

QUANTIFICATION OF METABOLIC PRODUCTS FROM LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FERMENTED DAIRY PRODUCTS

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Abstract

The nature of fermented products is different from one region to another. This is depending on the local indigenous microflora, which in turn reflected the climate condition of the area. The lactic acid bacteria (LAB) are counted by pour plate method obtained from traditional fermented food products such as

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Curd, Buttermilk, Cheese and Yoghurt. Four isolates were identified as lactic acid bacteria. In curd, Gram staining shows long curved rods, occur usually single or in pairs and purple in color. It was identified as Lactobacillus acidophilus. In buttermilk, Gram staining shows small curved rods occur in chains or in pairs and purple in color. It was identified as Lactobacillus bulgaricus. In cheese, Gram staining shows cocci occur in short chains and purple in color. It was identified as Streptococcus thermophilus. In yoghurt, Gram staining shows cocci, occur singly in pairs or in short chains and purple in color. It was identified as Pediococcus acidilactici. To determine the metabolic products from four isolates such as protein, reducing sugar and lactic acid. Protein concentration was determined by the Lowry's method using bovine serum albumin as a standard. Reducing sugar was determined by the dinitro-salicylic acid method, using glucose as a standard. Lactic acid production was done by fermentation process. Compared to all the four isolates, Lactobacillus acidophilus was found to be producing higher amount of metabolic products.

Key words: Lactic acid bacteria, Fermented milk products, Metabolic products and

Fermented food products being rich source of nutrients for human, can also serve as a good medium for the growth and multiplication of microorganisms. The most lactic acid bacteria (LAB) inhibit pathogenic, non-pathogenic and spoilage organism in fermenting foods and beverages. Lactic acid bacteria have been used successfully in all fermented food products. The lactic acid bacteria (LAB) comprise a clade of Gram positive, acid tolerant, non-sporulation, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria are usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end product of carbohydrate fermentation. This trait has historically linked LAB with food fermentation as acidification inhibits the growth of spoilage agents. The LAB group comprises the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Alloicoccus*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactospaera*, *Oenococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus* and *Weissella*. Historically, the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* form the core of the LAB group (Saranraj *et al.*, 2013).

Members of these genera *Lactobacillus* plays an essential role in the fermentation of food and feed. The most important characteristics of the lactic acid bacteria are their ability to ferment sugars to lactic acid. This may desirable in making products and these organisms have been isolated and screened by using fermented foods such as curd, buttermilk, cheese and yoghurt. Different antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, carbon-di-oxide and bacteriocins produced by these bacteria can inhibit pathogenic and spoilage microorganisms extending the shelf-life and enhancing the safety of food products. There are many potential

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applications of protective cultures in various food systems. These organisms have been isolated from grains, dairy and meat products, fermenting vegetables and the mucosal surface of animals. The lactic acid bacteria not only have their effect on food and flavor but they are also known to produce and excrete compounds with antimicrobial activity, such as bacteriocins. Bacteriocins of LAB are considered as safe natural preservatives or biopreservatives as it is assumed that they are degraded by the proteases in gastrointestinal tract (Padmanabha Reddy *et al.*, 2006).

Materials and Methods

Collection of Samples

Curd, Buttermilk, Cheese and Yoghurt were collected individually in suitable sterile containers from the place of their availability and brought to the laboratory for further study.

Isolation and Identification of Lactic acid Bacteria

Serial dilutions were prepared using each sample of Curd, Buttermilk, Cheese fermented idly dough and Yoghurt as inoculum. 0.1 ml of the serially diluted inoculum from 10^{-4} to 10^{-6} were added on MRS agar plates. The plates were kept for incubation at 37°C for 48 hours. After the incubation period, observed the colony development, morphology and biochemical characteristics.

Maintenance of Lactic Acid Bacterial Cultures

The isolated Lactic acid bacterial cultures (*Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Pediococcus acidilactici*) were maintained by sub-culturing routinely in MRS Agar slants at every 15 days and stored in refrigerator at 4°C.

Maintenance of Pathogenic Microorganisms

Pathogenic bacterial isolates *viz.*, *Vibrio cholerae*, *Shigella sonnei*, *Bacillus cereus* and *Staphylococcus aureus* and were maintained by sub-culturing routinely in Nutrient Agar slants at every 15 days and stored in refrigerator at 4°C.

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Estimation of Protein

Protein estimation was done using crude bacteriocin by Lowry's method. 2% of Sodium carbonate in 0.1N Sodium hydroxide (Regent – A) was prepared and 0.5% of copper sulphate solution was prepared in 1 % potassium sodium tartarate (Reagent – B). Alkaline solution was prepared by mixing 50 ml of Reagent – A and 1 ml of Reagent – B. Commercial Folin-ciocalteau reagent was diluted with an equal volume of distilled water. Bovine serum albumin was used as standard solution to determine the protein in the bacteriocin. In a test crude bacteriocin and 5ml of alkaline solution was taken. It was kept for 10 minutes at room temperature. After that 0.5 ml of Folin-

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ciocalteau reagent was added and OD value of sample was taken after 30 minutes. Protein presents in the sample form a blue coloured complex and protein was estimated using Bovine serum albumin as standard solution (Lowry *et al.*, 1956).

Estimation of Reducing Sugar

The Reducing sugars present in the Lactic acid bacterial isolates were determined by Di-nitrosalicylic acid method. Hundred mg of the sample was weighed and the sugars was extracted with hot 80% ethanol twice (5 ml each time). The supernatant was collected and evaporated by keeping it on a water bath at 80°C. 10 ml of water was added and the sugars were dissolved. Three ml of the extract was pipette out in the test tubes and equalized the volume to 3 ml with water in all the tubes. Then, 3 ml of DNS reagent was added. The contents were heated in a boiling water bath for 5 minutes. The contents of the tubes were added with 1ml of 40% Rochelle salt solution. The tubes were cooled and the intensity of dark red color was measured at 510 nm (Stephen Wessels *et al.*, 2004).

Estimation of Lactic Acid Production

The Lactic acid production by fermentation process was carried out by inoculating the test organisms in MRS broth and incubated at 37°C for 72 hours. The Lactic acid bacterial cultures were centrifuged at 3000 rpm for 15 minutes. To the supernatant 5 mg/ml each of catalase and protease was added to neutralize the influence of Hydrogen peroxide and Bacteriocin respectively, known volume of the supernatant was used for the titration at 24 hours interval to determine the quality of Lactic acid produced. To 25 ml of the supernatant of the test organism, three drops of phenolphthalein were added as indicator, from a burette 0.1M NaOH was slowly added to the samples until a pink color appeared. Each ml of 0.1M NaOH is equivalent to 90.08 mg of lactic acid (Michel Bakar Diop *et al.*, 2007).

Results and Discussion

The lactic acid bacteria were isolated from traditional fermented foods such as Curd, Buttermilk, Cheese and Yoghurt and the isolates were *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Pediococcus acidilactici*. In this study, the lactic acid bacteria were characterized from the traditional fermented foods. The lactic acid bacteria were enumerated by pour plate and spread plate method. By the method of Gram staining the morphological characterization were observed under microscope. The lactic acid bacteria (LAB) are counted by pour plate method obtained from traditional fermented food products such as Curd, Buttermilk, Cheese and Yoghurt. The total lactic acid bacterial counts in curd was 12×10^7 cfu/ml, in buttermilk was 10×10^7 CFU/ml, in cheese was 11×10^7 cfu/ml, and in yoghurt was 9×10^7 cfu/ml (Table – 1).

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The lactic acid bacteria (LAB) are counted by spread plate method obtained from traditional fermented food products such as curd, buttermilk, cheese and yoghurt. The total lactic acid bacterial counts in curd was 6×10^7 cfu/ml, in buttermilk was 6×10^7 cfu/ml, in cheese was 8×10^7 cfu/ml, in yoghurt was 5×10^7 cfu/ml. The cheese shows the highest number of microbial count that is 8×10^7 cfu/ml and yoghurt shows the lowest number of microbial count that is 5×10^7 cfu/ml (Table – 2). Marilingappa Hamuna and Ko and Ahn, 2000 were isolated and characterized lactic acid bacteria from traditional fermented foods such as appam, batter and Yoghurt. Two *Lactobacillus* were isolated and used for the extraction of bacteriocin and used as biopreservatives.

Lactic acid bacteria was first isolated from milk (Metchnikoff and William Heinemann, 1908; Todorov, 2005; Catherine Lewus and Alan Kaiser, 1991; Carr *et al.*, 2002) had isolated lactic acid bacteria from curd which were tested for inhibitory activity against psychrophilic pathogens *via*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus plantarum* had been isolated from various habitats *via.*, cheese and milk (Gonzales *et al.*, 1994; Rekhif *et al.*, 1995). Wild lactic acid bacteria strains were isolated from Egyptian raw milk and were screened based on their yield of biomass production during fermentation process (Ayad *et al.*, 2004). Eight stains of lactic acid bacteria were isolated from brukino faso fermented milk sample (Strompfova *et al.*, 2005). The isolated *Lactobacillus plantarum* strain from boza, a traditional drink produced by fermentation of different cereals with yeast and lactic acid bacteria (Todorov, 2005). Salim Ammor *et al.*, 2006 reported the occurrence of lactic acid bacteria (36 *Lactobacillus sake*, 22 *Enterococcus faecium*, 16 *Lactobacillus graviorae*, 11 *Vagococcus caniphilus* and 2 *Enterococcus* sp).

Table – 1: Estimation of Total Microbial counts by Pour plate Method

S. No	Sources	Dilution	Total Microbial count (CFU/ml)
1.	Curd	10^7	12×10^7
2.	Buttermilk	10^7	10×10^7
3.	Cheese	10^7	11×10^7
4.	Yoghurt	10^7	9×10^7

Table – 2: Estimation of total Microbial counts by Spread plate Method

S. No	Sources	Dilution	Total Microbial count (CFU/ml)
1.	Curd	10^7	6×10^7
2.	Buttermilk	10^7	6×10^7
3.	Cheese	10^7	8×10^7
4.	Yoghurt	10^7	5×10^7

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After morphological identification the identified strains were treated for physiological, carbohydrate and biochemical test. In physiological various temperatures viz., 15, 37 and 45°C in broth were determined by visual turbidity after 72 hours incubation and different pH 4.4 and 8.6 were given for their growth. In carbohydrate various sugars Arabinose, Lactose, Maltose, Mannitol, Mannose, Sucrose and Xylose were given to the isolated strains to conform the particular genus and the biochemical test also analyzed for the isolated strains and Oxidase test given the positive result. The isolated strains were identified on basis of their morphological, physiological and biochemical characteristics, the lactic acid isomer produced, the ability to ferment sugars and 16S rDNA analysis. The genus was *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Lactococcus* (Harun *et al.*, 2007). The sources used are curd, buttermilk, cheese and yoghurt to identified the lactic acid bacteria under microscope observation to observe morphological characteristics of lactic acid bacteria. The *Lactobacillus acidophilus* was isolated from curd, *Lactobacillus bulgaricus* was isolated from buttermilk, *Streptococcus thermophilus* was isolated from cheese, *Pediococcus acidilactici* was isolated from yoghurt. Bacteriocin was extracted from four isolates. The bacteriocin activity was determined its antimicrobial activity.

The characteristics of *Lactobacillus* are rods, usually long and slender, that forms chains in most species. They are microaerophilic, but some are strict anaerobe is knowing, catalase negative and Gram positive, and they ferments sugars to yield lactic acid as the main product. They ferment sugars chiefly to lactic acid if they are homofermentative, with small amount of acetic acid, carbon-di-oxide and trace products, if they are heterofermentative, they produce appreciable amounts of volatile products, including alcohol, in addition to lactic acid. Most species of this non spore forming bacterium ferment glucose into lactate hence the name *Lactobacillus* is industrial production of fermented food production of fermented food products (Sozzy and Smiley, 1980).

The characteristics of cocci occur in pairs, short chains or in long chain, depending upon the species and the conditions of growth and all are homofermentative. *Streptococcus* lactic grows well in milk and ferment the lactose to 0.8 to 1.0 percent acid of which L(+) lactic acid constitutes nearly all of the acid formed, although traces of acetic and propionic acid may be present. Optimum temperature of 30°C and a temperature range of 10°C to 40°C were required (Orberg and Sandline, 1985).

The characteristics of cocci occur in single, in pairs or in short chains or in tetrads and are Gram positive, catalase negative and microaerophilic. They are homofermentative, fermenting sugars to yield 0.5 to 0.9 per cent acid, mostly lactic and they grow fairly well in salt brines upto 5.5 per cent and poorly in concentrations of salt up to about ten per cent. They grow in the temperature 45°C but the best in 32°C.

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Pediococcus have been found growing, during the fermentation of brined vegetables (Piva and Headon, 1994).

To determine the metabolic products from four isolates such as protein, reducing sugar and lactic acid. Protein concentration was determined by the Lowry's method using bovine serum albumin as a standard (Table – 3). Reducing sugar was determined by the dinitro-salicylic acid method, using glucose as a standard (Table – 4). Lactic acid production was done by fermentation process (Table – 5). Compared to all the four isolates, *Lactobacillus acidophilus* was found to be producing higher amount of Lactic acid production.

Adesokan *et al.*, 2009 isolated seven species of lactic acid bacteria (LAB) namely *Lactobacillus fermentum*, *Lactobacillus casei*, *Lactobacillus delburekii*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Leuconostoc mesenteroides* were isolated from ogi, burukutu and retted cassava. The isolates were screened for quantitative production of lactic acid using normal MRS broth and modified MRS broth under varying conditions of growth such as temperature and pH and influence of carbon and nitrogen sources. It was observed that all the test isolates best utilized glucose and yeast extract at concentrations of 20g/lit and 5g/lit respectively for production of 30°C and pH 5.5. *Lactobacillus plantarum* produced the highest quantity of lactic acid production.

Table – 3: Determination of Protein from Lactic acid Bacteria

S. No	Organisms	Absorbance	Protein (mg/ml)
1.	<i>Lactobacillus acidophilus</i>	1.08	1.20
2.	<i>Lactobacillus bulgaricus</i>	1.01	1.11
3.	<i>Streptococcus thermophilus</i>	0.96	1.05
4.	<i>Pediococcus acidilactici</i>	0.90	1.00

Table – 4: Determination of Reducing Sugar from Lactic Acid Bacteria

S. No	Organisms	Absorbance	Reducing sugar (mg/ml)
1.	<i>Lactobacillus acidophilus</i>	0.78	1.00
2.	<i>Lactobacillus bulgaricus</i>	0.50	0.95
3.	<i>Streptococcus thermophilus</i>	0.47	0.90
4.	<i>Pediococcus acidilactici</i>	0.41	0.80

Table – 5: Determination of Lactic acid from Lactic acid bacteria

S. No	Organisms	Lactic acid production (ml)
1.	<i>Lactobacillus acidophilus</i>	1.35
2.	<i>Lactobacillus bulgaricus</i>	1.09
3.	<i>Streptococcus thermophilus</i>	0.98
4.	<i>Pediococcus acidilactici</i>	0.85

Conclusion

From this present study, it was concluded that the bacteriocin produced by Lactic acid bacteria can be applied both in, food industry and medical sector. They can be used in cheese, fermented sausage, beer, etc., yielding food quality and food safety advantages. Bacteriocins of Lactic acid bacteria can play a role in human gastrointestinal track, hence contributing to human health.

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