Screening of Lactic Acid producing bacteria and assessment of the Probiotic Effect of Plant Extracts on the selected bacteria.

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Abstract: Lactobacilli are gram positive, coccobacilli or rods, non-spore forming cocci. Lactic acid can be produced by carbohydrate fermentation or chemical synthesis. Lactic acid bacteria produce lactic acid as an end product by carbohydrate fermentation. In the present study lactic acid bacteria from different samples like fermented foods, Milk, Curd, Curry leaves, Tomato leaves, Fermented Milk were isolated and characterized. Effect of pH, temperature, carbon sources and bile salts on lactic acid production was determined and estimation of Lactic acid was carried out**.** Efficiency and evaluation of the probiotic effect of plant extracts of bilva (*Aegle marmelos*) and *Aloevera* were observed on selected isolates.

Key words: 1. Lactobacilli 2. Fermentation 3. Probiotic 4. Lactic acid bacteria

Introduction:

Lactic acid bacteria is a gram positive, non- sporing, devoid of cytochromes, acid tolerant, low gas content, catalase negative, nonaerobic habit but aerotolerant, fastidious and strictly fermentation (Khalid, 2011). Either rod shaped (bacilli), or spherical (cocci) bacteria These organisms have the property of producing lactic acid from lactose and other carbon sources by fermentation they are classified as lactic acid bacteria (LAB). LAB produces lactic acid as an end product by carbohydrate fermentation. The production of lactic acid can be done in two ways which are carbohydrate fermentation and chemical synthesis (Narayanan *et al.,* 2004).LAB can be classified based upon their morphology (cocci or rods, tetrad formation), growth at different salt concentrations and temperatures, fermentation of the lactic acid production (D, L, or both) (Axelsson, 2004).Lactic acid bacteria can be generally divided into two groups based upon the glucose fermentation pathways, those organisms which produce acetic acid, CO_2, and ethanol in addition to lactic acid are termed as heterofermentative lactic acid bacteria, and those organisms which produce exclusively lactic acid as end product are termed as homofermentative lactic acid bacteria (Lalam *et al.*, 2015).

Homofermentative LAB and Heterofermentative LAB are showed in table 1 (Lalam *et al*., 2015).

Table 1: Homofermentative and heterofermentative LAB

Homofermentative LAB	Heterofermentative LAB
Pediococcus	*Carnobacterium*
Lactococcus	*Oenococcus*
Streptococcus	*Enterococcus*
Vagococcus	*Leuconostoc*

Lactobacilli *Lactosphaera*

Lactobacilli

Lactic acid bacteria can be obtained from various sources as shown in the following table 2. (Shiphrah *et al.*, 2013).

Table 2: Sources of lactic acid bacteria

Various sources of lactic acid bacteria	
Milk	Cheese
Fermented Milk	Curd
Ragi	Buttermilk
Mango pickle	Chilli pickle
Curry Leaves	Tomato Leaves
***Solanum melongena* (Brinjal)**	*Alium cepa* (Onion)
Fruit juices	Sweet yoghurt
Sour yoghurt	Bitter gourd grass
Curd	Long grass,
South Indian fermented food (koozh),	Indian fermented food like idli batter, Sambhar, chutni

Probiotic is the maintenance of healthy gut micro flora, which may provide protection against gastrointestinal infections, gastrointestinal disorders and inflammatory bowel diseases. It was suggested that the efficiency of probiotics to offer a proper alternative to the use of antibiotics in the treatment of enteric infection (Marteau *et al.*, 2001) or to reduce the symptoms of antibiotic - associated diarrhoea (Rastall *et al.*, 2005). Lactic acid bacteria are microorganisms that play a major role in the production of fermented food. Lactic acid bacteria have many properties like: i. Anticarcinogenic ii. Antimutagenic activities iii. Hypocholesterolemic properties iv.Antagonistic action.

LAB generally utilized for enhancing the food safety and extended shelf life of food products because lactic acid bacteria mostly known to inhibit food borne pathogen and food spoilage microorganisms by producing various substances like ethanol, acetic acid, bacteriocins, CO_2 lactic acid, several aromatic compound and hydrogen peroxide. Lactic acid bacteria produce bacteriocins it can be easily degraded by the human gastrointestinal proteases because of this important feature LAB generally gained importance in the recent years in food preservation. Bacteriocins are nothing but extracellular bacterial proteins secreted by the cells (Cleaveland *et al*., 2001). LAB has a long history of safe use and members of the genera *Lactococcus* and *Lactobacillus* have been given generally regarded as safe (GRAS) status (Salminen *et al*., 1998). Lactic

acid has larger applications in various industries like Pharmaceutical industries, Food industries,Cosmetic industries and Chemical industries

Production of lactic acid by fermentation processes: Fermentations for lactic acid production are continuous- batch, repeated-batch, fed-batch. But batch and fed-batch cultures achieved higher concentration of lactic acid as compared with others, whereas higher productivity has been obtained by continuous cultures.

a) **Submerged Fermentation can be of the following types:**
   i. **Batch fermentation**
   ii. **Fed-batch fermentation**
   iii. **Continuous fermentation.**
b) **Solid-state Fermentation**
c) **Anaerobic Fermentation**
d) **Aerobic fermentation**

Lactic acid bacteria as probiotics

Probiotics are termed as "living micro-organisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition" (Guarner and Schaafsma, 1998; Tannock, 2002). Most probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as *Enterococcus sp, Lactobacillus sp,* and *Bifidobacterium spp.* (Klein *et al.,* 1998).Importance of *Lactobacillus sp.* in fermented foods one also needs to consider their importance as a probiotics. It was later defined as a live microbial feed supplement, which is beneficial to the host animal through improving its intestinal microbial balance. *Lactobacillus spp.* has been used as probiotic organisms. Wide range of lactobacilli has been used as in probiotic preparation these include *L.delbreuckii sub spp., L.casei, L.brevis, L.fermentum, L.planterum, L.reuteri, L.cellobiosus* (Steinkrause, 1995; Vinderola *et al.,* 2002). The pre-existing flora of the digestive tract is defined and complex which makes it very difficult to determine how the probiotic influence the intestinal eco system. The presence of microbial flora is necessary for the normal function of the digestive system. Elimination of severe perturdations of the flora leads to diarrhoea or constipation and the maintenance of healthy bacterial flora is therefore desirable.

Probiotics have been administered to patients with acute diarrhoea of various etiologies in several studies. Particularly in studies in children one can assume that enterotoxigenic *E.coli* (ETEC) and rotavirus dominate as enteric pathogens. *L. acidophilus* LB added to the oral rehydration therapy during acute diarrhoea reduced the duration of diarrhoea in treated children compared to the placebo group (Simakachorn *et al.,* 2000).Pancreatic juice inhibits growth of multi-resistant bacteria strain and some probiotic bacteria. However, individual strains tolerate growth in media supplemented with pancreatic juice independent of proteolytic activity (Kruszewska *et al.,* 2004). Mechanisms of adherence to an epithelial surface involve both receptor-specific binding and charge and hydrophobic interaction commonly express cell surface hydrophobicity (CSH) as measured through the Salt Aggregation Test (SAT).

In recent years, multiple reports have described beneficial effects from various aspects on important diseases, like inflammatory bowel disease (IBD), intestinal infection and allergy by addition of selected strains to food products, often together with fibre or a probiotic substance. In many countries, there are now several probiotic products in the market but the documentation is often based upon case reports, animal studies or uncontrolled small clinical trials. Furthermore, there is no general acceptance on how to characterize probiotic microorganisms, and few products declare the actual content of live microorganisms.

The present study has been undertaken to isolate lactic acid bacteria and characterize those that produce lactic acid, from fermented foods, Milk, Curd, Curry leaves, Tomato leaves, Fermented Milk. Production of lactic acid was estimated and optimisation of various parameters were checked and determination of the probiotic effect of plant extracts on the selected isolates was also carried out.

Applications of lactic acid bacteria (LAB)

Lactic acid has larger application in cosmetic, food, pharmaceuticals and chemicals industries and it is precursor to several products. Lactic acid has also found applications in leather tanning industry, in descaling processes. Even esters, salts and lactic acid derivatives are used as plasticizers, emulsifiers and solvents (Trindade, 2002). This organic acid (lactic acid) is also used in the production of acetaldehyde, 2, 3-pentanedione, lactide, polylactic acid, propylene oxide, ethyl lactate, acrylic acid and propanoic acid.Lactic acid acts as a mordant (fixative) for dyeing.In the cosmetic industry, lactic acid is used in the manufacture of aesthetic products and hygiene because of its antimicrobial, moisturizing and rejuvenating on the skin. As well as it is used in oral hygiene products, (Martinez *et al.*, 2013).In the pharmaceutical industry, lactic acid is used in dialysis, surgical sutures, implants, pills and controlled drug release systems. Lactic acid have been developed new application, such as production of biocompatible and biodegradable PLA polymers (Abdel-Rahman *et al.*, 2013) solvents and oxygenated chemicals.

Materials and methods

Sample collection:-

Various samples were collected from various regions. Milk sample (buffalo milk) was collected from Laskana region (21.255˚N, 72.932˚E), Surat. Tomato leaves were collected in sterile zip lock bag and brought to the laboratory from Sarakadiya village (21.0956˚N, 71.2867˚E), district: Amreli. Curry leaves were collected from Surat (21.1702˚N, 72.8311˚E), Gujarat. Sumul curd sample was collected from local dairy, Sarthana region (21.2306˚N, 72.9015˚E), Surat, Gujarat. Ragi and Sporolac tablet samples were collected from local market. All samples are used to isolate lactic acid bacteria.

Isolation and screening of lactic acid producing bacteria:

Medium used

De Man Rogosa Sharpe (MRS) medium was used for isolation of LAB. It is a selective medium for isolation of LAB. The MRS medium was sterilized by autoclaving at 121°C for 20 minutes and cooled to 50° C. The pH of the medium was adjusted to 6.5 and used.

Sample preparation and isolation

All samples were subjected to streak plate method for isolation of bacteria. (four flame streaking method).

1. For Curd sample, five ml of curd sample was added into the 5 ml sterile water and streak on MRS agar plate.
2. For Milk and Fermented milk sample, (fermented milk was prepared by keeping pure fresh milk in a clean bottle. The milk was capped tightly and kept at room temperature for 2 days and then used) (Hnin *et al.,* 2015) 4.5 ml sample was added in to 4.5 ml of sterile water, mixed well and streaked on MRS agar plate.
3. 60 gm Ragi flour was added into the 50 ml sterile water under aseptic conditions and incubated room temperature for 2 days to ferment the Ragi. After the incubation 4.5 ml fermented Ragi was added into the 4.5 ml sterile water, mixed well and streaked on MRS agar plate.
4. The samples consisted curry and tomato leaves was cut with the cutter or scissors and added in 5 ml normal saline. The above mixture was allowed to stand at room temperature for atleast 15 to 20 minutes and then streaked on MRS agar plate.
5. Sporolac tablet was added into the MRS broth and incubated for 10 minutes till the tablet dissolved, then a loopful of broth was streaked on MRS agar plate.

All the plates were incubated in an inverted position for 2 days at 37°C in incubation. Bacterial colonies growing on incubated plates were picked up carefully and streaked on the MRS agar medium following the four flame streaking technique for further purification. Single colonies were picked up and streaked on MRS slant and the grown out cultures were maintained at 4˚C in refrigerator for further studies. The isolates were assigned the code numbers. Isolated bacteria were streaked on MRS agar plates containing 0.8% calcium carbonate to distinguish lactic acid producing bacteria, from other bacteria and incubated at 37˚C for 48 hours. Colonies of acid producing bacteria are identified by a clear zone around each streak.

Morphological and biochemical characterization of the lactic acid bacteria

Morphological characterization of bacteria was examined by cell shape (such as size, shape, margin, surface, opacity, elevation, consistency, pigmentation) and gram staining reaction. The following biochemical tests, viz., Catalase test, Oxidae test, Indole test, Methyl Red test, Voges-Proskauer's test, Simmon Citrate, Fermentation test of Carbohydrates **(**Glucose, Maltose, Xylose, Lactose, Mannnitol), and Gelatin liquification tests were carried out for biochemical characterization of lactic acid producing bacteria.

Production of lactic acid

MRS broth was prepared and autoclaved it at 121˚C for 20 min. Cooled down at room temperature and inoculate with 24 hour old bacterial culture. Incubated at 37˚C and lactic acid was estimated by titration method at 24 hour, 48 hour and 72 hours.

Optimization of fermentation conditions.

Effect of pH on lactic acid production

Lactic acid bacteria were grown into broth with various pH values such as 6, 7, and 8 and then autoclaved. Then 50 ml MRS broth was inoculated with 2 ml of culture and incubated at 37˚C. After the incubation, measurement of the lactic acid by titration method was done at 24 hour and 48 hours.

Effect of temperatures on lactic acid production

50 ml MRS broth was inoculated with 2 ml of culture and incubated at different temperatures 4˚C, 25˚C and 37˚C. After the incubation, lactic acid was estimated by titration method carried out at 24 and 48 hours.

Effect of carbon sources on lactic acid production

Lactic acid bacteria were grown in broth amended with different carbon sources. 50 ml MRS broth was prepared with various carbon sources like Starch, Glucose and Lactose. One gram of different carbon sources was added into the sterile 50 ml broth. Inoculated with culture and incubated at 37˚C for 24 and 48 hour. Measurement of the lactic acid by titration method was done at 24 and 48 hours.

Effect of bile salt on lactic acid bacteria

MRS broth was prepared with bile salt (0.3%) and without bile salt and then autoclaved. Broth was inoculated with bacterial culture and incubated at 37˚C for 24 hours. The growth of the bacteria in presence and absence of bile salt was measured by spectrophotometer at 660 nm after 1, 2 and 3 hours.

Estimation of Lactic acid

The amount of lactic acid in fermentation broth was determined by transferring 25 ml of culture broth of LAB isolates into 100 ml flask. One ml of phenolphthalein indicator (0.5% in 5% alcohol) was added into the flask. This was titrated with 0.25 M NaOH for the appearance of pink color. The titratable acidity was calculated as lactic acid % W/V. Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid (Fortina, 1973).

Probiotic effect of medicinal plants [Bilva (*Aegle marmelos*) and *Aloevera*] on the selected isolates

Extract preparation:-

For preparation of Bilva (*Aegle marmelos*) and *Aloevera* extract, 20 gm powder of specific medicinal plant (*Aegle marmelos* (Bilva) and *Aloevera*) add into the 100 ml distilled water and then incubated in shaker at 120 rpm for 2 days. Then filtered through Whattman filter paper no.1 and prepared extract.

To assess the probiotic effect of medicinal plant extracts:-

1ml extract of specific medicinal plant was added into the 5 ml MRS broth, and inoculated with 24 hour young bacterial culture of the selected isolates. Then incubate it overnight at 37˚C and next day the growth was observed at 560 nm by using spectrophotometer.

Results and discussion:

Collection of samples

Sample collection sites are shown in figure 1.

Collection of samples such as Milk, Curry leaves, Tomato leaves, Ragi, Fermented Milk, Curd, Sporolac tablet and Fruit sample (Papaya) shown in figure 2. A total of 17 gram positive bacteria were obtained by using de Man Rogosa Sharpe (MRS) agar medium.

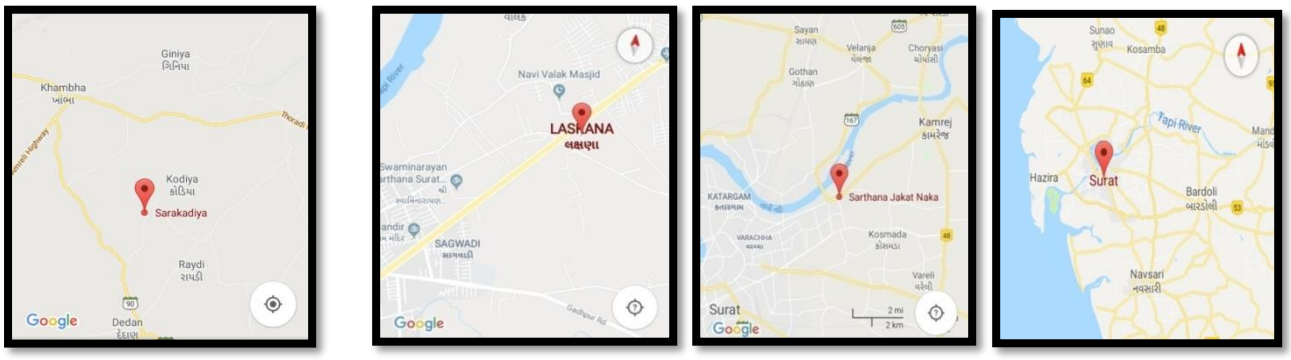


Figure 1: Sample collection site

Figure 2 : Collection and preparation of samples

The following table 3 shows bacteria isolated from different sample with the codes provided.

Table 3: List of LAB isolates from different sources with code numbers .

Sample	Sample No.	Code number of isolates	Total number of isolates
Papaya fruit	1	LAB 1	2
	2	LAB 2	
Curry leaves	1	LAB 3	1
Milk	1	LAB 4	1
Fermented milk	1	LAB 5	2
	2	LAB 6	
Ragi	1	LAB 7	3
	2	LAB 8	

	3	LAB 9	
Tomato leaves	1	LAB 10	1
Sporolac tablet	1	LAB 11	1
Curd	1	LAB 12	2
	2	LAB 13	
Curd	1	LAB 14	2
	2	LAB 15	
Curd	1	LAB 16	1
Curd	1	LAB 17	1

Isolation and Morphological characterization of bacteria on MRS Agar

Isolation of bacteria was carried out and several colonies showing different morphologies were selected. The colony characteristics of selected isolates were studied on de Man Rogosa Sharpe (MRS) agar plate. The results of obtained bacterial colonies are shown in figure 3.

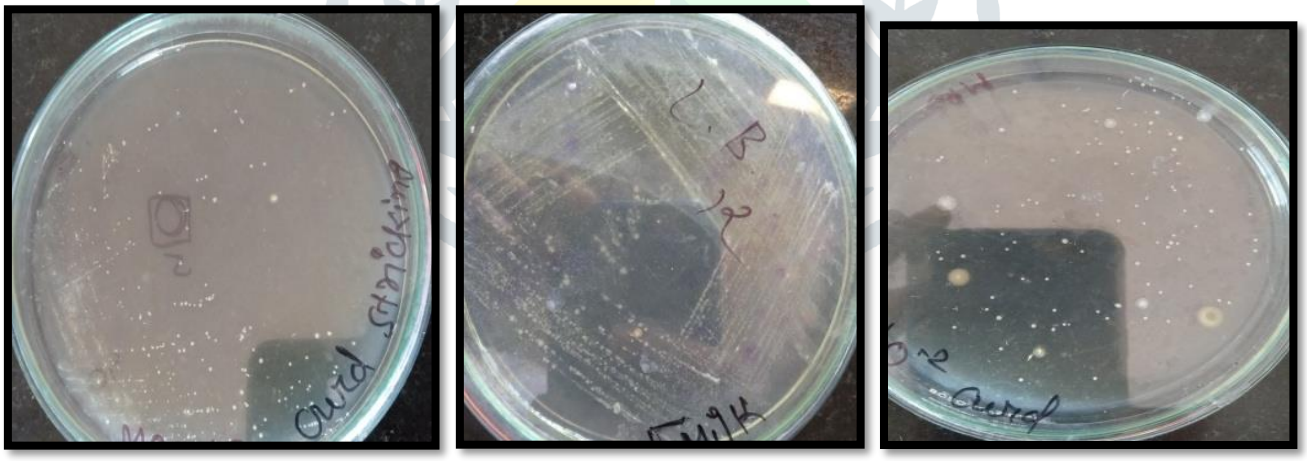


Figure 3: Growth of bacteria on MRS agar plate

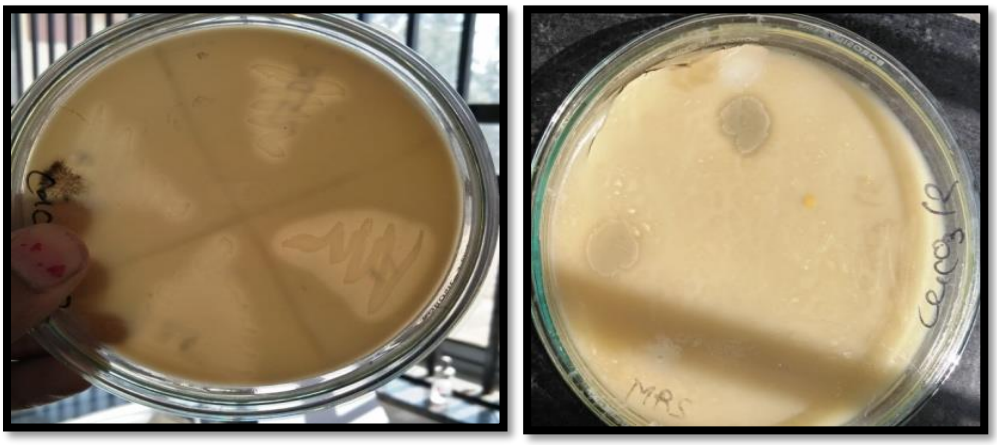
Generally all lactic acid producing bacteria are gram positive cocci or short rods and they were non motile. The morphological characterization of all isolates is given in following table 4.

Table 4 : Morphological characterization of all isolates growing on MRS agar plate

No.	Sample	Size	Shape	Edge	Elevation	Texture	Consistency	Opacity	Pigmentation	Gram staining
LAB-1	Papaya Fruit	Small	Circular	Lobate	Convex	Smooth	Moist	Opaque	none	Gram positive filamentous bacteria
LAB-2	Papaya Fruit	Pin point	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci
LAB-3	Curry leaves	Small	Round	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive diplo
LAB-4	Milk	Medium	Irregular	Entire	Convex	Rough	Moist	Opaque	None	Gram positive cocci
LAB-5	fermented	Small	Circular	Entire	Convex	Smooth	Dry	Opaque	Yellow	Gram positive
LAB-6	fermented milk	Medium	Irregular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in clusters
LAB-7	Ragi	Small	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in clusters
LAB-8	Ragi	Pin point	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in chain
LAB-9	Ragi	Small	Circular	Entire	Convex	Smooth	Dry	Opaque	None	Gram positive cocci
LAB-10	Tomato leaves	Small	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive rods
LAB-11	Sporolac Tablet	Medium	Circular	Entire	semi-submerged	Rough	Dry	Transluce nt	None	Gram positive rods
LAB-12	Curd	Pin point	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in clusters
LAB-13	Curd	Small	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in singly
LAB-14	Curd	Medium	Circular	Entire	Low-Convex	Smooth	Moist	Opaque	None	Gram positive cocci occurring in chains
LAB-15	Curd	Small	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive rods occuring singly
LAB-16	Curd	Large	Circular	Entire	Raise	Smooth	Dry	Opaque	None	Oval cells
LAB-17	Curd	Small	Circular	Entire	Convex	Smooth	Moist	Opaque	None	Gram positive Short and long rod

Clear zone of solubilization on MRS agar

Results of selected bacteria streaked on MRS agar plate, containing 0.8 % $CaCO_3$ and incubated at 37˚C for 48 hours. Lactic acid producing bacteria shows the clear zones of solubilization surrounding the colonies as observed in figure 4 and table 5 .



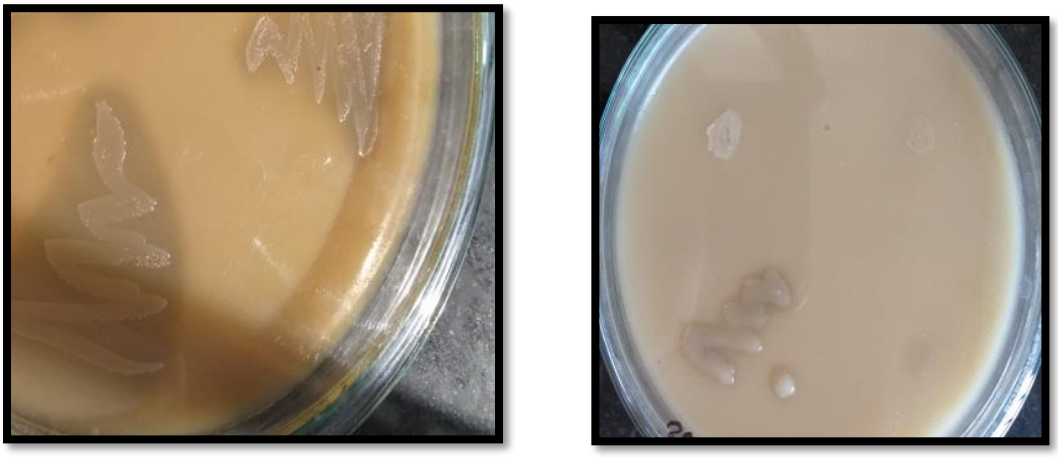


Figure 4: Zones of solubilization on MRS + 0.8% $CaCO_3$ plate

Table 5: Observation of clear zone on MRS + 0.8 % $CaCO_3$

Sample	Isolate no.	Zone observed on MRS + 0.8%$CaCO_3$
Papaya Fruit	LAB 1	NO
Papaya Fruit	LAB 2	NO
Curry Leaves	LAB 3	NO
Milk	LAB 4	YES
Fermented Milk	LAB 5	NO
Fermented Milk	LAB 6	YES
Ragi	LAB 7	YES
Ragi	LAB 8	YES
Ragi	LAB 9	YES
Tomato Leaves	LAB 10	NO
Sporolac tablet	LAB 11	YES
Curd	LAB 12	YES
Curd	LAB 13	NO
Curd	LAB 14	NO
Curd	LAB 15	YES
Curd	LAB 16	NO
Curd	LAB 17	YES

Biochemical characterization of isolates

The biochemical tests like Catalase test, Oxidae test, Indole test, Methyl Red test, Voges-Proskauer's test, Bile esculin test, Simmon Citrate, Gelatin hydrolysis, Fermentation test of Carbohydrates **(**Glucose, Maltose, Xylose, Lactose, Sorbitol, Mannnitol), were performed. Biochemical characterization of selected isolates is given in table 6.

Table 6: Biochemical characterization

	Tests								Carbohydrates					
Isolates no.	Catalase test	Oxidase test	MR test	V-P test	Citrate	Indole	Gelatin	Hydrolysis Bile – esculin	Glucose	Maltose	Lactose	Sorbitol	Xylose	Mannitol
LAB 4	-	-	-	-	-	-	-	+	+	+	+	-	-	+
LAB 6	-	-	-	-	-	-	-	-	+	+	+	-	+	+
LAB 8	-	-	-	-	-	-	-	-	+	+	-	+	-	+
LAB 9	-	-	-	-	-	-	-	-	+	+	-	-	-	+
LAB 11	-	-	-	-	-	-	-	-	+	-	-	-	-	-
LAB 12	-	-	-	-	-	-	-	+	+	+	-	-	-	-
LAB 15	-	-	-	-	-	-	-	-	+	+	+	+	-	-
LAB 17	-	-	-	-	-	-	-	+	+	+	-	-	-	-

Positive: +, Negative: -

The bacteria selected for further studies showed clear zone of solubilisation and were identified according to the morphological and biochemical characteristics as LAB 6 is *Lactococcus lactis*, LAB 8 is *Lactococcus cremoris*, LAB 12 is *Streptococcus sp.* and LAB 15 is *Lactobacillus lactis spp.*. The selected isolates were used for the fermentation and optimization studies.

Production of lactic acid

- Culture was inoculated with MRS broth and incubated at 24 hour, 48 hour and 72 hours. Lactic acid was quantitatively measured by titration method after 24, 48 and 72 hours. Result gives yellow to pink color at the end point of titration is shown in figure 5 and figure 6.

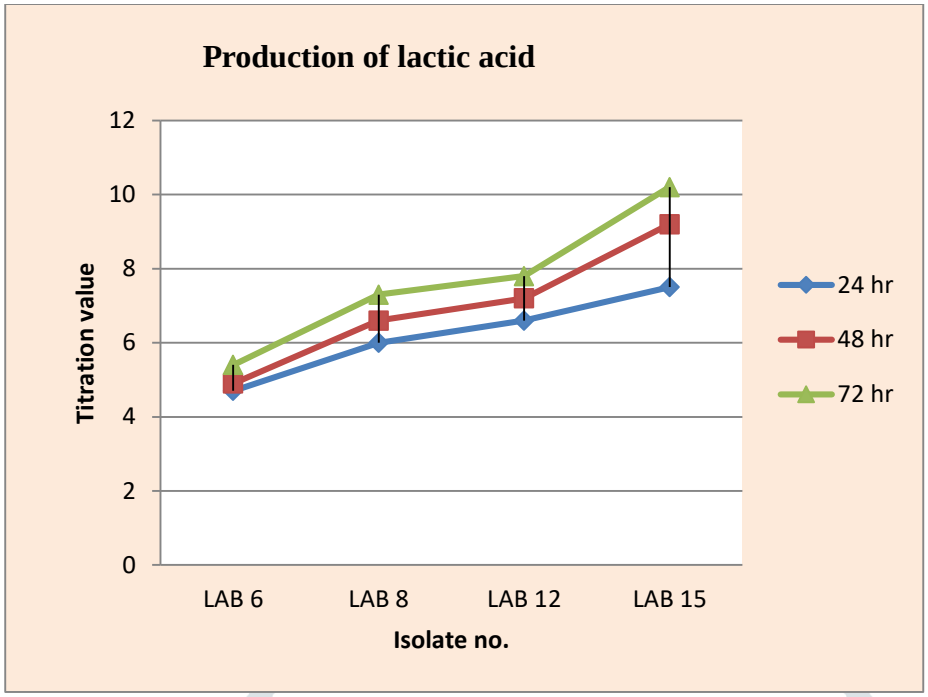


Figure 5: Production of lactic acid

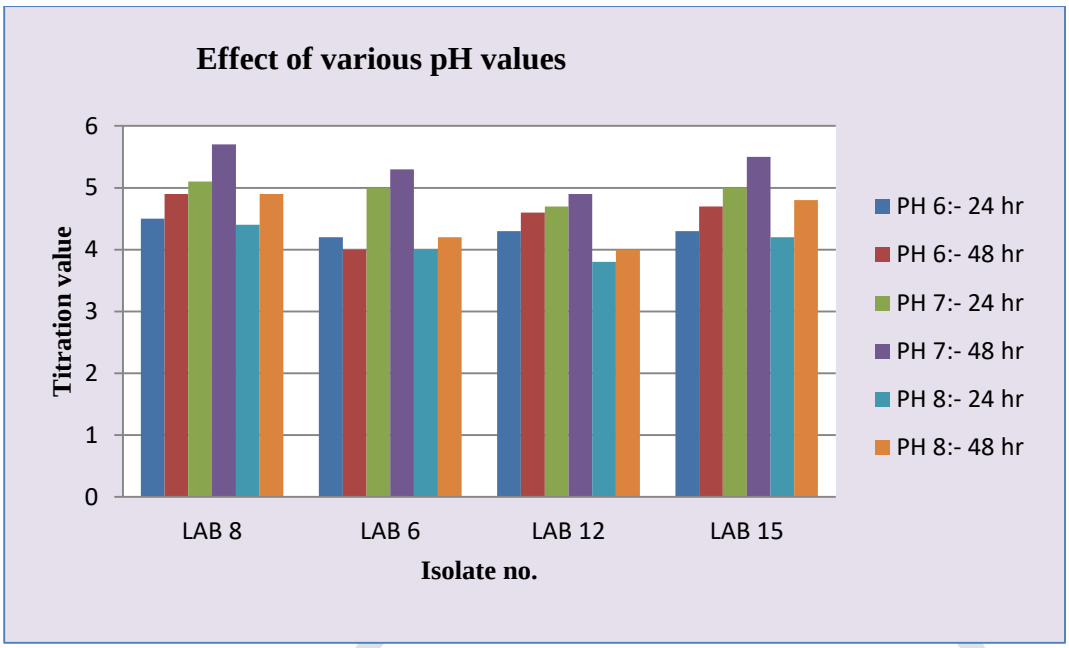
As evident from the results, an increase in lactic acid production was found up to 72 hour and thereafter no improvement of the lactic acid production was observed this could be attributed due to the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. A maximum lactic acid production of 0.22gm/L in LAB 15 was observed after 72 hour of incubation.

Therefore, fermentation time of 72 hour was considered optimal for lactic acid production. The fermentation period of 48 hour has been generally used for lactic acid production using different *Lactobacilli* cultures (Chiarni *et al.*, 1992; Gandhi *et al.*, 2000; Kumar *et al*., 2001). Hujanen *et al.*, (1996) selected homofermentative strain of *Lactobacillus casei* subsp *Rhamnosus* NRRL B-445 for lactic acid production using grass extract as a nitrogen source. Lactic acid was produced at 73 hours of fermentation period. In the present investigation the same result that lactic acid was maximum in the fermentation period of 72 hours but in the presence of glucose as carbon source, when compared with 24 and 48 hours was revealed.

Figure 6: Results of lactic acid estimation by titration method

Optimization of various parameters

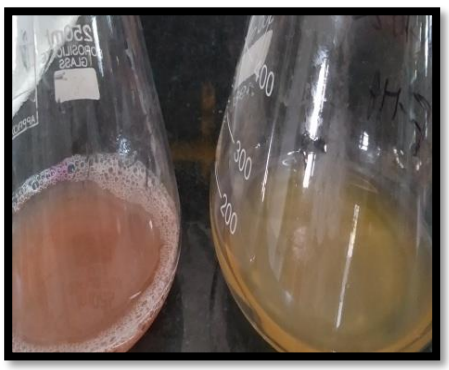
Figure 7: Effect of various pH values on lactic acid production



Effect of various pH values on lactic acid production:

The effect of pH on lactic acid production was estimated in MRS broth having different PH values of 6, 7 and 8 is showed in figure 10 and color change from yellow to pink by using titration method is shown in figure 8.Fortina, suggested in 1973, that each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid means 1000 ml of 1 N NaOH is equivalent to 90.08 gm of lactic acid. In our study the maximum lactic acid production was 5.7 ml of 0.25 M NaOH which is equivalent to 0.12 gm of lactic acid observed in LAB 8 isolate at pH 7. However, at higher and lower pH levels, there was decrease in lactic acid production at pH 5.0 and pH 8.0.A pH range of 6.5- 7.0 has been reported optimal for lactic acid production. The growth of four isolates LAB 8, LAB 6, LAB 12, and LAB 15 were higher in pH 7, when compared with pH 6 and pH 8.Pansuer *et al*., (2010) reported that maximum lactic acid production (33.48gm/l) was observed at pH 6.5.Ghaly *et al.,* (2004) found that pH 5.5 has been use for lactic acid production using *L. helveticus*. Krischke *et al.,* (1991) reported that a pH range of 6.0-6.5 has been optimal for lactic acid production using *L. casei* strain.

pH:6



pH:7

pH:8

Figure 8: Results of various pH values

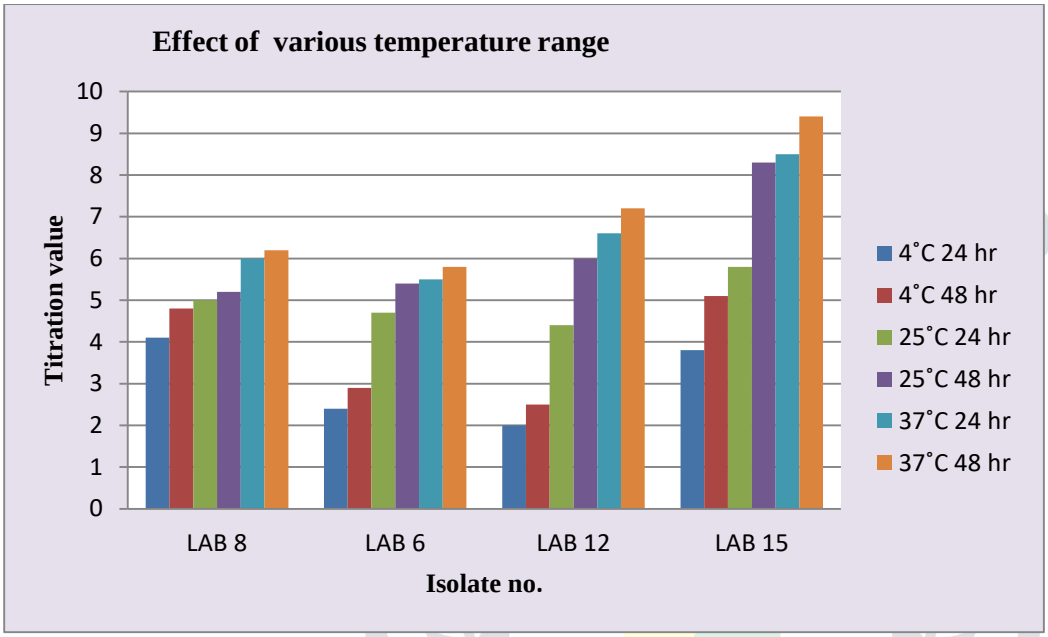


Figure 9: Effect of various temperatures on lactic acid production:

The effect of temperature on lactic acid production was estimated by cultures inoculated in MRS broth and incubated at different temperatures like 4˚C, 25˚C and 37˚C is showed in figure 9 and results of colour change from yellow to pink by using titration method is shown in figure 10.

In our study, the maximum lactic acid production was 9.4 ml of 0.25 M NaOH which is equivalent to 0.21 gm of lactic acid observed in LAB 15 isolate at 37˚C. However, at higher and lower temperature level, a decrease in lactic acid production at temperature 4˚C and 45˚C in all isolates was observed. The growth of four isolates LAB 8, LAB 6, LAB 12 and LAB 15 were higher in the temperature of 37˚C. When compared with 4˚C and 25˚C. In our study the isolated bacterial species were able to grow within 25-37˚C and the optimum temperature for maximum growth was found at 37˚C.

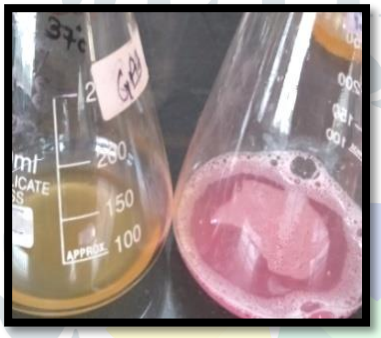
The temperature is also one of the important factors, which influences the activity of metabolic cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. However, below and above optimal temperature reaction rate is decreased which cause problems in cell metabolism. In fermentations using *L. delbrueckii*, and *L. bulgaricus* a temperature of 45°C, or higher may be maintained (Buchta, 1983).

Holzapful *et al.,* (1995) suggested that optimal temperature for growth of lactic acid bacteria varies between the genera from 20 to 45˚C. Krischke *et al.,* (1991) used 37˚C temperature for lactic acid production using *L. casei.* However, a temperature of 28°C has also been reported optimal for *L. casei* in a separate study (Nabi *et al.,* 2004). Guha *et al.,* (2013) observed maximum lactic acid production of 2.53 gm/L at 42°C. Panesar *et al.,* (2010) observed maximum lactic acid production of 33.72 gm/L was observed at 37°C is similar to the above perform study result.

Figure 10: Results of various temperatures

4˚C

25˚C



37˚C

The effect of carbon sources on lactic acid production was estimated by MRS broth having different carbon sources lactose, glucose and starch are shown in figure 11 and colour change from yellow to pink was determined by titration method as shown in **figure 12.** In our study, the maximum lactic acid production was 7.2 ml of 0.25 M NaOH which is equivalent to 0.16 gm of lactic acid observed in LAB 12 containing glucose as carbon source. However, other carbon sources lactose and starch, a decrease in lactic acid production was observed. Carbon source of glucose has been reported optimal for lactic acid production. The growth of four isolates LAB 8, LAB 6, LAB 12, and LAB 15 were higher in the carbon sources of Glucose as compared with lactose and starch. Glucose was used as a substrate for D-lactic acid production using *Bacillus (Lactobacillus) laevolacticus*. The result indicated that 97% of D- lactic acid was produced from 50 kgm^{-3} of glucose in a chemostat culture at pH 6.0 (De Boer *et al.,* 1990). *Lactobacillus coryniformis* sub sp. *torquens* produced 39 kgm-3 of D-lactic acid from 40 kgm^{-3} of glucose at pH 6.4 Varay *et al.,* 1996). Glucose can be selected for lactic acid production to get maximum yield of lactic acid and reduce the cost of purification process.

Serna cock reported that (2006) Glucose was used as a substrate for lactic acid production using *Lactococcus lactis* sub sp. *lactis*. The fermentation process was carried out at 32˚C with 60 gl^{-1} of

glucose and a pH of 6.0. The results indicated that 54 gl^{-1} of lactic was produced. In comparison with the above study, our results showed that best production at 37˚C with 20gl^{-1} of glucose and a pH of 7.0.

Present study revealed that the growth of five LAB isolates viz., *Lactococcus cremoris*, *Streptococcus sp.*, *Lactobacillus lactis*, *Lactococcus lactis* was higher in glucose as a substrate when compared with starch and lactose.

Figure11: Effect of various carbon sources on lactic acid production

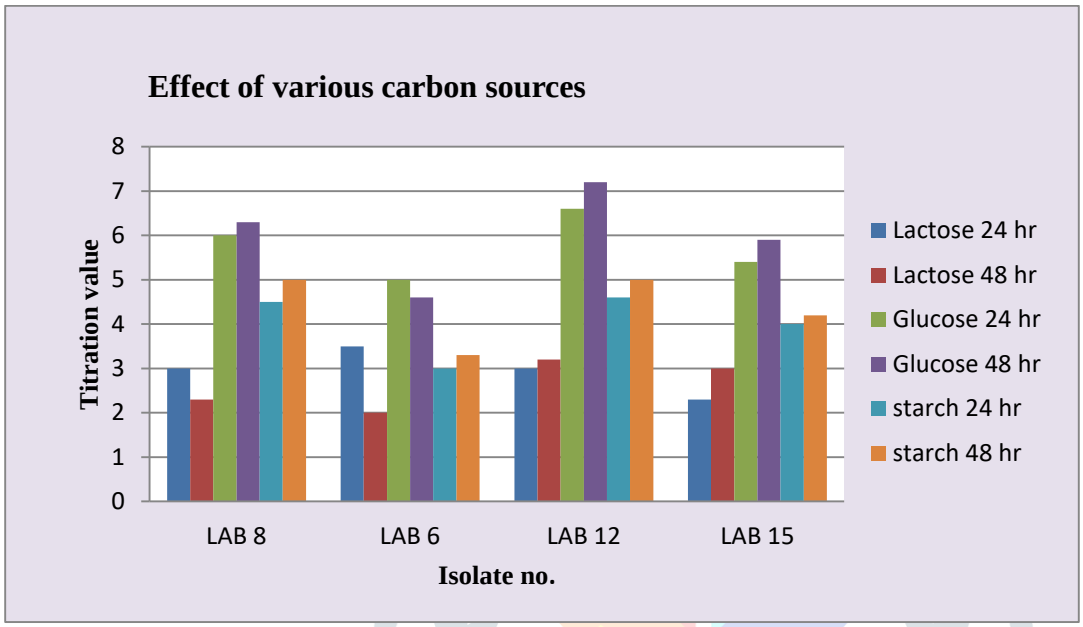
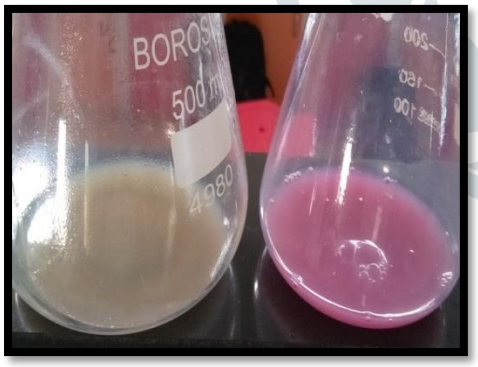
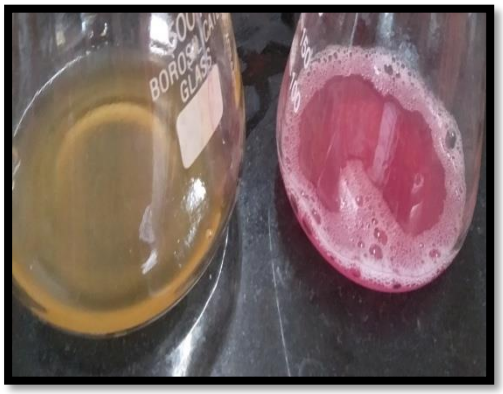


Figure 12: Result of various carbon sources



Starch

Lactose



Glucose

The effect of bile salt on lactic acid production was estimated by MRS broth with bile salt and without bile salts. Results are as shown in figure 12 and figure 13. Tolerance to bile salt is usually considered a basic property for LAB strains to survive in the small intestine. In the human gastrointestinal tract, the mean bile concentration is about 0.3% and is considered crucial and high enough to screen resistant strains (Gilliland *et al.*, 1985). The growth of the bacteria in presence of bile salt was maximum in LAB 15 at 3 hour as shown in graph. As well as minimum was observed in LAB 8 at 1 hour. The growth of the bacteria in absence of bile salt was maximum in LAB 15 at 3 hour as shown in graph. As well as minimum was observed in LAB 6 at 1 hour.Tolerance to bile salt is considered as an important property needed for potential probiotic isolates to shoe their viability in the intestinal tract (Succi *et al.*, 2005). Present study has revealed that all four selected isolates showed resistance to 0.3% bile concentration. Figure 13 shows the bile salt tolerance of bacteria and production of lactic acid.

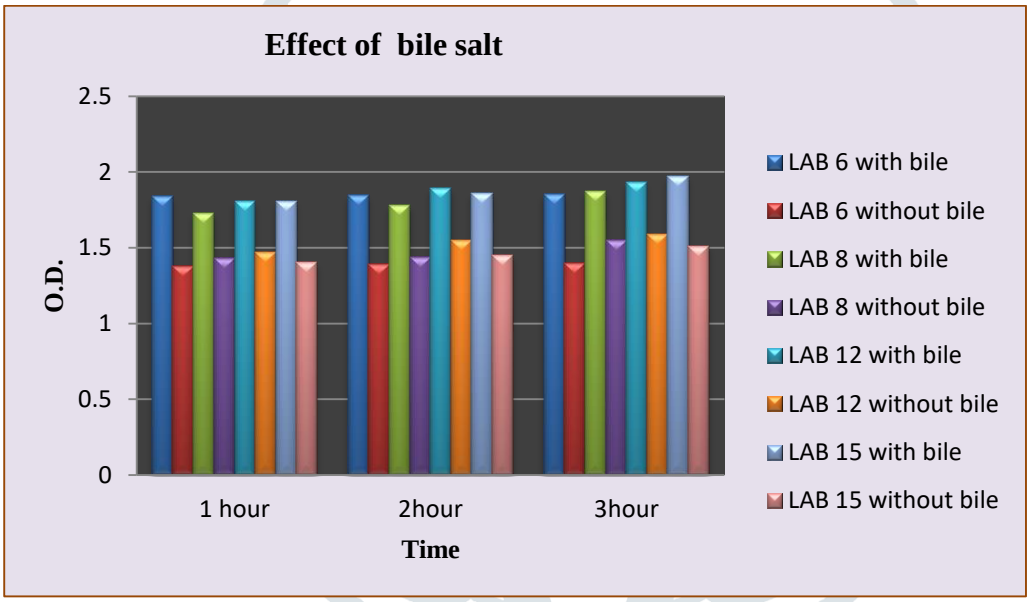


Figure 12: Effect of bile salt tolerance on lactic acid production:

Figure 13: Results of bile salt tolerance test.

Determination of probiotic effect of Bilva (*Aegle marmelos*) and Aloevera on selected bacterial isolates

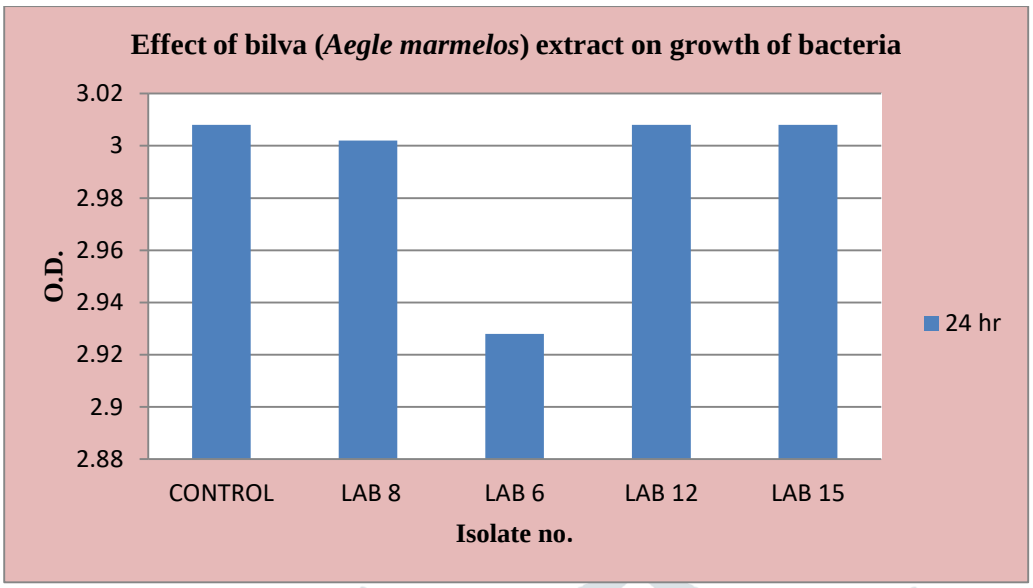


Figure 14: Effect of Bilva extract (*Aegle marmelos*) on selected bacterial isolates.

The probiotic effect of Bilva (*Aegle marmelos*) on the growth of lactic acid producing bacteria was observed in MRS broth containing bilva extract as shown in figure 14 and figure 15. In comparision to control, LAB 8 and LAB 6 showed slight decrease growth in the presence of Bilva (*Aegle marmelos*) extracts. This may be attributed to the presence of various phytochemical agents present in the bilva (*Aegle marmelos*) extracts. In comparision to control, LAB 12 and LAB 15 showed stimulated growth in the presence of Bilva *(Aegle marmelos*) extracts.

Figure 15: Result of bilva (*Aegle marmelos*) on selected isolates

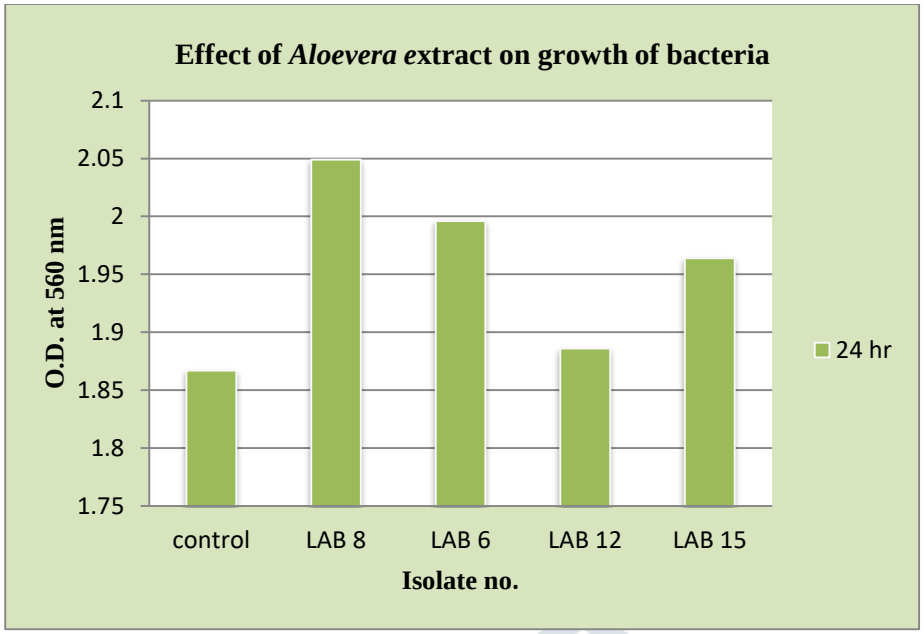


Figure 16: Effect of *Aloevera* extract on selected isolates

Effect of *Aloevera* on the growth of lactic acid producing bacteria was observed in MRS broth containing *Aloevera* extract as showed in figure 16.In the present study there was an increase in the growth of all isolates when *Aloevera* extract was used. Isolates LAB 8, 6, 12 and 15 showed good growth in presence of *Aloevera* extract as compared to control and therefore it may be incorporated in food items as probiotic. In comparision to control, LAB 8 showed maximum growth in the presence of *Aloevera* extract, whereas, LAB 12 showed minimum growth.

Probiotic lactobacilli are gaining enormous attention because of their established health effects such as, anti-diarrheal, anti- pathogenic, anti- diabetic, anti- cholesterol and anti- cancer activities. *Aloevera* also is considered to possess high content of vitamins, minerals, amino acids, trace elements, antimicrobial agent.

Conclusion

In this research, lactic acid producing bacteria were isolated, and characterized by morphological, biochemical and sugar fermentation tests. Lactic acid bacteria have the potential to produce lactic acid in large quantity at the optimized fermentation condition at 37°C and pH 7 in the MRS broth. Glucose was used as carbon source for lactic acid production, because it reduces the cost of purification process and also has less chance to contamination during the recovery process. The lactic acid produced by these organisms has antagonistic activity against pathogenic microorganisms associated with food, thereby serving as a means of preservation of food fermented with these organisms and invariably serving as a prophylactic means of checking proliferation of intestinal pathogens.

In this study, we also attempted to observe the probiotic effect of LAB using several plant extracts bilva (*Aegle marmelos*) and *Aloevera* and results showed that stimulatory *Aloe vera* stimulated the growth of Lactic acid bacteria as compared to bilva (*Aegle marmelos*) .

Conflict of Interest: The authors declare that there is No conflict of Interest.

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