative) for soil isolates

Name	Type	Morphology	Starch hydrolysablity
SI 1	Bacteria	Orange	-
SI 2	Bacteria	Bright yellow	-
SI 3	Bacteria	Orange slimy	-
SI 4	Bacteria	Yellow	-
SI 5	Bacteria	White	-
SI 6	Bacteria	White slimy	-
SI 7	Bacteria	Creamish	-
SI 8	Fungi	Green spore forming	-
SI 9	Fungi	Maroon, bordered by pink, spore forming	-
SI 10	Fungi	Centrally white, -n spore forming	-
SI 11	Fungi	White -n spore forming	+

2. Starch hydrolysablity Test (qualitative) for sewage treatment isolates

Name	Type	Morphology	Starch hydrolysablity
STI 1	Bacteria	White slimy	-
STI 2	Bacteria	Bright red	-
STI 3	Bacteria	White	+

3. Starch hydrolysablity test (qualitative) for food processing effluent isolates

Name	Type	Morphology	Starch hydrolysablity
FPEI 1	Bacteria	White	-
FPEI 2	Bacteria	White	-
FPEI 3	Bacteria	White slimy	-
FPEI 4	Bacteria	White	+

In addition to soil, sewage treatment effluent and food processing effluent isolates*, Lactobacillus plantarum* MTCC2621 and *Bacillus brevis* was subjected to starch hydrolysablity test and they both were found to degrade starch.

4. Lactic acid production test of the isolated microorganisms and the standard culture.

Name	Type	Morphology	Starch hydrolysablity	Lactic acid productivity
SI 11	Fungi	White, -n spire forming	+	-
STI 3	Bacteria	White	+	-
FPEI 4	Bacteria	White	+	-
Bacillus brevis	Bacteria	White	+	-
Lactobacillus plantarum MTCC2621	Bacteria	Grayish	+	+

[+ signifies detected while – signifies not detected]

Isolation of amylolytic lactic acid bacteria (ALAB)

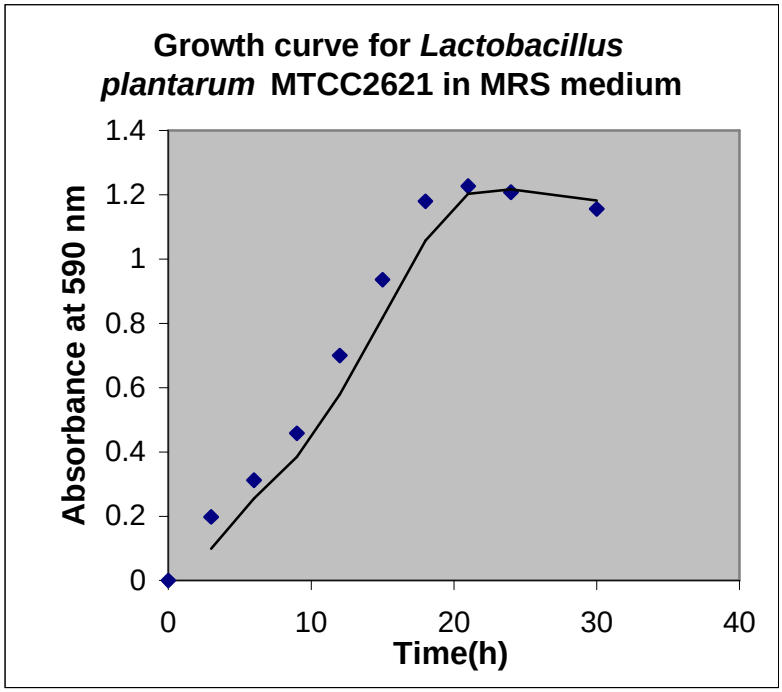
Altogether 18 organisms were isolated from sewage treatment effluent, food processing effluent and potato cultivation soil and 3 of them were found to be amylolytic as was evident from qualitative starch hydrolysablity test; however, we could not isolate a single lactic acid producing bacterium from them, as they did not respond positively in the titrimetric tests. In addition to these isolates, both *Bacillus brevis* and *Lactobacillus plantarum* MTCC2621 was found to be amylolytic. However, only *Lactobacillus plantarum* was actually found to be lactic acid producing. Thus the further experiments of this study were carried out using this standard strain of *Lactobacillus.*

5. Approximate Yield coefficient for starch hydrolysis by the isolated microorganisms and the standard cultures

Name	Medium	Initial Starch conc. of the broth (g/l)	Final Starch conc. of the broth (g/l)	Amount of Starch degraded (g/l)	Initial biomass (g/l)	Final biomass (g/l)	Amount of Biomass produced (g/l)	Yield coefficient (l^{-1})
SI 11	PDB	6.87	0.526	6.344	0	30.7	30.7	0.206
STI 3	NB	6.48	6.39	0.09	0	0.8	0.8	0.11
FPEI 4	NB	6.48	1.08	6.372	0	2.45	2.45	2.6
B.brevis	NB	6.48	4.65	1.83	0	8.05	8.05	0.227
*Lp*MTCC2621	MRS	6.45	4.765	1.685	0	17.65	17.65	0.095

From the table it was found that *Lactobacillus plantarum* MTCC2621 was the least efficient w.r.t. starch hydrolysis; however it is the only ALAB among all the isolates that makes it indispensable for further experiments**.**

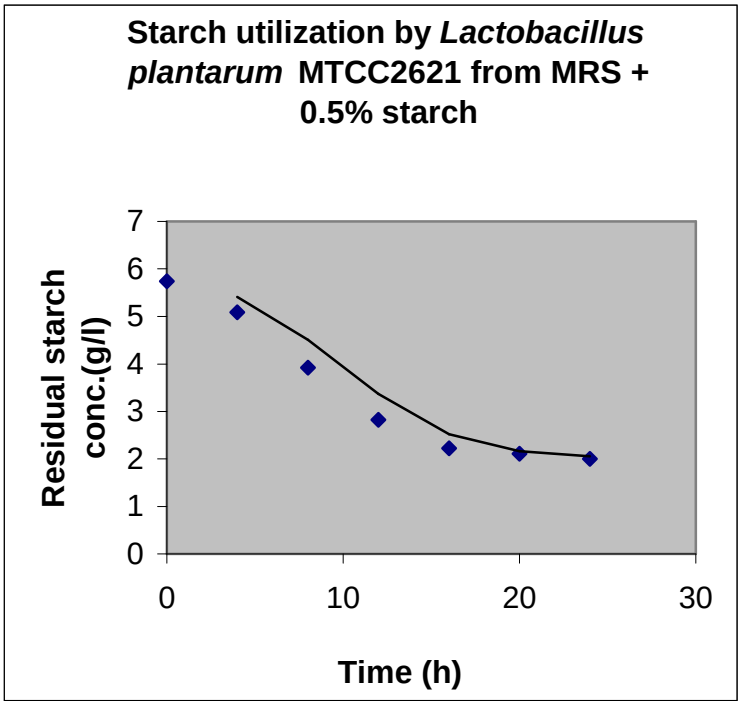
6. Growth curve of *Lactobacillus plantarum* MTCC2621 in MRS medium



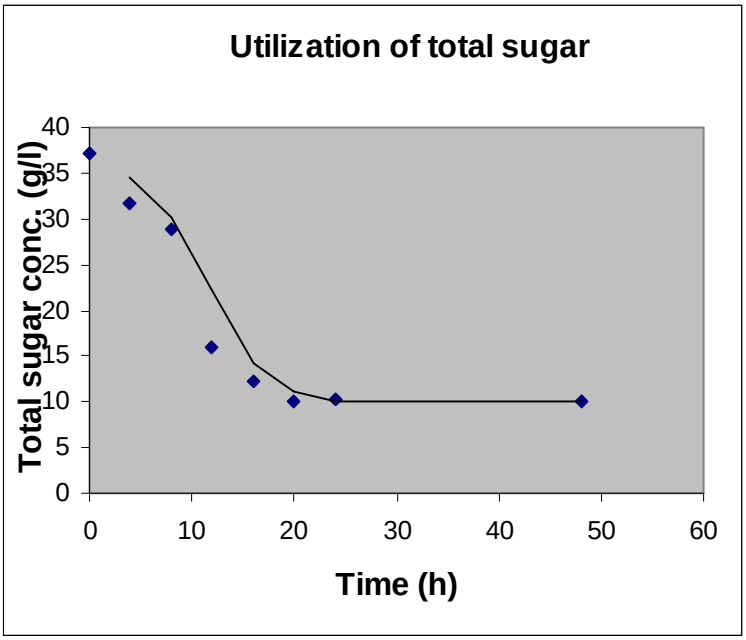
As evidenced from the absorbance values, the growth curve of *lactobacillus plantarum* is characterized by lag, exponential, stationary and death phase. The lag phase starts right after the inoculation and rests for 2-3 h. In this phase the bacteria adapts to the nutrient medium. After the lag phase is over the maximum division rate is observed in the exponential or logarithmic phase of growth. This phase rests for almost 17-18 h. The stationary phase, which continues for about 8 h begins after the log phase; here the cells in the culture no longer reproduce as because the concentrations of the nutrients vastly decrease during the exponential growth phase. 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 sufficient time has elapsed the culture shows a decline in growth in the death phase, where the bacterial cells die may be because of scarcity of the nutrients, accumulation of acid and other metabolic waste products. Approximately the phase starts 26-28 h after the incubation and continues

thereafter. Similar growth profiles are reported for other *Lactobacillus plantarum* isolates (Giraud et. al 1998)

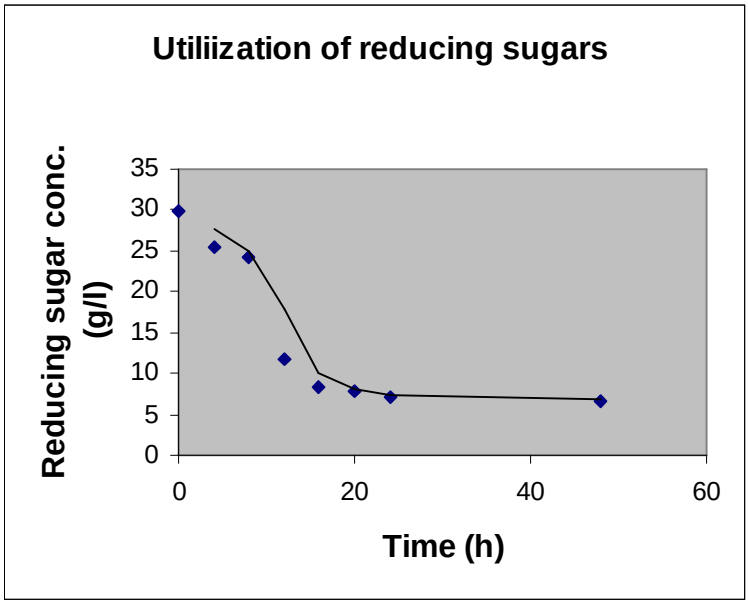
7. Starch hydrolysis by *Lactobacillus plantarum* from MRS + 0.5% starch



8. Utilization of total sugar from MRS + 0. 5% starch



9. Utilization of reducing sugar from MRS + 0.5% starch



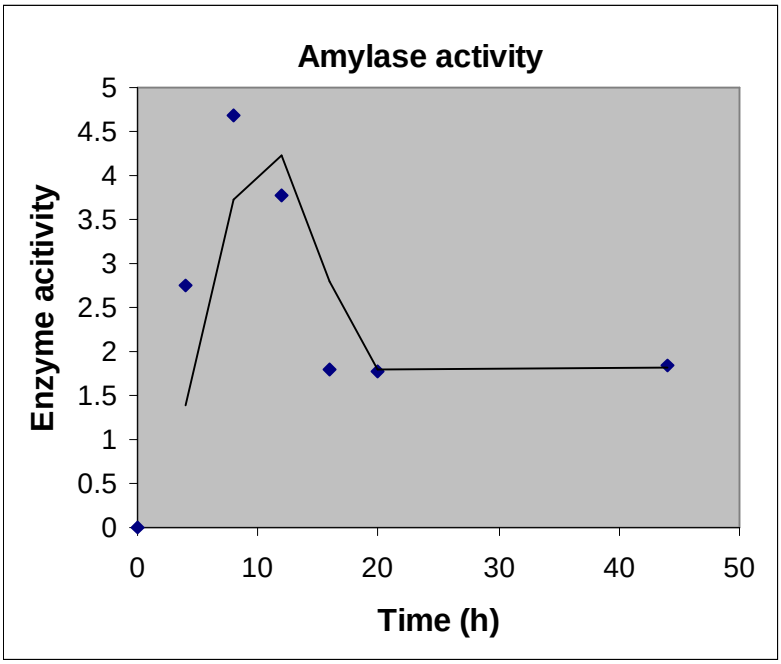
Estimation of different constituent utilization:

All the three quantities starch, total and reducing sugars shows individual utilization pattern over the period of exponential phase of growth of the culture. In each case, due to the entry in the stationary phase, even after completion of the experiments there remains some amount of constituents, which cannot be used by the bacteria. The initial and left over concentrations of starch, reducing and total sugar were 5.74, 29.86 and 37.21 g/l and 2, 6.57 and 10 g/l respectively. The rate of utilization in case of starch was much less compared to the other two quantities. This is evidenced by the steepness of the curve for reducing and total sugars. The rate of utilization of any of these quantities can be quantified by using the equation

Rate = - d[substrate]/dt = - (x_1 - x_2)/(t_1 - t_2), where x_1 and x_2 are the concentrations of the respective substrates at time t_1 and t_2 respectively. Thus the rate of utilization for starch, residual and total sugars between 8 and 12 h were 0.275 $gl^{-1}hr^{-1}$, 3.146$gl^{-1}hr^{-1}$ and 3.192 $gl^{-1}hr^{-1}$ respectively, which clearly

shows the difference. The rate of degradation of reducing sugars and total sugars, however, was almost equal.

10. Amylase activity of *Lactobacillus plantarum* in MRS broth + 0.5% starch



Quantification of amylase activity:

The extracellular amylase activity of *Lactobacillus plantarum* in MRS broth shows three distinct phases. In the first phase (up to 12 h of incubation) the activity sharply increases to a certain peak value (approx 4.7), then decreases steadily from 12 to 20 h of growth and finally becomes constant with a value of 1.8. This can be explained as follows. Firstly the ratio of starch hydrolysis and protein content increases because of quick and efficient removal (greater rate of degradation) of the starch in the exponential phase of growth of the bacteria. After 12 h, although the exponential phase yet not completed, there is a decrease in the rate of hydrolysis of starch and also accumulation of other extracellular proteins along with amylase, which decreases the ratio. After the completion (after almost 20h of incubation) of the exponential phase there is

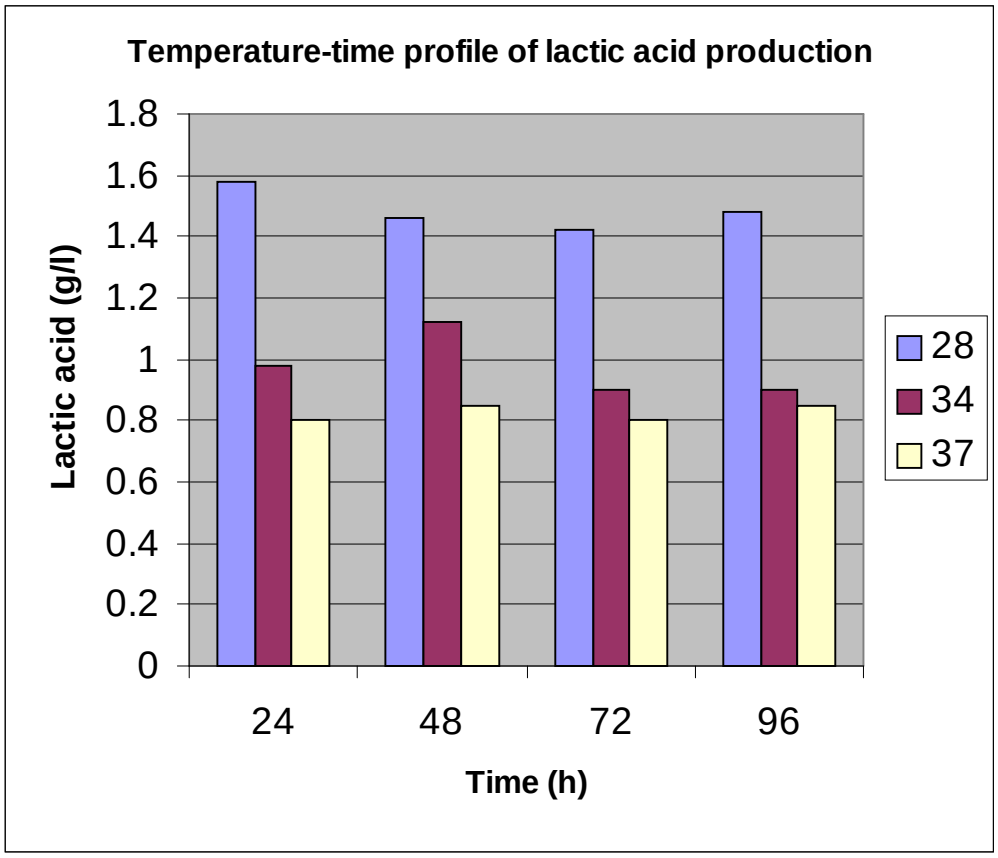
negligible amount of starch hydrolysis, and the residual starch content of the broth becomes almost constant. Also there is little amount of enzyme production, which makes protein content of the broth almost constant after 20 h. This produces a constant ratio of amylase activity for next 24 h (20 to 44).

11. Temperature optimization for Lactic acid production by *Lactobacillus plantarum* MTCC2621 at pH 6

Lactic acid production (g/l)

Temperature	After 24 hours	After 48 hours	After 72 hours	After 96 hours
28° C	1.58	1.46	1.42	1.48
34° C	0.98	1.12	0.9	0.9
37° C	0.8	0.85	0.8	0.85

The optimization profile can be represented as follows

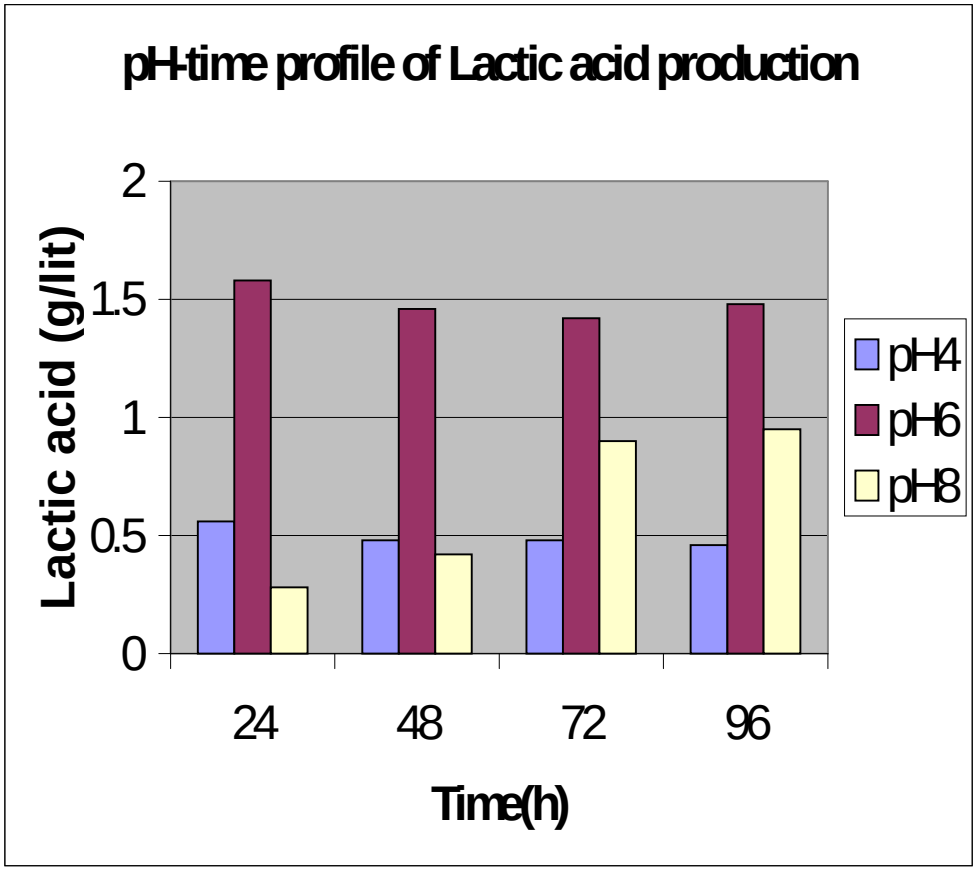


12. pH optimization for Lactic acid production by *Lactobacillus plantarum* MTCC2621 at temperature 28°C

Lactic acid production (g/l)

pH	After 24 hours	After 48 hours	After 72 hours	After 96 hours
4	0.56	0.48	0.48	0.46
6	1.58	1.46	1.42	1.48
8	0.28	0.42	0.9	0.98

The optimization can be represented as follows



Temperature and pH optimization:

As evident from the above figures for optimization, the suitable temperature and pH for lactic acid production was 28^0C and 6 respectively. For example lactic acid concentration at 28^0C (1.58g/l) after 24 h is over 60% more than the same at 34^0C (0.98g/l). Again lactic acid concentration at pH 6 after 24 h (1.58g/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 within first 24 h. After 24 h there is only a negligible change in the extracellular concentration of lactic acid. This may be due to the completion of the exponential as well as stationary phase and starting of the death phase (as inferred from the growth kinetics of *lactobacillus plantarum*) in which the bacteria shows no metabolic activity in general. The case for 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 cause may be the decrease 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.

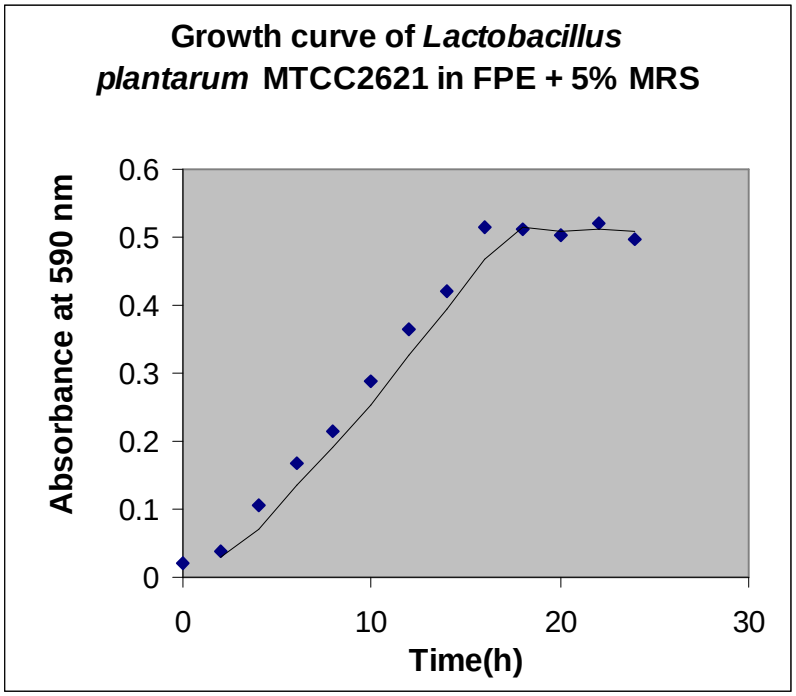
13. Substrate optimization for *Lactobacillus plantarum* MTCC2621 at temperature 28°C and pH 6

Percentage of MRS medium supplemented with Food processing effluent (FPE)	Bacterial growth after 18 h of incubation in g/l
5	5.976
10	7.6
20	9.296
40	9.490
60	12. 082
80	14.264

Simulation medium:

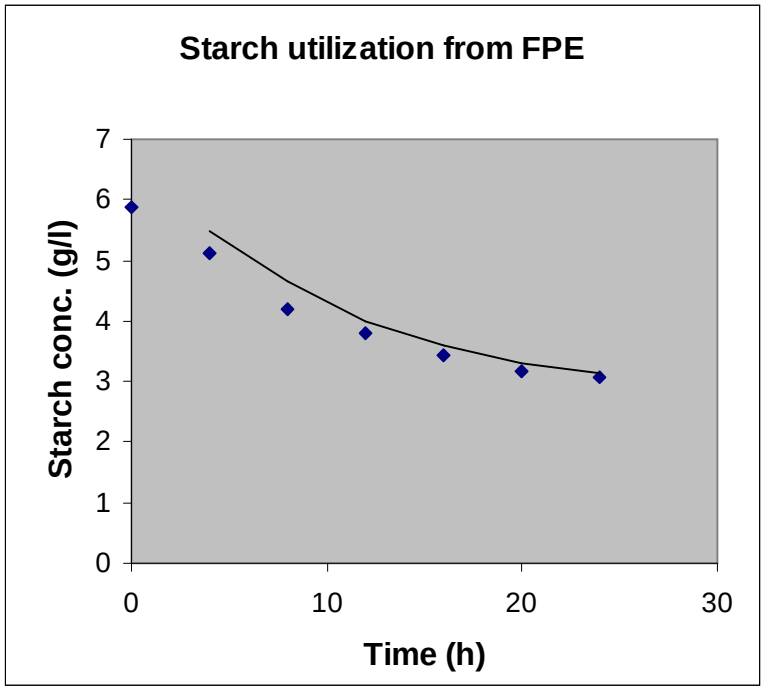
From the table it can be seen that the bacteria is well capable of survival and growth in the food processing effluent (FPE) when supplemented with as low as 5% MRS media (the viability of the palletized biomass was checked by plating after serial dilution). Thus this combination of FPE and MRS is the simulation medium from which the lactic acid can be produced. Since the medium would inherently contain nutrient concentrations that are sub optimal as compared to MRS itself, it will be of interest to study what effects does it bring on growth kinetics, starch utilization and the production of lactic acid.

14. Growth curve for *Lactobacillus plantarum* MTCC2621 in Food processing effluent (FPE) supplemented with 0.5%MRS at 28°C and ph 6



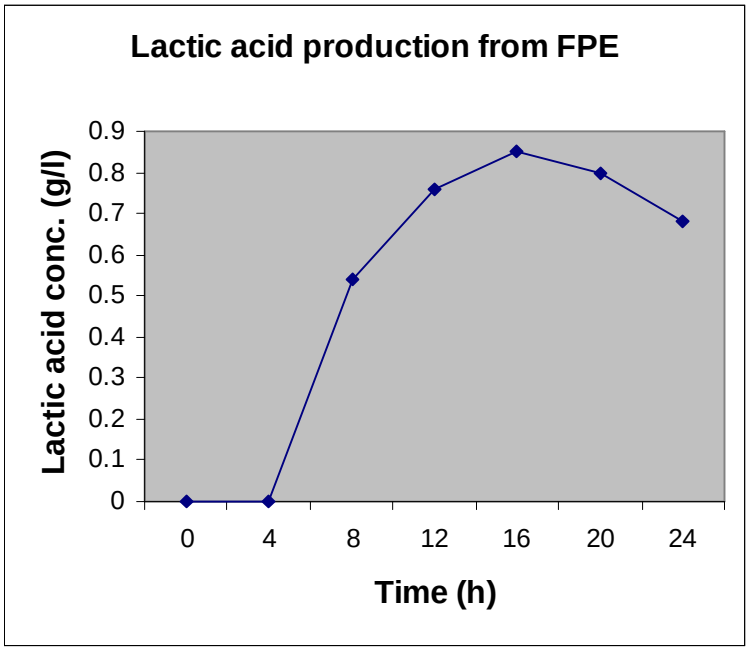
As in the case with MRS medium, the growth curve of *lactobacillus plantarum* in simulation medium is also characterized by lag, exponential, stationary and death phase with similar durations. The significant difference from the MRS medium is, however, the extent of growth of *Lactobacillus is* evident from the absorbance value. Since the duration of each phase is similar to MRS medium, it can be inferred that the reproductive machinery of *lactobacillus* in simulation medium is sluggish as compared to MRS. This is due to the sub-optimal nature of the nutrients in FPE.

15. Starch utilization by *Lactobacillus plantarum* MTCC2621 from FPE supplemented with 0.5% starch and 5% MRS



The general pattern of starch hydrolysis by *lactobacillus plantarum* in FPE is similar to that of MRS medium. However starting with a concentration of 5.86 g/l the left over starch after 24 h is more than 3 g/l which is significantly high than 2 g/l, the concentration of left over starch in MRS medium. The starch hydrolysis rate between 4 to 8 h was 0.10125 $gl^{-1}hr^{-1}$, which is again less than that of MRS medium 0.2685 $gl^{-1}hr^{-1}$. This again may be attributed to the sub optimal nature of the nutrients in simulation medium.

16. Production of lactic acid from FPE + 5% MRS + 0.5% starch



The time profile for lactic acid production was carried out for a 24 h period at regular interval of 4 h. The experiment suggests that lactic acid was not produced after 4 h of incubation. It is only after 8 h of incubation lactic acid could be detected and measured. At the 8th hr the concentration of lactic acid was about 0.5 g/l which rises up to 0.85 g/l during 16th hr and finally falls down to about 0.7 g/l after 24 h, which means that lactic acid was produced at a lower yield within 16 h of the incubation.

17. Lactic acid production under controlled pH condition

The lactic acid produced in the fermentation broth obtained from the bioreactor with pH controlled, was estimated titrimetrically and it was found that lactobacillus have produced 1.85g/l of lactic acid in it. This is significantly higher than the highest amount of lactic acid produced previously (1.58 g/l in case of temp 28^0C and pH 6 after 24h of incubation) which clearly indicates that controlling the pH has a definite positive effect on production of lactic acid for the organism.

18. L-lactate quantification using biochemical kit

The same fermentation broth that was used for determination of lactic acid (total) under controlled pH condition was also used here and the biochemical kit yielded a value of 0.368 g/l (following the calculation as provided by the manufacturer in the user's manual). This may be explained by assuming that the strain of present study, *lactobacillus plantarum* MTCC2621, is actually producing a DL mixture of lactic acid, of which L-lactate contributes by 20% (0.368 g/l out of 1.85 g/l of total lactate) approximately. In other words the bacteria is producing D:L isomers in a 4:1ratio.

Conclusions

1. Altogether 18 organisms were isolated from 2 different effluents and a potato cultivation soil and 3 of them were found to be amylolytic, however, not a single lactic acid producing strain could be isolated from them.

2. *Lactobacillus plantarum* MTCC2621 was the least efficient among all the amylolytic microorganisms w.r.t. starch hydrolysis; however it is the only ALAB among all the isolates and standard strains that makes it indispensable for further experiments**.**

3. The starch utilization by *Lactobacillus plantarum* MTCC2621 was brought by extracellular amylase as was evident by the lowering of starch conc. In the extracellular broth. The enzyme failed to utilize all the extracellular starch that was available to it and there was always some amount of starch that remained as left over. This was also the case with total and reducing sugar.

4. The optimal temperature and pH for lactic acid production was 28^0C and 6 respectively. The general trend of lactic acid production except in the case of pH 8 was that the production of the acid is completed within first 24 h. In the case for pH 8, lactic acid concentration increased with time of incubation.

5. From feasibility studies, it was clear that the bacterium was capable of survival and growth in the food processing effluent (FPE) when supplemented with as low as 5% MRS media. Thus this combination of FPE and MRS can be used as the medium for value addition in suitable food industry.

6. As in the case with MRS medium, the growth curve of *lactobacillus plantarum* in simulation medium shows similar trend, however, here the bacteria grow to much lesser extent as compared to MRS. The general pattern of starch hydrolysis by *lactobacillus plantarum* in FPE is also similar to that of MRS

medium but the left over starch after 24 h is more than the concentration of left over starch in MRS medium.

7. From the time profile for lactic acid production in FPE, it was found that lactic acid was not produced during first 4 h of incubation. It is only after 8 h of incubation lactic acid was produced. Here also lactic acid was produced at a lower yield when compared to the pure MRS growth medium.

8 under controlled pH condition, there is a significant increase in the level of production of lactic acid when compared to pH uncontrolled condition. Through biochemical kit measurement, it was found that *lactobacillus plantarum* MTCC2621, is actually producing a DL mixture of lactic acid with D:L isomers in a 4:1ratio.

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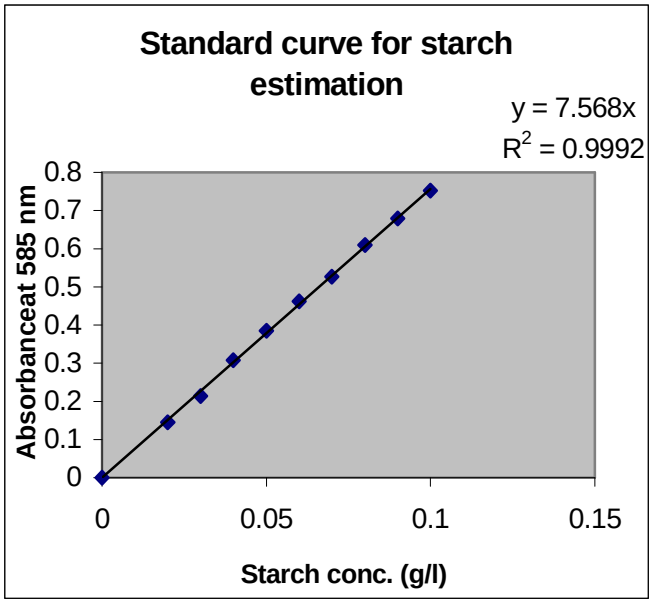
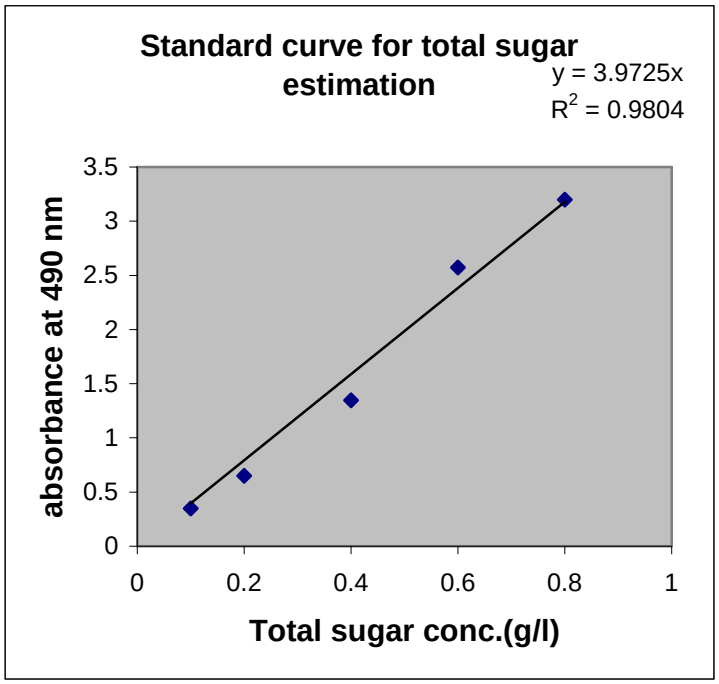
Annexure1

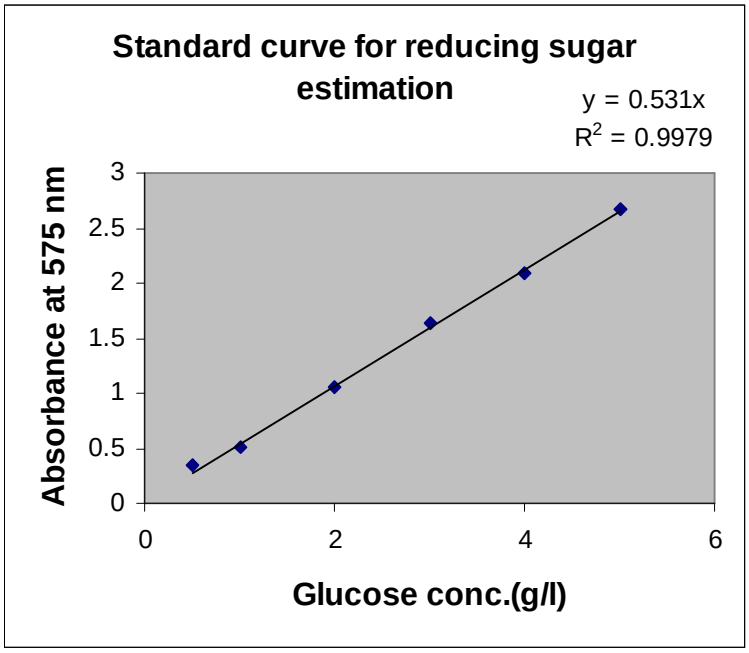
1. Standard curve for Starch estimation



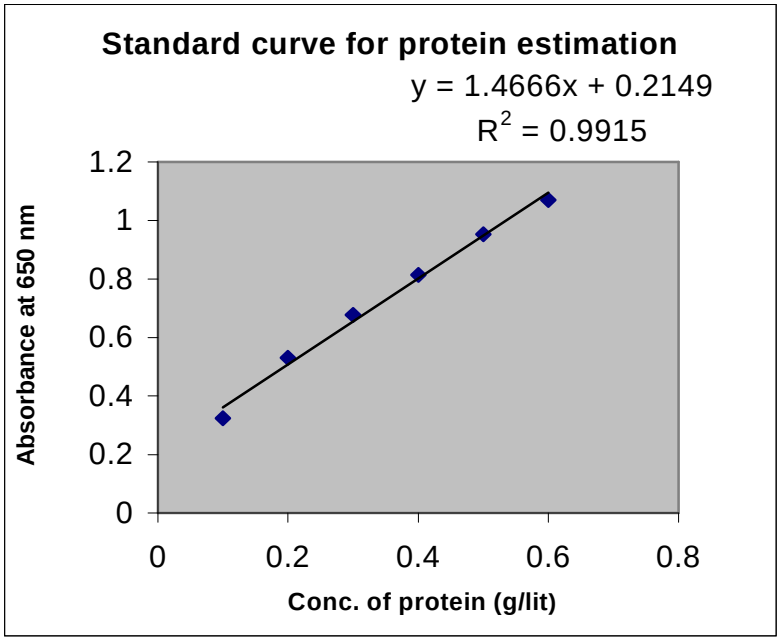
2. Standard curve for total sugar estimation



3. Standard curve for reducing sugar estimation



4. Standard curve for protein estimation



Annexure2

1. Composition of MRS medium

Peotone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
Tween 80	1.0 ml
Na_2HPO_4	2.0 g
sodium acetate	5.0 g
triammonium citrate	2.0 g
$MgSO_4$, $7H_2O$	0.2 9
$MnSO_4$, $4H_2O$	0.2 g
Agar	15.0 g
Distilled water	1.0 L
Adjusted pH to	6.2-6.6

2. Composition of Potato Dextrose Broth (PDB) medium

Potato (scrubbed and diced)	200.0 g
Dextrose	20.0 g
Distilled water	1.0 L
Adjusted pH to	5.6

3. Composition of Nutrient broth (NB) medium

Peptone	5.0 g
Beef extract	3.0 g
Distilled water	1.0 L
Adjusted pH to	6.6-7.0