CHAPTER 3

MATERIALS AND METHODS

3.0 Materials and Methods

3.1 Sources for Isolating Indigenous Lactic Acid Bacteria

Sources from which indigenous lactic acid bacteria were isolated were centred on local fermented food items such as 'Tapai' (fermented tapioca) and 'Tempoyak' (fermented durian) and other local food such as 'chili boh' (blended chili) and fresh goat's milk. All of the items were obtained from local market.

3.2 Isolation Through Enrichment

The medium used in the enrichment process was MRS broth (Appendix A.1). In the isolation procedure, 10 grams of either tapai, tempoyak or chili boh were blended with 100ml 0.85% NaCl saline. 10ml of the blended samples were inoculated into 100ml MRS broth in a 250ml Erlenmeyer flask. The flask was shaken (Hotech 718 Orbital shaker) at 100rpm at 37°C for 7 days. For samples from tempoyak and tapai, various sugars were tested individually as carbon source in the enrichment process. Sugars that had been tested were mannitol, sorbitol, lactose, sucrose and glucose at 2% concentration. For sample from chili boh, only glucose had been used as a substrate. For fresh goat's milk, no isolating medium was used. Instead, 100ml of the milk was fermented in a sterile 250ml Erlenmeyer flask and shaken (Hotech 718 Orbital Shaker) at 100rpm at the temperature of 45°C for 7 days. After 7 days, the cultures from each of the sources were subjected to a series of serial dilution up to 10^6. 0.1ml of the 10^6 dilution was applied on a MRS agar plate (Appendix A.2) and was spread evenly with a sterile spreader. The MRS agar plates were incubated

for 3 days and at temperature of 37°C. Colonies that developed on the plates were purified by repeated dilution streaking on fresh MRS plates to obtain single colonies. Isolates were maintained on MRS slants and in 20% glycerol; the latter stored at –20°C.

3.3 Screening for Lactic Acid Bacteria

3.3.1 Catalase Production

Individual colonies from each isolate were chosen at random and were sub-cultured on MRS agar plates. A single colony was streaked on a glass slide and a drop of 3% hydrogen peroxide was applied on the cells. The droplet was observed for bubble formation.

3.3.2 Gram's Staining

The isolates were subjected to Gram's staining (Appendix B.1). A thin smear of bacteria was made on a glass slide. The smear was fixed onto the slide by passing it over a flame. Crystal violet solution was placed onto the slide for 20s. The slide was gently rinse off with tap water. Gram's iodine solution was applied onto the smear for 20s. The slide was decolourised quickly in solution of equal parts of acetone and 95% ethanol. Again, the slide was washed gently with tap water. The slide was counter-stained with safranine for 10s. Lastly, the slide was washed with tap water and blotted dry. The slide was observed under light microscope (Olympus BH-2). The shape of the cells was also noted.

3.3.3 Gel Plug Test

To identify the homofermentative isolates, the gel plug test described by Gibson and Ab-del Malek (1945) was employed. In this procedure, 10ml of nutrient gelatine with 5% glucose concentration (Appendix A.3) was distributed into test tube and autoclaved (Tomy) for 15 minutes at 121°C. Glucose was added aseptically to test tubes. A 24-hours MRS broth culture was used as the inoculum. The cells were centrifuged at 300rpm for 10 minutes. The pellet was washed twice with 0.85% NaCl saline and finally suspended in 5ml of 0.85% NaCl saline. 1 ml of this suspension was used as an inoculum. After the test tubes were inoculated, sterile agar (appendix A.4) was poured into test tubes creating air tight gel plug above the culture. The test tubes were incubated for 7 days in a waterbath (Memmert) set at 30°C.

3.4 Identification of Lactic Acid Isomers Produced by the Isolates

3.4.1 Recovery and Purification of Lactic Acid

Each lactic acid bacteria isolate was cultured in 100ml basal MRS medium contained in 250ml Erlenmeyer flask and shaken at 100rpm at temperature of 37°C for 48 hours. The cells were separated by centrifugation (Beckman J2-MI) at 4000rpm and temperature of 4°C and the supernatant was filtered, using 0.22μm filter (Millipore). The method used to recover and purify lactic acid from fermentation broth was described by Vaccari *et al.* (1993), with several modifications. First the filtered broth was subjected to a strong anion exchange column (Amberlite IRA-400, Fluka Chemika AG) in carbonate

form. The broth was percolated in a downward flow at a rate of 0.4ml/min. The column was rinsed by passing two volumes of 35ml ultra pure water. This to remove compounds which were not adsorbed to the column. Ammonium carbonate was then passed down the column. Lactic acid was recovered as ammonium lactate in the eluate whilst the resin was at the same time regenerated to the carbonate form. After washing with water, the column was ready to be used again. After 15 cycles, the resin was purged of built up impurities by first passing 1M NaOH and then recharged back to carbonate form with 1M sodium carbonate.

The eluate containing ammonium lactate was passed through a strong cation exchange resin in hydrogen form (Amberlite IR-120(H), BDH Chemicals) at the rate of 0.7 ml/min. The resultant eluate from the treatment was concentrated by vacuum evaporation under 1 atm pressure. The cation resin was later regenerated by using 5% HCl. The recovery and purification of lactic acid from the fermentation broth were carried out in a cold chamber set at 10°C (figure 3.1).

3.4.2 The Identification of Lactic Acid Isomer(s) by Thin Layer Chromatography

The identification of lactic acid isomer(s) produced by the isolates was performed using thin layer chromatography (TLC) method described by Cecchi and Malaspina (1991). Glass based thin layer plates 10 cm by 20 cm coated with silica gel (Silica gel 60, 0.25mm,Merck) were used. The plates were dipped in 160ml of a 1% aqueous solution of copper (II) acetate monohydrate (Fluka Chemika AG) for 10 minutes and dried for 24hours at room temperature using electric fan and kept dry in a dessicator. Before using, the TLC

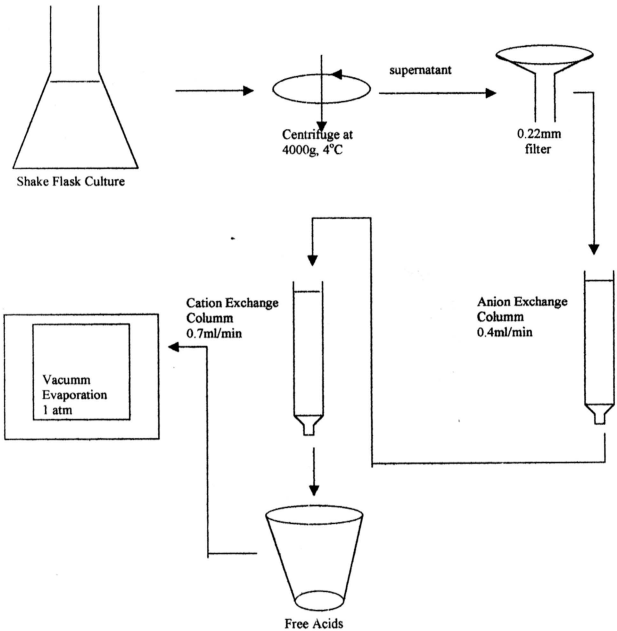

Figure 3.1: Shows the schematic diagram of the process of recovery and purification of lactic acid from the fermentation broth

plates were activated by drying with hair dryer for 20 minutes. The lactic acid samples and standards, D and L lactic acid (Fluka Chemika AG) were dissolved in 500µl acetone and applied as 3mm spot on the plate, which was then placed in a glass development tank. Separations were carried out with an aqueous solution of dioxane (Fluka Chemika AG)(ratio of dioxane/H_2O:9/1) as the mobile phase, and the process took approximately 40 minutes. The lactic acid isomers appeared as dark blue spots, and their positions were marked with pencil. The R_f of the spots were calculated and compared with the R_f of standards. R_f of the standards were calculated as follows:

R_f = Distance traveled by the spots/ Distance traveled by the solvent front.

3.5 Identification of the Bacterial Isolates

Seven of the homofermentative/ facultative heterofermentative isolates were chosen on the basis of the source of isolation. The isolates were identified by using API 50CHL identification kit (Biomerioux). The isolates were cultured on MRS agar plates at 37°C for 3 days. The single cell colonies on the agar plates were used according to the manufacturer's instruction. The API carbohydrate strips was inoculated and incubated for 48 hours at 37°C. The fermentation profiles of the isolates were noted and processed using the software provided by the manufacturer.

3.6 Physiological Characterisation of the Isolates.

Each of the lactic acid bacteria isolates was subjected to several tests to determine some of their physiological characteristics. All the tests were performed in universal bottles using basal MRS broth with bromocresol purple as a pH indicator. The pH of the broth was adjusted to 7 using 1M NaOH. As the production of lactic acid is growth related under normal conditions, therefore cell growth and acid production was determined by colour changes in the cultures. The colour changed from purple to yellow, indicating the change of pH caused by the production of acids that are formed during metabolic activities. Inocula for the test were prepared from 18 hours actively growing cultures that were centrifuged (Jouan C312) and the cells suspended in 0.85% (w/v) NaCl saline.

3.6.1 Various Temperature Conditions

In this procedure, 20ml of basal MRS broth with bromocresol purple in universal bottles were used. The carbon source used was 2% (w/v) glucose and pH of the medium was adjusted to 7. The basal MRS broth with bromocresol purple and glucose were autoclaved separately at 121°C for 15 minutes. 50µl of cell suspension in saline was inoculated into the bottles. The bottles were incubated in a shaking water bath (Techne) for 48 hours at various temperatures i.e. 15°C, 30°C, 37°C 45°C and 50°C. The colour changes of the broth were noted and the pH was taken. Each treatment was tested in triplicates.

3.6.2 Lactic Acid Tolerance

20ml of basal MRS broth with bromocresol purple and with various concentrations of DL-lactic acid (BDH Chemicals Ltd.) were used in this test. The pH of the broth was adjusted to 7 with 4N NaOH. The carbon source used was 2% (w/v) glucose and 50µl of cell suspension in saline was inoculated into the bottles. The various concentrations of lactic acid tested were (w/v) 2.5%, 5%, 7.5%, 10% and 15%. The bottles were incubated in a shaking water bath (Techne) with the temperature set at 37°C for 48hours. The colour changes of the broth were noted and the pH was taken. Each treatment was tested in triplicates.

3.6.3 Various NaCl Concentrations

Basal MRS broth with bromocresol purple and with various NaCl (BDH Chemical Ltd.) concentrations was used with pH adjusted to pH7. 2% (w/v) of glucose was used as carbon source. 50µl of cultures were inoculated into the bottles The various NaCl concentrations tested were (w/v) 1.5%, 2.5%, 5%, 7.5% and 10%. The bottles were incubated in a shaking water bath (Techne) with the temperature set at 37°C for 48hours. The colour changes of the broth were noted and the pH was taken. Each treatment was tested in triplicates.

3.6.4 Various pH Conditions

Basal MRS broth with different initial pH conditions were used. The various initial pH tested were pH 4.5, 7 and 9. 1M phosphoric acid and 1M NaOH were used to adjust the pH to each of the conditions. The bottles were incubated in a shaking water bath (Techne) for 48 hours at 37°C. Turbidity of the cultures was compared with the controls and the pH value was noted. Each treatment was tested in triplicates.

3.7 Biomass, End Point pH Values, Glucose Consumption and Lactic Acid Production Profiles of Selected Isolates Grown in a Range of Environmental Conditions

In this set of experiment, the component of the broth, the volume and the concentration of glucose remain the same. 20ml of basal MRS broth with 2% (w/v) glucose was used. Several parameters of growth conditions were studied. The parameters studied were temperature, NaCl concentration and initial pH. The media used in this set of experiment was basal MRS media with 2%(w/v) glucose concentration. The isolates were cultured overnight in 100ml basal MRS broth with 2% (w/v) glucose concentration. The cells were harvested using centrifugation and washed twice with 0.85% (w/v) saline and resuspended in distilled water 50µl of suspended cells was inoculated in the universal bottles containing 20ml of medium. Three isolates studied were TapLac, TapSuc and FM.

3.7.1 Effects of Various Temperature Conditions

Various temperatures tested for this procedure were set at 15°C, 30°C, 37°C 45°C and 50°C. The initial pH was set at pH7. The bottles were incubated in a shaking water bath (Techne) for 48hours. 10 ml of the broth was taken for cell dry weight and pH determination. 1ml was taken for glucose determination, and 1 ml for lactic acid determination. Each treatment was tested in triplicates.

3.7.2 Effects of Various Initial pH Conditions

The various initial pH tested were at pH 4.5, 7 and 9. 1M phosphoric acid and 1M NaOH were used to adjust the pH to each of the condition. The bottles were incubated in a shaking water bath (Techne) with the temperature set at 37°C for 48 hours. 10 ml of the broth was taken for cell dry weight and pH determination. 1ml was taken for glucose determination, and 1 ml for lactic acid determination. Each treatment was tested in triplicates.

3.7.3 Effects of Various NaCl concentrations

Basal MRS broth with various NaCl (BDH Chemical Ltd.) concentrations was used with pH adjusted to pH 7. The various NaCl concentrations were set (w/v) 1.5%, 2.5%, 5%, 7.5% and 10%. The bottles were incubated in a shaking water bath (Techne) with the temperature set at 37°C for 48 hours. 10 ml of the broth was taken for cell dry weight and

pH determination. 1ml was taken for glucose determination, and 1 ml for lactic acid determination. Each treatment was tested in triplicates.

3.8 Analysis of Cultures Cultivated at Various Environmental Conditions

3.8.1 Cell Dry Weight Determination

The biomass of the cultures was measured gravimetrically using cell dry weight. The 10ml sample were centrifuged for 10 minutes at 4000rpm (Jouan C312) and washed twice with 0.85% (w/v) NaCl saline. The biomass was dried on aluminum cup for 2 days at temperature of 80°C in an oven (Memmert) until constant weight was achieved and weighed on an analytical balance (Denver Instruments).

3.8.2 The pH Determination of the Broth

The supernatants from the centrifuged samples were used to measure the pH of the cultures using a pH meter. (Hanna Instruments)

3.8.3 Lactic Acid Quantification of the Fermentation Broth

Lactic acid concentration in the fermentation broth was determined using a method modified from Barker and Summerson (1941) and Lawrence (1975). A standard curve was

obtained by using several different concentrations of DL-lactic acid (Sigma). The standard graph was used to determine the lactic acid concentration of test samples. (Appendix C.1) The concentrations used were:

a) 0.29 μg/ml

b) 0.56 μg/ml

c) 1.11 μg/ml

d) 1.67 μg/ml

e) 2.00 μg/ml

3.8.3.1 Materials

Materials that were used in this procedure were:

a) Sodium DL Lactate (Sigma)

b) 4% (w/v) $CuSO_4.5H_2O$ solution (BDH Chemicals)

c) 20% (w/v) $CuSO4.5H_2O$ solution (BDH Chemicals)

d) 25% (w/v) $Ca(OH)_2$ solution (BDH Chemicals)

e) H_2SO_4-$CuSO_4.5H_2O$ (300ml 98%H_2SO_4 (Merck)+ 0.5ml 20% (w/v) $CuSO_4.5H_2O$)

f) 1.5%(w/v) p-hyroxydiphenyl (Sigma) in 0.5% (w/v) NaOH (BDH Chemicals)

3.8.3.2 Methods

2.3ml of distilled water, 0.5ml 20% $CuSO4.5H_2O$, 2ml 25% (w/v) $Ca(OH)_2$ and 0.2ml sample were mixed together in a test tube and were allowed to stand for 10 minutes in room temperature. The mixture was then centrifuged using a centrifuge (P-Selecta

Meditronic) with a swing out rotor for 10 minutes at 2000rpm. 0.2ml of the supernatant and 0.8ml of distilled water were mixed in a new test tube. The test tube was cooled in ice water. 6ml of H_2SO_4-$CuSO_4.5H_2O$ solution was added very slowly and mixed well to avoid localised superheating of the solution which can lead to erratic colorimetric development. The tube was then heated to 100°C in boiling water bath for 5 minutes. After 5 minutes, the tube was allowed to cool to room temperature under running water. 0.1ml of 4% (w/v) $CuSO_4.5H_2O$ and 0.1ml p-hydroxydiphenyl solution were added to the sample and mix well and quickly. The mixture was then cool under running water for 15 minutes. The mixture was then heated to 100°C for 90 seconds and cooled to room temperature under running water. The absorbance of the mixture was measured in glass cuvette at 570nm using a spectrophotometer (Shimadzu UV1601). The sample was diluted tenfold with distilled water if the absorbance was found to be greater than 0.6. Distilled water was used as a blank.

3.8.4 Quantification of Residual Glucose in the Broth

The residual glucose in the fermentation broth was determined using an enzymatic kit, which is Glucose (HK) procedure no. 16-UV, obtained from Sigma Diagnostics.

3.8.4.1 Sample Preparation

1 ml of the culture broth was centrifuged (model 2K15, Sigma Laborzentrifugen GmbH) at 14000g and at 4°C for 10 minutes to separate the cell debris and protein from the

culture broth. The supernatant was diluted tenfold with ultra pure water. The diluted supernatants were assayed for glucose according to the manufacturer's instruction.

In this procedure, glucose was phosphorylated by adenosine triphosphate (ATP) in the reaction catalysed by the enzyme hexokinase. The glucose-6-phosphate (G-6-P) formed was then oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenosine dinucleotide (NAD). This reaction was catalysed by glucose-6-phosphate dehyrogenase. (G-6-PDH). During this oxidation an equimolar amount of NAD was reduced to NADH. The consequent increase in absorbance at 340nm is directly proportional to glucose concentration.

3.8.4.2 Method

1 ml of the reagent was pipetted into a test tube. 0.1ml of sample was added, mixed by gentle inversion and incubated for 5 minutes at 37°C in water bath. For the blank, distilled water was added in place of the sample. The absorbance of the samples was measured using a spectrophotometer (model UV-1601, Shimadzu). A standard curve was obtained by using several different concentrations of glucose as standards plotted against their value of absorbance. The concentrations used were:

1) 0.2 g/100ml
2) 0.1 g/100ml
3) 0.05 g/100ml
4) 0.025 g/100ml

The concentration of residual glucose in the samples was determined from the standard curve (Appendix C.2). The amount of glucose consumed in the cultures was determined by subtracting the mean amount of glucose present in the controls (uninoculated broth) against the residual glucose in culture broths.

3.9 Time Course Study of Selected Isolates Using Shake Flasks.

Shake flasks studies of selected isolates were done using 400ml basal MRS broth in a 1000ml Erlenmeyer flask. The carbon source used was 2% glucose. The basal MRS broth and glucose solutions were autoclaved separately at 121°C for 15 minutes. A 50ml 18hour culture was used as inoculum. The inoculum was centrifuged (Jouan) and washed twice with 0.85% NaCl saline before it was inoculated into the cultures. Cultures were incubated in an incubator shaker (LH Fermentation, U.K) with the temperature set at 37°C and a shaking speed of 100rpm. The cultures were sampled at every 6 hours interval until the 54th hour. For every sampling, 20ml of the cultures were taken. The time course study was done in triplicate for each of the isolate.

3.10 Fermentation Broth Analysis of the Shake Flask Cultures

3.10.1 Cell Dry Weight Determination

10ml of sample were centrifuged. The cells were measured for cell dry weight while the supernatant was measured for pH. Methods used were as in section 3.8.1

3.10.2 pH Determination of the Broth

Methods used were as in section 3.8.2

3.10.3 Quantification of Lactic Acid in the Broth.

A 1ml aliquot of sample was centrifuged at 14000rpm at 4°C. The supernatant was diluted tenfold with ultra pure water. The quantification of lactic acid in the culture was performed using high performance liquid chromatography (HPLC).

3.10.3.1 Apparatus and Operating Conditions

The HPLC equipment consists of Waters 501 pump, tunable absorbance detector (model 486, Waters) set at 214nm, system interface module (SIM), and autosampler (model 717 plus Autosampler, Waters) running on Waters Millenium[32] software. The column used for the analysis was an ion exchange chromatographic column, Resolve 5μm spherical C18 3.9 X 150mm (Waters) maintained at room temperature. Analysis was performed isocratically at a flow rate of 0.5 ml/min.

3.10.3.2 Reagents

The mobile phase used was 0.5% diammonium hydrogen phosphate (BDH Limited) prepared by dissolving 5g of reagent grade diammonium hydrogen phosphate in 1000ml ultra pure water in one litre volumetric flask. The pH of the solution was adjusted to 3 with 60% reagent grade phosphoric acid (Ajax Chemicals). Several concentration of L- lactic

acid (Fluka Chemika AG) were used to obtained a standard curve with 0.25% (v/v) propionic acid (Sigma) as an internal standard. Standard curve was obtained by using several different concentrations of L- lactic acid. The concentrations of lactic acid in the samples were measured by comparing their peak area against the standard curve (Appendix C.3).

Those concentrations used were:

a) 0.03% (w/v)

b) 0.06% (w/v)

c) 0.13% (w/v)

d) 0.25% (w/v)

e) 0.50% (w/v)

3.10.4 Quantification of Residual Glucose in the Broth

The methods used were as in section 3.8.4

3.11 Statistical Analysis

All of the experiments were done in triplicate, the mean values was calculated as:

$\frac{x_1 + x_2 + x_3}{3} = \bar{x}$ and he standard deviation was calculated as

$$sd = \sqrt{\sum_{X=1}^{n} \frac{(x_i - \bar{x})^2}{n - 1}}$$

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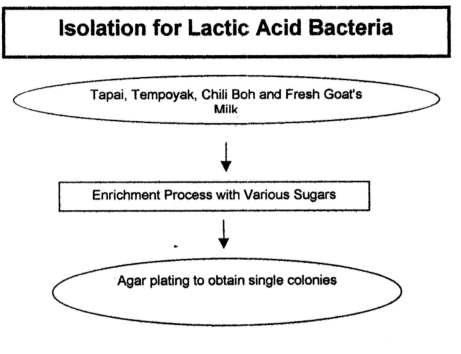
PART I



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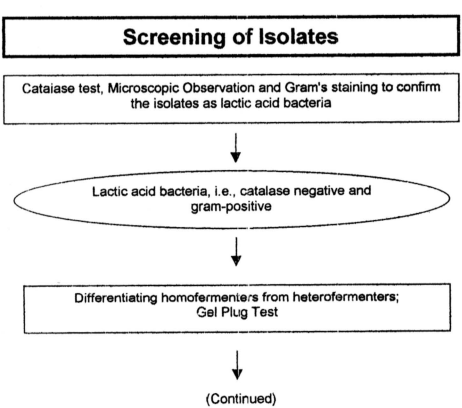

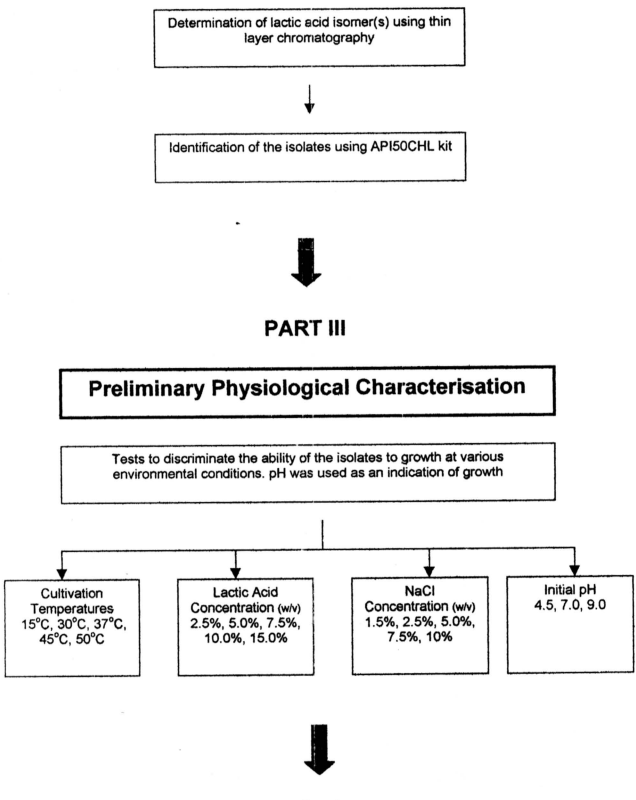
Determination of lactic acid isomer(s) using thin layer chromatography
Identification of the isolates using API50CHL kit
PART III
Preliminary Physiological Characterisation
Tests to discriminate the ability of the isolates to growth at various environmental conditions. pH was used as an indication of growth
Cultivation Temperatures 15°C, 30°C, 37°C, 45°C, 50°C
Lactic Acid Concentration (w/v) 2.5%, 5.0%, 7.5%, 10.0%, 15.0%
NaCl Concentration (w/v) 1.5%, 2.5%, 5.0%, 7.5%, 10%
Initial pH 4.5, 7.0, 9.0
continued

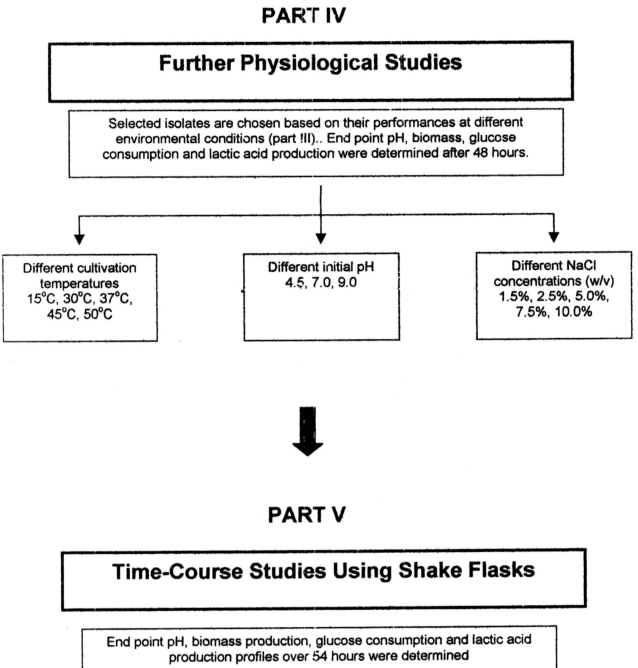

Figure 3.2: A schematic overview of the methods used in isolation, screening and characterisation of lactic acid bacteria isolated from local food sources