Chapter 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Most of the general chemicals used were procured from different companies such as: Himedia (Mumbai, India); Merck (India); Qualigens Fine Chemicals Ltd. (India); S.D. Fine Chemicals (India). Chemicals of high purity and analytical grades were procured from Sigma Aldrich (St. Louis, USA).

3.1.2 Microorganisms

The following microbial cultures as listed in Table 3.1 were used throughout the studies. They were procured from National Collection of Industrial Microorganisms (NCIM) of National Chemical Laboratory (Pune, India) and Microbial type culture collection and Gene bank (MTCC) of Institute of Microbial Technology (Chandigarh, India) etc.

Table 3.1 List of microbial cultures procured from different culture collections

Sl. No.	Name of bacteria	Culture identity
1.	*Streptococcus thermophilus*	NCIM 2904
2.	*Streptococcus thermophilus*	NCIM 2412
3.	*Streptococcus lactis*	NCIM 2114
4.	*Streptococcus lactis*	NCIM 2180
5.	*Lactobacillus helveticus*	NCIM 2733
6.	*Lactobacillus delbruccki subsp. bulgaricus*	NCIM 2025
7.	*Lactobacillus acidophilus*	NCIM 2902
8.	*Lactobacillus acidophilus*	NCIM 2909
9.	*Lactobacillus bulgaricus*	NCIM 2056
10.	*Lactococcus lactis subsp. lactis*	MTCC 3041
For Microbiological Assay of folate		
11.	*Lactobacillus casei*	NCIM 2364

3.1.3 Media

The following media were frequently used throughout the study. The compositions of the media used were as follows:

3.1.3.1 MRS agar (De Man *et al.*, 1960)

MRS agar medium was used for the purpose of bacterial maintenance and their storage.

Table 3.2 Composition of MRS medium

Components	**Composition (g/L)**
Peptone	10.0 g
Yeast extract	5.0 g
Beef Extract	10.0 g
Dextrose	20.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulphate	0.1 g
Manganese Sulphate	0.05 g
Dipotassium Phosphate	2.0 g
Agar	20 g
	pH =6.5

3.1.3.2 Folic acid Assay medium (Masuda *et al.*, 2012)

Folic acid assay media (Himedia) was utilized for the preliminary assessment of folate production by the procured strains.

Table 3.3 Composition of Folic acid assay medium

Components	**Composition (g/L)**
Casein acid hydrolysate, vitamin free	12.0 g
Dextrose	40.0 g
Sodium Citrate	0.200 g
L-Cystine	3.0 g
DL-Tryptophan	0.20 g
Adenine sulphate	0.020 g
Guanine hydrochloride	0.020 g
Uracil	0.020 g
Thiamine Hydrochloride	10.002 g
Pyridoxine hydrochloride	0.004 g
Riboflavin (Vitamin B_2)	0.002 g
Niacin	0.002 g
p-Amino benzoic acid (PABA)	0.0002 g
Biotin	0.0000008 g
Calcium Pentothenate	0.0004 g
Dipotassium phosphate	1.00 g
Monopotassium phosphate	1.00 g
Magnesium sulphate	0.400 g
Sodium chloride	0.020 g
Ferrous sulphate	0.020 g
Manganese sulphate	0.020 g
	pH = 6.8±0.2

3.1.3.3 Folic acid *casei* medium (Wilson & Horne, 1982)

Folic acid *casei* media (Himedia) was utilized microbiological assay of folic acid during the entire study for the quantitative assessment of folate produced.

Table 3.4 Composition of folic acid *casei* medium

Components	**Composition (g/L)**
Vitamin free Casein acid hydrolysate	10.00 g
Dextrose	40.00 g
Sodium acetate	40.00 g
Dipotassium phosphate	1.00 g
Monopotassium phosphate	1.00 g
DL-Tryptophan	0.20 g
L-Asparagine	0.60 g
L-Cystine hydrochloride	0.5 g
Adenine sulphate	0.01 g
Guanine hydrochloride	0.01 g
Uracil	0.01 g
Xanthine	0.02 g
Sorbitan monooleate complex	0.1 g
Glutathione reduced	0.005 g
Magnesium Sulphate	0.4 g
Sodium chloride	0.02 g
Ferrous Sulphate	0.02 g
Manganese Sulphate	0.015 g
Riboflavin	0.001 g
PABA	0.002 g
Pyridoxine hydrochloride	0.002 g
	pH = 6.5±0.2

3.1.3.4 Skimmed milk medium (Lin & Young, 2000)

Skimmed milk medium was used for folate production studies checked by the procured strains.

Table 3.5 Composition of reconstituted skim milk medium

Components	**Composition**
Skim milk powder	100.0 g
Distilled water	1000 mL
	pH = 7.0

3.1.3.5 Nutrient Agar (Schillinger & Lucke, 1989)

For the assessment of probiotic characteristics of folate producing strains, soft nutrient agar medium was used to culture some potential pathogenic indicator strains.

Table 3.6 Composition of nutrient agar medium

Components	**Composition**
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	5.0 g
Agar	20.0 g
Distilled water	1000 mL
	pH =7.0

3.1.3.6 MRS agar supplemented with bile salts (Dashkevicz *et al.*, 1989)

For the bile salt hydrolase activity to assess the probiotic characteristics of folate producing strains, MRS agar supplemented with bile salts is used.

Table 3.7 Composition of MRS medium supplemented with bile salts

Components	**Composition (g/L)**
Peptone	10.0 g
Yeast extract	5.0 g
Beef Extract	10.0 g
Dextrose	20.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulphate	0.1 g
Manganese Sulphate	0.05 g
Dipotassium Phosphate	2.0 g
Agar	20 g
Sodium salt of taurodeoxycholic acid	5 g
or	
Glycodeoxycholic acid	2 g

Sterilization of the media were done by autoclaving at 15 psig pressure for 15 min unless otherwise specified. Heat labile chemicals were filtered through membrane filters of varying porosity.

3.2 Methods

3.2.1 Maintenance of cultures

The bacterial cultures were subcultured in the MRS agar medium and incubated at 37°C for 24 h. Subculturing was done almost three times before the culture used as inoculum for the production studies. The bacterial cultures were maintained in MRS agar semi stabs at 4±1°C. The stock culture was transferred to fresh MRS medium in 3-4 weeks. For long term storage, the cells were preserved in 20% glycerol solution at (-20°C).

3.2.2 Screening of folate producing microorganisms

3.2.2.1 Strain screening for Folate production

The screening of microorganisms for having efficiency of folate production among the procured bacterial strains was done in two subsequent steps. Primarily it was done by qualitative and secondarily quantitative methods.

i) Primary screening (qualitative) by assessing growth in Folic acid assay media

The folate producing strains were screened initially by growth ability of procured strains in the folic acid assay medium which is folic acid free medium. Growth of the strains in vitamin free medium is used as an indicator or first screening of the folate production. For assessing this, procured strains were grown in 10 ml of MRS Broth at 37°C for 24 h without shaking condition. 5 ml of each culture was then centrifuged at 10,000 rpm for 10 min. supernatant was discarded and obtained cell pellet was washed twice and re-suspended in original volume of 0.90% sterilized saline solution. Cell suspension of each strain obtained was further inoculated in the 10 ml of the sterilized folic acid assay medium by taking 2%v/v inoculum and incubated at 37°C for 24 h without shaking condition. Centrifugation of the sample was done to obtain the cell pellet. Growth of all the procured strain was determined by measuring the absorbance or optical density at 600 nm (OD600nm). Strains showing growth more than or equal to 0.5

of OD600nm were considered for the probable potential for folate production ability (Masuda *et al.*, 2012).

ii) Secondary screening (quantitative method)

Microorganisms were used in shake flask studies, in 250 ml Erlenmeyer flasks having both MRS medium and reconstituted skim milk medium in order to further evaluate the folate production efficiency. First of all inoculum was prepared in the 50 ml of folic acid assay medium and then 2% inoculum was added in the MRS and reconstituted skim milk medium. All the flasks were incubated at 37°C for 24 h without shaking condition. After incubation sample was treated with human plasma solution for the extraction of folate from the sample. Quantitative estimation of folate was done by the microbiological assay using *Lactobacillus casei* as the indicator microorganism. All the potent cultures with folate production ability were maintained as stock cultures in MRS Semi stab at 4±1°C. Stock culture was transferred to fresh MRS medium every 3-4 weeks and used throughout the study for the production of folate.

3.2.3 Production of folate

The production of folate was performed as following steps:

i) **Shake flask Studies:** Primarily, the production of folate was carried out by the potent folate producers in shake flask using 250 mL Erlenmeyer flask containing MRS medium and reconstituted skim milk medium and comparative analysis of folate produced in both the medium by the individual strains.

ii) **Optimization of process parameters:** Under the shake flask conditions, various factors such as physical such as temperature, pH, inoculum age and inoculum volume and some nutritional parameters are known to be optimized for the maximum production.

iii) **Microbial fortification for the enhanced production:** Further, the folate production was done by the method of microbial fortification using probiotic strain

which has the dual advantage of enhanced folate production along with the probiotic benefits.

3.2.3.1 Inoculum preparation

The best screened folate producers *Streptococcus thermophilus* NCIM 2904 and *Lactobacillus helveticus* NCIM 2733 from the stock culture was allowed to grow in MRS agar semistab at 37°C for 24 h. The grown culture from the MRS semi stab was transferred to sterile folic acid assay broth in 250 mL Erlenmeyer flask and incubated at 37°C for 24 h.

3.2.3.2 Shake flask production studies

The production studies of folate were performed in a MRS medium containing: 10.0 g/L peptone, 5.0 g/L yeast extract, 10.0 g/L beef extract, 20.0 g/L glucose, 2.0 g/L ammonium citrate, 5.0 g/L sodium acetate, 0.1 g/L magnesium sulphate, 0.05 g/L manganese sulphate and 2.0 g/L dipotassium phosphate at pH 6.5 and reconstituted skim milk medium containing: Skim milk powder (10%) and distilled water. The 250 mL Erlenmeyer flasks containing 100 mL of both the production medium were then inoculated with individual inoculum (2% v/v) from 24 h grown seed culture and allowed to grow at 37°C till 24 h without shaking condition. The samples were collected at regular time interval, kept at 4°C until extraction and analysis. Folate from the samples was extracted and quantification was done by microbiological assay.

3.2.4 Extraction of folate

Modified extraction procedure was followed for the extraction of folate from the food matrix for the total folate (extracellular and intracellular) estimation (Lin & Young, 2000b). For this first of all human plasma solution was prepared according to the method described in a previous report (Bassett *et al.*, 2010). 0.25 mg of dried human plasma (Sigma, Mumbai) was dissolved in 1.25 ml of distilled water. 35µl mercaptoethanol was added in this dissolved solution and this suspension was homogenized at room

temperature for 1.5 h. after this, centrifugation was done at 1000 rpm for 1 min. supernatant was collected and fractioned and this solution was used immediately.

For the extraction procedure, six milliliters of the fermented production broth and or fermented milk was taken and 10 mL of extraction buffer (0.1 M phosphate buffer containing 0.5% sodium ascorbate as reducing agent) was added into the sample. The mixture was kept in a boiling water bath for 15 min and then centrifuged at 4000 rpm for 10 min. The mixture was allowed to cool down and then 0.4 mL of human plasma solution was added into it. Polyglutamyl deconjugase enzyme present in the Human plasma converts the polyglutamyl forms of folates to monoglutamyl forms. The mixture was then incubated at 37°C for 1 h under continuous rotation. Finally the reaction was stopped by placing the samples in boiling water for 5 min. Then, the extract was centrifuged at 10000 rpm for 20 min. Supernatant was then filtered through a 0.45µm filter and used directly for further quantification by microbial analysis or high performance liquid chromatography.

3.2.5 Analytical methods

3.2.5.1 Microbiological Assay

Microbiological assay was based on the fact that growth of some microorganisms is severely affected by the absence of some specific nutrients in the medium and they do not have the capability to synthesize these components themselves. So the addition of these nutrients can result in growth of those particular microorganisms such as the growth of *Lactobacillus rhamnosus* and *Streptococcus lactis* is influenced by the presence of folate in the medium which is present in beef and yeast extract (Stokstad, 1943). For the microbial assay first of all the folic acid *casei* media, standard folic acid solution, inoculum were prepared as follows:

i) **Folic acid *casei* media preparation:** 9.4 gm of this media and 50 mg ascorbic acid was added in 100 ml distilled water and dissolved by heating (Flynn *et al.*, 1951). The final pH of the media should be around 6.7. Then, 5 ml of this media was taken in each test tube and 0.5 ml of the sample to be tested or standard folic

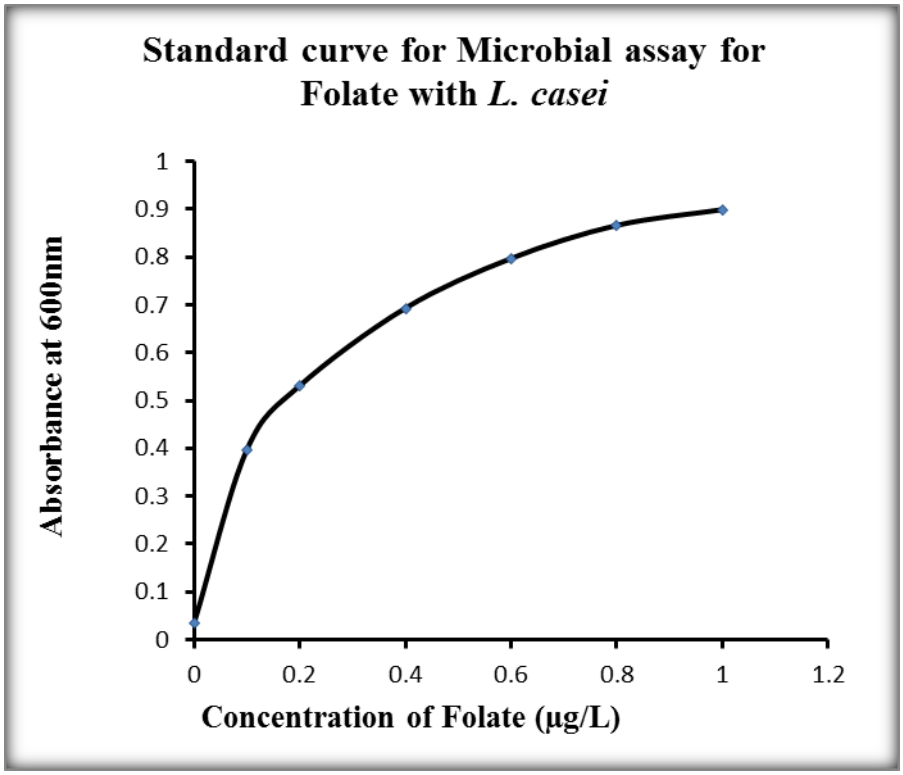
acid solution was added in each test tube. Distilled water was added in each test tube to make up the final volume upto 10 ml. This media was sterilized by autoclaving at 15 psig for 5 min.

ii) **Standard folic acid solution:** folic acid solution (0.2 µg/L phosphate buffer) was prepared. Volume ranging from 0.1 ml to 1.0 ml of this solution was taken in test tubes and 5 ml folic acid *casei* media having ascorbic acid was added into this. Distilled water was added to make up the final volume 10 ml.

iii) **Inoculum preparation:** Sterilized MRS media was prepared and inoculated with loopful inoculum of *Lactobacillus casei* NCIM 2364 under aseptic condition. Incubation was done at 37°C for 24 h at static condition. MRS broth was centrifuged at 6000 rpm for 20 min to obtain the cell pellet under aseptic condition. Cell pellet was washed twice and resuspended in 0.9% saline solution. This saline cell suspension was further used as inoculum for performing microbial assay.

Finally the 0.2% inoculum was allowed to grow in the folic acid *casei* media containing the desired samples to be quantified as well as standard solution of folic acid at 37°C for 18 h. Then the absorbance of the samples was taken at 600 nm (OD600nm) (Wilson and Horne, 1982).

A standard curve between absorbance (600 nm) and folic acid concentration (µg/L) was plotted. The unknown concentration of folic acid in desired samples could be calculated by just measuring the absorbance of the broth having desired sample at 600 nm and the calculation from the standard curve.



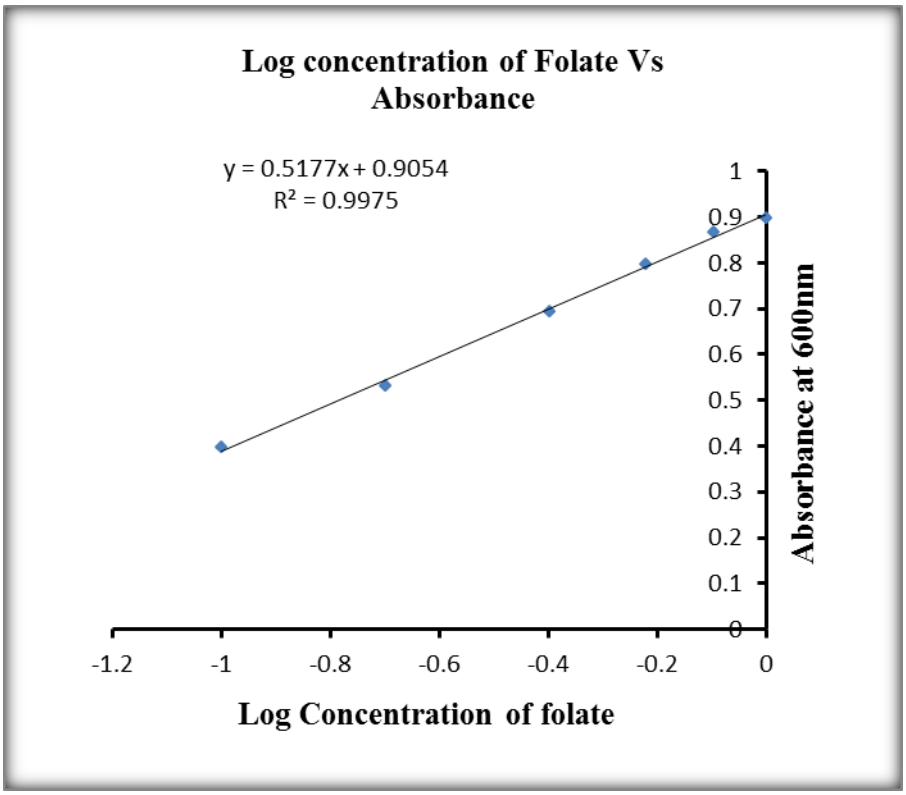


Fig 3.1 a) Folic acid concentration vs absorbance at 600 nm standard curve **b)** Linearization of the standard curve for the ease of the calculation

3.2.5.2 High Performance liquid Chromatography

A modified reversed-phase gradient HPLC method was performed (Shimadzu 20AD, UFLC). The samples were separated isocratically on the C-18 (5 µm particle size, 4.6x250 mm i.d.) analytical column (Keystone Scientific). The mobile phase consisted of 50% solution A, 0.02 M Phosphate buffer (pH 5) and acetonitrile in 97:3 ratio followed by a linear gradient of 50% solution B , 0.02 M Phosphate buffer (pH 5) and acetonitrile in 80:20 ratio. The mobile phase was filtered through a 0.22-µm pore membrane filter and degassed before use. The column was calibrated by the mobile phase at 25°C at a flow rate of 1 mL/min. The volume of injection was 20 µL. The absorbance of folic acid was monitored with a UV detector set at 200 nm. Peak identification was based on a combination of the retention time and the spectral characteristics. At 45 min, the column was equilibrated for 5 min in the initial conditions and another sample analysis could be initiated immediately (Lebiedzinska *et al.,* 2008).

3.2.6 Evaluation of probiotic efficiency of the folate producer strains

After the production studies, it has been observed that potent folate producer strains showed better production ability in skimmed milk medium than the MRS medium. Production in natural fermentation medium can have the advantage of the consumption of fermented product as it is thus no downstream processing is required. But in this case microorganism is ingested along with the product itself and generally the microorganisms are harmful for the human health. So the folate producer strain should exhibit the beneficial effects on human health such as the probiotics does. Thus in case of use of natural fermentation medium, probiotics efficiency of the producer strain should also be checked and the probiotic strain should be preferential over the normal strain. Probiotic efficiency can be checked by both the *in vitro* methods and *in vivo* methods. In this work, evaluation of probiotic efficiency was demonstrated by the *in vitro* methods given in Joint FAO/WHO and ICMR-DBT guidelines (Ganguly *et al.,* 2011) such as resistance to acidic condition and bile salts, antimicrobial activity, antibiotic sensitivity tests and degree of hydrophobicity described below.

3.2.6.1 Resistance to Gastric Acidity

To evaluate the resistance of gastric acidity, MRS broth was adjusted to different pH, i.e. 1, 2, 3, 4 and pH 7 as control by using 0.1N HCl and then sterilized in autoclave at 121°C for 15 min. Seed culture of the folate producers *S. thermophilus* and *L. helveticus* were inoculated (1% v/v) into the pH adjusted MRS broth and incubated at 37ºC for at least 3 h to simulate the acidic environment of the human stomach. After that cell growth was measured by plating method for the viable cell count (Hydrominus *et al.*, 2000).

3.2.6.2 Resistance to Bile Salts

Effect of bile salts on the growth of folate producing strains was studied by the method described in a previous study (Gilliland *et al.*, 1984). MRS broth having the different concentrations of bile salt (Sigma) (0.5, 1.0, 1.5 and 2.0%) was prepared. MRS media without any bile supplementation is used as control. After this, 0.1 ml inoculum was transferred to MRS broth and incubated for 37°C for minimum 4 h to stimulate the human intestinal environment. After that cell growth of the strains on the MRS agar plate is indicator for the strain to be bile salt tolerant.

3.2.6.3 Antimicrobial activity against potential pathogenic bacteria

Cell free supernatant (CFS) of the *S. thermophilus* and *L. helveticus* were prepared by inoculating the strains in 100 ml of MRS broth at 37°C till the early stationary phase (8-10 hours). Cells were separated by centrifugation at 10,000 rpm for 10 min at 4°C. The acid present in the supernatant was neutralized to pH 6 or 7 with 1M NaOH and filter sterilized with 0.2 µm filters. For the detection of antimicrobial activity of the substances in the resulting CFS, the agar well diffusion assay was performed (Schillinger & Lucke, 1989). Test pathogens selected for the study were *Escherichia coli* MTCC 443, *Salmonella typhi* MTCC 734, *Klebsiella pneumonie* MTCC 2653, *Shigella flexneri* MTCC 1457, *Vibrio cholera* MTCC 3906 and *Staphylococcus aureus* NCIM 5021. Indicator strains were inoculated and grown in a sterile petri dish containing solidified soft nutrient agar (0.8%, w/v). Wells were made in the nutrient agar and aliquots of 50 µl of supernatant were poured in the wells. After 24 h incubation at the optimal growth

temperature of indicator strains, a clear zone of inhibition of at least 2 mm in diameter around the wells was recorded as positive.

3.2.6.4 Antibiotic Resistance activity

Antibiotics susceptibility test was performed by the disc diffusion method with standard guidelines of antimicrobial resistance or sensitivity given by the Clinical and Laboratory Standards Institute (Bauer *et al.,* 1966). MRS agar was used instead of Muller Hinton agar for the testing. The antibiotics discs used were ampicillin, amoxycillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, tetracycline, norfloxacin and vancomycin. The results were expressed in term of inhibition zone around the discs and expressed as resistant (0 < 12 mm), intermediate (13 < 16 mm) or sensitive (17 < 33 mm) according to the observed interpretative points issued by CLSI.

3.2.6.5 Bile salt hydrolase activity

BSH activity was tested by the method described previously (Dashkevicz *et. al.,* 1989). 10 mL aliquots of overnight cultures (10^8-10^9) was spreaded on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TCDA) or 0.2% (w/v) glycodeoxycholic acid (GDCA) (Sigma) and 0.37 gL^{-1} $CaCl_2$. Plates were incubated anaerobically by using anaerobic jars for 72 h at 37ºC and strains forming precipitation zones were regarded BSH positive (Schillinger *et al.,* 2005).

3.2.6.6 Cell surface hydrophobicity test

The degree of hydrophobicity of the strains was tested by the method described in a recent study (Aswathy *et al.,* 2008). Culture to be tested was grown in 10 ml MRS broth, centrifuged at 6,000 rpm for 5 min. The cell pellet obtained was washed and re-suspended in 10 ml of Ringer solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$ and 0.01% $NaHCO_3$). The absorbance at 600 nm was measured to check the turbidity of the suspension. Equal volume of N-hexadecane and xylene was added slowly in the cell suspension and incubated at 37°C for 10 min followed by vortexing for 2 min. The hydrocarbon layer was allowed to separate and rise completely for about 30 min, than aqueous phase was removed carefully by the pipette and absorbance was measured at 600

nm. The percentage hydrophobicity of strains adhering to n-hexadecane and xylene was calculated using the equation:

$$\text{Hydrophobicity (\%)} = \frac{OD_{600}\ (\text{initial}) - OD_{600}\ (\text{final})}{OD_{600}\ (\text{initial})} \times 100$$

3.2.7 Optimization of process parameters

Different process parameters like temperature, pH, inoculum age, and inoculum volume were optimized by shake flask studies for production of folate. Folate concentration was investigated in production medium by varying one factor at a time, while other parameters were kept constant. The production of folate was also further optimized by employing response surface methodology (RSM) that is a statistical technique.

3.2.7.1 Optimization using one- factor-at-a-time (OFAT) method

As a primary step to enhance the folate production optimization, the process parameters were tested by varying one factor at a time and keeping others constant.

The following parameters were considered for the optimization:

i) Physical parameters

Incubation temperature	33, 35, 37, 40, 42 and 45 °C
pH	5.5-8.0
Inoculum age	6-24 h
Inoculum size	1-10% v/v

ii) Nutritional parameters

Carbon sources: Glucose, fructose, galactose, sucrose, lactose, maltose, independently at concentration (Carbon content was maintained at 4.0 g/L)

Precursor Compounds: Para amino benzoic acid, L-glutamate (50µM)

Prebiotics compounds: Sorbitol and Mannitol (0.4% w/v), Fructooligosaccharides and Galactooligosaccharides (10 g/L).

3.2.7.2 Optimization through Response Surface Methodology (Statistical design and analysis)

After OFAT, further optimization of process parameters was done by statistical methods using Response Surface Methodology (RSM). Implementation of RSM was done into two steps: first screening of significant process parameters using Plackett-Burman design (PBD) and later, the screened significant factors were utilized by Central composite design at different concentration levels to achieve experimental composition. The experimental design and statistical analysis of the data were done by using statistical software Minitab version 15.1.0.0, USA.

3.2.8 Immobilization of microbial cells for folate production

It has been observed that milk fermentation is traditionally carried out by the freely suspended microbial cells for the production of fermented dairy products. But now a days lot of attention is given also on the application of immobilized cells of lactic acid bacteria and probiotics for the production of dairy products as immobilized cells offer several advantages. For the preparation of immobilized cells, sterilized MRS broth was prepared as mentioned earlier and inoculated with 5% of inoculum under aseptic condition. Cells were allowed to grow at 40°C for 18 h at static condition and then centrifuged at 6000 rpm for 15 min at 4°C. Cell pellet was washed twice and suspended in of 0.90% sterile saline solution and mixed with 4% sodium alginate solution in the ratio of 1:1. The cell-alginate mixture was then added dropwise to 0.02 mM $CaCl_2$ solution through a syringe and kept for 30 min for jellification and hardening (Denkova *et al.,* 2004). The beads were recovered and rinsed with 0.90% sterile saline solution. The beads, with an average size of 2 mm, were obtained and stored at 4°C. The immobilized beads containing *S. thermophilus* was inoculated in the previously optimized reconstituted skimmed milk media under sterile conditions. Folate extraction and quantification was done by microbial assay as mentioned earlier.

3.2.9 Microbial fortification in food products to enhance the natural folate content

Food fortification is the process of addition of some specific micronutrients having significant scientific value in the foodstuffs. It is process chosen by the scientist and industries to reduce the number of people with deficiency of the particular micronutrient. There are 4 main methods: biofortification, commercial fortification, home fortification and microbial fortification. As observed from the study, folate production by the probiotic strain *S. thermophiles* was found to be more in natural fermentation medium that is skimmed milk medium in comparison to MRS medium. This kind of production can also be referred as microbial folate fortification i.e. addition of probiotic bacteria to foodstuff.

3.2.9.1 Microbial folate fortification in fruit cake

Inoculum for microbial fortification studies was prepared in folic acid assay medium and centrifuged to obtain the cell pellet. Cell pellet was washed twice and resuspended in 0.9% saline solution. 0.5 ml of this cell suspension was added as inoculum in 1x1 inch size equal pieces of cake by syringe. Cake pieces were incubated at 40°C for 24 h at static condition. Samples were taken at 0 h, 6 h, 12h, 18 h and 24 h. At the end of incubation period, extraction of all the samples and microbial assay was done by the methods described earlier.

3.2.9.2 Microbial folate fortification in orange juice

Orange juice was prepared using kitchen blender and filtered through 0.45 µm filter. Inoculum was prepared as mentioned above. 5% of *S. thermophilus* inoculum was inoculated in filtered fruit juices and incubated at 40°C for 24 h at static condition. Samples were taken at different time intervals and processed for extraction and analysis of folate (Gangadharan & Nampoothiri, 2011).

3.2.9.3 Microbial folate fortification in Tomato juice

Tomato juice was also prepared using kitchen blender and filtered through 0.45 µm filter. Inoculum is prepared in folic acid assay medium as described previously. 5% of *S. thermophilus* inoculum was added in filtered fruit juices. Incubation was done at 40°C for 24 h at static condition. Samples were taken at 0 h, 6 h, 12h, 18 h and 24 h. At the end of incubation period, extraction of all the samples and microbial assay was done. PABA and gluatamte was also added in both orange juice and tomato juice to check the effect on folate production (Gangadharan & Nampoothiri, 2011).

3.2.10 Efficacy and Stability studies of fortified food products

All the microbial folate fortified food products were checked for the stability on long term storage and viable cell count for the probiotic effects. Probiotic inoculated foodstuff were incubated at 40°C for 6 h. After that samples were stored in cold room at low temperature at 4°C for 28 days. Subsequently after each one week stability and efficacy of fermented milk, fruit cake, orange and tomato juices were checked in terms of folate content, viable cell count and pH to determine the shelf-life of the product for the intension of human consumption. Folate content was measured by performing microbiological assay with *L. casei* and pH was determined by pH meter. Viable cell count (log cfu/ml or log cfu/g) was determined by plating the serially diluted sample on MRS agar plate at 37°C for 48 h (Daneshi *et al.*, 2013).