

CHARACTERIZATION AND IDENTIFICATION OF *LACTOBACILLUS ACIDOPHILUS* USING BIOLOG RAPID IDENTIFICATION SYSTEM

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ABSTRACT

Objective: The present study was carried out to isolate and identify *L. acidophilus* and to compare the effect of inoculation methods and cultivation conditions on the yield of the bacteria.

Methods: Probiotic *Lactobacillus* strain was isolated from a commercial yogurt and the characterization of the bacteria was performed using gram stain, motility, catalase, biochemical tests and morphological features were confirmed using scanning electron microscope (SEM). Finally the identification was confirmed by BioLog system. Effect of inoculation methods and cultivation conditions on the growth and yield of the bacteria were studied.

Results: The isolated strain was Gram positive cocco-bacilli, nonmotile and catalase negative. It ferment maltose, lactose, sucrose, and glucose, but unable to ferment arabinose and sorbitol. The SEM examination displayed the ranging of the cells varied from $2.02 - 5.49 \times 0.50 - 0.59 \mu\text{m}$. From all the results it is confirmed that the species is *Lactobacillus acidophilus*. In addition, Biolog rapid identification system revealed the presence of *L. acidophilus* in the prepared samples with 90% indexed probability. Pour plate provided a relatively higher viable count than the spread plate, while no significant differences were observed between aerobic and anaerobic conditions.

Conclusion: The bacterial strain was successfully isolated after series of purification. It was identified as *Lactobacillus acidophilus*. It can be concluded that, a carbon utilization microplate assay system developed by Biolog, has the potential to simplify the identification scheme of lactic acid bacteria to the genus level.

Keywords: *Lactobacillus acidophilus*; Probiotics; SEM; Biolog.

INTRODUCTION

The relationship between certain food and health benefits has been investigated for many years. In recent years, there has been a lot of active research in the field of probiotics, due to the growing commercial interest in the probiotic food. The research work has also resulted in the understanding and ability to characterize specific probiotic organisms and their health benefits [1].

Probiotic microorganisms are often incorporated in food in the form of yoghurt and yoghurt type fermented food. Recently, there are probiotic ice cream, cheese, infant formulas, breakfast cereals, sausages, luncheon meats, chocolate and puddings. Nondairy food also has been manufactured with the addition of the same types of microorganisms. In fact, there are also medical probiotics in the form of capsules and tablets [2].

Lactobacilli represent a significant part of our intestinal microflora, and their friendship with the general state of human health is under serious investigation [3]. The genus *Lactobacillus* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. Strains of *L. acidophilus* were introduced into dairy products because of the potential advantage of consuming active LAB adapted to the intestine and to produce mildly acidified yoghurts [4].

The taxonomy of *L. acidophilus* group has been subjected to huge changes during latest years and may have caused some confusion [5]. A large number of studies have been carried out for the identification and classification of LAB, which include conventional biochemical tests such as carbohydrate fermentation patterns using commercially available kits [6], physiological tests [7] as well as the more complex techniques using molecular biology-based methods [5, 8]. More recently, API Kit and Bio-log have been used to a large degree for LAB identification. Several culture media have been developed and evaluated for the selective enumeration of probiotic LAB in yoghurts and fermented milk [9-12]. Harris-Baldwin and Gudmestad [13] developed a rapid identification method for phytopathogenic bacteria based on the utilization of 95 carbon sources using the Biolog automated identification system.

The aim of this study is to identify *L. acidophilus* using gram stain, biochemical tests, scanning electron microscopy and automated system for rapid identification of bacteria (BioLog identification system) and optimize the maximum growth of the bacteria using different inoculation methods (pour plate and spread plate method) and cultivation conditions (aerobic and anaerobic conditions).

MATERIALS AND METHODS

Identification of *Lactobacillus acidophilus*

The isolated colony formed on the MRS agar (Difco, USA) plates was identified using gram stain, biochemical tests, scanning electron microscopy and automated system for rapid identification of bacteria (BioLog identification system). The identification was performed according to Bergey's manual of determinative of bacteriology [14]. The culture was kept in MRS agar slant and stored at 4 °C. For long term storage, one loop of bacteria was mixed in Microbank (a sterile vial containing porous beads kept in glycerol as cryopreservative and serves as carriers to support microorganisms) and stored at -20 °C.

Gram staining test

The isolated bacteria were examined using gram staining kit (Becton, Dickinson and Company, USA) according to Collins and colleagues technique [15], and was observed under light microscope (Olympus BX 50, Japan) with a magnification of 1000x.

Motility test

Two methods namely, hanging-drop wet method [16] and Carigie's technique [17] were performed. The slide was observed under a light microscope with 40x magnification to check the motility of the bacteria. On the other hand, in Carigie's technique, the bacteria were inoculated into the centre of a tube having motility medium (Becton, Dickinson and Company, USA) using stabbing method. The medium was incubated at two different temperatures of 25 °C and 37 °C for 48 h. The motility of the bacteria was inferred by observing the spreading growth in the incubated semisolid agar.

Catalase test

To perform this test, a single isolated colony was streaked on a glass slide and one drop of 3 % hydrogen peroxide (Merck, Germany) was added on to it. The effervescence of oxygen indicated the positive response of the bacteria to catalase test [18].

Carbohydrate fermentation test

Phenol red broth base medium was used as a medium for this test. Different sugar substrates namely, arabinose, sucrose, maltose, lactose (BDH, UK), sorbitol (GPR, UK) and glucose (R & M Chemicals, UK) were used. 0.1 g (0.1 % w/v) of each sugar substrate was added to 100 ml of the medium. 5 ml of each mixture was transferred into each tube. For gas detection, Durham tube was inserted into the test tube containing glucose. All the tubes were sterilized for 15 min at 121 °C. The tubes were inoculated with a single colony of the bacteria under study. The positive reaction of the bacteria was indicated by the changes in the colour of the medium [19].

Scanning electron microscopy

The scanning electron microscope (SEM) was used to observe and identify the shape of the bacteria under study, prepared by liquid culture method [20]. In this method, bacterial suspension was centrifuged at 5000 rpm for 15 min (Microfuge E, Beckman, UK). The supernatant of the bacteria was discarded and the bacterial cells were fixed in McDowell-Trump fixative reagent pH 7.2 (Agar Scientific Limited, UK) for at least 2 h. The bacterial cells were washed with 0.1 M of phosphate buffer and again centrifuged at 5000 rpm for 10 min. The pellet again was re-suspended in the phosphate buffer and centrifuged. The pellet was fixed for one hour in 1.0 % osmium tetroxide (Agar Scientific Limited, UK) prepared in phosphate buffer. The sample was washed two times with distilled water for 10 min and centrifuged. The sample was dehydrated for 10 min in ethanol (Merck, Germany) at concentrations of 50 %, 75 %, 95 % and 99.5 %. After that, 1 ml of hexamethyldisilazane (Agar Scientific Limited, UK) was added to the sample tube for 10 min. Hexamethyldisilazane was decanted from the tube and the cells were air-dried at room temperature. The sample specimen was coated with gold and viewed under SEM (Leo Supra 50 VP equipped with Oxford INCA 400 energy dispersive x-ray microanalysis system, Germany).

Biolog rapid automated system

Biolog micro plates and databases were first introduced in 1989. Biolog scientists developed a proprietary carbon-source utilization test methodology and placed it in a convenient micro Plate™ test format. The Biolog anaerobic micro plate is designed for the identification of a wide number of anaerobic bacteria.

The following procedure was followed to identify the isolated bacteria. The isolated bacteria were cultured on MRS agar plates at 37 °C for 48-72 h. A single cell colony from MRS agar was sub-cultured in BHI medium for 36-48 h. The cultured bacteria were suspended in anaerobic Biolog fluid (Biolog, Hay Ward, USA). The turbidity of the suspension was monitored and measured using Biolog turbidity meter until reaching 65% of transmittance. 100 µl of the suspension was poured into each of the 99 holes of the Biolog Micro Plate™ (Biolog, Hay Ward, USA). The plate was incubated for 24 h at 37 °C in an aerobic jar containing only CO₂ gas using hydrogen free atmosphere kit (Oxoid, UK). The plate was inserted into the Biolog automatic system and the identification process was carried out using Biolog software [21].

Characteristics of *Lactobacillus acidophilus*

Growth at different pH

A single isolated colony was subcultured in MRS broth adjusted to different pH using NaOH (1.0 M) or HCl (1.0 M) and incubated at 37 °C for 24 h to observe the ability of the growth of *L. acidophilus* under different pH values.

Bile salt tolerance

The ability of the strains to tolerate bile salts was determined according to the method described by Gilliland and colleagues [22]

and Walker and Gilliland [23]. *Lactobacillus acidophilus* was tested for rapid growth in MRS broth medium with and without the addition of bile salts (Sigma, Germany). MRS broth was prepared with different concentrations of bile salts at 0, 0.1, 0.3, 0.5 and 1.0 % w/v and dispensed in 10 ml volume test tubes and sterilized at 121 °C for 15 min. Three tubes of each concentration were inoculated with 0.1 ml of *L. acidophilus* culture and incubated at 37 °C for 48-72 h. The total viable counts of *L. acidophilus* were obtained for all concentrations. The results were expressed as the percentage of growth in the presence or absence of bile salts. The bile tolerance (%) was calculated using the equation below:

$$\text{Bile tolerance (\%)} = \frac{\text{No of viable cells with bile salts} \times 100 \%}{\text{No of viable cells without bile salts}}$$

Evaluation of parameters affecting *Lactobacillus acidophilus* growth

Effect of inoculation method

The viable counts of *L. acidophilus* bacteria were obtained in MRS medium using two different methods namely pour plate and spread plate methods. The two methods were compared to find out which method was more suitable for counting the number of *L. acidophilus*. Both methods were conducted by ten fold serial dilution from original culture. The pour plate method was applied by transferring 1 ml from 10⁻⁵ diluted *L. acidophilus* into a sterile petri-dish and 20 ml of MRS agar was then poured into the petri-dish. The plate was left for approximately 2 h to solidify and kept in an incubator at 37 °C for 72 h. On the other hand, the spread plate method was conducted by adding 0.1 ml from 10⁻⁴ dilution onto the surface of the MRS agar plates and spread using a sterile spreader. The number of colony was counted using a colony counter (Technical lab instrument, USA). The experiments were repeated five times.

Effect of cultivation condition

The growth of *L. acidophilus* in MRS medium prepared using pour plate method was studied under aerobic and anaerobic conditions. For both conditions, five plates were used. The plates were incubated in aerobic incubator or kept inside an anaerobic jar containing carbon dioxide generating system (Oxoid kit, UK) before being incubated in aerobic incubator at 37 °C for 72 h.

RESULTS AND DISCUSSION

Identification of *Lactobacillus acidophilus*

The isolated bacteria were observed by light microscope. It is clear that the bacteria was gram positive, rod shaped coccobacilli, occurring singly or in chains. The gram staining results indicated that the isolated bacteria could be identified as lactobacilli [14].

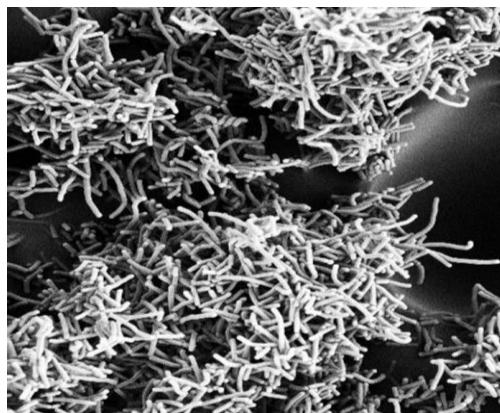
Hanging-drop wet method showed that the isolated bacteria were nonmotile. In the other hand, Carigie's technique also showed that the bacteria grew only along the stab line in the medium. Therefore, these methods conformed that the bacteria under investigation was nonmotile. The nonmotile behavior is a characteristic of *L. acidophilus* [24].

The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. In performing catalase test, no bubble was observed indicating that the isolated bacterium is catalase negative and could not mediate the decomposition of H₂O₂ to produce O₂. It is well known that *Lactobacillus acidophilus* is catalase negative [25, 26].

The main task of Carbohydrate fermentation test is to investigate the ability of bacteria to ferment different types of carbohydrate. Phenol red broth base medium was used as an indicator to differentiate the bacteria according to their patterns of carbohydrate utilization. Table 1 show that the isolated bacteria could ferment maltose, lactose, sucrose and glucose, but not sorbitol and arabinose. No bubble was detected from the glucose inserted with Durham tube indicating that no gas production could be associated with the growth. Thus, the results obtained coincided with *L. acidophilus* strain characteristic [14, 27].

Table 1: Biochemical tests results

Test	Observation
Arabinose	No fermentation
Sorbitol	No fermentation
Maltose	Fermentation (acid production)
Lactose	Fermentation (acid production)
Sucrose	Fermentation (acid production)
Glucose (acid)	Fermentation (acid production)
Glucose (gas)	No gas formation (no bubble in Durham tube)

Fig. 1: Scanning electron microscope photomicrograph of *L. acidophilus*. (Magnification 10,000x.)

Scanning electron microscopy

The scan electron microscope showed that the cells were coccobacilli in shape. The length of the cells varied from 2.02 μm to 5.49 μm and the diameter ranged from 0.50 μm to 0.59 μm (Fig.1). The shape and the range of the measured dimension obtained for the isolated bacteria were in good agreement with the results of other researchers [28, 29].

Micro station Biolog system

The MicroPlate "Metabolic finger print" when read against a Biolog MicroLog database showed a high positive probability that the microorganism was *L. acidophilus* (Table 2.), whereas other bacteria showed very low probability.

Table 2: Biolog micro plate readings of isolated bacteria

Species	PROB	SIM	DIST *
<i>Lactobacillus acidophilus</i> BGA	61	0.66	4.89
<i>Lactobacillus acidophilus</i> BGB	35	0.65	4.62
<i>Lactobacillus buchneri</i>	2	0.37	6.95
<i>Lactobacillus casei</i>	1	0.26	8.40
<i>Lactobacillus plantarum</i>	1	0.05	8.81
<i>Lactobacillus delbrueckii ss lactis</i>	0	0.01	7.59
<i>Lactococcus lactis ss lactis</i>	0	0.01	4.27
<i>Lactococcus plantrum</i>	0	0.01	9.20
<i>Lactobacillus delbrueckii ss bulgaricus</i>	0	0.01	8.93
<i>Lactobacillus amylovorus</i>	0	0.01	6.96

PROB = Probability

SIM = Similarity

DIS = Distance Between #1 and # 2 species

Characteristics of *Lactobacillus acidophilus*

Growth at different pH

Table 3 shows the results of the growth of *L. acidophilus* at different pH values. The turbidity observed for pH values in the range of 4.0 to 7.0 indicating that the bacteria preferred to grow in acidic and neutral environment.

Bile salt tolerance

The bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract such as bile salts

[304]. For this purpose, the effect of different concentrations of bile salts on the growth of *L. acidophilus* bacteria in MRS broth was investigated and the results are presented in Table 3. The total viable count of *L. acidophilus* decreased with an increase in the bile salt concentration when compared with the control. *L. acidophilus* showed reasonable growth at all bile salt concentrations studied. The bile tolerance results were 87.41%, 75.49%, 69.53% and 54.96 % for the 0.1%, 0.3%, 0.5% and 1.0 % of bile salt concentrations. The ability of *L. acidophilus* to resist bile salts was studied by other researchers [31, 32].

Table 3: Effects of pH and different concentrations of bile salts on the growth of *L. acidophilus*

pH	Result
3.0	-
4.0	+
5.0	+++
6.0	+++
7.0	++
8.0	-
9.0	-
Bile salt concentration (%)	Bile tolerance (%)
0.0	100.0
0.1	87.4
0.3	75.5
0.5	69.5
1.0	55.0

(-) No growth, (+) slightly growth, (++) good growth, (+++) very good growth,

Evaluation of culture condition affecting *L. acidophilus* growth

Effect of inoculation method

Fig. 2 shows the viable counts of *L. acidophilus* cultivated by pour plate and spread plate methods in MRS agar. The viable count data in pour plate were higher than that of the spread plate method. The viable counts between the two methods were significantly different ($p < 0.05$).

The higher count in pour plate method could be because this method is more suitable for facultative anaerobic bacteria [33], and *L. acidophilus* is known to have such characteristics [34]. The results showed that the pour plate method was superior to the spread plate method.

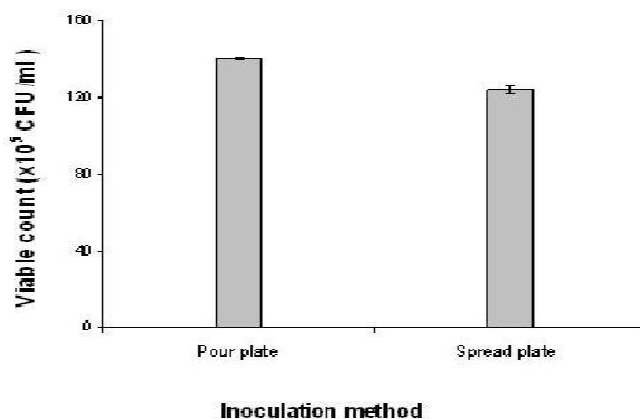


Fig. 2: Viable count of *L. acidophilus* in pour plate and spread plate methods. Mean \pm SD, N=5

Effect of culture condition

The growth result of *L. acidophilus* in aerobic and anaerobic conditions was shown in Fig. 3. The number of counts appeared to be comparable between aerobic and anaerobic condition. There was no statistically significant difference in the bacterial counts in both media under

aerobic and anaerobic conditions. *L. acidophilus* is facultative and can grow in both aerobic and anaerobic conditions [28]. The result was similar to the finding of Kelly and colleagues [34]. They reported no difference in bacterial count for MRS agar when incubated under aerobic and anaerobic conditions. Hence, both aerobic and anaerobic conditions are suitable to grow *L. acidophilus*.

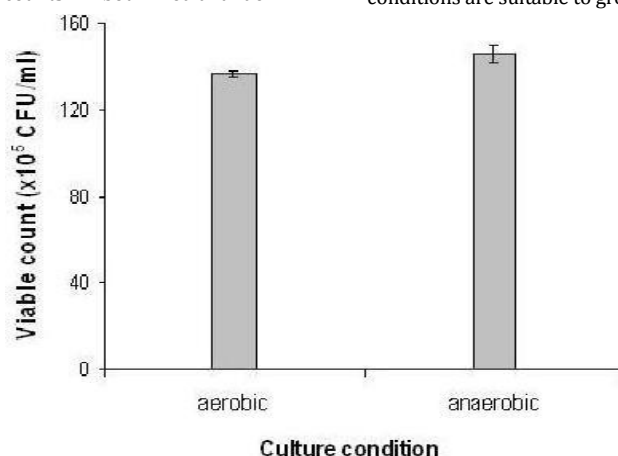


Fig. 3: Viable count of *L. acidophilus* cultured under aerobic and anaerobic conditions. Mean \pm SD, N=5.

CONCLUSION

The bacteria were successfully isolated from a commercial yoghurt drink and identified as *L. acidophilus* using gram staining, scanning electron microscope, motility, catalase and carbohydrate fermentation tests. The identity of *L. acidophilus* was further confirmed using Biolog rapid identification system. The bacterial strain showed remarkable growth at pH between 5 and 6. The pour plate method was preferred over the spread plate method as the former provided a relatively more counts suggesting that *L. acidophilus* could be cultivated under both of these conditions. Altogether, it can be concluded that the Biolog system is credible for morphological identification of bacteria.

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REFERENCES

1. Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch P, Ross R. Market potential for probiotics. *American Journal of Clinical Nutrition* 2001; 73: 476s.
2. Chan W, Boswell C, Zhang Z. The effect of different in-vitro solutions in the dissolution of calcium pectinate beads intended for the delivery of live cells to the human large intestine. *Minerva Biotechnology* 2000; 12: 271-278.
3. Salminen S, Isolauri E, Salminen E. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie van Leeuwenhoek* 1996; 70: 347-358.
4. Schillinger U. Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. *International journal of food microbiology* 1999; 47: 79-87.
5. Pot B, Hertel C, Ludwig W, Descheemaeker P, Kersters K, Schleifer K. 1993. Identification and classification of *Lactobacillus acidophilus*, *L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. *J Gen Microbiol.* 1993; 139: 513-7.
6. Milliere J, Abidi F, Letebure G. Taxonomic characterisation of *Lactobacillus delbrueckii subsp. bulgaricus*, isolates from a Cameroonian zebu's fermented raw milk. *J. App. Bact.*1990; 80: 583-588.
7. Axelsson L: Lactic Acid Bacteria: Classification and Physiology In: S. Salminen and A. V. Wright eds. *Lactic Acid Bacteria*, Morcel Dekker, Inc. 1993: 1-64.
8. Collins M, Samelis J, Metaxopoulos J, Wallbanks S. Taxonomic studies on some Leuconostoc-like organisms from fermented sausages: description of a new genus Weissella for the Leuconostoc paramesenteroides group of species. *J. Appl. Bact.*1993; 75: 595-603.
9. Dave R, Shah N. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *International Dairy Journal* 1997; 7:31-41.

10. Roy D, Sirois S, Vincent D. 2001. Molecular discrimination of lactobacilli used as starter and probiotic cultures by amplified ribosomal DNA restriction analysis. *Current microbiology* 2001; 42: 282-289.
11. Talwalkar A, Kailasapathy K. A review of oxygen toxicity in probiotic yoghurts: Influence on the survival of probiotic bacteria and protective techniques. *Comprehensive Reviews in Food Science and Food Safety* 2004; 3: 117-24.
12. Vinderola C, Reinheimer J. Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. *International Dairy Journal* 2000; 10: 271-275.
13. Harris-Baldwin A and Gudmestad NC. Identification of phytopathogenic coryneform bacteria using the Biolog automated microbial identification system. *Plant Disease* 1996; 80: 874-878
14. Holt G, Krieg N, Sneath P, Staley J, Williams S. *Bergey's Manual of Determinative of Bacteriology*. 9th edition. Williams and Wilkins. USA, 1994: 560-570.
15. Collins, C. H., Lyne, P. M. and Grange, J. M. Collins and Lyne's microbiological methods. 8th edition. Butterworth-Heinemann, London, 2004.
16. MacFaddin J: *Biochemical Tests for Identification of Medical Bacteria*. 3rd edition. Philadelphia, USA. 2000
17. Barrow G, Feltham R: *Cowan and steel's manual for the identification of medical bacteria*, 3rd edition. Cambridge university press, UK. 2004.
18. Nelson, G. and George, S. Comparison of media for selection and enumeration of mouse fecal flora populations. *J. Microbiol. Methods* 1995; 22: 293-300.
19. Thoesen, J. C. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th edition. Version 1, fish Health Section, American Fisheries Society, 1994.
20. Nation, J. L. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technology* 1983; 58: 347-351.
21. MicroLog system user guide. A powerful automated system for the rapid identification of bacteria, yeasts and filamentous fungi. Hayward, USA. 2001.
22. Gilliland S, Staley T, Bush L. Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *J. Dairy Sci.*1984; 67: 3045-3051.
23. Walker D, Gilliland E. Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *L. acidophilus*. *J. Dairy Sci.*1993; 76: 956-961.
24. Tamang J, Sarkar P. Microbiology of mesu, a traditional fermented bamboo shoot product. *Int. J. of Food Microbiology* 1996; 29; 49-58.
25. Schillinger U. Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. *International Journal of Food Microbiology*1999; 47: 79-87.
26. Robinson R: *Dairy Microbiology* Volume (1) The microbiology of milk. 2nd eds Elsevier science publishing Co .INC. USA. 1990
27. Klaenhammer T, Russell M: *Lactobacillus acidophilus* In: Robinson R, Batt C, Patel D eds. *Encyclopedia of Food Microbiology*. Academic press, London, UK. 2000.
28. Ray B: *Fundamental food microbiology*, 2nd eds. CRC press, USA.2001
29. Gilliland S, Walker D. Factors to Consider When Selecting a Culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in Humans. *J. Dairy Sci.* 1990; 73: 905- 911.
30. Brashears M, Gilliland S, Buck L. Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *J. Dairy Sci.*1998; 81: 2103-2110.
31. Corzo G, Gilliland S. Bile salt hydrolase activity of three strains of *Lactobacill acidophilus*. *J. Dairy Sci.*1999; 82: 472-480.
32. Soestbergen A, Ching H. Pour plates or streak plates? *Applied Microbiology* 1969; 18: 1092-1093.
33. Mombelli B, Gismondo M. The use of probiotics in medical practice. *Int. J. Antimicrob. Agents* 2000; 16:531-536.
34. Kelly W, Asmundson R, Harrison G, Huang C. Differentiation of dextran-producing *Leuconostoc* strains from fermented rice cake (*puto*) using pulsed-field gel electrophoresis. *Int. J. Food Microbiology* 1995; 26: 345-352.