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## Comparative study of fermentative capacity of different lactobacilli strain to produce lactic acid from tamarind kernel powder

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### Abstract

In present work four different lactic acid producing bacteria (LAB) were used for the production of the lactic acid from tamarind kernel powder (TKP) and checked its feasibility to be used as raw material instead of glucose. Hydrolysis of tamarind seed powder is carried out by acid catalyst, sulfuric acid and it is used as carbon source for lactic acid production. The different fermentation parameters were studied for four L-lactic acid producing bacteria (LAB) i.e Lactobacillus bulgaricus-2056 (LB-S1), Lactobacillus delbrueckii-2025 (LD-S2), Lactobacillus casei -2125 (LC-S3), Lactobacillus casei var. rhamnosus - 2364 (LC-S3) using glucose standard culture and tamarind kernel hydrolysate (TKH) sugar culture. Out of the four strains used, Lactobacillus casei -2125 gave best results for the sugar consumption and lactic acid production. It gives nearly 85 % conversion in 70 hour of fermentation. It gives productivity of lactic acid up to 80 g.l<sup>-1</sup> with respect to TKH sugar and production rate of 1.08 g/l/hr. Productivity of 0.58 g.g<sup>-1</sup> of tamarind kernel powder was observed in the experiment. It shows that the Lactobacillus casei -2125 strain can give higher production of lactic acid using tamarind kernel sugar.

**Keywords:** Agro waste, Tamarind kernel powder, L-lactic acid, comparative study

### 1. Introduction

Lactic acid is a versatile chemical having numerous applications in pharmaceuticals, cosmetics, food industry<sup>[1,2]</sup>. It is used as pH regulating agent or inhibitor for bacterial spoilage in wide variety of processed food. Lactic acid (LA) is considered as one of the most useful chemical used in food industry. In contrast to the other acids it has mild acidic taste, it is non volatile and odorless. Most importantly it is classified as generally regarded as safe (GRAS) by FDA in the US. It is a very good preservative and pickling agent<sup>[3]</sup>. It is also used as raw material for the production of the chemicals like, lactate ester, propylene glycol, 2-3 pentane dione, propionic acid, acrylic acid, acetaldehyde, acrylic acid and lactide. These chemicals find various applications in food, pharmaceutical, polymer, textile industry<sup>[4]</sup>. It also finds huge application as monomer for biodegradable polymer polylactic acid (PLA)<sup>[5]</sup>. Although the demand for PLA is increasing, its current production capacity of only 450,000 metric tons per year is dwarfed by the 200 million metric tons per year of total plastics produced<sup>[6]</sup>. The lactic acid market has been estimated to be 3.3 million tons by 2015, due to continuously increasing demand with the growth rate of 8% per annum<sup>[7]</sup>. Thus even this huge production is not able to fulfill the need of the biopolymer market due to the high manufacturing cost of raw material, that is, lactic acid monomer.

Furthermore, the primary costs associated with lactic acid production include the fermentative substrates of nutrients, expensive nitrogen sources and sugars required for the cell growth and the fermentation along with the downstream recovery and purification process. To meet recent applications of lactic acid for PLA and to be commercially viable, overall lactic acid production costs should be at or below \$ 0.8 per kilogram of lactic acid, as the selling price of PLA must decrease by roughly half of its present price to compete with fossil fuel-based plastics<sup>[6]</sup>.

To this end, a strain for the industrial lactic acid production should produce more than 100 g.l<sup>-1</sup> of lactic acid with a highest yield, near maximal theoretical value, a high optical purity of lactic acid (>99%), and a high productivity in cheap culture<sup>[1]</sup>.

This problem can be overcome by using the agro waste materials having the low market value and rich in carbohydrates and other nutrients. Recently, lactic acid has been produced from a variety of carbohydrates, including starchy and lignocellulosic biomasses, depending on the substrate availability in the producing country<sup>[1]</sup>.

In present work tamarind kernel powder (TKP) is used as the source of carbon for producing

lactic acid [8]. India is, one of the world's largest exporters of tamarind products, and make export of 0.3 million ton of tamarind pulp annually and has almost double production of it annually [9]. Tamarind fruit is a brown pod-like legume which contains a soft acidic pulp (about 55%), many hard-coated seeds (34%), 11% shell and fiber in a pod [10]. Each pods contains 1 to 12 fully formed seeds which are brownish shiny, hard and flattened. This sees comprised of seed coat (20-30%) and kernel or endosperm (70-80%). This kernel is important source of carbohydrates, proteins, fat and valuable amino acids [11]. Thus we have more than 75-80 thousand tones of tamarind kernel, which can be a cheap raw material for the production of Lactic acid. It has more than 70% of starch content. Tamarind seed Polysaccharides, also called glycans, consist of mono saccharides and their derivatives. It contains xylose, galactose and glucose in the ratio of 1: 2.5:2.8 respectively and about 2-3 % of the polyfuranose [12]. In this work, the fermentation capacity of different lactobacillus species to produce lactic acid from tamarind seed powder (TKP) was investigated. The tamarind kernel hydrolysate (TKH) produced from acid hydrolysis was used as carbon source. The capacity of different bacteria to utilize tamarind kernel hydrolysate sugar to produce lactic acid is studied comparatively with respect to the parameters like cell growth, sugar consumption and lactic acid production.

## 2. Materials and Methods

### 2.1. Materials

#### 2.1.1. Chemicals

L-lactic acid 99.9 %, Sodium dihydrogen phosphate dihydrate analytical reagent grade (A.R), Phosphoric acid A.R grade, MRS media, yeast extract, peptone. deMan, Rogosa and Sharpe (MRS) broth, MRS agar, glycerine, polypeptone D-glucose, potassium hydrogen phosphate  $K_2HPO_4$ , sodium acetate, 5; diamonium citrate,  $MgSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot H_2O$ , Tween-80 (poly sorbit-80) and other nutrients were purchased from HiMedia, india. All the other chemicals used are of A.R. grade and used as received.

#### 2.1.2. Tamarind Kernel Powder (TKP)

Tamarind kernel powder (TKP) was procured from local markets, of Mumbai, India (during season of 2013 March). It was pretreated by drying and extraction of lipids and stored in an airtight container at room temperature until use.

#### 2.1.3. Microorganisms

Four lactic acid bacteria (LAB) used for the study were of lactobacillus species. These strains were identified as, *Lactobacillus bulgaricus* (NCIM - 2056), *Lactobacillus delbrueckii* (NCIM - 2025), *Lactobacillus casei* (NCIM - 2125), *Lactobacillus casei* var. *rhamnosus* (NCIM -2364) was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, CSIR-India. For the sack of convenience all the strains were renamed as, LB S1, LD-S2, LC-S3 and LC-S4. All the LAB strains are anaerobic and producing mainly L-lactic acid. The strain was stored in MRS broth with 70% glycerine stalk at  $-80^\circ C$ .

### 2.2. Analytical Methods

Tamarind kernel powder (TKP) available from the market is dried in oven at  $60^\circ C$  for 8 hour. It is sieved to get  $100\ \mu m$  size, to get uniform particle size which is beneficial for the good interaction of reagents. It is made free of lipids (oil/ fat)

by following the extraction procedure mentioned in previous experiment [13]. This dried TKP has the composition as: moisture 0.67%, protein 15.20 %, fat/oil 0.32 %, crude fibre 4.60 %, ash 1.80 %, total sugar 77.50 % and reducible sugar 72.40 % [13].

Each culture sampling involved a 2 ml extraction of culture liquid with a sterile pipette. An aliquot of each sample was divided into two parts. One part is acidified with 1N sulfuric acid to precipitate calcium sulfate and to liberate lactic acid from calcium lactate. It is centrifuged at 5000 rpm for 10 min. The supernatant liquid after filtration through  $0.2\ \mu m$  PES membrane is used for sugar analysis and for lactic acid detection. The second portion is acidified by hydrochloric acid to dissolve unreacted calcium carbonate and precipitated calcium lactate. It is diluted ten times and used for cell count determination by spectrophotometer, by measuring its optical density (OD) at 600nm.

Total reducible sugar was determined by 3,5 di nitro salicylic acid method (DNSA) [14]. Absorbance of the sample was read at 540 nm by double beam UV-Vis spectrophotometer. High performance liquid chromatography (HPLC) was employed to analyze lactic acid present in the fermentation broth, using HiQsil, C-18 HS reverse phase ODS column( KYA TECH, HPLC Column, Japan, 240 mm x 4mm,  $0.4\ \mu m$  particle size) maintained at  $40^\circ C$  by a column heater. The detector used is Waters 2489 UV/Visible detector at 210 nm and 515 isocratic pump (Waters, Massachusetts, United States) The mobile phase was  $NaH_2PO_4 + H_3PO_4$  10 mM buffer system (pH 3) at flow rate of  $0.8\ ml\ min^{-1}$  [15]. For all the analysis  $20\ \mu l$  of diluted sample is injected. Concentrations of lactic acid were determined by comparison to a standard curve generated using L (+) lactic acid standard.

### 2.3. Experimental

#### 2.3.1. Hydrolysis of TKP

Hydrolysis of TKP was done as per the procedure in previous experiment [13]. Here, pretreated TKP was added to get 20 % w/v of TKP slurry in 0.5N sulfuric acid solution. It was incubated at  $121^\circ C$  at 15 psi pressure for 30 min. After cooling residue of the TKP is removed by filtration. The filtrate is neutralized by adding  $CaCO_3$ , with stirring to get proper mixing. This solution is heated to  $70^\circ C$  for 30 min and cooled to room temperature. This will help to react the  $CaCO_3$  and form complete precipitate of  $CaSO_4$  salt (Gypsum).It is removed by filtration and treated with 1% activated charcoal to remove any colored impurities. Reducible sugar concentration of the solution is checked by DNSA method, and solution is adjusted to get final sugar concentration of  $100\ g.l^{-1}$ . This solution is used as source of carbon for the fermentation study.

#### 2.3.1. Fermentation

LAB strains were grown on MRS Broth (HiMedia), which was sterilized in MilliQ water to get  $21.0\ g.l^{-1}$  solution. It was filtered through a sterile  $0.22\ \mu m$  poly ether sulfon (PES) membrane and stored in the 2 ml eppendorf tube in a 70% glycerol at  $-80^\circ C$  until use. This seed stalk vial is suspended in 20 ml of growth medium with composition as follow ( $g.l^{-1}$ ): protease peptone -10; beef extract -10; yeast extract - 5; dextrose - 100; polysorbate-80 - 1; ammonium citrate - 2; sodium acetate -5,  $MgSO_4$  - 0.10;  $MnSO_4$  - 0.05 and  $K_2HPO_4$  - 2; Final pH is adjusted to approximately 6.5. Growth media was sterilized by autoclaved at  $121^\circ C$  and 15 psi pressure for 15 min. It is incubated at  $38^\circ C$  for 48 hours with shaking

at 140 rpm for 48 hours.

10% v/v of viable cell is transferred to the standard production media of glucose with the similar composition as growth media and working volume of 100 ml in 250 ml Erlenmeyer flask. For all the shake flask experiment, the concentration of the sugar is kept approximately to 100 g l<sup>-1</sup>. Calcium carbonate (CaCO<sub>3</sub>) (50 % of weight of sugar) is used as neutralizing agent for the experiment. It was added to the flask while inoculating the bacteria, maintaining aseptic conditions. It is incubated at 38°C with rotation speed at 140 rpm.

Comparative study of four LAB strains for fermentative capacity to produce lactic acid was done by using similar conditions as the for the glucose standard culture, while replacing glucose carbon source with TKH reducible sugar. Fermentation kinetics was studied by observing different fermentation parameters as optical density, reducible sugar concentration and lactic acid. For this samples were taken at intervals of 10 hours up to 100 hour of reaction time. After each sample point, flasks were flushed with nitrogen gas, resealed and returned to the shaker platform.

All the experiments were carried out in triplicate and values were expressed as (±) standard deviation (SD) for numerical values in table and graphs with error bars.

### 3. Results and Discussion

#### 3.1. Fermentation with Standard Glucose Culture

**Table 1:** Fermentation parameters for the four different stain of lactobacillus in glucose standard culture

Parameters	LB - S1	LD - S2	LC - S3	LC - S4
Process Time ( Hr)	70	70	70	70
Max. O.D. <sub>600nm</sub>	9.4 ± 0.12	9.0 ± 0.21	10.4 ± 0.11	10.0 ± 0.24
Conversion ( g.g <sup>-1</sup> )	93.2 ± 1.37	96.0 ± 0.80	95.0 ± 1.33	93.7 ± 1.55
Lactic acid Yield (g.g <sup>-1</sup> )	91.7 ± 0.94	95.3 ± 0.47	94.6 ± 1.12	91.6 ± 0.51
Rate of production (g/l/hr)	1.31	1.36	1.35	1.31

All the data reported as a mean of triplicate analysis with standard deviation (taking 70 hour of reaction time)

#### 3.2. Tamarind Kernel Hydrolysate (TKH) Sugar Culture

Fermentation of a TKH sugar culture was performed in similar condition and environment as that of standard sugar culture. The observations obtained were reported with respect to the cell growth, sugar consumption and lactic acid production by four LAB stains. The results were summarized in table. 2, for the reaction time of 70 hours, as there is a nearly steady state after this fermentation time.

**Table 2:** Fermentation parameters of Lactobacillus strains in Tamarind Kernel Hydrolysate Sugar Culture

Parameters	LB-S1	LD-S2	LC-S3	LC-S4
Process Time ( Hr)	70	70	70	70
Max. O.D. <sub>620nm</sub>	8.6 ± 0.28	7.9 ± 0.41	9.4 ± 0.75	9.5 ± 0.28
Conversion ( g.g <sup>-1</sup> )	64.4 ± 1.73	62.1 ± 0.66	82.3 ± 1.09	80.5 ± 1.16
Lactic acid Yield (g.g <sup>-1</sup> ) <sup>a</sup>	62.9 ± 1.58	61.9 ± 1.16	75.3 ± 0.90	67.9 ± 1.98
Lactic acid Yield (g.g <sup>-1</sup> ) <sup>b</sup>	45.6 ± 1.15	44.9 ± 0.56	54.6 ± 0.65	49.2 ± 1.43
Rate of production (g/l/hr)	0.90	0.88	1.08	0.97

a - Lactic acid yield per gram of reducible tamarind kernel sugar,

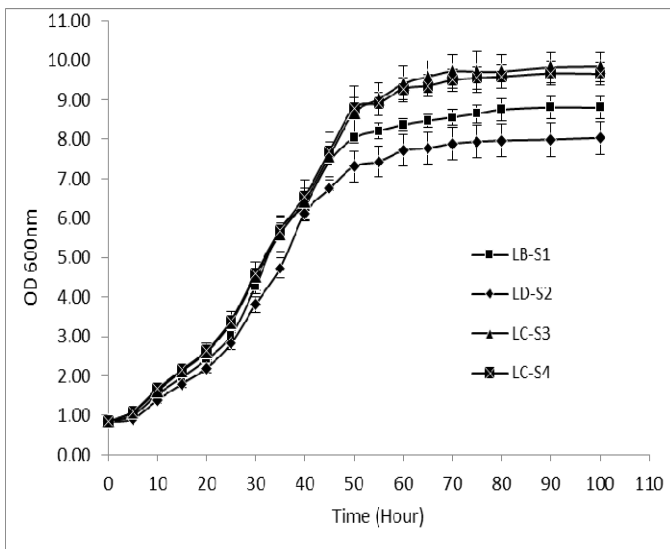
b - Lactic acid yield per gram of tamarind kernel powder

The four lactic acid producing bacteria (LAB) strains used in this study were selected on the basis of their special characteristics as: 1) they produce a single isomer of lactic acid, 2) they are commonly used in fermentation of agro wastes 3) they were most tolerant of the micro aerobic environment of the shake flasks [15]. **Table 1** represents the observations of the fermentation using standard glucose culture. It showed that there is nearly similar growth for the all four strains with more than 90 % of conversion in 70 hours of reaction time, hence this time is taken as optimum time of maximum production. Out of four strains LC- S3 has give highest growth followed by LC- S4, LB-S1 and then LD - S2. Glucose consumption rate was following the different trend than that of the cell growth. It shows highest rate of conversion of 96 % for the LD -S2 strain while LC- S3, LC-S4 and LB - S1 following it. There is relatively small difference for the glucose conversion for all the strains. This trend is continued in the lactic acid production and it is the general trend for lactobacillus. Lactic acid yield was 90 - 94 % for all the LAB strains used with LD -S2 and LC-S3 giving highest.

In the present study, two strains namely, LB -S1 and LD- S2 are homo fermentative bacteria, while the LC- S3, and LC - S4 are the facultative bacteria. Still they have given similar trend in cell growth, glucose consumption and lactic acid production. Hence they all follow the similar pathway for the fermentation of glucose.

#### 3.2.1. Cell growth, Glucose consumption and Lactic acid Production

The growth rates of all the LAB strains were measured by taking optical density (O.D.) at regular interval of time (figure.1). Out of for strains LC-S3 had shown maximum O.D. value followed by LC-S4. While LB-S1 and LD-S2 shows lower value of O.D. It shows nearly linear increment in the O.D with time, up to 50 hours for all four strains. After this there was negligible growth in O.D. up to 100 hour or reaction time. It shows the steady phase of the bacterial growth. This trend is similar to the trend obtained with the standard glucose culture. But in this process, bacterial growth reaches the steady state there is still more than 30 % of reducible sugar is present in culture. Generally the cell growth is found to get ceased, when there is an inadequate supply of the nutrients relative to the metabolic need of the bacterial cells. As TKH sugar is composed of galactose, xylose, and glucose in the proportion of 1: 2.25:2.8 respectively and about 2-3 % of the polyfuranose [12]. Thus, there is 60 % of the hexose (galactose and glucose) sugar and nearly 40 % of the pentose (xylose) sugar is present in the culture. Homo fermentative bacteria cannot consume xylose, while studies showed the presence of glucose affected the consumption of xylose, which was recognized as the repression of xylose uptake by glucose [17]. Even, as LC-S3 and LC-S4, an facultative hetero fermentative bacteria had shown lower growth after this phase.

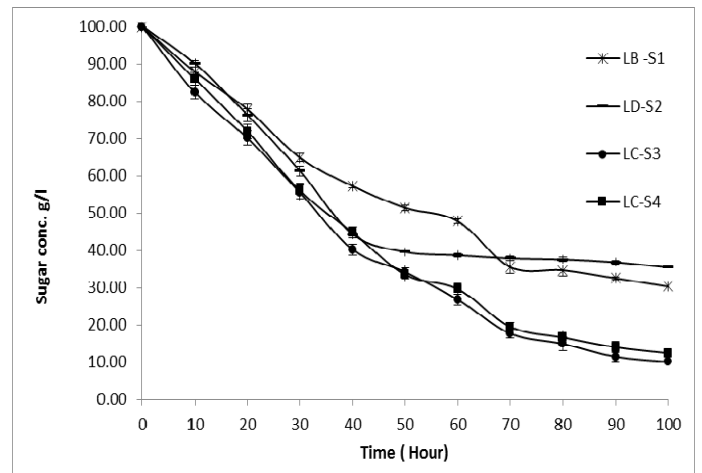


**Fig 1:** Growth rate of four Lactobacillus strains in TKH sugar culture.

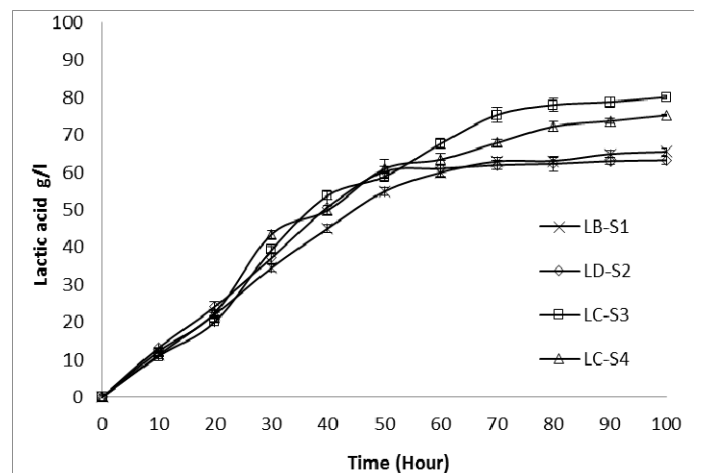
Glucose consumption and lactic acid production of LC-S3 and LC-S4 was greater than LB-S1 and LD-S2. The higher lactic acid production for a hetero fermentative species is usual, and it generate minimal amounts of acetic acid and formic acid with aldose sugar, at micro aerobic condition. (typical of homo fermenters). Homo fermenters utilize hexose sugar via the Embden–Meyerhof pathway (pentose phosphate/glycolytic pathway) [18]. Homo fermentative LAB possess aldolase enzymes and produce lactic acid as the major end product [19]. Therefore, as TKH sugar has hexose sugar (glucose and galactose) content of 60 % it is entirely feasible for all four strains to metabolize it by pentose phosphate/glycolytic pathway. Thus, up to this phase conditions were similar to that of the standard glucose culture. While the remaining xylose sugar part is difficult to consume for homo fermentative LB-S1 and LD-S2 bacterial strains. Hence they give a very slow rate of consumption of glucose with only 68 % of consumption.

While the LC-S3 and LC-S4, which are facultative hetero fermentative bacteria, consume the remaining xylose sugar by phosphoketolase pathway. Compared to glucose, the conversion of xylose requires additional enzymatic steps. Hence, when cells are exposed to xylose there is a lag of time before the enzymes required for ingestion appear, as some enzymes are inducible [6]. In the presence of glucose, genes required for xylose utilization are repressed. The several studies have shown that most microorganisms could not metabolize xylose and glucose simultaneously. Xylose began to be consumed when there was no glucose in the [20, 21]. It gives slower metabolism for xylose. LC-S3 and LC-S4 gives the conversion of nearly 90 % in 100 hour of fermentation time.

Lactic acid production for the LC-S3 and LC-S4 give highest production of lactic acid, giving a trend similar to the glucose consumption. LC-S3 given yield of 80 g l<sup>-1</sup> in 100 hour of reaction 100 hour of time while LC-S4 gives 80 g l<sup>-1</sup> in 100 hour. While the production of lactic acid was only 65 g l<sup>-1</sup> and 63 g l<sup>-1</sup> for LB-S1 and LD-S2 strain. The productivity was found to be 75 g l<sup>-1</sup> at reaction time of 70 hour with productivity of 1.08 g.l<sup>-1</sup> hour<sup>-1</sup> with respect to the TKH reducible sugar content (Table.2). With respect to dry TKP the yield of lactic acid was found to be, 54.6 g l<sup>-1</sup>.



**Fig 2:** Consumption of TKH Sugar by Lactobacillus strains in Tamarind Kernel Hydrolysate Sugar Culture



**Fig 3:** Production of lactic acid Lactobacillus strains in Tamarind Kernel Hydrolysate Sugar Culture

#### 4. Conclusion

In conclusion, fermentation of tamarind kernel hydrolysate sugar with homo and hetero lactic acid producing bacteria gave good yield of lactic acid. Out of the four strains used for this study, Lactobacillus casei (NCIM - 2125 (LC-S3)), gives highest conversion of almost 90 % of a available sugar. It gives lactic acid productivity of 80 %. The production of lactic acid was 0.58 g.g<sup>-1</sup> of tamarind kernel powder. Thus it is best to use this strain for the lactic acid production from tamarind kernel powder.

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