CHAPTER 2

Materials and General Methods

2.1. Materials

2.1.1. Microorganisms

The folate producing lactic acid bacteria (LAB) used for the present study was isolated locally and the details are provided in chapter 3. *Lactobacillus casei* NCIM 2364 was used for folate quantification by microbiological assay, *Escherichia coli* DH5α (Invitrogen, CA, USA) was used for transformation related to recombinant DNA work. *Lactococcus lactis* NIZO 9000 was purchased from NIZO food research laboratory (Netherlands). *E. coli* (MTCC 739), *Staphylococcus aureus* (MTCC 96) were used as test organisms for detecting antimicrobial activity of the LAB strains. *Raoultella planticola* (isolate from NIIST) was included as test pathogen for studying coaggregation of the LAB strains.

2.1.2. Plasmid DNA and Primers

The expression vector pNZ8148 was from MoBiTec (Germany). All the oligos used for amplification of 16s rDNA and the desired folate biosynthetic genes by Polymerase Chain Reactions (PCR) were custom synthesized from Integrated DNA Technologies (IDT), USA. The respective genes and oligos are mentioned in the appropriate chapters.

2.1.3. Culture Media, Reagent Kits and Chemicals

Lactobacillus MRS (de Man, Rogosa and Sharpe) agar and broth were used for the isolation of LAB and for the growth of *Lb. casei*, M17 broth for growing *Lactococcus* strains, Luria Bertani (LB) broth and agar for *E. coli* strains, Folic acid casei (FAC) medium, Folic acid assay (FAA) medium and mucin for adhesion studies were procured from Himedia (India). Some specific media used for microbial identification like Differential media for classification of homo-and heterofermentors, Simmons citrate agar for citrate utilization were also from Himedia (India). The respective medium compositions are provided in Annexure I.

TA cloning, plasmid isolation, PCR clean up and gel extraction kits were obtained from Fermentas (USA). Hi Carbo kit for carbohydrate utilization tests was purchased from Himedia (India). All the kits were used as per the protocol described by the manufacturer.

Restriction enzymes (*Nco*1, *Kpn*1, *Hind*III and *Xba*1), T4 DNA ligase and dNTPs were purchased from Fermentas (USA). Taq DNA polymerase for PCR, lysozyme, RNaseA, folic acid standards and human plasma were procured from Sigma-Aldrich, India. Antibiotics like ampicillin and chloramphenicol were purchased from Himedia (India). All other molecular biology grade chemicals were from Sigma-Aldrich (India).

2.1.4. Cell Lines

HT-29 cell line was procured from ATCC and Caco-2 cell line was from Riken Culture Collection Center (RIKEN BioResource Center, Ibaraki, Japan). The details of propagation and maintenance of the cell lines are provided in chapter 3.

2.2. General Methodology

2.2.1. Microbiological Methods

a. Gram Staining

Gram staining is a differential staining procedure developed by Hans Christian Gram in 1884 that helps to characterize bacteria as gram positive or gram negative.

Principle

The staining technique is based on the difference in cell wall composition of different bacteria. Gram positive bacteria have thick layer of protein-sugar complexes and relatively low lipid content and are able to retain the crystal violet-iodine complex during staining and appears purple in colour. Whereas, gram negative bacteria have thin layer of peptidoglycan and high lipid content which allows the crystal violet-iodine complex to leach out of the cells during decolourization and hence take up the counter stain safranin and appears red.

Reagents

1. Crystal violet
2. Gram’s iodine
3. Alcohol (95% v/v)
4. Safranin

Protocol

i. Prepared a thin smear on a clean grease free slide with a loopful of sample
ii. Flooded the air-dried, heat-fixed smear of cells for 1 min with crystal violet staining reagent
iii. Rinsed off the crystal violet with distilled or tap water
iv. Flooded the slide with mordant (Gram's iodine) for 1 min and washed with distilled or tap water
v. Flooded the slide with decolourizer (95% alcohol) for 10-15 s
vi. Rinsed off the decolourizer with distilled or tap water
vii. Added safranin and allowed to remain for about 30 s and washed with distilled or tap water
viii. Blot dried with absorbent paper and observed under the microscope

b. Catalase Test

Principle

This test demonstrates the presence of catalase, an enzyme that mediates the breakdown of hydrogen peroxide into oxygen and water. This can be used to differentiate bacteria that produce catalase from non catalase producers.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2 + O_2$$

Procedure

A small amount of culture was transferred on a clean, dry glass slide. Added a few drops of H_2O_2 onto the smear. A positive result is indicated by the rapid evolution of oxygen as evidenced by air bubbles whereas no bubbling indicated a negative result.

c. Differentiation of Homo- and Heterofermentative LAB

Lactic bacteria differential broth formulated as per McDonald et al. (1987) was used for differentiation of homofermentative and heterofermentative LAB. Medium constituents like casein acid hydrolysates, papaic digest of soyabean meal and yeast extract supply all the necessary nutrients for the growth of LAB. Fructose is the fermentable carbohydrate in the medium and bromocresol green is the pH indicator. Heterofermentative LAB produce CO_2, lactic acid, acetic acid, ethanol, and mannitol from hexoses, and the homofermentative LAB produce primarily lactic acid from hexose. The

LAB isolates were inoculated into Lactic Bacteria Differential Broth (5 mL) and incubated at 37 ºC for 24 h. Homofermentative LAB was indicated by a green colour formation. Heterofermentative LAB induced lesser acidification and thus varied in the colour formation by the indicator in the medium.

d. Citrate Utilization Test

This test detects the capability of the organism to utilize citrate as the sole carbon source. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced resulting in an increase in pH which is indicated by a colour change. The LAB isolates were individually streaked into Simmons citrate agar slants and incubated for 24 h at 37 ºC. A positive test is indicated by a colour change from green to blue and in negative test the colour remained green (Simmons, 1926).

e. Carbohydrate Utilization by the Selected Isolates

The carbohydrate utilization by the selected isolates was checked using HiCarbo Kit. It is a standardized colorimetric identification of carbohydrate utilization tests based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes which are indicated by a spontaneous colour change in the media. Overnight cultures were washed in saline and 50 µL was inoculated into each well of the kit under aseptic conditions and incubated at 37 ºC for 24 h. The results were interpreted by the colour change.

f. Culture Maintenance

The newly isolated *Lactococcus* strains as well as *L. lactis* NIZO 9000 were propagated in M17 medium supplemented with 0.5% (w/v) glucose. Other LAB isolates were propagated in MRS medium. The fully grown plates were stored at 4 ºC. The strains were sub cultured once in every three weeks. *Lb. casei* NCIM 2364 was grown in MRS medium and sub cultured every two weeks. *E. coli* DH5α was maintained in LB medium. For long time storage of cultures glycerol stocks (40% v/v) were made and stored at -80 ºC.

2.2.2. Analytical Methods

a. Folate Analysis by Microbiological Assay

Folate analysis was carried out using a folate auxotroph *Lb. casei* as the indicator organism (Horne, 1997; Horne & Patterson, 1988; Wilson & Horne, 1982). The growth of *Lb. casei* will be proportional to the folate concentration in the medium.

Preparation of folate standard

i. 5 mM solution of folinic acid was prepared [(6RS)-5-formyl tetrahydrofolate] i.e., 3 mg/mL in 0.1M potassium phosphate buffer pH 7.0.

ii. To estimate actual concentration, the solution was diluted to 500 times using 0.1M potassium phosphate buffer pH 7.0. OD of this solution at 285nm was taken.

Therefore actual concentration of 3 mg/mL = $\frac{\text{OD} \times \text{dilution factor}}{\text{Molar extinction coefficient of folinic acid}}$

The solution was then diluted to 2 mM with sterile water ($M_1V_1=M_2V_2$). This stock solution was stored at -20 °C (2 mM is approximately 1 mM because bacteria can use only the S-isomers)

iii. The working standard was prepared by further dilution to 1.5 nM, filter-sterilized and used for assay

Preparation of inoculum of indicator organism

i. *Lb. casei* NCIM 2364 was streaked in MRS agar plate supplemented with 1% (w/v) $CaCO_3$

ii. Single colony from the plate was used to inoculate FAC medium (5 mL), incubated at 37°C for 18 h. This step allows removal of residual folate stored by the cell

iii. 1% (v/v) of the above culture was inoculated in FAC medium (20 mL) supplemented with 30 pg/mL calcium folinate, incubated at 37 ºC for 18 h.

iv. 1% (v/v) of the above culture was inoculated into two tubes (5 mL FAC medium), one containing 60 pg/mL folate (positive control) and one without folate (negative control). If growth was positive in positive-control and negative in negative-control, glycerol stocks of the 20 mL culture which was unable to grow in FAC medium in absence of external folate were made (equal volumes of culture and 80% (v/v) glycerol).

All the steps were shown in a flow sheet (**Fig. 2.1**).

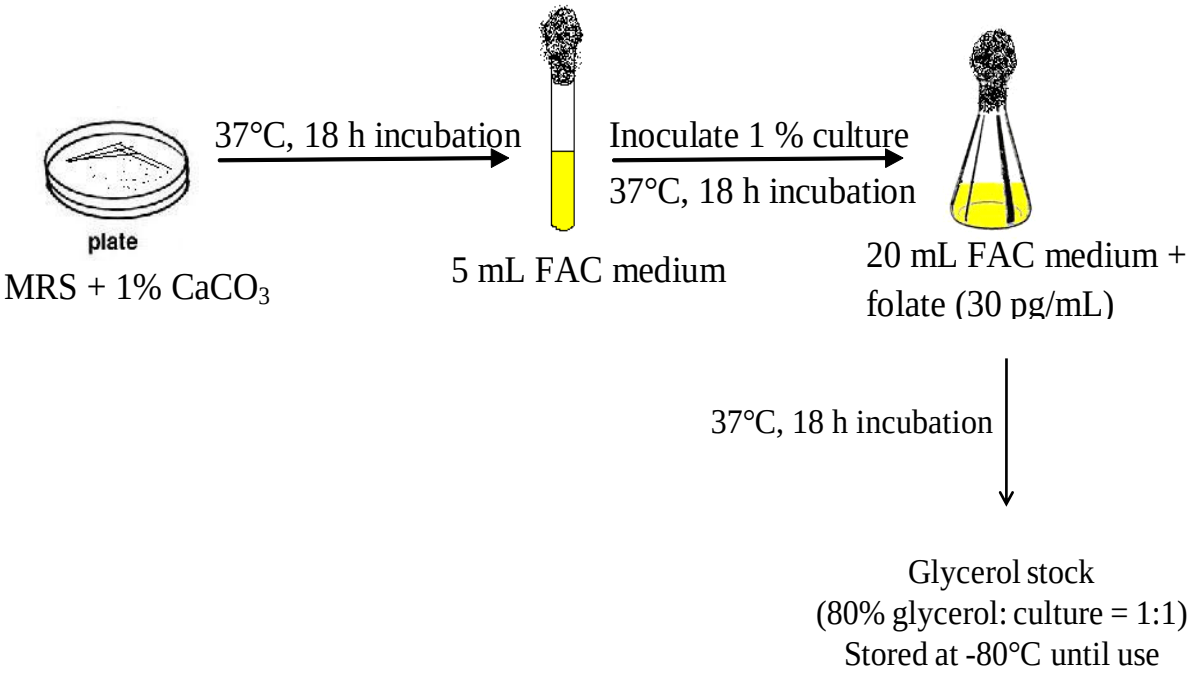

Fig. 2.1. Preparation of inoculum for microbiological assay

Preparation of folate test sample for quantification

To measure intra- and extracellular folate, the culture was centrifuged (13000 x g, 10 min, 4 ºC) and the cells and supernatant were collected. For extracting extracellular folate the supernatant was diluted with extraction buffer (0.1M phosphate buffer, pH 6.1 with 0.5% (w/v) sodium ascorbate) boiled for 15 min, centrifuged, filtered (0.22 µ filter) and stored at -20 ºC until use. For intracellular folate the cell pellet was washed with extraction buffer and resuspended in the same. This was followed by the addition of 20 µL lysozyme (50 mg/mL stock) and kept in ice for 1 h. The samples were then sonicated (Sonics, USA, amplitude 47% and 30 S ON and OFF cycle for 5min on ice) and boiled for 5 min and centrifuged (13000 x g, 10 min, 4 ºC). Supernatant was filtered and stored at -20 ºC until use. In addition to maximum folate release, heating inactivates the folate producing bacteria thereby preventing it from interfering with the microbiological assay.

Microbiological assay gives nearly equal responses to mono- di- and triglutamyl folate, while the response to longer chain polyglutamyl folate decreases markedly in proportion to chain length. Hence, the total folate can be determined only after deconjugation of the polyglutamyl chains. The samples were enzymatically deconjugated using human plasma, as a source of γ-glutamyl hydrolase, at 37 ºC and pH 4.8 for 4 h. To 1g human plasma, 0.1 M 2-mercaptoethanol (5 mL) with 0.5% (w/v) sodium ascorbate

(Sigma-Aldrich) was added and clarified the supernatant by centrifugation. To 20 µL sample, 1 µL of the above enzyme preparation was added and incubated at 37 ºC for 4 h in dark (Sybesma et al., 2003b). Samples were diluted and 10 µL was used for the assay

Reagents

Working Buffer: The composition is as follows

1M Potassium phosphate buffer, pH 6.1 : 0.5 mL

Sodium ascorbate : 1.6 g

Milli Q water : 9.5 mL

Solution was sterilized by filtration (0.22µ filter)

Protocol

Folate standards with concentration ranging from 50 pM – 300 pM were used for standardization of the assay. The reaction mixture consisted of working buffer, folate standards of different concentration, double strength FAC medium, sterile water and the *Lb. casei* inoculum (**Table 2.1)**.

Table 2.1. Reaction mixture for folate detection by microbiological assay

SI No	Reagents	Volume (µL)
1	Working buffer	8
2	Folate standard solution	0-60
3	Sterile water	122-62
4	FAC medium (2X)	150
5	*Lb. casei* inoculum	20
	Total volume	300

After the addition of all the reagents and inoculum the plates (96 well) were incubated at 37 ºC for 18 h and OD was read at 600 nm using microtitre plate reader (Tecan-NanoQuant, Infinite® 200 PRO series, Switzerland). The absorbance measured was plotted on Y axis and the folate concentration on X axis (**Fig. 2.2**).

$$\text{Conc. of folate (ng/mL)} = \frac{\text{Molarity of folate x Molecular weight of folate x Volume of reaction mixture x Dilution factor}}{1000 \text{ x } 1000 \text{ x Volume of extract}}$$

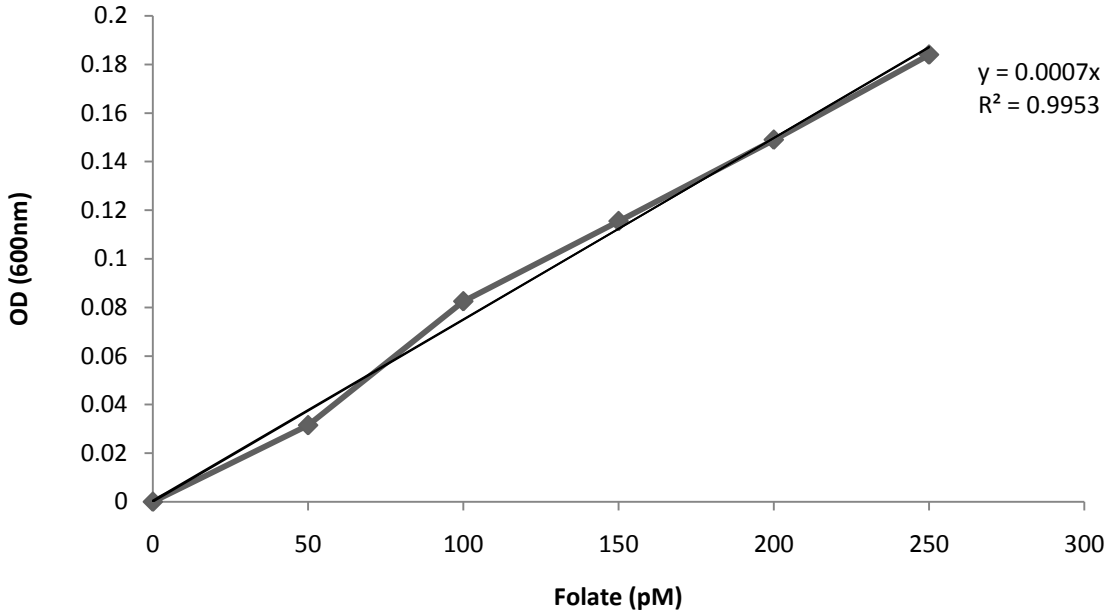

Fig. 2.2. Standard graph for folate detection by microbiological assay

b. Estimation of Lactic Acid

Lactic acid production by the selected LAB isolates in MRS medium is determined by colorimetric method of Barker and Summerson (1941).

Principle

Lactic acid is converted into acetaldehyde by heating with conc. H_2SO_4 in presence of copper. The acetaldehyde formed then reacts with p-hydroxy phenyl to yield a coloured complex which is read at 500 nm.

Reagents

1. $CuSO_4.5\ H_2O$ (4% w/v)
2. Conc. H_2SO_4
3. p-hydroxy phenyl reagent: The reagent was prepared by dissolving 1.5 g of p-hydroxy phenyl in 10 mL of 5% (w/v) NaOH solution by warming and stirring. It was then diluted to 100 mL with distilled water and stored in coloured bottle in a cool place.

Lactate standard

Lithium lactate (0.213 g) was dissolved in about 100 mL distilled water. To this 1 mL concentrated H_2SO_4 was added and the solution is further diluted to 1 L and mixed thoroughly. Every 5 mL of this contained 1 mg lactic acid. For preparing working

standard the stock solution was diluted to achieve a final concentration of 10 µg lactic acid/mL.

Procedure

i. Appropriate dilutions of the samples were prepared
ii. 1 mL of the diluted sample was transferred to test tubes
iii. Added 50 µL of 4% (w/v) $CuSO_4$ solution
iv. Added 5 mL conc. H_2SO_4
v. Kept in boiling water bath for 10 min and cooled the tubes
vi. Added 100 µL of p-hydroxy phenyl reagent while vortexing
vii. Incubated at room temperature for 30 min
viii. OD was read at 500 nm
ix. The absorbance was plotted on Y axis against the concentration taken on X axis **(Fig. 2.3)**

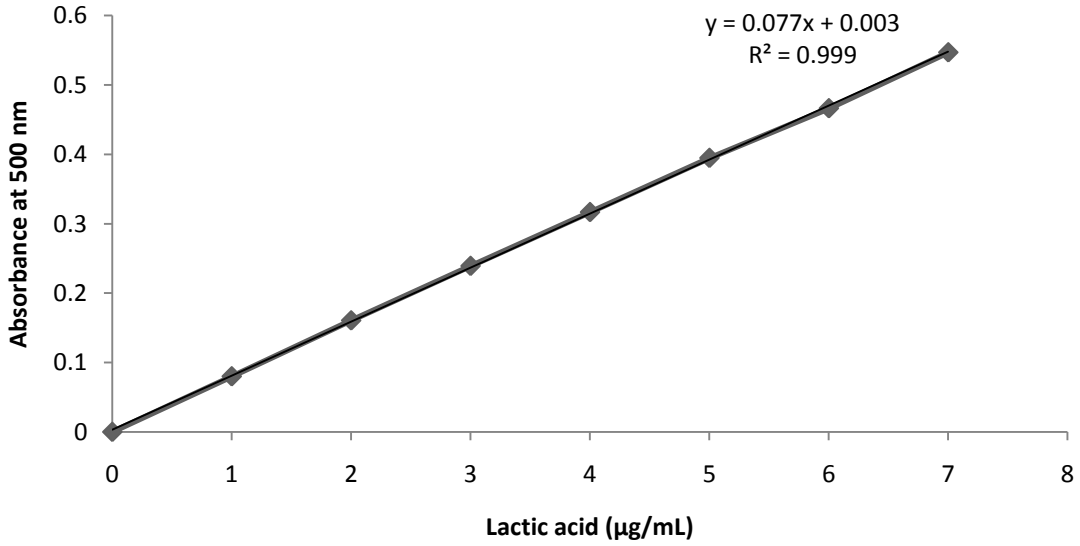

Fig. 2.3. Standard graph for lactic acid standard detection

c. Determination of Titratable Acidity

Titratable acidity (TA) provides an estimate of total acid content of a food. TA is determined by neutralizing the acid present in the food with a standard base and the end point is usually a target pH or a colour change of pH sensitive dye typically phenolphthalein. It is calculated using the volume of titrant, the normality of the base and the volume (weight) of the sample and is expressed in terms of the predominant organic

acid. The equivalent weights of common food acids are similar and hence TA is not substantially affected by mixed predominance of acids (Sadler **&** Murphy, 2010).

Procedure

Transferred 9 mL fermented sample to a small beaker. Added 1-2 drops of phenolphthalein indicator. The sample was titrated with 0.1 N NaOH to a pink endpoint (pH 8.2) and recorded the volume of titrant used (Friedrich, 2001). The percentage of lactic acid produced in the sample was determined from the following equation

$$\% \text{ acid} = \frac{(\text{NaOH used in mL} \times \text{Normality of NaOH} \times \text{Milliequivalent factor} \times 100)}{\text{Volume of sample (mL)}}$$

2.2.3. Molecular Biology Methods

a. Isolation of Genomic DNA

Genomic DNA isolation from LAB isolates was carried out as per the protocol described by Savadogo et al. (2004) with slight changes.

Principle

Initially the cell pellet was washed with TES buffer to remove medium components and cell exudates which otherwise could interfere with subsequent reactions and DNA yield. STET buffer, lysis buffer and SDS (sodium dodecyl sulphate) were used to disrupt the cell membrane and extraction of DNA. DNA was protected from endogenous nucleases by chelating Mg++ ions using EDTA. Phenol and chloroform were used to denature and separate proteins from DNA. Chloroform was also involved in the stabilization of the rather unstable boundary between an aqueous phase and pure phenol layer. Isoamyl alcohol acts as an antifoaming agent. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by ice cold ethanol.

Reagents

1. TES buffer: 6.7% (w/v) sucrose, 50 mM Tris HCl at pH 8.0 and 1mM EDTA
2. STET buffer: 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Tris HCl at pH 8.0
3. EDTA (50 mM)
4. Lysis buffer: TES containing 50 mg/mL lysozyme

5. 20% (w/v) SDS in TE buffer
6. Phenol/ chloroform/ isoamyl alcohol (49:49:1)
7. 70% (v/v) ethanol
8. TE buffer

Procedure

18 h old culture (mid-log phase) grown in MRS medium (10 mL) was taken and cells were collected by centrifugation (3000 x g, 10 min, 4°C). The cells were frozen for at least 1 h at -20°C. Thawed pellets were washed in 1 mL TES buffer. The pellets were then resuspended in 300 µL STET buffer. To this 75 µL of lysis buffer was added and the suspension was incubated at 37°C for 1 h. After addition of 40 µL pretreated (37 °C) 20% (w/v) SDS in TE buffer, cells were vortexed for 60 s and incubated at 37 ºC for 10 min followed by 10 min incubation at 65 ºC. TE buffer (100 µL) was added and the lysate was extracted with 1 volume phenol/ chloroform/ isoamyl alcohol. Aqueous phase was separated after centrifugation (18000 x g, 5 min) and the step was repeated once more. Double volume 70% (v/v) ethanol was added to the aqueous phase and kept at -80 °C for 1 h and centrifuged (18000 x g, 30 min, 4 °C) to obtain the pellet. This was followed by the addition of 200 µL of 70% (v/v) ethanol, centrifuged and obtained the pellet. The pellet was dissolved in 30 µL TE buffer and stored. Genomic DNA was analysed by electrophoresis in 1 % (w/v) agarose gels.

b. Competent Cell Preparation and Transformation

Competent cells of *E. coli* DH5α were prepared according to the modified protocol of Inoue et al. (1990).

Principle

In chemical competent cell preparation the cells were treated with divalent cations such as $CaCl_2$ and $MgCl_2$ which made them more permeable to plasmid DNA. It was suggested that the divalent cations formed coordination complexes with the negatively charged DNA and lipopolysaccharides. The heat shock step strongly depolarized the cell membrane of the $CaCl_2$ treated cells. This decrease in membrane potential lowered the negativity of the cell's inside potential and allowed the movement of negatively charged DNA into the cell's interior. A subsequent cold-shock raised the membrane potential to its original value (Panja et al., 2006).

Reagents

1. $MgCl_2$ (100mM)
2. $CaCl_2$ (100mM)
3. FT Buffer (0.1 M $CaCl_2$ solution in 15% (v/v) glycerol)
4. LB broth and agar with suitable antibiotic

Procedure

Single colony of *E. coli* DH5α from LB plate was inoculated into 5 mL LB broth and incubated overnight (37 °C, 200 rpm). Overnight culture (1% v/v) was inoculated in 50 mL LB broth and incubated (37 °C, 200 rpm) until OD_{600} was 0.6. The culture was chilled on ice for 15 min. All other solutions were also chilled on ice. Culture was then centrifuged at 1500 x g (4 °C) for 5 min (Kubota, Japan). The medium was discarded aseptically and the pellet was resuspended in ice cold 100mM $MgCl_2$ (10 mL) and centrifuged at 1500 x g for 5 min. Resuspended in 100 mM $CaCl_2$ (10 mL) and kept in ice for 40 min and centrifuged at 1500 x g (4 °C) for 5 min. The pellet was resuspended in 1 mL FT buffer and aliquoted into sterile 1.5 mL micro-centrifuge tubes, frozen on dry ice and stored at – 80 °C until use.

For transformation, a vial of competent cells was thawed in ice and incubated with 10-30 ng of plasmid along with the competent cells on ice by gentle tapping and kept in ice for 40 min. Heat shock was applied for 90 s at 42 °C and then kept in ice for 2 min. LB medium (900 µL) was added and incubated in shaking condition at 37 °C for 1 h. The culture was then plated onto LB agar plates with suitable antibiotic and incubated overnight at 37 °C.

$$\text{Transformation efficiency} = \frac{\text{No. of transformants (colonies)} \times \text{Final volume at recovery}}{\mu\text{g of plasmid DNA} \times \text{Volume plated (mL)}}$$

c. Isolation of Plasmid DNA

Plasmid isolation was carried out by alkaline lysis method described by Birnboim and Doly (1979) using the plasmid Mini Preparation Kit (Fermentas) following the manufacture's protocol.

Principle

Pelleted bacterial cells were resuspended and subjected to SDS/alkaline lysis to liberate the plasmid DNA. The resulting lysate was neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column.

Chromosomal DNA, still in a very high molecular weight form, was selectively denatured and when the lysate was neutralized by acidic sodium acetate, the mass of chromosomal DNA renatures and aggregates to form an insoluble network. Cell debris, chromosomal DNA and SDS precipitate are pelleted by centrifugation, and the supernatant containing the plasmid DNA is loaded onto the spin column membrane. The adsorbed DNA was washed to remove contaminants, and is then eluted with a small volume of the elution buffer.

Reagents

1. Resuspension buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RnaseA
2. Lysis buffer: 200 mM NaOH and 1% SDS (w/v)
3. Neutralization buffer: 3.0 M potassium acetate, pH 5.5
4. Wash buffer: 1.0 M NaCl, 50 mM Tris-HCl, pH 7.0, added 96% ethanol (35 mL) prior to first use
5. Elution buffer: 10 mM Tris-Cl, pH 8.0.

Protocol

Overnight culture (1.5 mL) was pelleted. The pellet was resuspended in 250 µL resuspension buffer by vortexing. This was followed by the addition of 250 µL of lysis buffer and mixed by gently inverting the tube 4-6 times until the solution became viscous and slightly clear. To this 350 µL of neutralization buffer was added. This was mixed immediately and thoroughly by inverting the tube 4-6 times and centrifuged for 5 min at 12000 x g to clarify pellet cell debris and chromosomal DNA. The clarified supernatant was transferred to spin column and centrifuged for 1 min. Discarded the flow-through and place the column back into the same collection tube and added 500 µL of the wash buffer into the column and again centrifuged at 12000 x g for 1 min. The washing step was repeated using 500 µL wash buffer. The flow-through was discarded and column was centrifuged for an additional 1 min to remove residual wash buffer. The mini prep column was transferred into a clean 1.5 mL microfuge tube and the plasmid DNA was eluted with 50 µL elution buffer. The purified plasmid was visualized on an agarose gel and quantified at A_{260} using NanoDrop 1000 Spectrophotometer (Thermoscientific, USA). The purified plasmid DNA was then stored at -20°C.

d. Preparation of Electrocompetent L. lactis cells and Electroporation

Principle

The cells to be made electrocompetent were grown in an osmotically stabilized medium with glycine to weaken the cell walls followed by transformation by electroporation. Electroporation technique involves the use of high intensity electric pulse(s) to create transient pores in the cell membrane to facilitate the entry of exogenous molecules like DNA, RNA, protein etc. The removal of the applied electric field results in the resealing of the pores which ensures the survival of the electrically stimulated recipient cells (Prasanna, 1997).

Reagents

1. G/L-SGM17B: M17-Broth with 0.5 M sucrose 2.5% (w/v) glycine 0.5% (w/v) glucose
   Sucrose and glycine were added to the M17-B and sterilized for 15 min at 121 °C. Sterile glucose was added after cooling the medium
2. 0.5 M sucrose/ 10% (v/v) glycerol
3. 0.5 M sucrose/ 10% (v/v) glycerol/ 0.05 M EDTA
4. 2 mM $CaCl_2$
5. 20 mM $MgCl_2$

Protocol

i. Inoculated *L. lactis* NIZO 9000 into 2.5 mL G-SGM17B and incubated at 30 ºC for 24 h
ii. Diluted the 2.5 mL culture in 25 mL G-SGM17B, incubated at 30 ºC for 24h
iii. Diluted the 10 mL culture in 90 mL G-SGM17B
iv. Incubated until OD_{600} reached 0.2 - 0.3
v. Cells were centrifuged for 10 min (2000 x g, 4 ºC)
vi. Washed the cells with 20 mL 0.5 M sucrose, 10% (v/v) glycerol (4 ºC) and spinned down (2000 x g)
vii. Resuspended the cells in 200 mL 0.5 M sucrose, 10% (v/v) glycerol, 50 mM EDTA (4 ºC) and the suspension was kept on ice for 15 min and spinned down
viii. Repeated the washing step
ix. Resuspended the cells in 1 mL 0.5 M sucrose, 10% (v/v) glycerol (4 ºC) and stored as 100 µL aliquots at -20 ºC until use.

Electroporation

Competent cells (100 µL) was added into a prechilled electroporation cuvette with 5 µL DNA and mixed gently by tapping. Then it was electroporated at 2000V, 200Ω and 25 F (Biorad Genepulser) and immediately added 700 µL of ice cold M17 broth supplemented with 0.5% glucose, 2 mM $CaCl_2$ and 20 mM $MgCl_2$. The cuvette was kept on ice for 5 min and incubated at 37 ºC for 1 – 1.5 h. The cells were then plated onto M17 agar with 0.5% glucose and chloramphenicol (10 µg/mL) and incubated at 37 ºC for 2 days.

e. Isolation of Recombinant Plasmid from L. lactis

Plasmid isolation from *L. lactis* was carried out as per the protocol described by Duan et al. (1999) with slight modifications.

Reagents

1. Solution I – 50 mM glucose, 25 mM Tris/HCl, 10 mM EDTA (pH 8.0), to which 20 mg/mL lysozyme and 100 mg/mL RNase were added just before use
2. Solution II – 0.2 M NaOH, 2% (w/v) SDS; prepared just before use
3. Solution III – 1.2 M Tris/HCl (pH 7.0), 2 M NaCl.
4. Phenol, chloroform and isoamyl alcohol were mixed at 25:24:1 (by volume)

Protocol

The culture was grown overnight in M17 broth supplemented with 0.5% (w/v) glucose and 10 µg/L cholramphenicol. The cells (1.5 mL) were harvested by centrifugation (1500 x g for 5 min) and washed with 400 µL ice cold acetone. It was then centrifuged (12000 x g for 10 min) and the pellet was resuspended in 200 µL solution I and incubated at 37 ºC for 20 min. The microfuge tubes were vortexed and 300 µL solution II was added. Mixed carefully by inverting the tubes and incubated at room temperature for 3 min. Then 170 µL of ice cold solution III was added and mixed by intermittent inversion for 3 min. To this 500 µL phenol was added, mixed by inversion and centrifuged (12000 x g, 10 min) and the upper phase was collected and transferred to a fresh microfuge tube. Phenol/chloroform/isoamyl alcohol (600 µL) was added, mixed and centrifuged (12000 x g, 30 min). This was followed by the addition of 600 µL isopropanol to the upper phase. Chilled in ice for 15 min and centrifuged (12000 x g, 30 min). The pellet was rinsed gently with 70% (v/v) ethanol, centrifuged (12000 x g, 15 min), dried

and then resuspended in 20 µL nuclease free water. The purified plasmid was visualized on an agarose gel and quantified at A_{260} using nanodrop.

f. Gel Extraction of DNA

The purification of DNA fragments from agarose gel was carried out using GeneJET gel extraction kit (Boom et al., 1990).

Principle

The DNA fragment of interest was excised from the agarose gel, placed in a microcentrifuge tube, solubilised in binding buffer and applied to the column. The chaotropic agent in the binding buffer dissolved agarose, denatured proteins and promoted DNA binding to the silica membrane in the column. The colour indicator in the binding buffer allowed easy monitoring of the solution pH for optimal DNA binding. Impurities were removed by washing. Purified DNA was then eluted from the column with the elution buffer.

Reagents

1. Binding Buffer
2. Wash Buffer
3. Elution Buffer (10 mM Tris-HCl, pH 8.5)

Procedure

The gel slice containing the DNA fragment was excised using a clean scalpel or razor blade and placed into a pre-weighed 1.5 mL tube and weighed. The weight of the gel slice was recorded and 1:1 volume of binding buffer was added to the gel slice (volume: weight). The gel mixture was incubated at 50 - 60°C for 10 min or until the gel slice is completely dissolved, and the tube was mixed by inversion to facilitate the melting process. The gel mixture was vortexed briefly before loading on the column and 1 gel volume of 100% isopropanol was added to the solubilised gel solution and mixed thoroughly. The solubilised gel solution was transferred to the GeneJET purification column and centrifuged for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. An additional binding step was carried out with 100 µL of Binding Buffer if the DNA was to be used for sequencing. Wash Buffer diluted with ethanol (700 µL) was added to the GeneJET purification column and centrifuged for 1 min. The flow-through was discarded and placed the column back into the same collection tube. The empty purification column was centrifuged for an additional 1 min to

completely remove residual wash buffer. The column was then transferred into a clean 1.5 mL microcentrifuge tube and added 50 µL of Elution Buffer to the centre of the purification column membrane and centrifuged for 1 min. The column was discarded and the purified DNA was stored at -20 °C.

g. Polymerase Chain Reaction (PCR)

PCR is a technique to amplify a DNA template to produce specific DNA fragments *in vitro*.

Principle

In PCR, the enzyme DNA polymerase adds nucleotides to the 3' end of a custom-designed oligonucleotide when annealed to a longer template DNA.

Reagents

1. Forward and Reverse Primers (10µM working stocks)
2. Template DNA
3. *Taq* DNA Polymerase
4. dNTPs (200 µM each)
5. $MgCl_2$ (1.5–2.0 mM)
6. 10 X Taq Buffer
7. Nuclease free water

Procedure

PCR reactions were carried out were performed by standard procedures as described by Sambrook et al. (1989). It involves repeated cycling (30 – 35 cycles) of three steps carried out in a thermo cycler (Mastercycler EP Gradient, Eppendorf, Germany). During the initial denaturation at 94 °C the double stranded DNA melts open to single stranded DNA. This will be followed by an annealing step where the mixture is cooled to a temperature of 50 – 70 °C resulting in the binding of primers to their complementary sequence in the template DNA. The reaction is then heated to 72 °C which is the optimal temperature for the action of DNA polymerase. The DNA polymerase then extends the primers by adding nucleotides onto the primer in a sequential manner, using the target DNA as a template resulting in an exponential increase of the number of copies of the gene. The steps will be then repeated. The PCR conditions and concentration of reagents were optimized individually for each gene of interest and were discussed in detail in the corresponding chapters (chapter 3 and 8).

h. Agarose Gel Electrophoresis

Principle

Agarose gel electrophoresis is a method of separating and analyzing DNA fragments ranging in size from a few hundred base pairs to about 20 kb. An agarose gel is a complex network of polymeric molecules and the average pore size depends on the buffer composition and the type and concentration of agarose used. The negatively charged DNA molecules will migrate to the positive pole through the highly cross-linked agarose matrix under the influence of an electric field. The rate of migration depends on the type and conformation of the DNA and also the ionic strength of the running buffer.

Reagents

1. Agarose
2. Tris-Acetic acid-EDTA (TAE) buffer, pH 8.0 (1X)
3. Ethidium bromide (10 µg/mL)
4. 6x loading dye
5. DNA marker
6. DNA samples

Procedure

Agarose (1% w/v) was melted in TAE buffer to form transparent solution. After cooling ethidium bromide was added, mixed and then poured into a mould and allowed to harden. Sample slots were formed on the gel by placing a comb on the gel before hardening. After setting the gel was mounted on electrophoresis tank with TAE buffer. DNA samples were mixed with loading dye and loaded on the sample slot. Closed the lid of the gel tank and run the gel. After running the gel the separated samples were visualized under UV light (Sambrook et al., 1989).

2.3. Major Instruments

The major instruments used to carry out this study are listed in Annexure II

2.4. Conclusion

The chapter briefly explained the general materials and methods used in this study. All protocols were of standard practice and the corresponding references are provided. More detailed methodologies for each aspect of the studies are mentioned in respective chapters.