International Journal of Advanced Technology in Engineering and Science
Vol. No.4, Issue No. 06, June 2016
www.ijates.com
ijates
ISSN 2348 - 7550

IDENTIFICATION AND ANALYSIS OF PROBIOTIC PROPERTIES OF LACTOBACILLUS SPP. (LACTOBACILLUS PLANTARUM & LACTOBACILLUS LACTIS)

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ABSTRACT

In the last few decades, lactic acid bacteria have attracted enormous attention for food manufacturers due to their potential associated with health-promoting effects. They are classified as probiotics which do not pose any health risk to the man and some are designated as "GRAS" (Generally Recognised As Safe) organisms. The purpose of this study is characterization, identification and analysis of probiotic properties of Lactobacillus plantarum and Lactobacillus lectis in different growth conditions and in various biological fluids. These microorganisms showed similar characteristics as probiotic organism. Bacteria were identified as Lactobacillus spp. by observing their colony morphology, physiological and as well as some biochemical characteristics. pH is an important factor which can dramatically affect bacterial growth. In our experimental design we have observed the growth of our isolated Lactobacillus spp. in various pH values ranges from 2.5 to 8.5. NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, pH of the media decreased with the increasing acid production. This investigation indicates that, there is a minor variation in organic acid production by Lactobacilli due to their regional variation. Results indicate that there were no losses of viability of cell in simulated gastrointestinal condition.

Keywords: Gram positive Bacteria, Lactobacilli, Probiotic, and gastrointestinal condition.

I. INTRODUCTION

The beneficial effects of food with added live microbes (probiotics) on human health are being increasingly promoted by health professionals. Probiotic products available in the markets today, are usually in the form of fermented milks and yoghurts; however, there is also a demand for the probiotic products derived from cereals and non dairy products (Fuller. 1989 & 1992). And, owing to health considerations, from the perspective of cholesterol in dairy products, alternative raw materials for probiotics need to be searched (Holzapfel *et. al.* 2001). The development of nondairy probiotic products is a challenge to the food industry in its effort to utilize the abundant natural resources by producing high quality functional products (Hasan *et. al.*, 2001). In this

respect, probiotic-containing baby foods or confectionery formulations have been developed by adding the strains as additives (Catanzaro and Green, 1997).

A better knowledge of the physiological characteristics of barley based fermented beverage can help in development of fermented product containing beneficial components of both barley and probiotic culture. The use of barley in cereal based fermented product has great potential due to its high nutritional value and other beneficial health benefits .The study of barley based fermentation and assessment of associated properties could lead into production of a healthy novel non dairy probotic beverage. Understanding of the effect of water to grain ratio, starter culture used to prepare barley based fermented beverage is also required.

Heat treatment of substrate mixture is necessary to achieve microbial safety, to enhance shelf life and to denature anti-nutritional compounds. It also helps to enhance nutritional value by denaturing trypsin inhibitors, haemaagglutinins, sapofins and other non essential compounds (Kwok *et. al*., 2002). Lactic acid bacteria (LAB) which have been used for food fermentation since the ancient time, can serve a dual function as it act as food fermenting agent and also provides potentially health benefits (Vernoux *et. al*., 2003). LAB are GRAS (general recognized as safe) that means they do not have any pathogenic and virulent property. They provide health benefits to human by maintaining normal intestinal microflora (microbiota) (Azcarate-Peril *et. al.*, 2001).

The plant-based beverage or fermented plant beverage is non alcoholic fermented beverage produced for various plants by LAB fermentation. Recent report proved that plant-based beverage could promote good health and even can cure some diseases (Krinos *et. al.*, 2001). Moreover, fermentation reduces the amount of non digestible material in plant foods, leading to improved bio-availability of minerals and trace elements (Kalantzopolous *et. al.*, 1997).

II. MATERIALS AND METHODS

1.1 Maintenance of bacterial culture: *Lactobacillus plantarum* and *Lactobacillus lactis* bacterium were used throughout the study and de Man, Rogosa, Sharpe (MRS) was used for the cultivation of the L*actobacillus plantarum*. Cultures were incubated at 30º C and 37ºC for 18 hour. These organisms were grown on the MRS medium containing (in grams/liters): peptone 10.0, yeast extract 4.0, glucose 20.0, tween 80 1.0, potassium di-hydrogen phosphate 2.0, tri-ammonium citrate 2.0, sodium acetate 5.0, magnesium sulphate.7H2O 0.2, $MgSO_4.4H_2O$ 0.05, agar 7.0 (pH-6.2±0.2) at 25ºC.

1.2 Growth and harvesting of bacterial cells

Preparation of primary culture: The inoculum was prepared by adding a loop full of freshly prepared pure culture from petridish into 25 mL of autoclaved above – said medium containg glucose as carbon source in a 100 mL shake flask and incubated at 30º C in incubator for 12- 18 h (to reach culture OD at 600 nm =0.6- 0.8).

Preparation of secondary culture: One percent of the inoculum from the above said culture was added to 25 mL of the medium in 100 mL shake flask. The flasks were incubated in a shaking incubator at 25ºC for 18h.

1.3 Isolation of Bacteria

One gram of each sample was dissolved into 100 ml of MRS broth at pH 6.5. After dissolving into MRS broth they were shaken homogeneously and were incubated at 37°C for 24 h in aerobic condition.. The cultures were

subjected to five subculture at 37°C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to remove unwanted bacteria. After seven subcultures, the bacterial culture was streak onto MRS agar media at pH 4.8. Finally, the single colony of lactobacillus was isolated by observing their colony morphology and some biochemical tests (Gram staining, catalase, endospore and motility test) and the culture were maintained in MRS broth at pH 5.5.

1.4 Identification:

The isolated bacteria were identified as *Lactobacillus spp*. by observing their morphological characteristics and by means Gram staining, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostactic phenol tolerance test and 1-10% NaCl tolerance test . MRS broth containing inhibitory substances such as 0.4% phenol and 1-10% NaCl were inoculated with 1% (v/v) 24h active culture of lactobacillus and incubated (anaerobically) for 24 h at 37°C in the presence of 10% CO_2 (Vernoux *et. al.*, 2003).

1.5 Determination of viable cell count:

50 µl activated culture which was serially diluted upto 10-16 times was spread on MRS agar and after incubating at 30ºC for 48 to 72 hours, the CFUs were counted. The log CFU/mL at any given dilution was calculating using the formula:

Log (CFU/mL) = Log {(Number of colonies*Dilution factor)/Sample volume}*1000

1.6 Determination of Optimal Growth and pH:

For the determination of optimal growth and pH of lactobacillus, 1% (v/v) fresh over night culture of lactobacillus were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37°C in the presence of 10% CO_2. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD 560) against the uninoculated broth.

1.7Assay for Bile Tolerance (Normal and fibrotic liver):

To study bile salt tolerance we take liver homogenate for the further study.

1.7.1 Normal liver homogenate:

Preparation of liver homogenate:-

To prepare liver homogenate firstly tissue must be washed in saline, soaked in filter paper, and then homogenized in 10 mL of 0.15M tris buffer (pH-7.4). After homogenization with the help of homogenizer centrifused this solution at 3000 g at 4ºC for 30 min and after centrifugation take out the supernatant for the assay and discard the pallet . Now the homogenate is ready to use to check bile tolerance of *L*actobacillus.

Procedure:-

Take 2mL of liver homogenate in a test tube or falcon. Add 10 µl of lactobacillus culture in this liver homogenate. Now incubate the solution at 37ºC. Growth pattern was observed by taking Optical density at 600 nm or by spreading different dilutions on MRS agar plates at different time period.

1.7.2 Fibrotic liver homogenate:

Preparation of liver homogenate:-

To prepare liver homogenate firstly tissue must be washed in saline, soaked in filter paper, and then homogenized in 10 mL of 0.15M tris buffer (pH-7.4). After homogenization with the help of homogenizer centrifuged this solution at 3000 g at 4ºC for 30 min and after centrifugation take out the supernatant for the assay and discard the pallet. Now the homogenate is ready to use to check bile tolerance of *Lactobacillus*.

Procedure:-

Take 2mL of liver homogenate in a test tube or falcon. Add 10 µl of *Lactobacillus* culture in this liver homogenate. Now incubate the solution at 37ºC. Growth pattern was observed by taking Optical density at 600 nm or by spreading different dilutions on MRS agar plates at different time period.

1.8 Assay for Gastric Juice Resistance:

Preparation of synthetic gastric juice:-

2g NaCl, 3.2 mg of pepsin dissolved in 500 mL of water. Add 7 mL HCl. Make total vol.1L by adding water and pH is 1.2.

Procedure:-

The juice was heated to 37ºC for 30 min and filtered in a sterile manner before use .To test tubes containing 10 mL of juice, 10 µl of lactobacillus culture was added. Samples were taken at 0, 30, 180 min and the survival rate was measured by spreading 100 µl of different dilutions on MRS agar plates , which are incubated anaerobically at 37ºC for 48 h.

1.9 Assay for simulated body fluid tolerance:

Preparation of simulated body fluid :

Potassium phosphate –dibasic trihydrate , tris, magnesium chloride hexahydrate, Sodium chloride, Sodium bi carbonate, potassium chloride, calcium chloride, sodium sulphate were dissolved in 500 mL of water according to the known protocol. Add 1M hydrochloric acid (40 mL). Make total vol. 1L by adding distilled water.

Procedure:-

Take 9 mL simulated body fluid in a test tube or falcon. 10µl *Lactobacillus* culture was added in this body fluid test tube. Growth pattern was observed by taking optical density with the help of spectrophotometer at 600 nm at different time periods. Growth can be observed by spreading 100 µl of different dilutions on MRS agar plates, which are incubated anaerobically at 37ºC for 48 h.

1.10Assays for NaCl Tolerance:

For the determination of NaCl tolerance of isolated lactobacillus 10 test tube containing

MRS broth was adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

1.11Quantification of Organic Acid and Determination of pH Value:

One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from Milk Vita Co-operative Bangladesh Ltd. and initial pH (6.68) was determined by a digital electrode pH meter (Hanna, Model No. 211). The inoculated skim milk was incubated at 37°C for 72 h and samples were collected in every 24 h, 48 h and 72 h and liquids of coagulated milk were separated by filtration. pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1 N NaOH.

1.12 Lipid peroxidation assay:

Preparationn of liver homogenate:

To prepare liver homogenate firstly tissue must be washed in saline, soaked in filter paper, and then homogenized in 10 mL of 0.15M tris buffer (pH-7.4). After homogenization with the help of homogenizer centrifuged this solution at 3000 g at 4ºC for 30 min and after centrifugation takes out the supernatant for the assay and discard the pallet. Now the homogenate is ready to use to check bile tolerance of lactobacillus.

10µl of bacterial sample was taken and mixed it with 2 mL of liver homogenate. 0.5 mM ferrous sulfate (50µl) and 0.5mM H_2O_2 (50µl) was added. Now incubate the solution at 37ºC for 60 minutes. After incubation we added 1mL of 15% trichloro acetic acid and 1mL of 0.67% thiobarbituric acid to the solution .Now heated the solution in boiled water for 15 minutes and the absorbance of the mixture was measured at 532 nm. Lipid peroxidation percentages were measured by using following formula:

$$\% \text{ lipid peroxidation} = 1 - \frac{(\text{Abs. of sol. with extract} - \text{Abs. of sol. without liver homogenate}) \times 100}{\text{Absorbance of control without extract}}$$

III. RESULTS AND DISCUSSION

1.1 Identification

Bacteria isolated were identified as *Lactobacillus spp. (Lactobacillus plantarum* and *Lactobacillus lactis)* by observing their colony morphology, physiological and as well as some biochemical characteristics. Microscopically they were Gram-positive (Fig. 1), rod shaped (Fig. 2), non- motile, catalase negative and absence of endospore (Fig. 3). The isolates have the abilities to coagulate milk and were able to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. Their characteristics have shown in Table 1.

Biochemical and physiological characteristics	**Isolate-1 (Lactobacillus plantarum)**	**Isolate-2 (Lactobacillus lactis)**
Gram stain	+	+
Motility test	Non motile	Non motile
Catalase test	_	_
Endospore test	_	_

0.4%phenol	+	+
Milk cogulation	+	+
NaCl tolerance	+	+

Table.1: Biochemical and physiological characteristics of *Lactobacillus plantarum* and *Lactobacillus lactis*

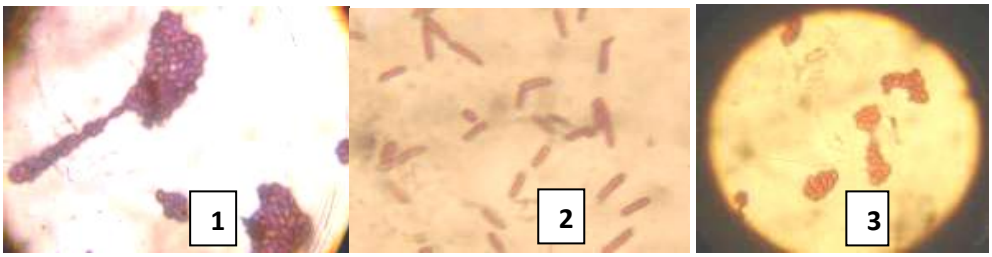

Fig.1: Gram-+ve *Lactobacillus spp*. Fig.2: Isolated rod-shaped *Lactobacillus spp*. Fig.3: Non-spore forming *Lactobacillus spp.*

1.2 Determination of viable cell count

By incubating the *Lactobacillus plantarum* at 30ºC for 48 to 72 hours, the CFUs were counted and the results showed as follows (Fig. 4).

Days CFU/ml	0 days	20 days	40 days
L. plantarum	4.5×10^6	4.97×10^6	4.75×10^6

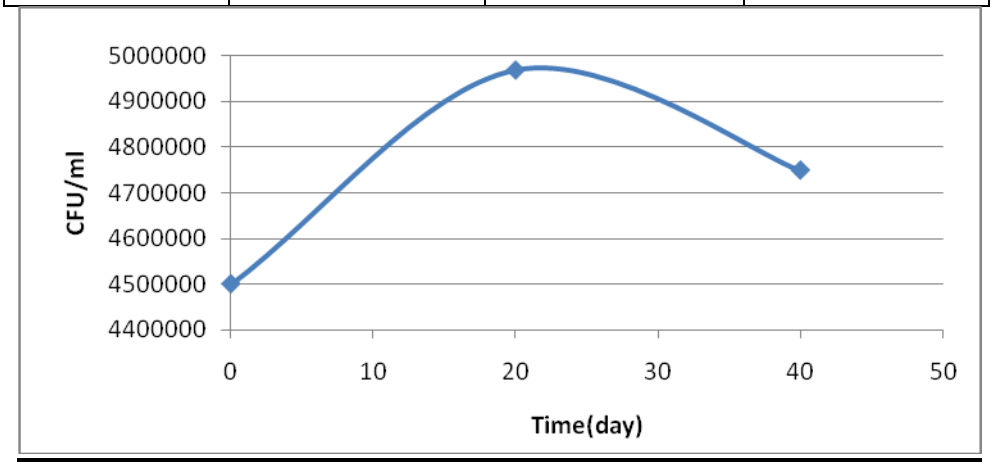

Fig.4: Viable cell count of *Lactobacillus plantarum*.

1.3 Assay for bile tolerance (Normal and fibrotic liver)

Isolated *Lactobacillus spp.* (*Lactobacillus plantarum*) was able to survive in normal and fibrotic liver homogenate so *Lactobacillus plantarum* can survive in bile acid. The isolated *Lactobacillus spp*. was also able to multiply in above mentioned concentrations of bile acid. In the Fig. 5&6 the optical density values against incubation time are shown for survival in normal liver homogenate.

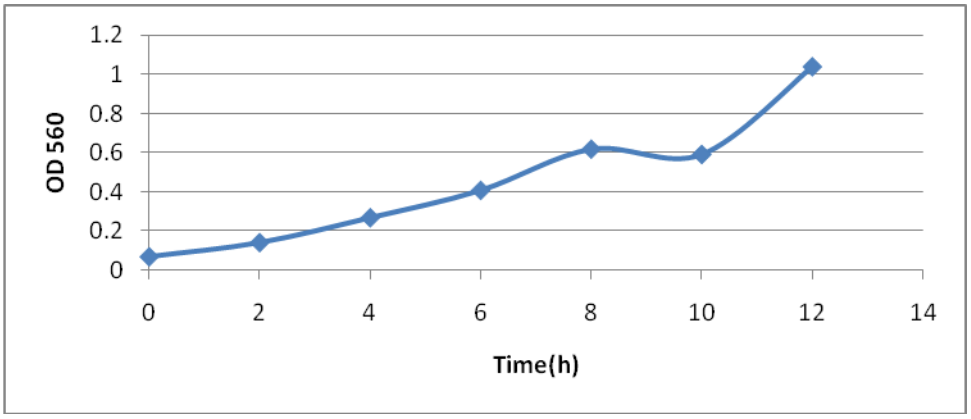

Fig. 5: Bile acid tolerance of *Lactobacillus plantarum* in normal liver homogenate.

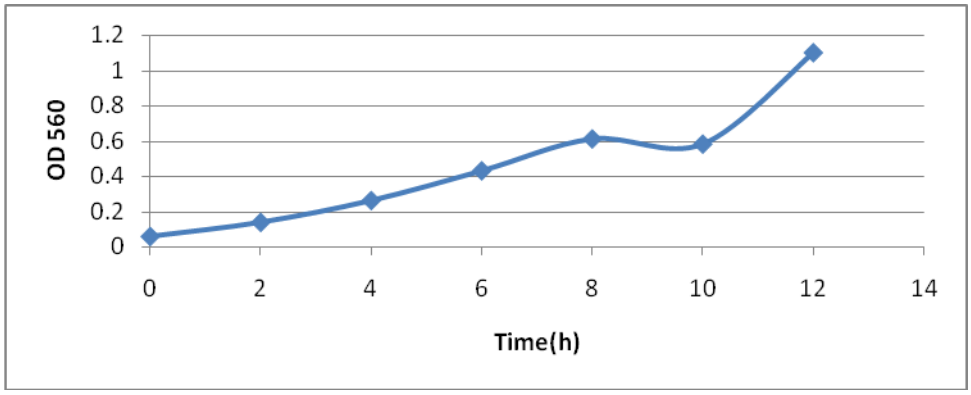

Fig.6: Bile acid tolerance of *Lactobacillus plantarum* in fibrotic liver homogenate.

1.4 Assay for simulated body fluid tolerance

Results for simulated body fluid tolerance are shown in following Fig. 7 with respect to time and optical density.

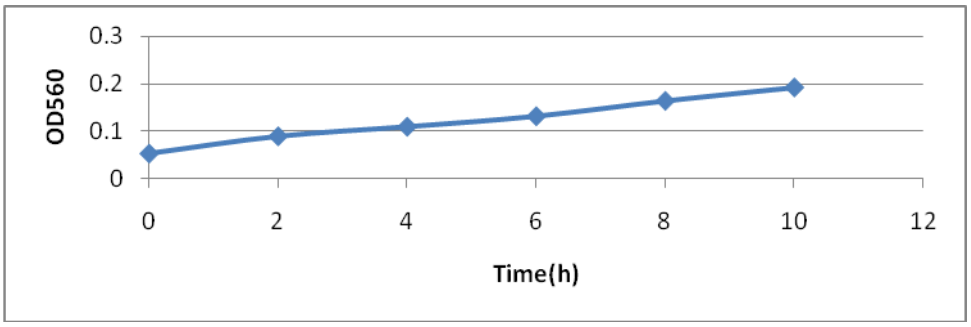

Fig.7: Simulated body fluid tolerance of *Lactobacillus plantarum* at diff. time points.

1.5 Lipid peroxidation assay:

The results obtained from the analysis of lipid peroxidation are shown in Fig. 8. The lipid peroxidation assay is done to assess the oxidative damage caused by reactive oxygen species (ROS) that causes oxidative stress and damage to cell membranes. The lipid peroxidation lowest content was observed in *L. lactis* (30 %) and highest in *L.plantarum* (41.6%) at 0^{th} day. At 20^{th} day and 40^{th} day of storage highest peroxidation percentage was fond in *L. lactis.* There was no significant decrease in lipid peroxidation percentage during storage

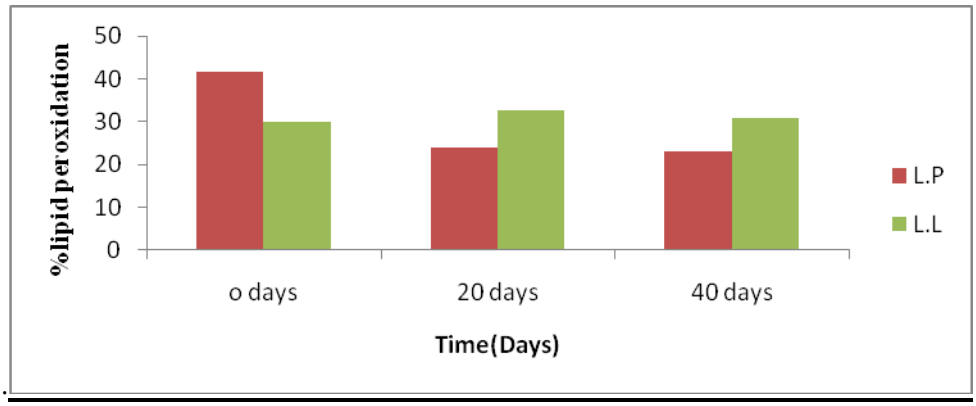

Fig.8: % lipid peroixidation of *Lactobacillus plantarum* & *Lactobacillus lactis*

IV CONCLUSION

This work aimed to characterize, identify, analyse the probiotic properties of *lactobacillus sp. (Lactobacillus plantarum)* and to investigate the capabilities of this strain of lactic acid bacteria, for the development of functional foods. Results showed that this test strain has ability to produce high amount of lactic acid ranged and they exhibited tolerance to acid as well as alkali with the viable cell numbers of 10^6 CFU/ml after 14 hours of fermentation. This strain can be survived in low pH. So this strain of lactic acid bacteria have possible role of probiotics in improvement of functions and reducing the risk of diseases.

FUTURE PROSPECTS

This work conclusively showed good probiotic potential of *Lactobacillus sp*. (*Lactobacillus plantarum* and *Lactobacillus lactis*) in probiotic based drinks and foods. The enhanced health- beneficial potential of these bacteria is paved the way for the development of future functional food.

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