"PREPARATION AND PREFORMULATION STUDIES OF ORAL DOSAGE FORM OF PROBIOTICS"

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INTRODUCTION

For many years, *Lactobacillus acidophilus*, which is one of the widely used probiotics, is available in the form of culture milk and added in many food products due to the many beneficial health effects in humans. Lately, *Lactobacillus acidophilus* has been formulated into pharmaceutical dosage forms such as capsules, for the treatment of diseases such as diarrhea and to improve the immune system. Products containing *Lactobacillus acidophilus* has to be refrigerated as the bacteria can not survive when kept at room temperature. Moreover, without proper protection from the highly acidic environment of the gastric and the presence of bile salts, the bacteria may have died before reaching the small intestine. In view of these limitations, the present study is conducted with the aim to prepare *Lactobacillus acidophilus* capsule which is stable at room temperature and the bacteria remains viable until reaching the small intestine.

OBJECTIVE OF STUDY

The aim of the study is to prepare granules containing viable *Lactobacillus acidophilus* which can be packed into capsules and stable when stored at ambient room temperature.

The study was conducted in various stages:

1) Isolation and identification of *Lactobacillus acidophilus* strain from commercial yoghurt.
2) Evaluation and optimization of the cultivation condition for the enhancement of the growth of *Lactobacillus acidophilus*.
3) Preparation and formulation of *L. acidophilus* into capsule.

MATERIALS AND METHODS

Materials

Man-Rogosa–Sharpe (MRS) broth (Criterion, U.S.A); Rogosa agar medium (Oxoid, UK); LAMVAB agar medium (MRS agar with vancomycin and cysteine as selective and elective agents and named as *Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green medium); Nutrient agar, nutrient broth, hydrogen peroxide and trypton soy broth (Merck, Darmstadt, Germany); Gram staining reagents, Bacto agar and phenol red broth base medium (Becton, Dickinson and Company, USA); Hydrochloric acid (Fisher Chemicals, UK); Brain heart infusion agar and carbon dioxide

generating system free from H_2 (Oxoid, UK); Biolog micro plates and inoculating fluid (Biolog, Hay Ward, USA); Bile salt (Sigma, Germany); Sulfuric acid (Sigma, USA); Sodium hydroxide (R & M Chemicals, UK); Arbinose, sucrose and lactose (BDH, UK); Sorbitol (GPR, UK); McDowell-Trump fixative reagent, phosphate buffer, osmium tetroxide and hexamethyldisilazane (Agar Scientific Limited, UK). Microbank (Pro-LAB Diagnostic, Canada). Barium chloride and calcium chloride hydrate ($CaCl_2$. $2H_20$) (Merck, Darmstadt, Germany); Vancomycin hydrochloride (Abbott Labs, North Chicago, USA); L-cystine-HCl (Fluka, Switzerland); Tween 80 (Sigma, UK); Glucose, sodium hydrogen carbonate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate (R & M Chemicals, UK); Yeast extract (Fluka, Germany); Bromocresol-Green, sodium acetate and magnesium sulphate hydrate (AJAX Chemicals, Sydney, Australia); Lactose monohydrate impalpable grade (HMS, Holland); Microcrystalline cellulose [Avicel PH-101]) (Asahi Chemical Industry, Tokyo, Japan). Corn starch (Euro Chemo, China); Magnesium stearate and Aerosil (Uitgest, Holland); Polyvinylpyrrolidone K29 (ISP Technology, USA); Eudragit L30D-55 (Pharma Polymer, Rohm, Darmstadt, Germany); Triethylcitrate and monobasic potassium phosphate (Merck, Darmstadt, Germany); Pancreatin and pepsin (Sigma, Germany); Hard gelatin capsules (Halagel (M), Malaysia).

Isolation and enumeration of *Lactobacillus acidophilus*

A commercial yoghurt drink (Vitagen, Denmark) was taken as a source of *Lactobacillus acidophilus* for this study. For the isolation of *Lactobacillus acidophilus*, 5 ml of the yoghurt was inoculated into 95 ml of nutrient medium (general medium) as well as MRS medium (selective medium for *Lactobacilli*) and incubated at 37 °C for 24 hours (Memmert, Germany).

The enumeration of viable cells was conducted by taking 1.0 ml of the yogurt drinks and diluted serially in ten folds (10^{-1} -10^{-8} with MRS broth as diluent) (Koch,1994). 0.1 ml of each dilution was taken and spread on MRS agar plate. The plates were incubated at 37 °C for 72 hours and the number of colonies on the plate was counted and recorded using a colony counter (Technical Lab Instrument, USA).

Identification of *Lactobacillus acidophilus*

The isolated colony formed on the MRS agar 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 (Holt *et al.*, 1994). 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 (http://www.pro-lab.com/products-microbank.php).

(a) Gram staining test

The isolated bacteria were examined using gram staining technique. The bacterial smear was prepared by applying a thin smear of bacteria on the glass slide and left to dry (Collins *et al.*, 2004). The smear of bacteria was fixed on the slide by passing the slide a few times over a flame. A crystal violet solution was then put on the slide for 1 min and the excess crystal violet solution on the slide was gently rinsed off with tap water. The gram's iodine solution was applied on the slide for 1 min and the slide was quickly decolourized with 95% ethanol. The slide was stained with a counter stain safranin for 1 min and then washed and dried. Finally, the slide was observed under light microscope (Olympus BX 50, Japan) with a magnification of 1000x. The shape and the colour of the cell were observed and photographed using the camera attached to the microscope.

(b) Motility test

Two methods namely, hanging-drop wet (MacFaddin, 2000) and Carigie's technique (Barrow and Feltham, 1999) were performed. In hanging-drop wet method, drops of culture containing live bacteria were placed on a clean dry cover slip and the cavity of the slide was inverted over the cover slip to ensure that the drop was in the center of the cavity and the slide was pressed gently. 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 a centre of a tube having motility medium using stabbing method. The medium was incubated at two different

temperatures of 25 °C and 37 °C for 48 hours. The motility of the bacteria was inferred by observing the spreading growth in the incubated semisolid agar.

(c) Catalase test

To perform this test, a single isolated colony was streaked on a glass slide and one drop of 3 % hydrogen peroxide was added on it. The effervescence of oxygen indicated the positive response of the bacteria to catalase test (Nelson and George, 1995).

(d) Carbohydrate fermentation test

Phenol red broth base medium was prepared as described in section 2.2.2(j) and used as a medium for this test. Different sugar substrates namely, arbinose, maltose, lactose, sucrose, sorbitol and glucose were used. 0.1 g (0.1 % w/v) of each sugar substrate was added to 100 ml of the medium. The mixture of 5 ml 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 (Thoesen, 1994).

(e) 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 (Nation, 1983). In this method, *L. acidophilus* suspension was centrifuged at 5000 rpm for 15 min (Microfuge E, Beckman, UK). The supernatant of the bacteria was discarded and the bacterial cells fixed in McDowell-Trump fixative reagent (pH 7.2) for at least 2 hours. 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 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 at concentrations of 50 %, 75 %, 95 % and 99.5 %. After that, hexamethyldisilazane (HMDS) was added to the sample tube for 10 min. HMDS 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).

(f) 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 hours. A single cell colony from MRS Agar was sub-cultured in BHI medium for 36-48 hours. The cultured bacteria were suspended in anaerobic Biolog fluid. 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™. The plate was incubated for 24 hours at 37 °C in an aerobic jar containing only CO_2 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 (MicroLog system user guide, 2001).

Characteristics of *Lactobacillus acidophilus*

(a) Growth at different pH

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

(b) Bile salt tolerance

The ability of the strains to tolerate bile salts was determined according to the method described by Gilliland *et al.* (1984) and Walker and Gilliland (1993). *Lactobacillus acidophilus* was tested for rapid growth in MRS broth medium with and without the addition bile salts. MRS broth was prepared with different concentrations of bile salts at 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. Two tubes of each concentration were inoculated with 0.1 ml of *L. acidophilus* culture and incubated at 37 °C for 48-72 hours. 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:-

$$\text{Bile tolerance (\%)} = \frac{\text{No of } \textit{L. acidophilus} \text{ in MRS broth with bile salts}}{\text{No of } \textit{L. acidophilus} \text{ in MRS broth without bile salts}} \times 100\ \%$$

Evaluation of parameters affecting *L. acidophilus* growth

(a) Different selective growth medium

The growth of *Lactobacillus acidophilus* in different selective medium namely, MRS agar (DeMan *et al.*, 1960), Rogosa agar (Rogosa *et al.*, 1951), LAMVAB agar (Hartemink, 1997) and edible agar was evaluated. 100 µL of *Lactobacillus acidophilus* suspension was spread on the plate of each medium and incubated under an anaerobic condition at 37 °C for 72 hours (Jackson *et al.*, 2002). The colonies were isolated and examined in terms of morphology and using gram staining. The viable count of *Lactobacillus acidophilus* in all the media was also obtained and compared using colony count method.

Lactobacillus acidophilus grown in MRS agar plate was also subjected to counting using McFarland standard. For this purpose, the bacteria were suspended in phosphate buffer and vortexed to emulsify the cells until sufficient turbidity equivalent to that of McFarland standard # 5 was obtained. One ml of the suspension was transferred into a petri dish and mixed with 20 ml of MRS agar. The culture was incubated at 37 °C for 72 hours. The viable count was obtained and the results were compared with the standard number in the McFarland sheet (www.hardydiagnostics.com).

(b) Effect of inoculation method

The viable counts of *Lactobacillus* bacteria were obtained in MRS agar and edible agar media using two different methods namely, pour plate and spreading method. The two methods were compared to find out which method was more suitable for counting the number of *Lactobacillus 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 *Lactobacillus acidophilus* into a sterile petri-dish and 20 ml of MRS agar and edible agar were then poured into the petri-dish. The plate was left for approximately 2 hours to solidify and kept in an incubator at 37 °C for 72 hours. On the

other hand, the spreading method was conducted by adding 0.1 ml from 10^{-4} dilution onto the surface of the MRS and edible 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.

(c) Effect of cultivation condition

The growth of *L. acidophilus* in MRS agar and edible agar media 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 anaerobic jar containing carbon dioxide generating system (Oxoid kit, UK) before incubated in aerobic incubator at 37 °C for 72 hours.

Growth profile of *Lactobacillus acidophilus*

Viable count data and opaque density (OD) measurements at a certain wavelength are very important to establish a microbial growth curve. The detection wavelength of 600 nm was selected as the optimum wavelength for measuring OD in all the experiments in the study. The same detection wavelength was also used by other researchers (Scragg, 1991; Leal-Sánchez *et al.*, 2003; Kiviharju *et al.*, 2005).

(a) Growth curve of *Lactobacillus acidophilus* before optimization

The growth curves of *Lactobacillus acidophilus* in MRS broth and edible broth media were investigated before optimization. *Lactobacillus acidophilus* was cultured in 100 ml of MRS and edible media using 250 ml Erlenmeyer flask. The pH of the medium was adjusted to 6.5 ± 0.5. The cultures were incubated at 37 °C for 72 hours (Memert, Germany). The growth of the bacteria was determined at 6-hour intervals by obtaining the viable counts and measuring the OD values at 600 nm. The experiment was repeated three times.

(b) Optimization of growth parameters

In order to optimize the growth of *Lactobacillus acidophilus* in the MRS broth and edible broth media, the growth parameters such as incubation temperature, pH of the medium, agitation speed and inoculum size, were investigated to achieve optimum condition for maximum growth.

The effect of the incubation temperature on the growth of *Lactobacillus acidophilus* was studied. Flasks containing 100 ml of the sterile medium were subjected to different temperature at 5 °C, 25 °C, 37 °C and 45°C for 36 hr. The initial pH of medium in each flask was adjusted to 6.5 ± 0.5 and the inoculum size was 1 % v/v with no agitation.

The effect of initial pH on the growth of *Lactobacillus acidophilus* was also studied. The pH was adjusted using either 1.0 M of HCL or 1.0 M of NaOH. The pH values of 4.0, 5.0, 6.0, 7.0 and 8.0 were selected. The flasks containing 100 ml of sterile medium were subjected to incubation temperature of 37 °C for 36 hours, keeping the inoculum size at 1 % v/v with no agitation.

In the study of the effect of agitation speed, the pH of the medium and the incubation temperature were fixed at 6.0 and 37 °C, respectively, and 1 % v/v inoculum size was used. The medium was incubated for 36 hours at different agitation speeds of 0, 50, 100 and 150 rpm using shaker water bath incubator (Memert, Germany).

In the study of the effect of inoculum size, various inoculum sizes of 0.5 %, 1.0 %, 3.0 %, 5.0 %, 7.0 % and 10.0 % v/v were used. The pH, incubation temperature and incubation time were maintained at 6, 37 °C and 36 hours, with an agitation speed of 100 rpm.

The turbidity and the viable count of the cultures were obtained. Each experiment was repeated three times.

(c) Growth curve of *L. acidophilus* after optimization

Once the optimum condition was obtained for both MRS broth and edible broth media, the growth curves of *L. acidophilus* under optimum condition was again determined. The viable counts and OD were recorded at every 6-hour intervals until 72 hours.

Preparation of *L. acidophilus* in edible medium with skim milk

100 ml of edible broth medium containing *Lactobacillus acidophilus* (10^9 cfu/ml) was added with skim milk powder at concentrations of 0.5% w/v, 0.8% w/v, 1.0% w/v, 1.2% w/v, 1.6% w/v, 1.8% w/v, 2.0% w/v, 2.2% w/v, 2.5% w/v, 2.7% w/v and 3.0%, w/v. The flasks were incubated at 37°C for 36 hr with an agitation speed of 100 rpm. I ml was

taken from each flask followed by serial dilution before the number of colonies was counted using pour plate method. The plates were incubated at 37°C for 72 hr and the viable counts of each concentration were obtained.

Preparation of *L. acidophilus* granules

Microcrystalline cellulose, lactose monohydrate and corn starch first were sieved through a 300-μm diameter sieve (Endecotts, UK) to break up lumps. 2 % w/w povidone was added as a binder. All these materials were blended in a planetary mixer (Kenwood, UK) for 5 min. Different amount of edible broth medium containing *Lactobacillus acidophilus* and 2.0 %w/v skim milk were mixed with the powder mass for 10 min. The following table shows the different formulations containing *L. acidophilus* and 2% w/v skim milk in edible broth medium.

Ingredient	Formulation		
	F(1)	F(2)	F(3)
L. acidophilus and 2% w/v skim milk in edible broth medium	35 ml	52 ml	65 ml
Corn starch	30 g	25 g	25 g
Lactose Monohydrate	38 g	35 g	25 g
Povidone	2 g	2 g	2 g
Microcrystalline Cellulose	30 g	38 g	48 g

Another set of experiment was prepared but using freeze-dried *L. acidophilus* .powder which was comprised of bacteria, edible medium and 0.5% w/v skim milk. Sufficient amount of distilled water was used as granulation fluid. The method of preparation was the same as described above. The different formulations containing freeze-dried *Lactobacillus acidophilus* and 0.5% w/v skim milk in edible broth medium prepared are shown as below:-

Ingredient	Formulation		
	F(4)	F(5)	F(6)

Freeze-dried *Lactobacillus acidophilus* and 0.5% w/v skim milk in edible broth medium	13 g	38 g	50 g
Corn starch	20 g	20 g	20 g
Lactose Monohydrate	65 g	40 g	28 g
Polyvinylpyrrolidone	2g	2 g	2 g
Distilled water	30 ml	41 ml	34 ml

For both types of formulations, the wet mass was screened through 1.70 mm diameter sieve (Endecotts, UK) and dried at 38 ± 2 °C for about 18 hours until a constant weight using an oven (Memert, Germany). The dried granules were screened through 1.00 mm diameter sieve (Endecotts, UK). Finally, magnesium stearate (1% w/w) and aerosol (0.5% w/w) were added and mixed with the granules. Granules within a size range of 0.71- 1.00 mm diameter were used in the preparation of capsules

Characterization of formulations

The granules were evaluated in terms of bulk density, tap density, moisture content, compressibility, flowability and viable count. Each experiment was repeated five times.

(a) Bulk and tapped densities

The bulk density was calculated by measuring the mass per unit volume of the granules. The mass of the volume was recorded and the corresponding volume of granules was measured using graduated measuring cylinder. The bulk density (ρ) was calculated using the following equation (Badawy *et al.*, 2000; Tüske *et al.*, 2005)

$$\rho = \frac{M}{V}$$

where,

M: mass of the granules.

V: volume of the granules.

For the tapped density measurement, the measuring cylinder was tapped 200 times which was sufficient for the granules to reach a constant volume. The volume was measured and the tapped density calculated using equation 6.1.

(b) Moisture content determination

The moisture content of the granules for different formulation was monitored and measured using an infra red moisture analyzer (Mettler LP16, Deltarange, type LP16 M, Switzerland).

(c) Compressibility test

The compressibility index was calculated from the bulk and tapped densities using the following equation (Keleb *et al.*, 2002):

$$compressibility\,(\%) = \frac{\rho_f - \rho_i}{\rho_f} x\,100\%$$

Where, ρ_f: tapped density and ρ_i: bulk density.

(d) Flowability test

The flowability corresponding to compressibility values of different granule formulations was determined by referring to standard values of relationship between compressibility and flowability table as shown below (Lachman *et al.*, 1987).

Compressibility	Flowability
5-15	Excellent
12-16	Good
18-21	Fair- possible
23-35	Poor
33-38	Very poor
<40	Very, very poor

(e) Viable count

Granules of 1 g and pour plate method were used in the study. The plate was left for about 2 hours to solidify and kept in an incubator at 37 °C for 72 hours. The counting of *Lactobacillus acidophilus* in the granules was carried out using colony counter (Technical lab instrument, USA).

Enteric coating of granules

To achieve the suitable processing conditions for the use of top-spray fluidized bed coater in the coating of granules (Uni-Glatt, Germany), different coating parameters such as (1) inlet temperature, (2) fluidization air flow rate, (3) atomizer pressure and (4) spray rate, were optimized by a one-at-a-time strategy, varying one parameter while keeping the others constant. First, the different inlet temperatures were selected and the optimum temperature was recorded when the granules dried and the bacteria was alive. The optimum temperature was then fixed and different fluidized air flow rates were selected to optimize the flow rate. The best fluidized air flow rate was fixed and the pressure was optimized. Two spray rates were tested after fixing all the other parameters and the spray rate in which no agglomeration was selected. Batches of 200 gram of granules were allowed to fluidize in the coating chamber until the inlet air temperature reached the required temperature. The granules were coated with aqueous coating solution containing Eudragit L30D-55 at 5.0 % w/v, 7.5 % w/v, 10.0 % w/v, 12.5 % w/v and 15.0 % w/v, plasticized with 5% v/v of triethylcitrate. After spraying, the coated granules were allowed to fluidize for 10 min to ensure complete drying. The coated granules of 500 mg were filled manually into hard gelatin capsules of size number zero.

In-vitro dissolution studies

The in-vitro dissolution of *Lactobacillus acidophilus* capsules under simulated gastric and intestinal fluids was studied. The simulated gastric fluid and intestinal fluid were prepared according to the method described in United States Pharmacopoeia 26 (2003).

The simulated gastric fluid was prepared by dissolving 2.0 g of sodium chloride and 3.2 g of purified pepsin (pepsin is derived from porcine stomach mucosa, with an activity of

800 to 2500 units per mg of protein) in 7.0 mL of hydrochloric acid and sufficient water to make up the volume to 1000 ml. The pH of the solution was about 1.1-1.2.

The simulated intestinal fluid was prepared by dissolving 6.8 g of monobasic potassium phosphate in 250 mL of water, mixed and added 77 mL of 0.2 M sodium hydroxide and 500 mL of water. 10.0 g of pancreatin was added and topped up with water to 1000 mL. The resulting solution was adjusted with 0.2 M sodium hydroxide to pH of 6.8 ±0.1.

The dissolution study was performed according to USP 26 (2003) dissolution test apparatus II paddle method (model PT- DT7, Pharma Test Apparatebau GmbH, Germany). The dissolution medium was 900 ml maintained at 37.0 ± 0.5 °C, and the rotation speed was set at 100 rpm. Samples of 5 ml were withdrawn after 2 hr and the number of *Lactobacillus acidophilus* in the dissolution medium was estimated using pour plate method.

Stability study

The capsules were stored over a 9 month-period at 5 °C, 28 °C and 40 °C. The stability of the capsule was evaluated by obtaining the viable counts using pour plate method.

Statistical analysis

The results were treated statistically using SPSS software (version 13, USA). Student's Independent T-Test and one-way analysis of variance were used to analyze the results. When there was a statistically significant difference, a post-hoc Tukey Honestly Significant Difference test was used. A statistically significant difference was considered when $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and enumeration of *Lactobacillus acidophilus*

The results obtained showed that the bacteria contained in yoghurt produced only one type of colony when isolated in MRS agar (selective medium for *Lactobacilli*). The colony was white in colour, circular in shape and large in size. On the other hand, isolation on nutrient agar (general medium) produced two types of colony. One was

circular in shape, large and white in colour, while the other was irregular in shape, smaller in size and yellow in colour.

The enumeration of the bacteria was directly counted from the yogurt diluted to ten fold serially in the MRS agar medium and the viability result was about 10^7cells/ml. The number of probiotics obtained was higher than the minimal number of probiotic bacteria required in a product (above 10^5 or 10^6 cells per g) that needed to be consumed to exert a health-promoting effect for the consumers (Speck, 1978; Kim, 1988).

Identification of *Lactobacillus acidophilus*

(a) Gram staining test

The isolated bacteria were observed by light microscope after gram staining and the photograph is shown in Fig. 1. It is clear that the bacteria was gram positive, rod shaped coccobacilli, occurring singly or in chains. The gram staining results indicate that the isolated bacteria could be identified as *Lactobacilli* (Holt *et al.*, 1994).

(b) Motility test

Hanging-drop wet method showed that the isolated bacteria were non-motile. 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 non-motile. The non-motile behaviour is a characteristic of *Lactobacillus acidophilus* (Tamang and Sarkar, 1996).

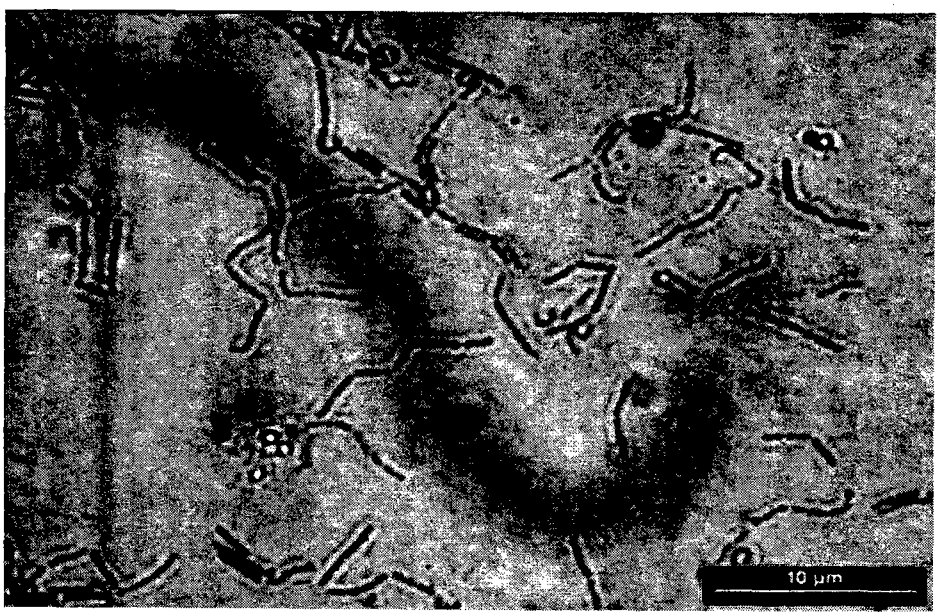


Fig. 1: *Lactobacillus acidophilus* as observed under light microscope.
(Magnification 1000x)

(c) Catalase test

No bubble was observed indicating that the isolated bacteria was catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2. It is well known that *Lactobacillus acidophilus* is catalase negative (Schillinger, 1999; MacFaddin, 2000).

(d) Carbohydrate fermentation

Table 1 showed that the isolated bacteria could ferment maltose, lactose, sucrose and glucose but not sorbitol and arbinose. No bubble was detected from the glucose inserted with Durham tube indicating that no gas production associated with the growth. Thus, the results obtained coincided with *L. acidophilus* strain characteristic (Holt *et al.*, 1994; Barrow and Feltham, 1999).

(e) Scanning electron microscopy

Fig 2 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. The shape and the range of the measured dimension obtained for the isolated bacteria were in good agreement with the results of other researchers (Robinson, 1990; Klaenhammer and Russell, 2000; Ray, 2001).

Table 1: Biochemical tests results

Test	Observation
Arbinose	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)

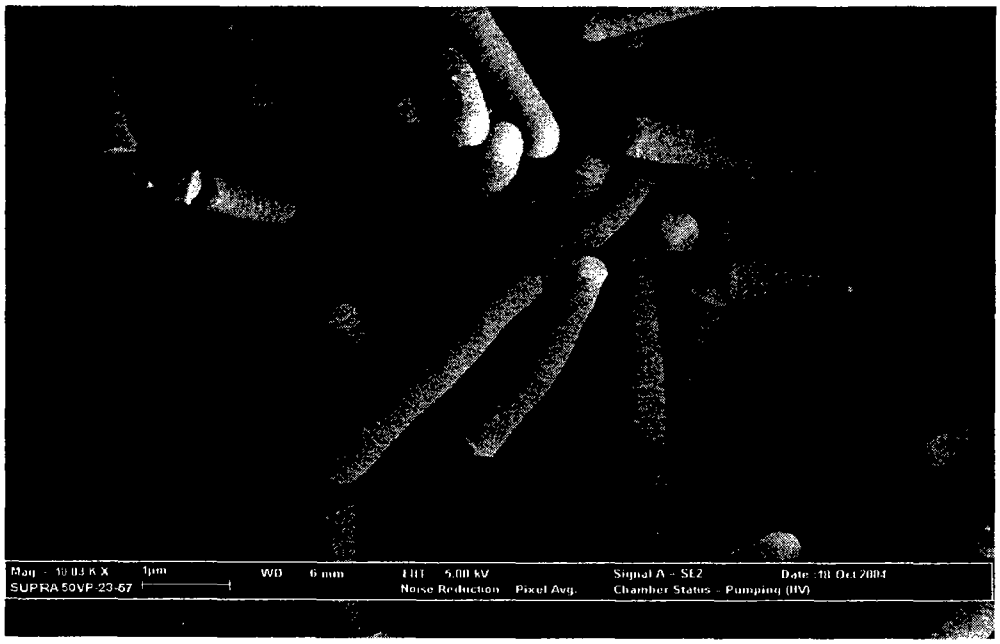


Fig. 2: Scanning electron microscope photomicrograph of *Lactobacillus acidophilus.* Magnification 10,000x.

(f) 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.

Characteristics of *Lactobacillus acidophilus*

(a) 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 between the range of 4.0 and 7.0 indicated that the bacteria preferred to grow in acidic and neutral environment.

(b) 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 (Gilliland and Walker, 1990). 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 4. 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 (Brashears *et al.*, 1998; Corzo and Gilliland, 1999).

Table 2: Biolog micro plate readings of isolated bacteria.

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

PROB = Probability

SIM = Similarity

DIS = Distance Between #1 and # 2 species

Table 3: Effects of pH on the growth of *Lactobacillus acidophilus*.

pH	Observation
3.0	Clear
4.0	Slightly turbid
5.0	Very turbid
6.0	Very turbid
7.0	Turbid
8.0	Clear
9.0	Clear

Table 4: Effect of different concentrations of bile salts on the growth of *Lactobacillus acidophilus*. Mean ± SD, N = 3.

Bile salt concentration (%)	Viable count (CFU/ ml) $\times 10^7$	Bile tolerance (%)
0.0	151 ± 3	100.0
0.1	132 ± 2	87.4
0.3	114 ± 2	75.5
0.5	105 ± 2	69.5
1.0	83 ± 1	55.0

Evaluation of culture condition affecting *L. acidophilus* growth

(a) Different growth medium

The morphology and characteristics of *Lactobacillus acidophilus* in different selective growth medium are presented in Table 5. The properties of the colony were similar in all the media except the colour of the colony which was green in LAMVAB medium while white in the other media.

The colony viable count results are shown in Fig 3 and Table 6. There was a statistically significant difference in the viable count results. Post-hoc test showed that there was a statistically significant difference in the viable count between edible medium and all the other three media, MRS, Rogosa and LAMVAB. There was no statistically significant difference in the colony count results among MRS, Rogosa and LAMVAB media. This suggested that *Lactobacilli* are not inhibited by vancomycin available in LAMVAB medium. Similar results were also obtained by Hartemink *et al.* (1997).

The results of morphology and viable count of *L. acidophilus* cultivated in MRS, Rogosa and LAMVAB media suggested that these media could be used consistent with the results reported by other researchers (Nelson and George, 1995; Hartemink and Rombouts, 1999; Silvi *et al.*, 2003; Minelli *et al.*, 2004). Although the viable count of *L. acidophilus* cultivated in edible medium was relatively lower as compared with other media, this number is greater than the minimal number of probiotic bacteria required to be consumed to exert a health-promoting effect as described earlier.

Lactobacillus acidophilus grown in MRS agar plate was also subjected to McFarland standard counting to examine the suitability of this counting method. The number of *L. acidophilus* which demonstrated the same turbidity as McFarland standard # 5 was calculated to be 13 $x10^7$ cell/ml. Clearly, the count obtained was lower than that of McFarland standard (the density of the culture of McFarland Standard # 5 is about 15 $x10^8$ cell/ml). McFarland Standard uses turbidity in the quantification which does not rule out the number of bacteria that has died. Hence, McFarland standard only provides a rough estimation of the number of bacteria which appears to be less accurate as compared to colony counting.

(b) Effect of inoculation method

Fig. 4 shows the viable counts of *L. acidophilus* cultivated by pour plate and spreading method in MRS medium and edible medium. The viable count data in pour plate were higher than that of the spreading 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 (Soestbergen and Ching, 1969) and *L. acidophilus* is known to have such characteristics (Mombelli and Gismondo, 2000). The results showed that the pour plate method was more superior than the spreading method.

Table 3.1: Evaluation of *L. acidophilus* in different growth medium.

Morphology and characteristics of *L. acidophilus*	Culture medium			
	MRS agar	Rogosa agar	LAMVB agar	Edible agar

i) Colony	Single	Single	Single	Single
ii) Shape	Circular	Circular	Circular	Circular
iii) Surface appearance	Smooth	Smooth	Smooth	Smooth
v) Size	Big	Big	Big	Big
vi) Colour	White	White	Green	White
vii) Gram staining	+ve	+ve	+ve	+ve

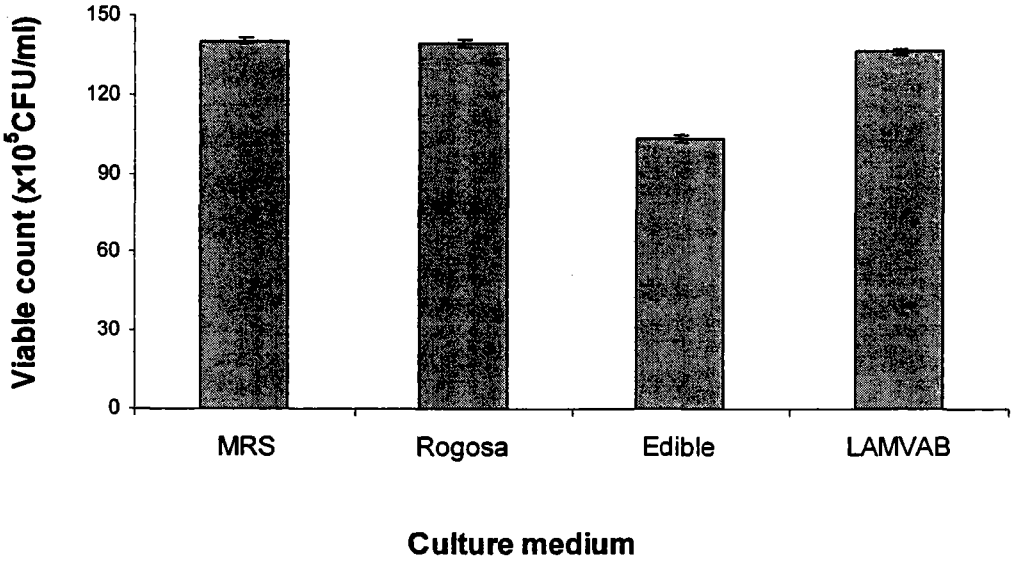


Fig. 3: Colony viable counts in different culture medium.

Table 6: Viable count in different growth medium. Mean ± SD, N=5.

Selective Medium	Viable count (CFU/ml) x 10^5
A) MRS	140.2 ± 1.92
B) Edible medium	103.2 ± 3.27
C) LAMVAB	136.2 ± 2.28
D) Rogosa	139.2 ± 3.76
Statistical analysis	$p<0.05$
Tukey HSD (Multiple Comparisons)	A & B ($p<0.05$) A & C ($p>0.05$) A & D ($p>0.05$) B & C ($p<0.05$) B & D ($p<0.05$) C & D ($p>0.05$)

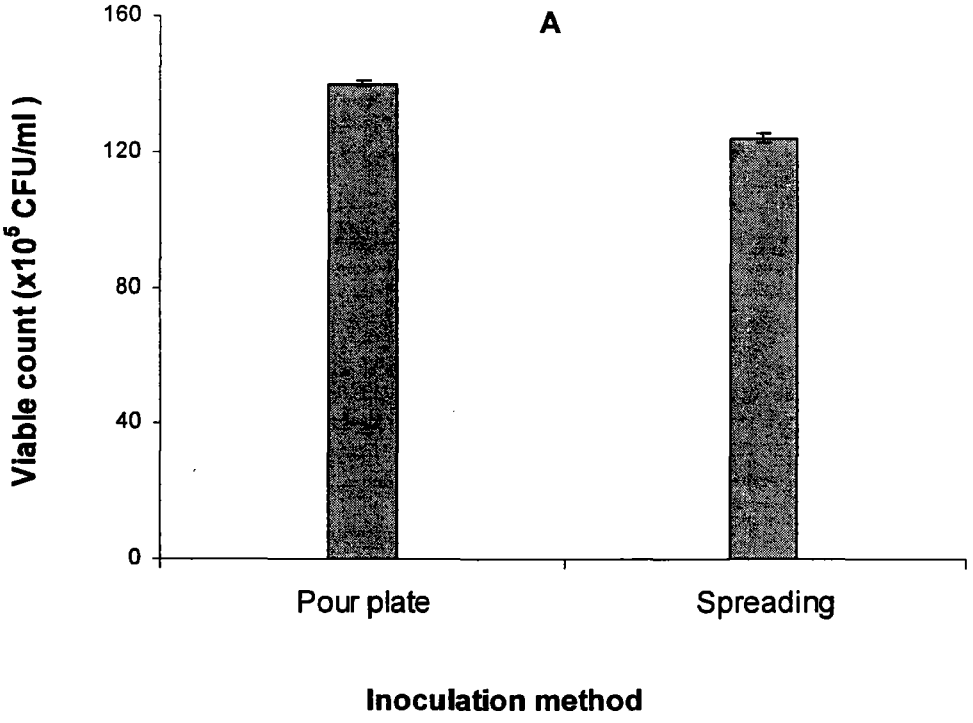
(c) Effect of culture condition

The growth results of *L. acidophilus* in aerobic and anaerobic conditions in MRS and edible media are shown in Fig. 5. 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. *Lactobacillus acidophilus* is facultative and can grow in both aerobic and anaerobic conditions (Klaenhammer and Russell, 2000). The present result was similar to the finding of Kelly *et al.* (1995). 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 *Lactobacillus acidophilus.*

Growth curve of *Lactobacillus acidophilus* before optimization

The growth profiles of *Lactobacillus acidophilus* in MRS and edible media before optimization are shown in Fig. 6. The growth parameters used in this experiment were selected based on parameters reported by other research groups (Heenan *et al.*, 2002; Helland *et al.*, 2004). These curves were established to find out the time required by the

bacteria to reach the logarithmic phase. It can be clearly seen that the growth increased and reached a stationary phase after 36 hours in both media. The higher growth of *L. acidophilus* in MRS medium than in edible medium suggested that MRS medium was more suitable to optimize the growth of *Lactobacillus acidophilus*.



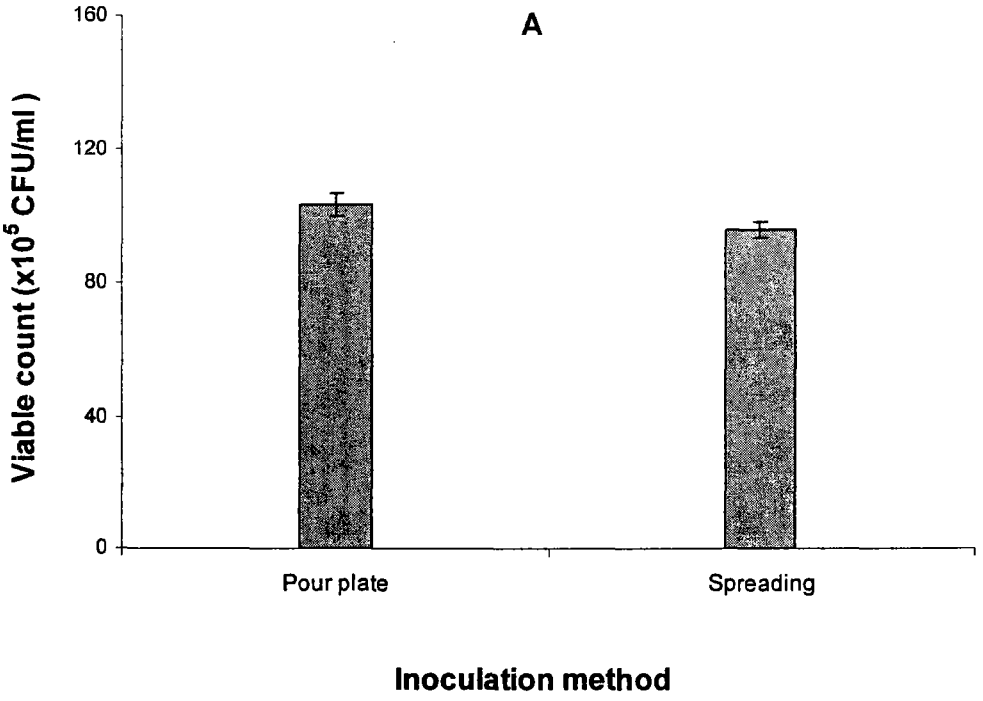
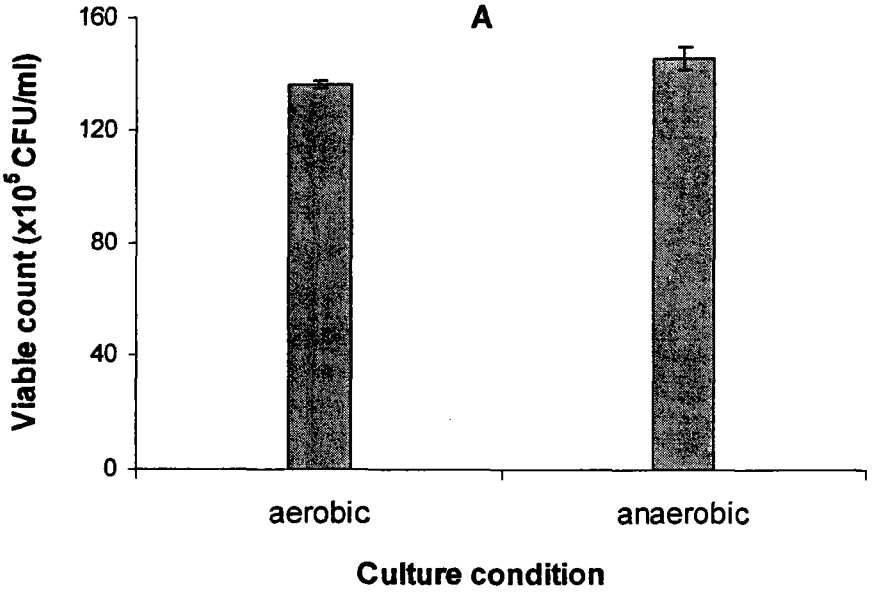


Fig. 4: Viable count of *L. acidophilus* in pour plate and spreading methods. (A) MRS medium; (B) edible medium. Mean ± SD, N=5.



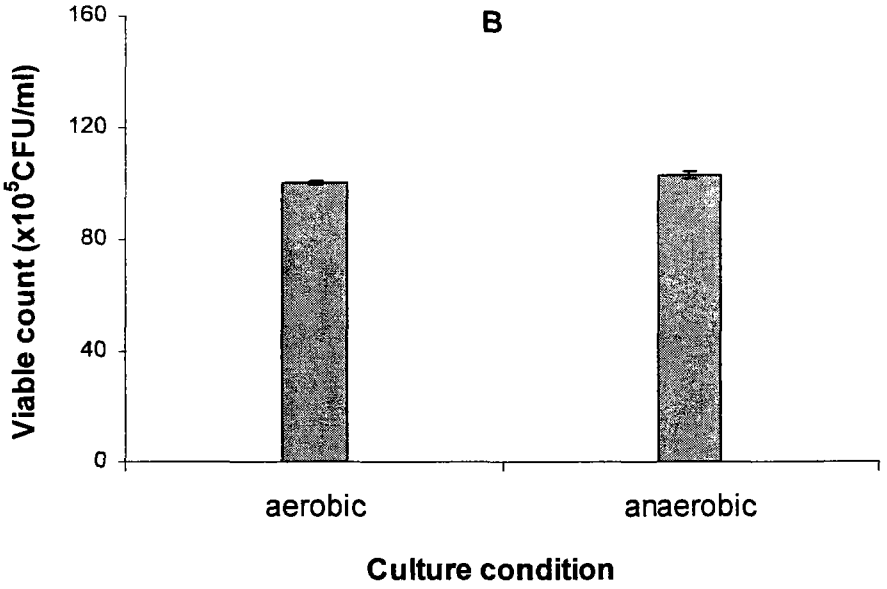
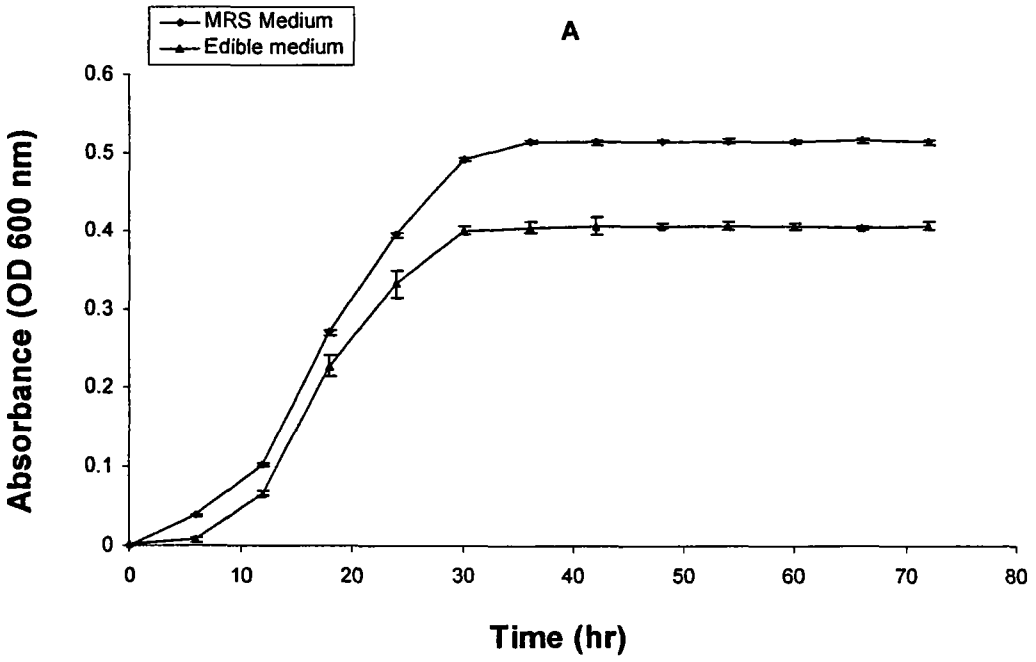


Fig. 5: Viable count of *L. acidophilus* cultured under aerobic and anaerobic conditions. (A) MRS medium; (B) edible medium. Mean ± SD, N=5.



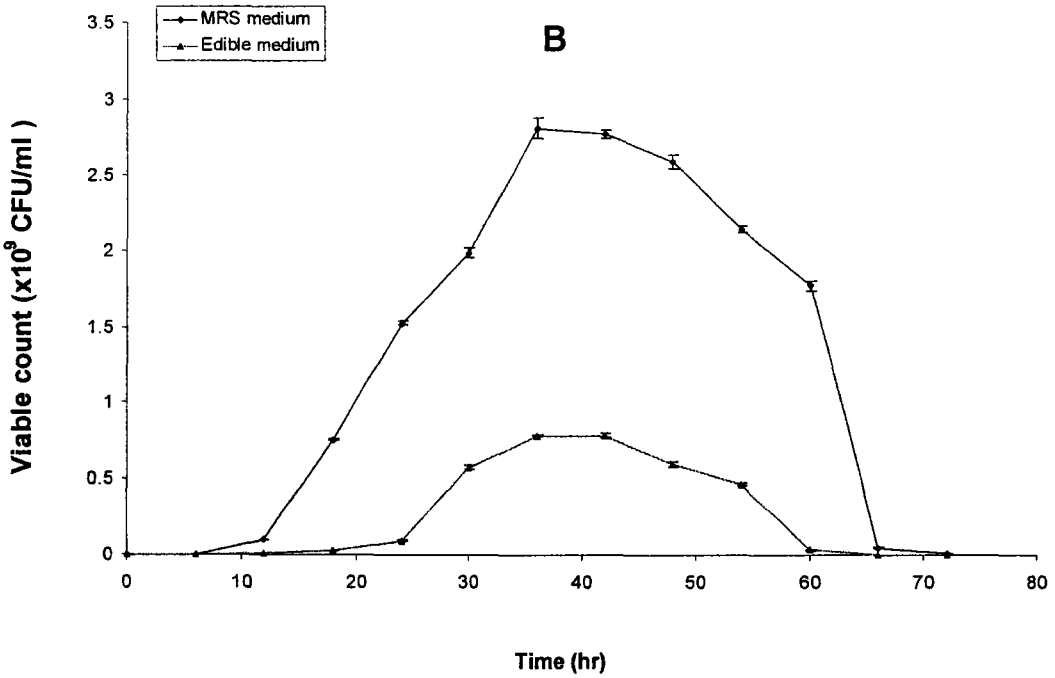


Fig. 6: Growth profiles of *L. acidophilus* cultivated in two different media before optimization. (A) absorbance, (B) viable count. Mean ± SD, N= 3.

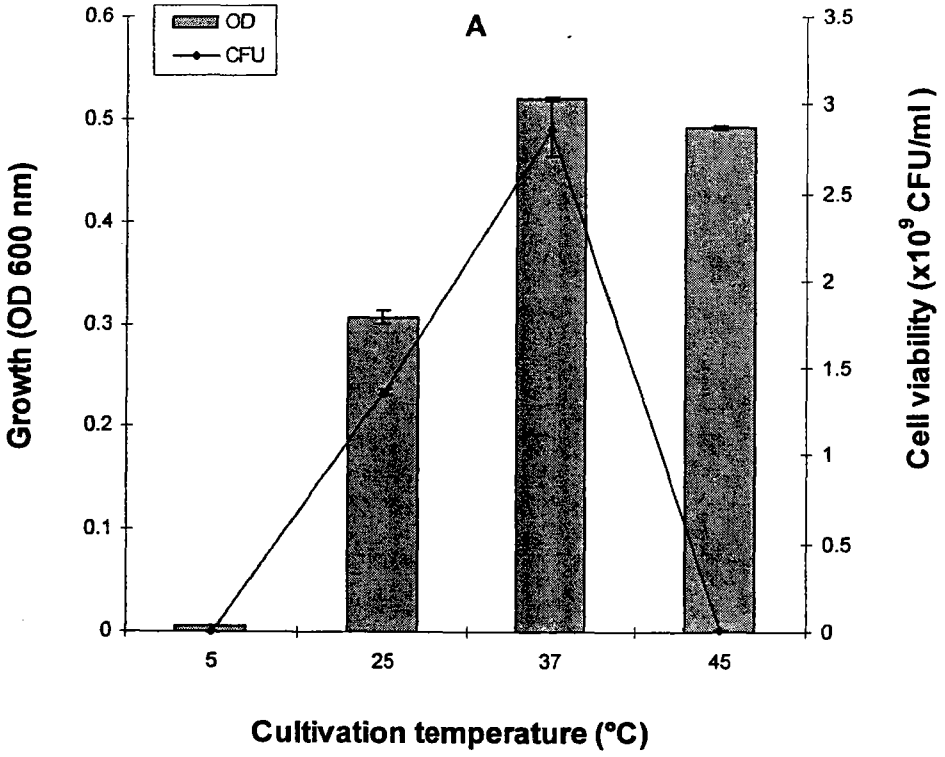
Optimization of culture condition in MRS and edible media

(a) Effect of cultivation temperature

Fig. 7 shows the effect of cultivation temperature on the growth of *Lactobacillus acidophilus*. For both media, *L. acidophilus* produced the maximum number of cells when cultivated at 37 °C ($p < 0.05$). The bacterial growth in both MRS and edible media declined at temperature of 45 °C. It was also observed that the growth decreased at temperature below 37 °C for both MRS and edible media.

The cultivation temperature is closely linked to the growth rate of microorganisms and maximum growth rate could be achieved at optimum temperature. The above results showed that the optimum temperature for maximum growth of *L .acidophilus* was 37 °C in both MRS and edible media. An optimum cultivation temperature at 37 °C was also reported by other research groups (Krischke *at al.*, 1991; Hujanen and Linko, 1996; Patrignani *et al.*, 2006). Gomes and Malcata (1999) and Batt (2000) reported that *L. acidophilus* could grow even at 45°C, with an optimum growth within 35-40 °C. Saarela *et al.* (2000) suggested that at 37-40 °C range, most of the probiotic strains multiplied well. The reduction or inhibition of the growth of microorganisms at temperature higher

than the optimum value could be due to the disruption of the membrane of microorganisms by heat (Prescott *et al.*, 1999).



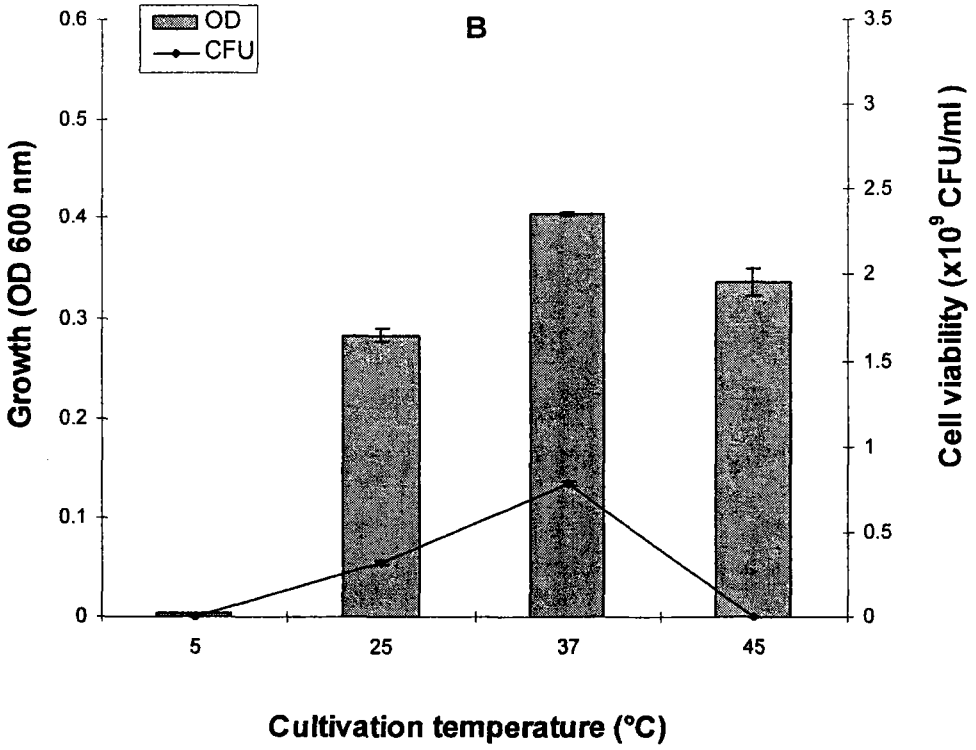
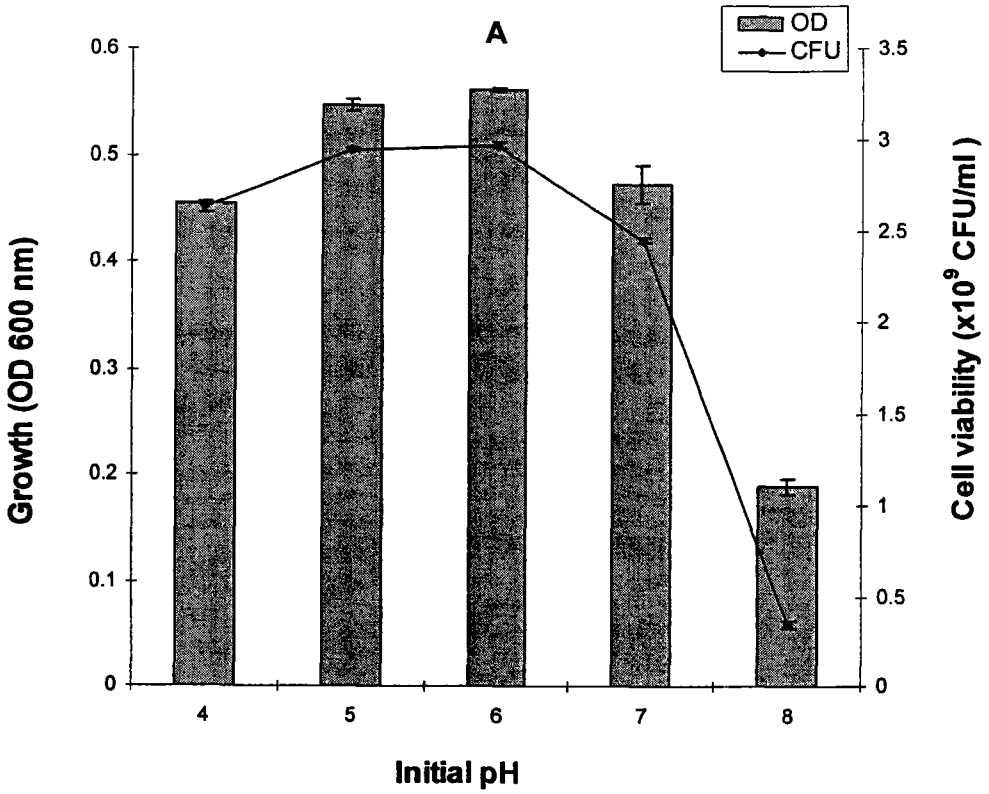


Fig. 7: Effect of temperature on the growth of *Lactobacillus acidophilus.*
(A) MRS medium; (B) edible medium. Mean ± SD, N=3.

(b) Effect of initial pH

The effect of the initial pH of the medium on the growth of *Lactobacillus acidophilus* and the results of absorbance and viable counts of *Lactobacillus acidophilus* in both MRS and edible media are shown in Fig. 8 . It can be observed that the growth pattern of *Lactobacillus acidophilus* as a function of initial pH of the medium in both media was quite similar. The growth of microorganism decreased at pH 7.0 and in alkaline medium at pH 8.0. The maximum growth was under acidic condition at pH between 5.0 and 6.0 with a slight drop at pH 4.0. The difference in OD was statistically significant ($p < 0.05$). The OD at a maximum growth was 0.56 for MRS medium (Fig. 3.7 a) and 0.41 for edible medium (Fig. 3.7 b) and the maximum cell counts were 2.97×10^9 CFU/ml and 0.81×10^9 CFU/ml for MRS and edible media, respectively. The initial pH of the cultivation medium plays a key role in the growth and viability of the bacteria. The pH range for optimum growth exists for each species of microorganisms. Although microorganisms often grow over a wide range of pH, they are limited by their tolerance and viability.

The present results were consistent with the findings obtained by others. Hartemink *et al.* (1997) showed that *Lactobacilli* were highly acid-resistant, and the growth was possible at an initial pH of 5.0. Piuri *et el.* (2005) used initial pH 6.5 for the growth of *Lactobacillus* species. On the other hand, *Lactobacilli* cells showed the ability to grow in a medium with pH 7.2 (Stile and Holzapfel,1997; Liew *et al.*, 2005). De man *et al.* (1960) reported a maximum growth when initial pH of the medium was between 6.2 and 6.5. Wenge and Mathews (1999) found that the optimum pH for *Lactobacillus* lied within the range of 5.0-6.0.



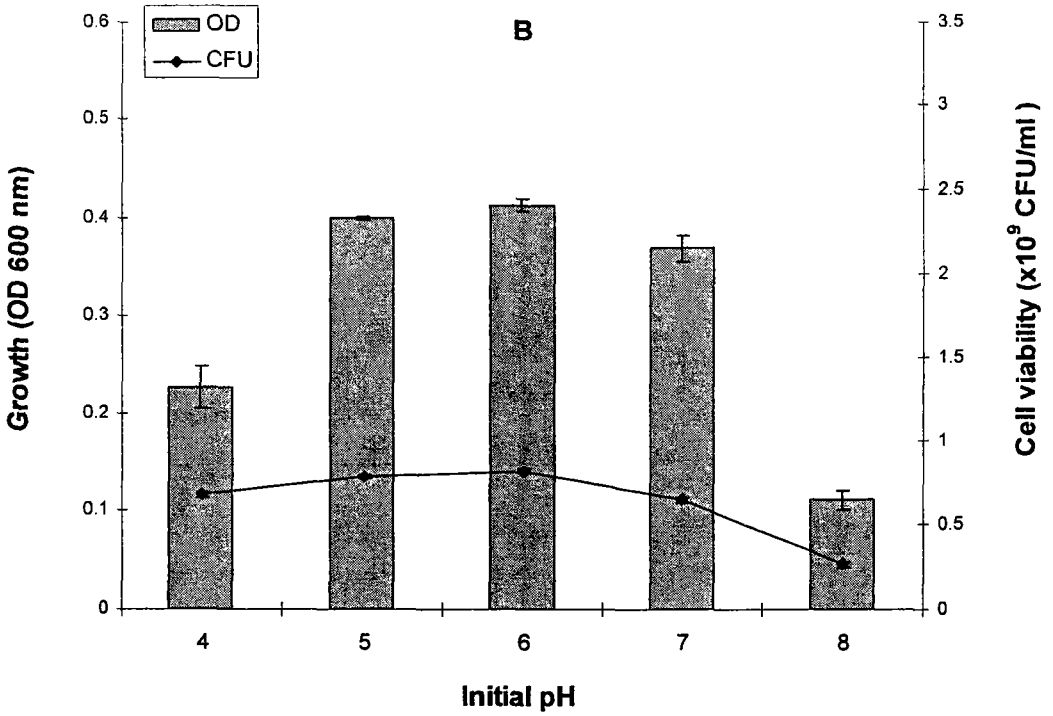


Fig. 8: Effect of initial pH on growth of *Lactobacillus acidophilus*
A) MRS medium; (B) edible medium. Mean ± SD, N=3.

Batt (2000) and Vamanu *et al.* (2001) mentioned that pH in the range between 5.5 and 5.8 was optimum for the growth of *Lactobacillus*. Taillandier *et al.* (1996) showed that pH 6.0 was the optimum value.

(c) Effect of agitation speed

The results of varying the agitation speed from 0 to 150 rpm are shown in Fig. 9. It is evident that there was growth without any agitation and the growth increased with an increase in agitation speed. A maximum growth was achieved at an agitation speed of 100 rpm and further increase in the agitation speed to 150 rpm decreased the growth by approximately 20 %. The difference in the values of OD and the cell viability among the various agitation speeds was significantly different ($p < 0.05$).

The bacteria can grow under a static environment (0 rpm) due to its facultative characteristics. The low growth in this case could be contributed by competition of the microorganism population for nutrients within a specified region in the medium (Kankaanpää *et al.*, 2001). With agitation, the bacteria could have been distributed more uniformly in the medium. As such, the nutrients might be sufficient to sustain the growth of a higher number of bacteria (Zisu and Shah, 2003). In this study, the

maximum growth occurred at 100 rpm possibly due to the maximum homogeneity of the medium at this speed. Further increase in the agitation speed above 100 rpm reduced the growth rapidly. When the agitation speed used was higher than the optimum value, a greater mechanical force or shear force was generated which led to a higher rate of cell destruction, which damaged and lowered the viability of cells of microorganisms (Venkatadri and Irvine, 1990; Shioya *et al.*, 1999). The use of agitation to improve the growth and viability was reported by many research groups. Vamanu *et al.* (2001) used agitation speed of 50 rpm for *Lactobacillus acidophilus* growth. Selma *et al.* (2004) incubated *Lactobacillus* species in a well-shaken water bath for 24 hours. Heenan *et al.* (2002) reported that cultures were incubated for 36 hours at 37 °C on a shaking incubator with an agitation speed of 100 rpm for *Lactobacilli.* Dalgaard and Koutsoumanis (2001) incubated the flasks in an incubator with an agitation speed of 100 rpm. Liew *et al.* (2005) mentioned that cultivation was conducted at 37 °C in an orbital shaker at 120 rpm. Planas *et al.* (2004) reported that the cultivation of lactic acid bacteria was carried out in a rotary agitator at 200 rpm. Hence, it is obvious that the use of an optimum agitation speed in the growth phase contributed to the homogeneity of the medium and thus increased the population of the growth and improved its viability. Agitation speed above the optimum speeds, caused cell damage of microorganisms and resulted in a low cell growth.

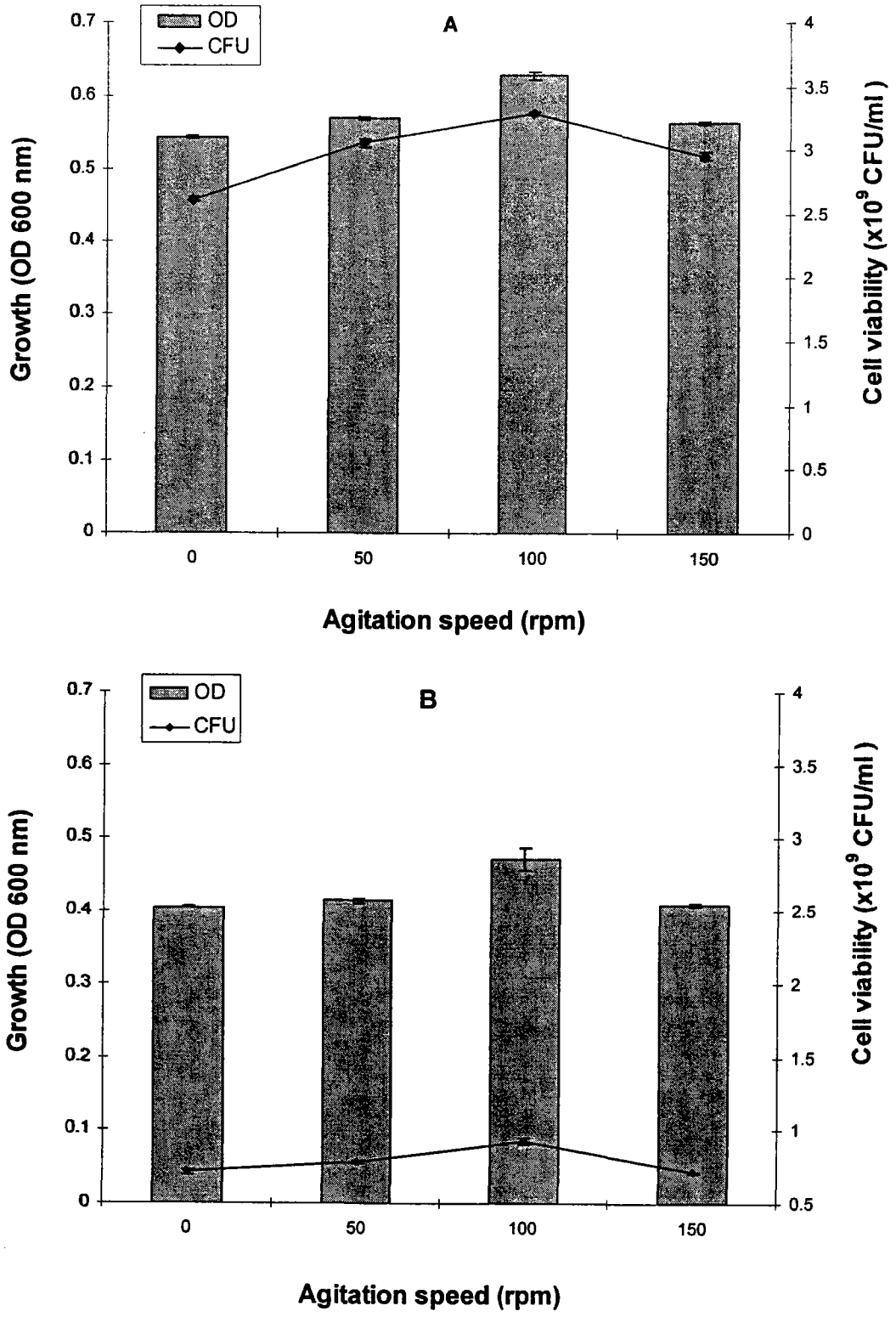


Fig. 9: Effect of agitation speed on growth of *Lactobacillus acidophilus*.
(A) MRS medium (B) edible medium. Mean ± SD, N=3.

(d) Effects of inoculum size

Inoculum size is considered as an important parameter and plays a key role in achieving maximum growth. Alteration in the inoculum levels of probiotic cultures could affect the population and viability of probiotic microorganisms (Dave and Shah, 1997). The results of the effects of inoculum size are presented in Fig. 10. The bacteria grew in both MRS and edible media and the maximum growth was achieved in MRS medium for inoculum size of 1.0 % v/v ($p<0.05$), while in the edible medium for inoculum size of 3.0% v/v ($p<0.05$). The maximum OD value and cell viability were 0.64 and 3.43×10^9 CFU/ml for MRS medium (Fig. 10a) and 0.51 and 0.934×10^9 CFU/ml for the edible medium (Fig. 10b). The maximum growth of the bacteria achieved at these inoculum sizes could possibly because the nutrients in these media were sufficient for the maximum growth. Further increase in inoculum size beyond the optimum values reduced the cell viability which might be due to the competition of the bacteria for the nutrients in the medium. On the other hand, lower percentage of inoculum size than the optimum value resulted in undesired low cell viability. The use of 1 % v/v inoculom size in MRS medium was also reported by other researchers (Ferreira and Gilliand, 1988; Liong and Shah, 2005; Maragkoudakis *et al.*, 2006).

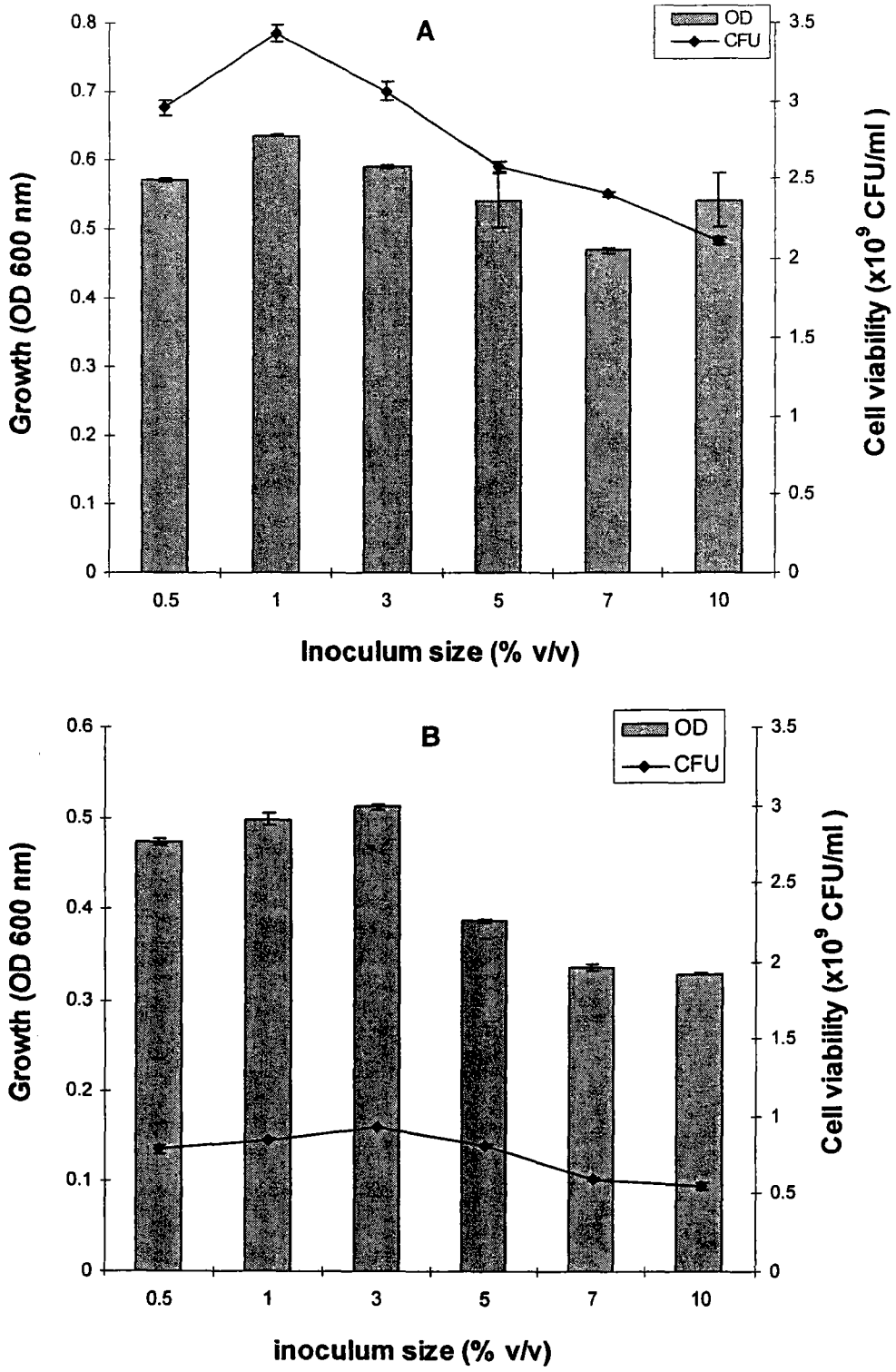


Fig. 11: Effect of inoculum size on the growth of *Lactobacillus acidophilus*. (A) MRS medium; (B) edible medium. Mean ± SD, N=3.

Growth profile of *Lactobacillus acidophilus* after optimization in MRS and edible media

The growth curves after selecting the optimum values of all parameters under study, namely, cultivation temperature at 37 °C, initial pH value of the medium at pH 6.0, agitation speed of 100 rpm and inoculum size of 1 % v/v for MRS and 3% v/v for edible media are shown in Fig. 11 for MRS and Fig. 12 for edible medium. The corresponding growth curves before optimization were also included in these figures for the purpose of comparison of the results. It can be noted that the growth population increased by about 20 % and 30 % for both MRS and edible media after optimization. The time to reach the initial stationary phase was the same between before and after optimization which was about 36 hours for both media. The optimum growth condition would be utilized in further study.

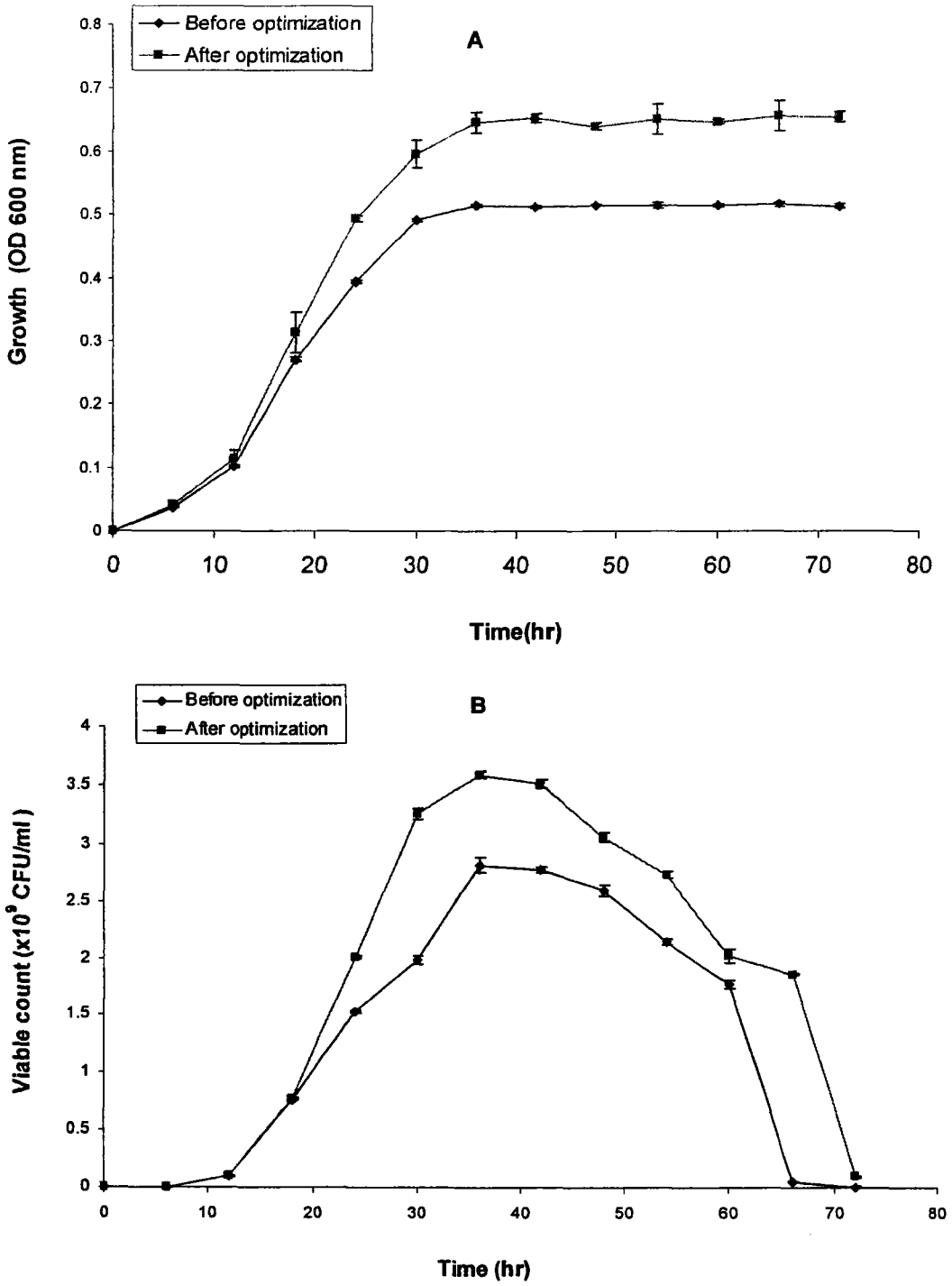


Fig. 11: The growth profile of *L. acidophilus* (cultivated in MRS medium) after optimization. (A) absorbance (B) viable count. Mean ± SD, N= 3.

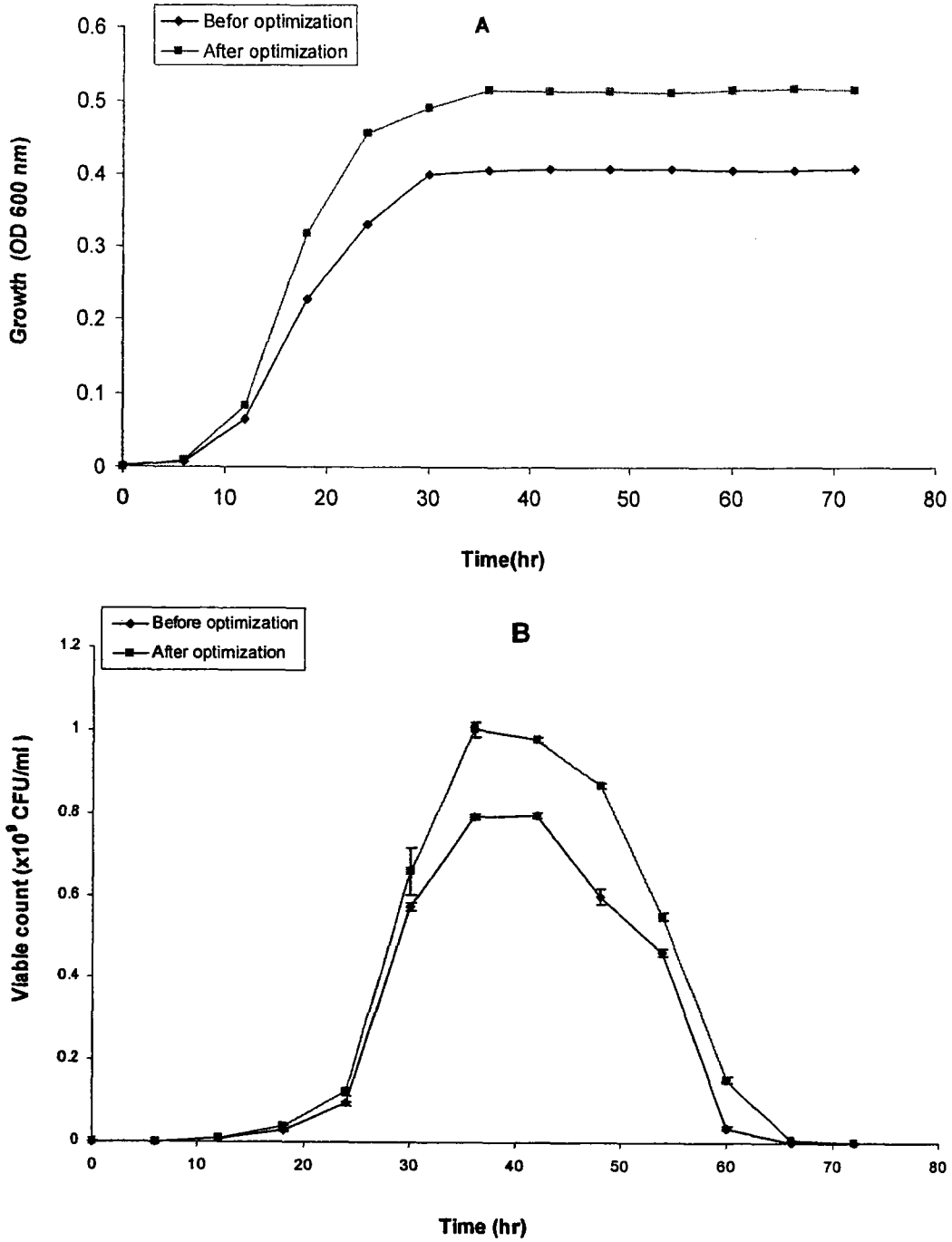


Fig. 12: The growth profile of *L. acidophilus* (cultivated in edible medium) after optimization. (A) absorbance; (B) viable count. Mean ± SD, N= 3.

Growth of *L. acidophilus* in edible medium with skim milk

Skim milk has been used as a growth medium for *L. acidophilus* (Mäyra- Makinen and Bigret, 1993). *Lactobacillus acidophilus* requires different amino acids to grow which are present in the skim milk but these amino acids alone are not sufficient to support the maximum growth rates (Monnet *et al.*, 1996). Combination of skim milk (contains amino acids) with edible medium that contains glucose (which is the source of carbohydrate), yeast extract (which is the source of nitrogen) and minerals stimulate the growth of *L. acidophilus* (Stahouder *et al.*, 1969; Sandine, 1996). The growth profile and the viable counts of *L. acidophilus* are shown in Fig. 13 and Table 7. The viable counts increased by increasing the concentration of the skim milk and reached a maximum of

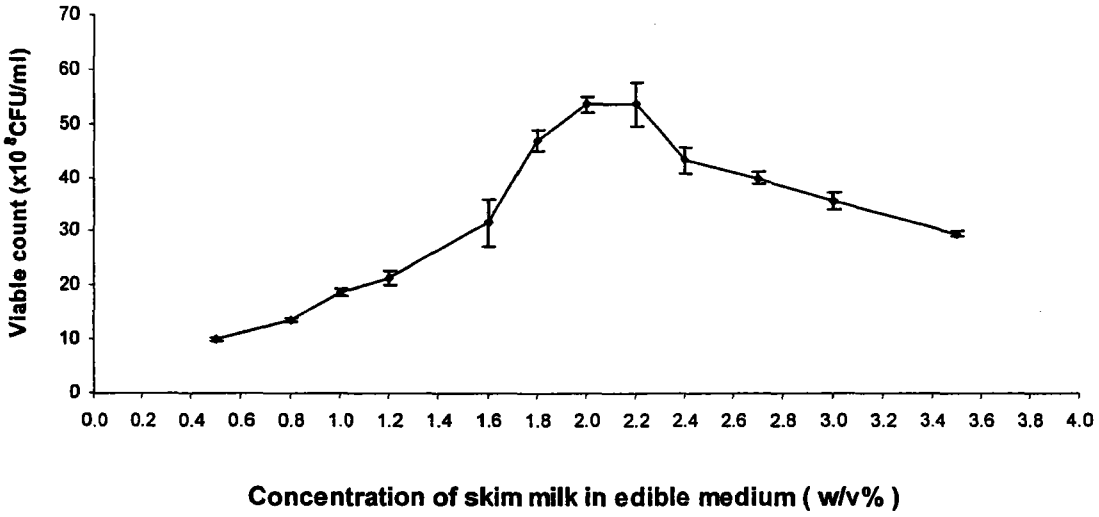


Fig. 13: Viable count of *L. acidophilus* in edible medium with skim milk

Table 7: Growth of *L. acidophilus* in edible medium with skim milk.

Concentration	Viable count ($x10^9$CFU/ml)	
A) 0.5	1.00 ± 0.04	
B) 0.8	1.36 ± 0.03	
C) 1.0	1.870 ± 0.070	
D) 1.2	2.133 ± 0.120	
E) 1.6	3.160 ± 0.452	
F) 1.8	4.700 ± 0.200	
G) 2.0	5.367 ± 0.153	
H) 2.2	5.367 ± 0.416	
I) 2.4	4.333 ± 0.252	
J) 2.7	4.000 ± 0.100	
K) 3.0	3.567 ± 0.153	
L) 3.5	2.960 ± 0.0529	
Statistical analysis	p<0.05	
Tukey-HSD (Multiple Comparisons)	A & B p>0.05	D &H p<0.05
	A & C p<0.05	D & I p<0.05
	A & D p<0.05	D & J p<0.05
	A & E p<0.05	D & K p<0.05
	A & F p<0.05	D & L p<0.05
	A & G p<0.05	E & F p<0.05
	A & H p<0.05	E & G p<0.05
	A & I p<0.05	E & H p<0.05
	A & J p<0.05	E & I p<0.05
	A & K p<0.05	E & J p<0.05
	A & L p<0.05	E & K p>0.05
	B & C p>0.05	E & L p>0.05
	B & D p<0.05	F & G p<0.05
	B & E p<0.05	F & H p<0.05
	B & F p<0.05	F & I p>0.05
	B & G p<0.05	F & J p<0.05
	B & H p<0.05	F & K p<0.05
	B & I p<0.05	F & L p<0.05
	B & J p<0.05	G & H p>0.05
	B & K p<0.05	G & I p<0.05
	B & L p<0.05	G & J p<0.05
	C &D p>0.05	G & K p<0.05
	C & E p<0.05	G & L p<0.05
	C & F p<0.05	H & I p<0.05
	C & G p<0.05	H & J p<0.05
	C & H p<0.05	H & K p<0.05
	C & I p<0.05	H & L p<0.05
	C & J p<0.05	I & J p>0.05
	C & K p<0.05	I & K p<0.05
	C & L p<0.05	I & L p<0.05
	D & E p<0.05	J & K p>0.05
	D & F p<0.05	J & L p<0.05
	D & G p<0.05	K & L p>0.05

$5.37.x10^9$ CFU/ml at 2.0 % concentration. The difference in the results of viable counts for different concentration of skim milk was significant statistically. There was no significant difference with a further increase from 2.0 % to 2.2 % ($p < 0.05$). Above 2.2 %, there was a significant decline in the viable counts. The increase in the viable counts of bacteria with an increase in skim milk concentration is obviously due to the larger supply of amino acids to sustain and enhance the growth of bacteria cells. The reduction in the growth rate with a further increase in skim milk concentration beyond optimum 2.2 % was possibly due to the osmotic effects which could have killed the bacteria.

Characterization of different types of formulation

The results of bulk density, tapped density, moisture content, compressibility, flowability and viable count of different *L. acidophilus* formulations are shown in Table 8.

The results of bulk and tapped densities of the granules provide useful information about the volume of the final dosage form. In addition, these two parameters could affect the potency of the final granules and also used to calculate the granule compressibility (Lachman *et al.*, 1987). Moisture content is a critical parameter for granulation process. Certain moisture content range aids in producing granules with optimum binding force. When the moisture content lied between 0.7 and 2.0 %, most of the granules were well formed. However, if the moisture content was above the

critical range, the granules became sticky and hard (Lachman *et al.*, 1987). The results of moisture content of all formulations investigated in this study lied within the acceptable range.

The compressibility of the granules affects the ability of powders to form compact under pressure. Powders which form hard compacts under applied pressure without exhibiting any tendency to cap or chip can be considered compressible. Compressibility ranges from 5 to 15 % indicated excellent flowability of the granules (Lieberman and Lachman, 1980; Lachman *et al.*, 1987). the compressibility obtained for all the formulations was between 5.08 %and 9.37 % and therefore the flowability of the granules was excellent.

The viable count was conducted for all the *L. acidophilus* formulations. The viable count results of all the *L. acidophilus* formulations were above 10^9 CFU/500 mg, which are sufficient for the humans (Kebary, 1996; Krasaekoopt *et al.*, 2006). Formulations #3 and #5 were selected for enteric coating.

Evaluation of coating process and capsulation of the granules

Enteric coating is important to protect *L. acidophilus* against highly acidic environment in the stomach (Conway *et al.*,1987; Berrada *et al.*, 1990). To optimize and achieve the suitable coating conditions, a series of experiments was carried out using blank granules (granules without *L. acidophilus*).

The coating parameters, which included inlet air temperature, fluidized air flow rate, atomizer pressure and spray rate, were very critical and necessitated optimization as these parameters could affect the viability of the probiotics during coating (Olsen, 1985;

Olsen, 1989). The observation obtained in the optimization of the coating parameters is summarized in Table 9.

The selected formulations were coated successfully using Eudragit L30D-55, which was capable to provide protection against digestive extremes of gastric acid and bile salts and to target drug release in the small intestine (Datta *et al.*, 1995; Amighi and Moes, 1996; Cole *et al.*, 2002). To improve the plasticity of the coating, triethyl citrate was introduced. This plasticizer not only affects the film formation process through the lowering of the glass transition temperature but also the film properties such as water vapour transmission and drug release (Pharmaceutical Coatings Bulletin, 1995, 1996).

Table 9: Evaluation of the coating parameters

Parameters	Condition	Observation
Inlet temperature °C	50-60	Bacteria died.
	28-30	Granules agglomerated.
	36-38	Granules dried and bacteria were alive.
air flow rate (m^3/h)	120-140	Granules disintegrated.
	90-110	Good fluidization.
	60-80	Granules could not be fluidized.
Atomizing air (bar)	1.4	Granules became powdery.
	1.0	Good fluidization.
	0.8	Agglomeration occurred.
Spray rate (ml/ min)	1.5	Agglomeration occurred.
	1.0	No agglomeration.

In-vitro dissolution studies

The in vitro dissolution results of *Lactobacillus acidophilus* coated with different levels of Eduraget L30D-55 are shown in Table 10. Formulation number # 3 showed release at coating level below 7.5% and no release at the coating level of 7.5 % and above. The coating level of below 7.5% was not sufficient to protect *Lactobacillus acidophilus* from the highly acidic stomach environment. On the other hand, formulation #5 showed a release at the enteric coating level below 10 % but no release at the coating level of 12.5 % and above, indicating that the coating level of at least 12.5% was necessary to adequately protect *Lactobacillus acidophilus* from the highly acidic stomach environment. The lower enteric coating level required by formulation #3 could be attributed to the presence of higher content of skim milk and the use of skim milk which was not exposed to freeze drying.

On the other hand, It was observed that once formulations #3 and #5 were in simulated intestinal fluid, *Lactobacillus acidophilus* was readily released with the dissolution of the enteric coat under alkali pH environment (Table 11).

These coating level was critical to ensure that probiotics survived during the passage through human gastrointestinal tract (Conway *et al.,* 1987; Goldin *et al.,* 1992) and reached the small intestine as viable organisms for potential health effects (Havenaar and Veld, 1992; Salminen *et al.,* 1998).

Table 10: *Lactobacillus acidophilus* release in simulated gastric fluid.

Formulation	(%)Coating level	Viable counts
F # 3	5.0	.68 x10^4 CFU/ Cap2
	7.5	No growth
	10.0	No growth
F # 5	5.0	6.1 x10^6 CFU/ Cap
	7.5	3.7 x10^5 CFU/ Cap
	10.0	1.05 x10^3 CFU/ Cap
	12.5	No growth
	15.0	No growth

Table 11: *Lactobacillus acidophilus* release in simulated intestinal fluid.

Formulation	(%)Coating level	Viable counts
F # 3	7.5	1.58x10^9 CFU/ Cap
	10.0	1.55x10^9 CFU/ Cap
F # 5	12.5	1.28x10^9 CFU/ Cap
	15.0	1.39x10^9 CFU/ Cap

Stability study

One of the most important aspects of *Lactobacillus acidophilus* product is stability of the bacteria (Brennan *et al.*, 1983; Hyun and Shin, 1998). The selected formulation #3 with coating level of 7.5% and formulation #5 with coating level of 12.5 % were subjected to stability test at different temperature and the results are depicted in Figures 14 and 15. Both formulations were stable over nine months at 5 °C and 28 °C but the viability of bacteria declined gradually and drastically after 3 months at 40 °C. This finding suggested that the capsules remained stable after 9-month storage at room temperature.

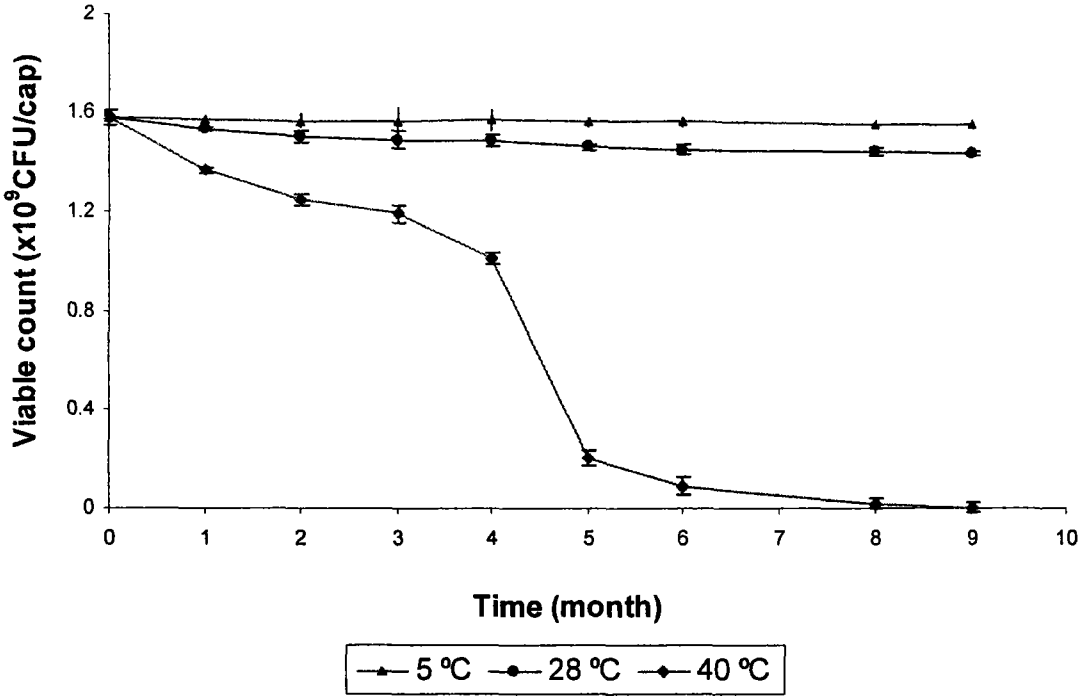


Fig. 14: Stability study of probiotic *Lactobacillus acidophilus* capsule (Formulation # 3)

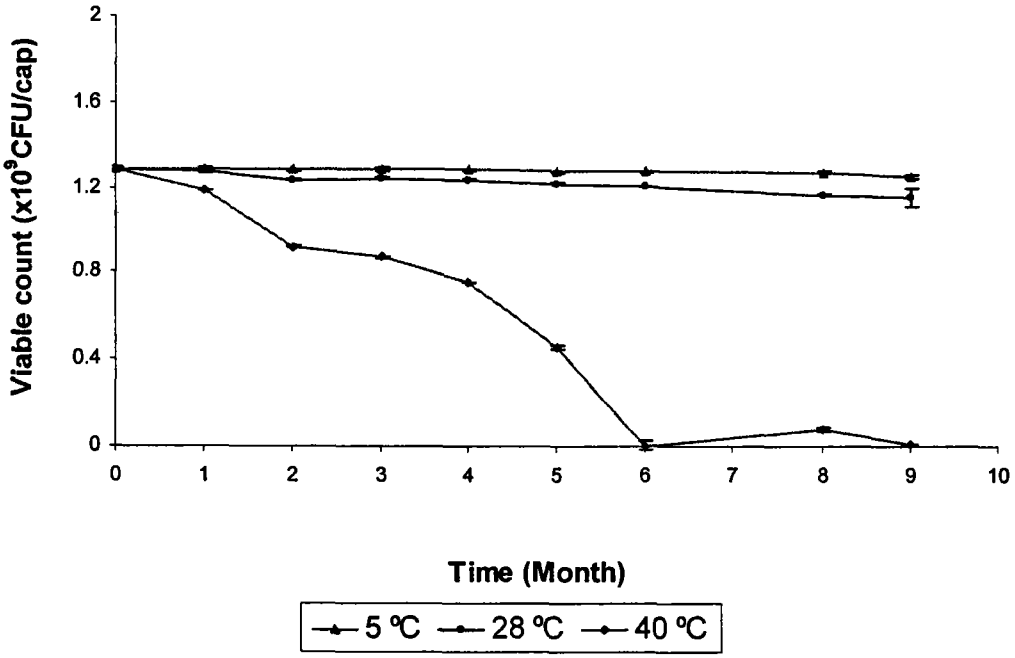


Fig. 15: Stability study of probiotic *Lactobacillus acidophilus* capsule (Formulation # 5)

CONCLUSION

In summary, the bacteria were successfully isolated from a commercial yoghurt drink and identified as *Lactobacillus acidophilus* using gram staining, scanning electron microscope, motility, catalase and carbohydrate fermentation tests. The identity of *Lactobacillus acidophilus* was further confirmed using Biolog rapid identification system. The study revealed that *Lactobacillus acidophilus* preferred to grow in medium with pH between 4 and 7. The presence of bile salt reduced the viable counts of *Lactobacillus acidophilus*. Moreover, the viable counts declined further with an increase in the bile salts, suggesting that the bacteria have to be protected from the bile salts.

Lactobacillus acidophilus showed similar morphology and characteristics in MRS, Rogosa, LAMVAB and edible agar media except that the colour of the colony was green in LAMVAB agar medium. The pour plate method was preferred over the spreading method as the former provided a relatively higher number of counts. There was no significant difference between aerobic and anaerobic conditions, suggesting that *Lactobacillus acidophilus* could be cultivated under both of these conditions. Counting using McFarland Standard might not be suitable and inaccurate as compared with colony counting method. The optimum growth conditions of *Lactobacillus acidophilus* in MRS broth medium were incubation temperature of 37 °C, initial pH of the medium of 5.0, agitation speed of 100 rpm and inoculum size of 1.0 %, while in edible medium the results were similar except the inoculum size was 3.0 %. The growth after optimization was improved by about 20 % for MRS broth medium and about 30 % for edible broth medium. MRS medium was more superior as a growth medium as the number of cell counts in this medium was comparatively higher than in edible medium.

Two types of granules, one containing *L. acidophilus* and skim milk in edible broth medium, while the other one containing freeze-dried *L. acidophilus* and skim milk in edible broth medium, were successfully prepared. The results of physical characterization of the two types of granules were satisfactory and the bacteria were alive. The enteric coating level of 7.5% was required for formulation # 3 and 12.5% for formulation # 5 to protect *L. acidophilus* from the detrimental effect of acidic environment in the gastric. Once the formulation transited into small intestine of alkali pH, the enteric coat dissolved and *L. acidophilus* was released in the small intestine. The stability study showed that *L. acidophilus* capsules were stable for at least nine months at 5 °C and 28 °C. Therefore, *L. acidophilus* capsules could be stored at room temperature without the hassle of stored refrigerated. In addition, the successful preparation of formulation # 3 suggests that *L. acidophilus* can be incorporated directly in the preparation of granules without the need to undergo freeze drying.

REFERENCES

Amighi, K. and Moës, A. (1996). Influence of plasticizer concentration and storage conditions on the drug release rate from Eudragit RS30D film-coated sustained-release the ophylline pellets. *Eur J Pharm Biopharm.* **42**, 29-35.

Badawy, S. F., Menning, M. M., Gorko, M. and Gilbert, D. L. (2000). Effect of process parameters on compressibility of granulation manufactured in a high-shear mixer. *International Journal of Pharmaceutics*, **198**, 51-61.

Barrow, G. and Feltham, R. (1999). Cowan and steeel's manual for the identification of medical bacteria, Cambridge university press, UK.

Batt, C. (2000). *Lactobacillus* /Introduction. In: R. Robinson, C. A. Batt, and D. Patel, *Encyclopedia of Food Microbiology*. Academic press, London, UK.

Berrada, N., Lemeland, J., Laroche, G., Thouvenot, P. and Piaia, M. (1990). *Bifidobacterium* from fermented milks: Survival during gastric transit. *J. Dairy Sci,* **74**, 409-413.

Brashears, M. M., Gilliland, S. E. and Buck, L. M. (1998). Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei. J. Dairy Sci.* **81**, 2103-2110.

Brennan, M., Wanismail, B. and Ray, B. (1983). Prevalence of viable *Lactobacillus acidophilus* in dried commercial products. *J. Food Prot.* **46**, 887-892.

Cole, E. T., Scott, R. A., Connor, A.L., Wilding I. R., Petereit H-U, Schminke, C. and Beckert T.,(2002). Cade D. Enteric coated HPMC capsules designed to achieve intestinal targeting. *Int. J. Pharm.* **231**, 83-95.

Collins, C. H., Lyne, P. M. and Grange, J. M. (2004). Collins and Lyne's microbiological methods. 8th ed. Butterworth-Heinemann, London.

Conway, P., Gorbach, S. and Goldin, B. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.* ***70***, 1-12.

Corzo, G. and Gilliland, S. E. (1999). Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* **82**, 472-480.

Dalgaard, P. and Koutsoumanis, K., (2001). Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. *Journal of Microbiological Methods* **43**,183-196.

Datta, R., Tsai. S., Bonsignore, P., Moon, S. and Frank, H. R. (1995). Technological and economic potential of poly (lactic acid) and lactic acid derivatives; *FEMS Microbiology Reviews* **16**, 221-231.

Dave, R. and Shah, N. (1997). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *International Dairy Journal* **7**, 31-41.

De Man, J. C., Rogosa, M. and Sharpe, M. (1960). A medium for the cultivation of *Lactobacilli. J. Appl. Bacteriol.* **23**,130-135

Ferreira, G. and Gilliand, S. (1988) Bacteriocin involved in premature death of *Lactobacillus acidophilus* NCFM during growth at pH 6. *J. Dairy Sci.* **71**, 306-315.

Gilliland, S. E. and Walker, D. K. (1990). Factors to Consider When Selecting a Culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in Humans. *J. Dairy Sci.* **73**, 905- 911.

Gilliland, S., Staley, T. and Bush, L. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *J. Dairy Sci.* **67**, 3045-3051.

Goldin, B., Gorbach, S. , Saxelin, M., Barakat, S., Gualtieri, L. and Salminen, S. (1992). Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Digestive Diseases and Sciences* **37**, 121-128.

Gomes, A. M. and Malcata, F. X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science and Technology* **10**, 139-157.

Hardy diagnostics (2003) [on line] [accessed on 20 May 2003] from World Wide Web https://www.hardydiagnostics.com/catalog2/user/Product.asp.

Hartemink, R. and Rombouts, F. M., (1999). Comparison of media for the detection of *Bifidobacteria, Lactobacilli* and total anaerobes from faecal samples. *J. Microbiol. Methods* **36**, 181–192.

Hartemink, R., Domenech, V.R., Rombouts, F.M. (1997). LAMVAB — A new selective medium for the isolation of *Lactobacilli* from faeces. *Journal of Microbiological Methods* **29**,77-84.

Havenaar, R. and Huis In't Veld J. H. J. (1992) Probiotics: A general view. In, Wood B. j. B.: The Lactic Acid Bacteria, Vol. 1: *The Lactic Acid Bacteria in Health and Disease*, Chapman and Hall, New York, p. 151-170.

Heenan, C. N., Adams, M. C., Hosken, R. W. and Fleet, G. H. (2002). Growth Medium for culturing probiotic bacteria for applications in vegetarian food products. *Lebensm.-Wiss. u.-Technol.* **35**, 171-176.

Heenan, C. N., Adams, M. C., Hosken, R. W. and Fleet, G. H. (2002). Growth Medium for culturing probiotic bacteria for applications in vegetarian food products. *Lebensm.-Wiss. u.-Technol.* **35**, 171-176.

Helland, M. H., Wicklund, T. and Narvhus, J. A. (2004). Growth and metabolism of selected strains of probiotic bacteria in milk- and water-based cereal puddings. *International Dairy Journal* **14**, 957–965.

Holt, G. J., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S.T. (1994). *Bergey's Manual of Determinative of Bacteriology*. 9th edition. Williams and wilkins. USA, p. 560-570.

Hujanen, M. and Linko, K. K. (1996). Effect of temperature and various nitrogen sources on L(+)-lactic acid production by *Lactobacillus casei. Applied Microbiol Biotechnoi.* **45**, 307-313.

Hyun, C. and Shin, H. (1998). Utilization of Bovine Blood Plasma Obtained from a Slaughterhouse for Economic Production of Probiotics. *Journal of Fermentation and Engineering* **86**, 34-37.

Jackson, M. S., Bird, A. R. and McOrist, A. L. (2002).Comparison of two selective media for the detection and enumeration of *Lactobacilli* in human faeces. *Journal of Microbiological Methods* **51**, 313-321.

Kankaanpää, P. E., Salminen, S. J., Isolauri, E. and Lee, Y. K. (2001). The infuence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiology Letters* **194**,149-153.

Kebary, K. M. (1996). Viability of *Bifidobacterium bifidum* and its effect on quality of frozen Zabady. *Food Research International* **29**, 431-437.

Keleb, E. I., Vermeire, A., Vervaet, C. and Remon, J. P. (2002) Continuous twin screw extrusion for the wet granulation of lactose. *International Journal of Pharmaceutics* **239**, 69-80.

Kelly, W.J., Asmundson, R.V., Harrison, G.L. and Huang, C.M. (1995). Differentiation of dextran-producing *Leuconostoc* strains from fermented rice cake (*puto)* using pulsed-field gel electrophoresis. *Int. J. Food Microbiology* **26,** 345-352.

Kiviharju, K., Leisola, M. and Eerikäinen, T. (2005). Optimization of a *Bifidobacterium ongum* production process. *Journal of Biotechnology* **117**, 299-308.

Klaenhammer, T. R. and Russell, M. W. (2000). *Lactobacillus acidophilus* In: R. K. Robinson, C. A. Batt and D. Patel (eds.), *Encyclopedia of Food Microbiology.* Academic press, London, UK.

Koch, A. (1994). Growth measurement. In: P. Gerhardt, R. Murray, W. Wood, N. Krieg (eds.). Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D C, p. 249-277.

Krasaekoopt, W., Bhandari, B. and Deeth, H.C (2006). Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. *LWT - Food Science and Technology* **39,** 177-183.

Krischke, W., Schroder, M. and Trosch, W. (1991). Continuous production of L- lactic acid from whey permeate by immobilized *Lactobacillus casei* subsp.*casei. Applied Microbiology and* Biotechnology **34**, 573-578.

Lachman, L., Lieberman, H. A. and Kanig, J. L. (1987). *Theory and practice of industrial pharmacy*, 3rd ed. Lea & Febiger, Philadelphia,USA.

Leal-Sánchez, M. V., Ruiz-Barba, J. L., Sánchez, A. H., Rejano, L., Jimēnez-Díaz, R., A. Garrido, A. (2003). Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. *Food Microbiology* **20**, 421-430.

Lieberman, H. A. and Lachman, L. (1980). Pharmaceutical dosage forms. Tablet volume 1, New York and Basel: Marcell Dekker Inc.

Liew, S. L., Ariff, A. B, Raha, A. R. and Ho, Y. W., (2005). Optimization of medium composition for the production of a probiotic microorganism, *Lactobacillus rhamnosus*, using response surface methodology. *International Journal of Food Microbiology* **102,** 137-142.

Liong, M. T. and Shah, N. P. (2005). Optimization of cholesterol removal, growth and fermentation patterns of *Lactobacillus acidophilus* ATCC 4962 in the presence of mannitol, fructo-oligosaccharide and inulin: a response surface methodology approach. *Journal of Applied Microbiology* **98,** 1115-1126.

MacFaddin, J. F. (2000). *Biochemical Tests for Identification of Medical Bacteria.* 3rd ed. Philadelphia, USA.

Maragkoudakis, P. A., Miaris, C., Rojez, P., Nikos, M., Magkanari, F., Kalantzopoulos, G. and Tsakalidou, F. (2006). Production of traditional Greek yoghurt using *Lactobacillus* strains with probiotic potential as starter adjuncts. *International Dairy Journal* **16,** 52-60.

Mäyra-Makinen, A. and Bigret, N. (1993). Industrial use and production of lactic acid bacteria, In: S. Salminen and A. Von wright (eds.), *Lactic acid bacteria.* Marcell Dekker Inc. New York, USA. p. 65-95.

MicroLog system user guide, (2001) A powerful Automated system for the rapid identification of bacteria, yeasts and filamentous fungi. Hayward, USA.

Minelli, E. , Benini, A., Marzotto, M., Sbarbati, A., Ruzzenente, O., Ferrario, R., Hendriks, H., Dellaglio, F., (2004). Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. *International Dairy Journal* **14**, 723-736.

Mombelli, B. and Gismondo, M. R., (2000). The use of probiotics in medical practice. *Int. J. Antimicrob. Agents* **16,** 531-536.

Monnet, V., Condon, S., Cogan, T. M. and Gripon, J. C. (1996) Metabolism of starter culture. In, T. M. Cogan, and J. P. Accolas, (eds.), *Dairy Starter Culture.* New York: VCH publishers, p. 47.

Nation, J. L. (l983). A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technology* **58**, 347-351.

Nelson, G. and George, S. (1995). Comparison of media for selection and enumeration of mouse fecal flora populations. *J. Microbiol. Methods,* **22**, 293-300.

Nelson, G. and George, S. (1995). Comparison of media for selection and enumeration of mouse fecal flora populations. *J. Microbiol. Methods,* **22**, 293-300.

Olsen, K. W. (1989). Fluid bed equipment, In, G. Isaac, P*harmaceutical pelletization Technology*, Dekker New York, p. 39-69.

Olsen, M. A. (1985) Fluid bed agglomerating and coating technology - state of the art. *Int J Pharm Technol Prod Manuf.*, **6**, 18-24.

Patrignani, F., Lanciotti, R., Mathara, R. L., Guerzoni, E. and Holzapfel, W., (2006). Potential of functional strains, isolated from traditional Maasai milk, as starters for the production of fermented milks. *International Journal of Food Microbiology.* **107**, 1-11.

Pharmaceutical Coatings Bulletin (1995). Influence of plasticizers on the dissolution and physical properties of ethyl cellulose films and coated beads, Morflex 102-103.

Pharmaceutical Coatings Bulletin (1996). Influence of triethyl citrate on the properties of tablets containing coated pellets, Morflex 102-104.

Piuri, M., Sanchez-Rivas, C. and Ruzal, S. M. (2005). Cell wall modifications during osmotic stress in *Lactobacillus casei. Journal of Applied Microbiology*, **98,** 84-95.

Planas, M., Vázquez, J. A., Marquē, R., Pērez-Lomba, M. P. and Murado G. M., (2004) Enhancement of rotifer (Brachionus plicatilis) growth by using terrestrial lactic acid bacteria. *Aquaculture*, **240**, 313-329.

Prescott, L, Harley, J. and Klein, D. (1999). Microbiology. 4th ed. Wm. C. Brown Publishers, Dubuque, Iowa, USA.

Pro-Lab diagnostics, delivering to day research for tomorrow (2004) [on line] [accessed on 10 April 2004] Available from world wide web http://www.pro-lab.com/products-microbank.php.

Ray, B. (2001). Fundamental food microbiology, 2nd ed. CRC press, USA.

Robinson, R. K. (1990). *Dairy Microbiology* Volume (1) The microbiology of milk. 2nd ed. Elsevier science publishing Co .INC. USA.

Rogosa, M., Mitchel, J. A., Wiseman, R. F. (1951). A selective medium, for the isolation of oral and faecal *Lactobacilli. J. Bacteriol.*, **62**, 132-133.

Saarela, M., Mogensen, M., Fondén, R., Mättö, J., and Mattila-Sandholm, T., (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology*, **84**,197-215.

Salminen, S., Bouley, C., Boutron-Ruault, M., Cummings, J., Franck, A., Gibson, G. Isolauri, E., Moreau, M., Roberfroid, and Rowland, I. (1998). Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition,* **80**, 147-171.

Sandine, W. E. (1996) Commericial production of dairy starters cultures. In, T. M. Cogan and J. P. Accolas, (eds.), *Dairy Starter Culture*. VCH publishers, New York, USA, p. 191.

Schillinger, U. (1999). Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. *International Journal of Food Microbiology,* **47**, 79-87.

Scragg, A. H. (1991). *Bioreactors in Biotechnology: A Practical Approach*. Ellis Hor Wood Limited, UK, p. 26-35.

Selma, M., Salmerón, M., Valero, M. and Fernández, P. (2004) Control of *Lactobacillus plantarum* and *Escherichia coli* by pulsed electric fields in MRS Broth, Nutrient Broth and orange–carrot juice. *Food Microbiology,* **21**, 519-525.

Shioya, S., Morikawa, M., Kajihara, Y., and Shimizu, H. (1999). Optimization of agitation and aeration conditions for maximum virginiamycin production. *Applied Microbiology and Biotechnology*, **51**, 164-169.

Silvi, S., Verdenelli, M. C., Orpianesi, C., and Cresci, A. (2003) Isolation and identification of *Lactobacillus* and *Bifidobacterium* strains from faecal samples of elderly subjects for a possible probiotic use in functional foods. *Journal of Food Engineering,* **56,** 195-200.

Soestbergen, A. A. and Ching, H. L. (1969). Pour plates or streak plates? Applied Microbiology, **18**, 1092-1093.

Speck, M. L. (1978). Enumeration of viable *L. acidophilus* organisms in dairy products. *J. Food Prot.* **41**, I35 -137.

Stahouder, J., Jansen J. A. and Hap, G. (1969) preservation of starters and mass production of starter bacteria. *Netherlands milk and dairy journal*, **23**,182-199.

Stile, M. and Holzapfel, W. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.*, **36**, 1-29.

Taillandier, P., Gilis, F., Portugal, F., Laforce, P. and Strehaiano, P. (1996). Influence of medium composition, pH and temperature on the growth and viability of *Lactobacillus acidophilus. Biotechnology Letters*, **18**, 775 -780.

Tamang, J. P. and Sarkar, P. K. (1996). Microbiology of mesu, a traditional fermented bamboo shoot product. *Int. J. of Food Microbiology*, **29**, 49-58.

The United States Pharmacopoeia, USP 26. Pharmacopoeial Convention, (2003), Asian edition , Webcom limited, Canada

Thoesen, J. C. (1994). *Suggested procedures for the detection and identification of certain finfish and shellfish pathogens.* 4th ed., Version 1, fish Health Section, American Fisheries Society.

Tüske, Z., Regdon, G. J, Erős, I, Srčič, S and Pintye-Hódi, K. (2005). The role of the surface free energy in the selection of a suitable excipient in the course of a wet-granulation method. *Powder Technology*, **155**, 139-144.

Vamanu, A., Câmpeanu, G., Popa, O., Vamanu, E., Steluța, G., Lucia, D., and Doina, D., (2001). Studies on obtaining a probiotic product based on mixed microbian biomass. *Roum. Biotechnol. Lett.* **7**, 569-572.

Venkatadri, R., and Irvine, R. L. (1990). Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium. Appl. Envir. Microbiol.*, **56**, 2684-2691.

Walker, D. K., and Gilliland, S. E. (1993). Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *Lactobacillus acidophilus. J. Dairy Sci.*, **76**, 956-961.

Wenge, F. and Mathews, A. P. (1999). Lactic acid production from lactose by *Lactobacillus plantarum*: kinetic model and effects of pH, substrate, and oxygen. *Biochemical Engineering Journal*, **3**, 163-170.

Zisu, B. and Shah, N. P. (2003) Effects of pH, Temperature, Supplementation with Whey Protein Concentrate, and Adjunct Cultures on the Production of Exopolysaccharides by *Streptococcus thermophilus* 1275. *J. Dairy Sci.* **86**, 3405-3415.