Chapter 4 RESULTS AND DISCUSSION

4.1 Strain screening for having ability of folate production

4.1.1 Primary screening by assessing growth in Folic acid assay media

Folic acid assay medium is the medium which contains all the necessary nutrients except the folic acid, for the growth of most of the lactic acid bacteria and the procured microbial strains. This medium is lacking in the folic acid concentration so the microorganisms which can synthesize the folic acid, can only grow in the medium. This is the principle of the preliminary screening of the potential folic acid producers. So the procured strains were initially grown in the MRS medium at 37°C for 24 h without shaking condition. After this centrifugation of the sample was done to obtain the cell pellet which is used as the inoculum for the folic acid assay media. Growth in the folic acid assay medium was checked by taking the absorbance of the sample at 600nm. In the preliminary screening, only the procured strains which showed growth more than or equal to 0.5 of OD600nm were considered as the probable folate producers (Masuda *et al.*, 2012).

After the 24 h of incubation, all the procured strains showed growth upto certain extent. Among the 10 procured strains (*Streptococcus sp., Lactobacillus sp. and Lactococcus sp.*), eight strains showed absorbance more than 0.5 OD at 600nm however only 2 strains i.e. *Streptococcus lactis* NCIM 2114 and *Streptococcus lactis* NCIM 2180 showed very less amount of growth equivalent to 0.189 and 0.212 OD (at 600nm) respectively (Table 4.1).This may be due to both of these microorganisms could not produce sufficient folic acid to support their growth further. Based on the results, it was concluded that rest of the eight strains which showed more than 0.5 absorbance has the probable potential of the folate production, however they showed variation in the absorbance. Hence, the further confirmatory studies were needed to choose the best folate producing microbial strains.

The results obtained during growth experiment are shown in Table 4.1. Based on the values of OD600nm, *Streptococcus thermophilus* NCIM 2904 has shown highest growth

data which is probably due to the maximum potential of folate production (OD value 0.823) followed by *Lactobacillus helveticus* NCIM 2733 (OD value 0.769). Whereas other procured strains have showed lesser OD values ranging from 0.578-0.723.

Table 4.1 Comparison of results from preliminary screening of folate production by the procured strains

Strain Name	**Strain I.D.**	**Growth in folic acid assay medium**	
		OD600 nm at 0h	**OD600nm at 24 h**
Procured Strains			
Streptococcus thermophilus	NCIM 2904	0.123	**0.823**
Streptococcus thermophilus	NCIM 2412	0.127	**0.621**
Lactobacillus delbrueckii subsp. bulgaricus.	NCIM 2025	0.125	**0.723**
Lactobacillus bulgaricus	NCIM 2056	0.126	**0.682**
Streptococcus lactis	NCIM 2114	0.128	0.189
Streptococcus lactis	NCIM 2180	0.134	0.212
Lactobacillus helveticus	NCIM 2733	0.130	**0.769**
Lactobacillus acidophilus	NCIM 2902	0.121	**0.578**
Lactobacillus acidophilus	NCIM 2909	0.124	**0.619**
Lactococcus lactis subsp. lactis	MTCC 3041	0.129	**0.712**

4.1.2 Secondary screening (Quantitative method)

All the procured strains listed in Table 4.1 were allowed to grow in MRS medium and reconstituted skim milk medium for 24 h at temperature 37°C to further evaluate the folate production potential of the microbial strains. Samples were withdrawn at 24 h and extraction of all the samples were done according to the procedure given in the section 3.2.4 of the materials and methods. After extraction of folate from the samples, quantitative estimation of folate was done by the microbiological assay using

Lactobacillus casei as indicator microorganism according to the procedure mentioned in the section 3.2.5.1 of the materials and methods. The data are shown in Table 4.2.

Table 4.2 A comparative chart of microorganisms for folate production in both MRS broth and reconstituted skim milk medium

Strain Name	**Strain I.D.**	**Folate concentration in MRS medium (µg/L) at 24 h**	**Folate concentration in reconstituted skim milk medium (µg/L) at 24 h**
Procured Strains			
Streptococcus thermophilus	NCIM 2904	**30.2**	**39.8**
Streptococcus thermophilus	NCIM 2412	16.32	20.12
Lactobacillus delbrueckii subsp. bulgaricus.	NCIM 2025	22.1	25.8
Lactobacillus bulgaricus	NCIM 2056	15.85	13.34
Streptococcus lactis	NCIM 2114	ND	ND
Streptococcus lactis	NCIM 2180	9.4	ND
Lactobacillus helveticus	NCIM 2733	**24.13**	**28.9**
Lactobacillus acidophilus	NCIM 2902	13.56	14.34
Lactobacillus acidophilus	NCIM 2909	16.3	19.39
Lactococcus lactis subsp. lactis	MTCC 3041	21.42	24.5

N.D. = not determined

From the Table 4.2 it is clear that the strain *Streptococcus thermophilus* NCIM 2904 had the highest potential for folate production in both the MRS medium and reconstituted skimmed milk medium (30.2 µg/L and 39.8 µg/L respectively) at 24 h, while *Lactobacillus helveticus* NCIM 2733 was second highest producer showing the folate production 24.13 µg/L and 28.9 µg/L respectively in MRS medium and reconstituted skimmed milk medium.

From both the preliminary screening and secondary quantitative screening it was clear that the *Streptococcus thermophilus* NCIM 2904 and *Lactobacillus helveticus* NCIM 2733, among the procured strains, are potential candidates in the cluster for folate production. Hence, these two microorganisms were used for further studies.

4.2 Time course study of folate production by the selected strains

In order to evaluate the folate production profile, both the selected strains *S. thermophilus* and *Lactobacillus helveticus* were allowed to grow in the different production medium. MRS medium and Reconstituted skimmed milk medium were chosen to evaluate the efficiency of folate production. Production studies were carried out in 250 mL Erlenmeyer flask containing 100mL of MRS medium and reconstituted skimmed milk medium. Both the strains were allowed to grow in the production medium at 37 °C without shaking condition for 30 h of incubation time. Samples were collected at different time interval and extraction of folate and quantification was done according to the protocol described in the materials and methods (section 3.2.4 and 3.2.5.1) respectively.

The profile of folate production in MRS medium by both the strains is shown in Figure 4.1. Time course study of the folate production by the selected strains depicted that folate production has been started at 3-4 h after culture inoculation. The production of folate was increased after 5-6 h and reached to a maximum level of 36.9 µg/L and 26.47 µg/L respectively by the *S. thermophilus* and *L. helveticus* at 18 h and was found to be continued till 30 h. The level of the folate production was increased up to 18 h however, after that folate production slightly decreased which might be due to decrease of the medium pH due to the acid formation which resulted in the folic acid degradation as it is very labile in acidic environment (Rao *et al.,* 1984).

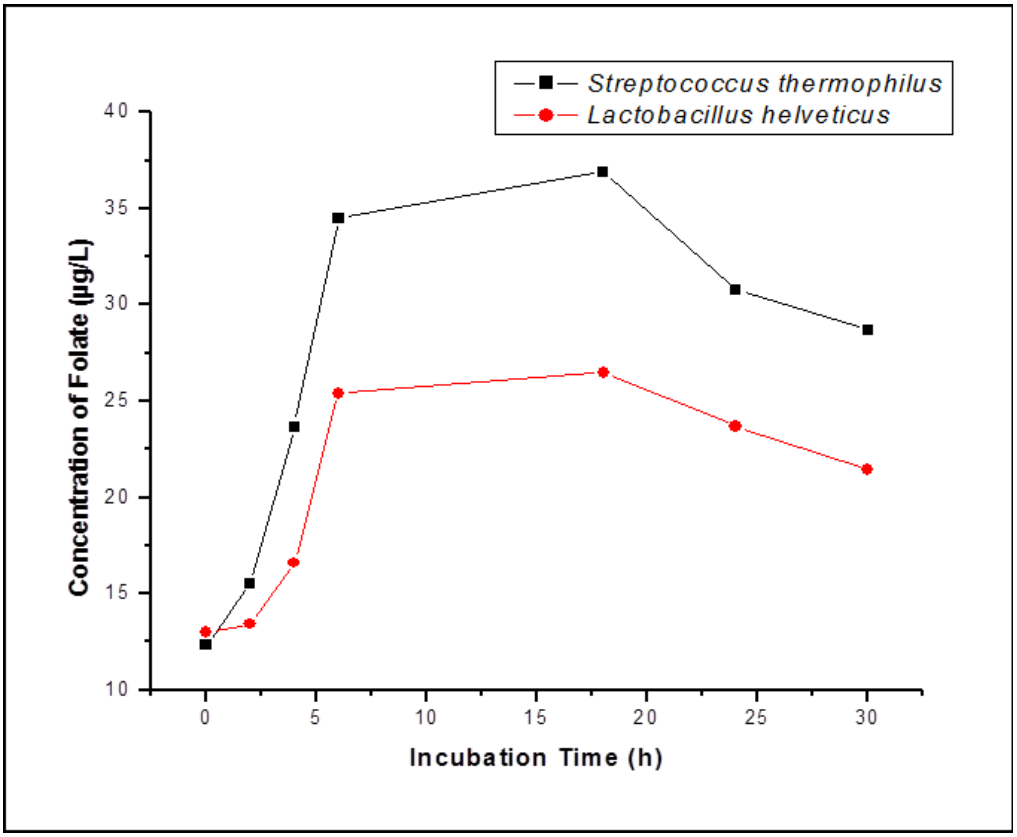


Fig. 4.1 Time course study profile of folate production by the *Streptococcus thermophilus* and *Lactobacillus helveticus* in MRS medium

The more or less similar observation was recorded in case of use of reconstituted skimmed milk medium as production medium (Figure 4.2). The folate production by both strains started at 3-4 h after the inoculation of seed cultures and production continues to increases till 6 h and obtain the maximum level of 48.28 µg/L in case of *S. thermophilus*. However, *Lactobacillus helveticus* produced the maximum folate level of 38.2 µg/L at 18 h. Afterwards, folate production in milk medium was also tends to decrease little bit as recorded in case of MRS medium. The decrease in folate concentration might be due to the result of low pH in the medium because of lactic acid formation. Another reason for this decrease may also be the behavior/nature of lactic acid bacteria and yogurt starter culture as they also consume folate for their own growth and metabolic activities (Lin and Young, 2000, Gangadharan & Nampoothiri, 2011).

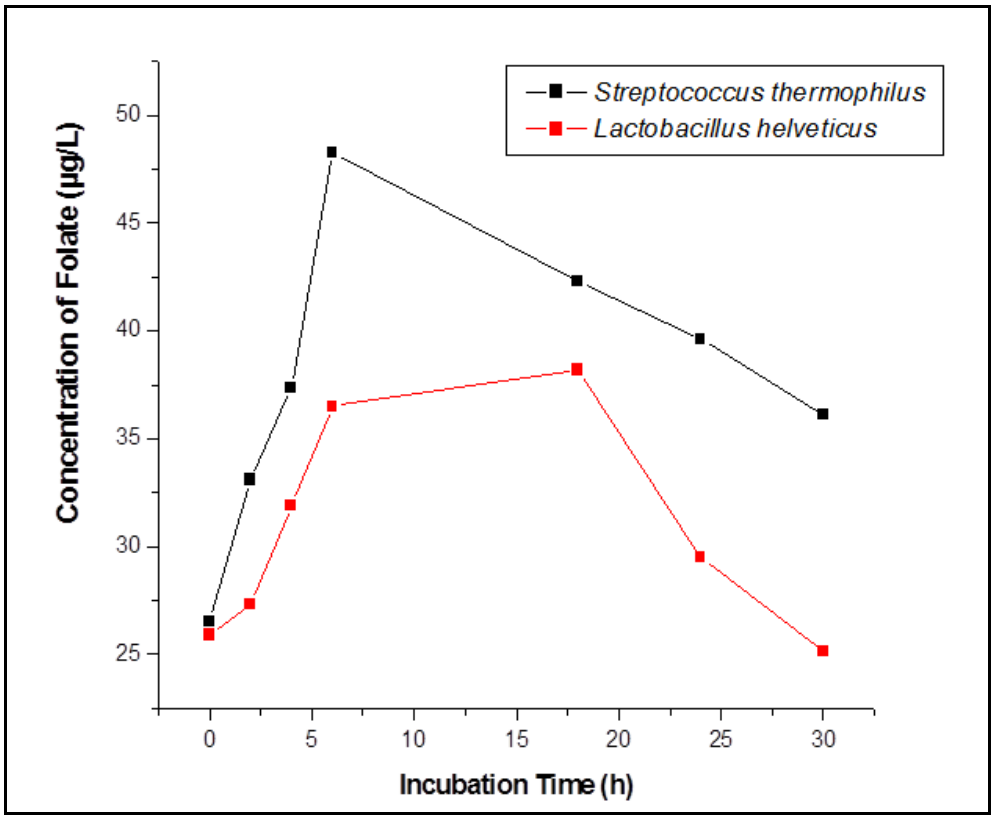


Fig. 4.2 Time course study profile of folate production by the *Streptococcus thermophilus* and *Lactobacillus helveticus* in reconstituted skim milk medium

In order to compare the results of folate production by the producer strains in the selected different production medium, a comparative bar chart is plotted for the individual strains. *S. thermophilus* produces the maximum folate in 6 h in the reconstituted skim milk medium however in MRS medium folate production was found to be maximum in 18 h. Although after comparison it was found that *S. thermophilus* produces maximum folate (48.28 µg/L) in 6 h in reconstituted skim milk medium than the MRS medium still in 18 h (36.9 µg/L) as shown in figure 4.3.

Similar observation was reported with the *L. helveticus* (Figure 4.4). Maximum folate was found to be produced at 18 h in both MRS and skim milk medium however folate production was found to be higher in reconstituted skim milk medium (38.2 µg/L) than the MRS medium (26.47 µg/L).

It has been confirmed from the observations reconstituted skim medium was better production medium than MRS medium and *S. thermophilus* showed better folate production potential rather than the *L. helveticus*. Hence, the reconstituted skimmed milk media was chosen for the rest of the studies.

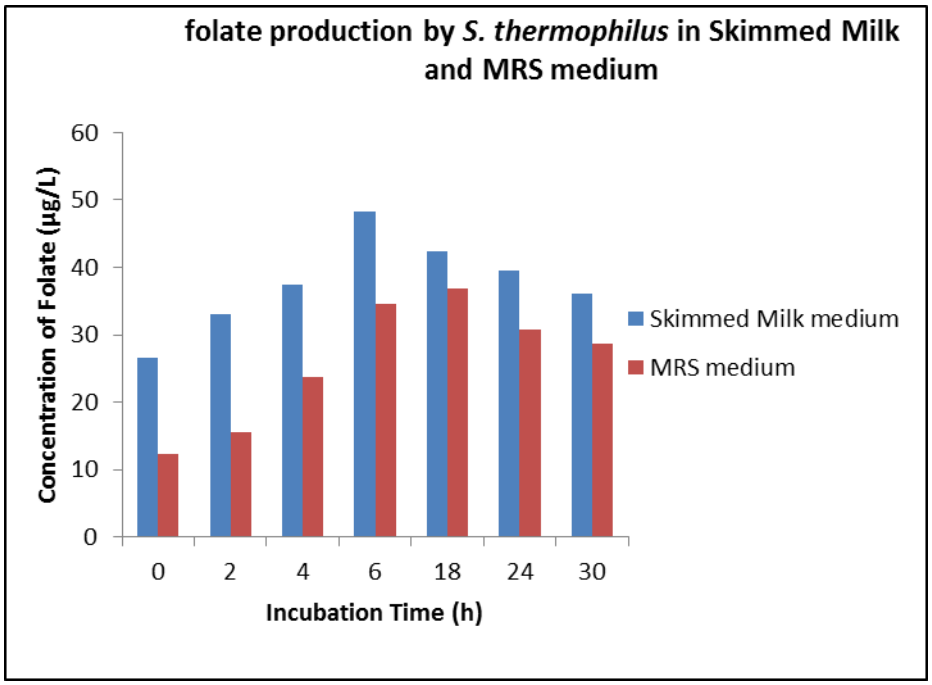


Fig. 4.3 Comparative chart of folate production by *Streptococcus thermophilus* in MRS medium and reconstituted skim milk medium

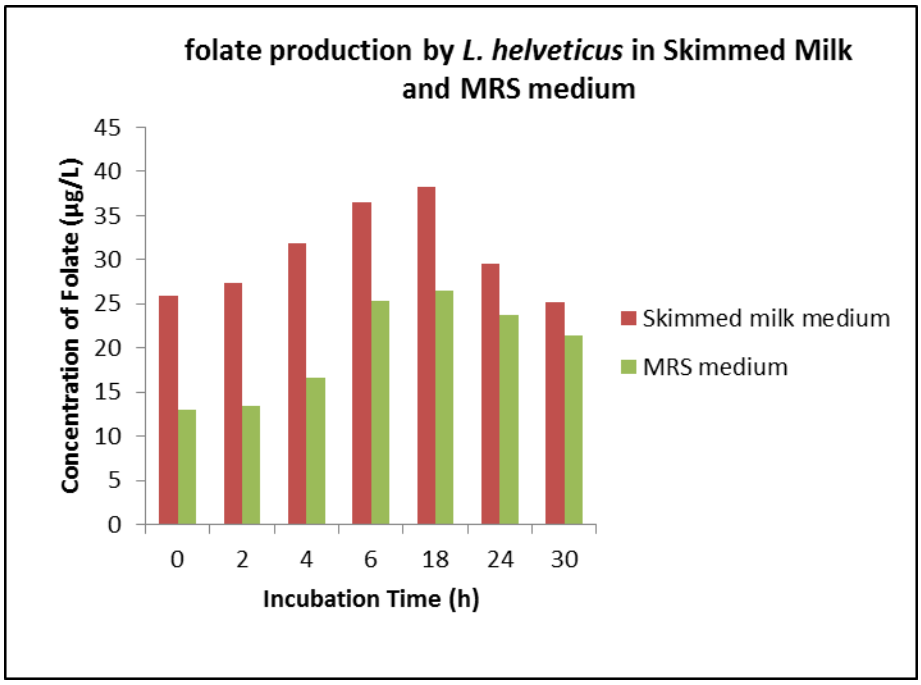


Fig. 4.4 Comparative chart of folate production by *Lactobacillus helveticus* in MRS medium and reconstituted skim milk medium

4.3 Growth kinetic study of folate production by the selected strains

S. thermophilus and *L. helveticus* grew well in reconstituted skim milk media at 37°C. Growth profiles of both the strains were almost similar. Exponential phase started from around 3 h and continued upto 18 h afterwards microorganisms entered in the stationary phase which continued till 28 hr. Growth profile of both the microorganisms were shown in figure 4.5. Specific growth rate was found to be 0.32 h^{-1} for *S. thermophilus* and 0.29 h^{-1} for *L. helveticus* after the calculation of the slope of the log X vs. time graph.

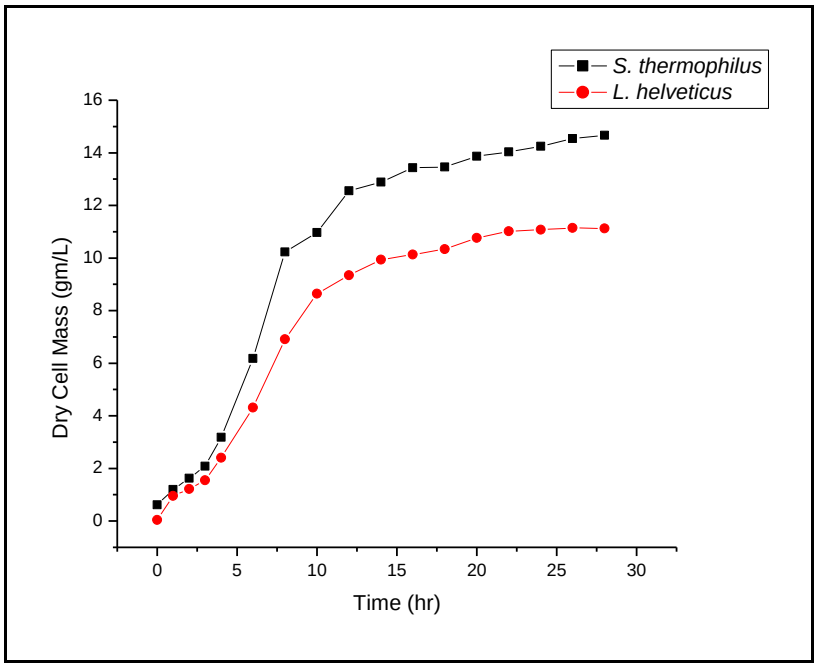


Fig. 4.5 Growth Kinetics profile of both the folate Producer strains

After the growth kinetics study of *S. thermophilus* and *L. helveticus*, it may be said that folate production in reconstituted skimmed milk medium showed the growth associated production pattern because maximum production of folate was found to be in the early exponential phase of the microorganism. It has also been observed that folate production tends to decline from 6 h- 24 h which may be due to the stationary phase from around the 18 h which ultimately supported the growth associated pattern of folate production.

Fermented milk is produced by the inoculation of these yogurt starter cultures in the reconstituted skim milk medium which is popular among the population as the healthy beverage to be consumed on regular basis for the several health benefits. Thus for the further studies, reconstituted skimmed milk media was used everywhere as it favored the higher production by both the strains. Reconstituted skimmed milk medium is also a natural fermentation medium thus it eliminates the need and cost of the downstream processing required for the purification of the desired product from the fermented broth as fermented milk itself is a consumable product. But the most important thing for this kind of the consumable fermented product is to have the probiotics microorganisms as

the inoculum so that culture present in the fermented product should not exert harmful effects instead should have the beneficial effects on the human beings after ingestion.

4.4 Evaluation of Probiotic efficiency of the selected folate producers

In order to evaluate the probiotic efficiency, *in vitro* and *in vivo* both methods were used. Generally the probiotics means the beneficial microorganisms which when administered in adequate amount confer health benefits to the host. Probiotic microorganism should remain alive during the whole gastrointestinal tract upon ingestion to confer the health benefits. Species belonging mainly to *Lactobacillus* and *Bifidobacterium* are considered as probiotics but some strains of *Streptococcus*, *Enterococcus, Propionibacterium* and *Saccharomyces* also show some probiotic properties. In this study folate production is checked in the fermented milk and the purpose is the consumption of folate rich fermented milk without any processing. So the probiotic efficiency of both the folate producers should be evaluated. In the current study for the efficiency evaluation, *in vitro* tests were taken under consideration which was done according to the guidelines given by the Joint FAO/WHO (2002) and ICMR-DBT guideline for the evaluation of probiotics in food. All the tests and their results are described as follows.

4.4.1 Resistance to gastric acidity

Probiotic potential of any microorganism is necessarily evaluated by its ability to grow in complex environment of the human digestive tract to impart its health benefits as the pH is too low to inhibit the growth of the most common human pathogens. The bacteria must first survive through the stomach (pH -1.5-2, acidic) before reaching to the intestine (Dunne *et al.,* 2001). Generally *Streptococcus* and *Lactobacillus* species, important in the fermentation of dairy and vegetable products, were found to be acid tolerant. The effect of different pH on the viability of two strains is presented in Table 4.3. *S. thermophilus* is able to survive very well at pH 1 however *L. helveticus* showed very less tolerance to low acidic pH after incubation for 3 h and 24 h. At pH 1.0, the growth of the *L. helveticus* showed very less viable cells 2.35±0.35 log c.f.u. ml^{-1} was

detected after 3 h. The *in vitro* probiotic evaluation reports of *Lactobacillus* species are available easily as studies on *Streptococcus* species are very rare. However this study was supported by the findings of Khalil, (2009) in which *S. thermophilus* CHCC showed better survival at pH 2, which could not grow at pH 1.5. There was a continuous reduction in viability at pH 1.0 than that in control (pH 7.0). In the present study, survival of *S. thermophilus* up to 6.0±0.14 log c.f.u. ml^{-1} at 3 h and 7.45±0.49 log c.f.u. ml^{-1} at 24 h at pH 2 and 4.7±0.56 log c.f.u. ml^{-1} and 2.35±0.35 log c.f.u. ml^{-1} at pH 1 indicated good degree of acid tolerance. Vinderola and Reinheimer, (2003) also found similar results i.e. better survival of *S. thermophilus* strains even at pH 2.0.

Table 4.3 Resistance of producer strains to gastric acidity in terms of acidic pH.

pH / Strains		7 (Control)	4	3	2	1
		Viable cell count (log cfu/ml)				
S. thermophilus	3 h	8.7±0.3	8.25±0.2	6.15±0.21	6.0±0.14	4.7±0.56
	24 h	10.5±0.07	9.55±0.35	7.0±0.14	7.45±0.49	5.65±0.63
L. helveticus	3 h	8.85±0.07	6.8±0.4	4.8±0.14	3.5±0.4	2.35±0.35
	24 h	9.95±0.5	7.4±0.14	5.25±0.2	3.7±0.84	3±0.14

*Values are reported in terms of mean of duplicates ± SD

4.4.2 Resistance to Bile Salts

Bile salt resistance is also required prior condition to be fulfilled by the tested strain to be probiotic as it is important for the metabolic activity and colonization of the strain in the small intestine of the host. Colonization of the strain in the small intestine ultimately leads the balance of intestinal healthy microflora (Havenaar *et al.,* 1992; Tambekar and Bhatuda, 2010). In this study, a trend of decreasing bacterial viability with increased concentration of bile salt was reported with the both strains. *S. thermophilus* and *L. helveticus* showed viable count of 7.15±0.2 log c.f.u. ml^{-1} and 4.8±0.14 log c.f.u. ml^{-1} respectively even on 2% bile salt addition in MRS media after incubation for 4 h (Table 4.4).

Table 4.4: Bile Salt tolerance study of folate producer strains.

Bile Salt concentration (%w/v) / Strains		Control (0%)	0.5%	1.0%	1.5%	2%
		Viable cell count (log cfu/ml)				
S. thermophilus	4 h	8.95±0.2	8.3±0.14	7.75±0.2	7.55±0.4	7.15±0.2
	24 h	13.9±0.3	9.6±0.4	8.5±0.2	8.3±0.28	7.7±0.14
L. helveticus	4 h	8.85±0.5	7.05±0.2	6.8±0.14	5.5±0.4	4.8±0.14
	24 h	11.7±0.3	8.15±0.07	7.95±0.35	6.95±0.35	5.05±0.07

*Values are reported in terms of mean of duplicates ± SD

4.4.3 Antimicrobial activity against potential pathogenic bacteria

Antimicrobial activity of the strains was checked by the agar well-diffusion method. Results (Table 4.5) showed that the CFS of *S. thermophilus* culture had moderate activity against most of the indicator strains tested. Maximum antimicrobial activity was observed with the *Escherichia coli* and *Staphylococcus aureus*, since these strains were strongly inhibited (zone of inhibition between 6 to 8 mm). Moderate activity of CSF of *S. thermophilus* was observed with the *Klebsiella pneumonie* and *Vibrio cholera* while no activity was recorded against the *Shigella Flexneri*. However *L. helveticus* showed moderate or less antimicrobial activity against only three indicator strains *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. No activity had been observed with other pathogenic indicator strains. Impact of antimicrobial activity depends on the strain, media components and physical parameters. Generally it had been suggested that growth of the pathogenic microorganisms is restricted by the production of inhibitory compounds such as lactic acid and organic acids and some kind of bacteriocins produced by the probiotic strains.

Table 4.5: Antimicrobial activity of strains against potential pathogenic bacteria

Strains	Indicator strains					
	Escherichia coli MTCC 443	*Salmonella typhi* MTCC 734	*Klebsiella pneumoniae* MTCC 2653	*Shigella flexneri* MTCC 1457	*Vibrio cholera* MTCC 3906	*Staphylococcus aureus* NCIM 5021
S. thermophilus	+++	+	++	-	++	+++
L. helveticus	++	+	-	-	-	+

Diameters of inhibition zone are the mean of duplicates: + diameter of inhibition zone <2 mm, ++ diameter of inhibition zone between 2 and 5 mm, +++ diameter of inhibition zone between 6 and 8 mm, - no effect detected

4.4.4 Antibiotic Resistance activity

Due to the adequate use of antibiotics in humans, generally microorganisms possesses antibiotic resistance gene. For the strain to be used as probiotics, presence of antibiotic resistant gene is major issue in terms of safety aspects due to transferrable genes mainly. Generally antibiotic susceptibility test is used to evaluate the presence of antibiotic resistance gene that could transfer to the human or animal and became a potential threat for the life. *S. thermophilus* were found either sensitive or moderately sensitive to ampicillin, amoxicillin, chloremphenicol, ciprofloxacin, gentamicin, kanamycin and vancomycin whereas resistant against erythromycin, tetracycline, norfloxacin and streptomycin and *L. helveticus* was found to be sensitive or moderate sensitive to ampicillin, amoxicillin, chloremphenicol and ciprofloxacin whereas resistant against rest of the antibiotics (Table 4.6). It has been reported that *Lactobacilli* generally had antibiotic resistance gene naturally (Charteris *et al.,* 1998b). Strains which are safe in the sense of antibiotic resistance may be used as a potential candidate in the development of future probiotics. In this study *S. thermophilus* showed sensitivity to most of the antibiotics studied thereby showing probiotic potential.

Table 4.6: Antibiotic susceptibility profiles of folate producer strains

Strains	A	Am	C	Cf	E	G	K	T	N	Va	S
S. thermophilus	S	S	MS	S	R	S	S	R	R	S	R
L. helveticus	S	S	S	MS	S	R	R	R	R	R	R

Antibiotics: ampicillin-A; amoxicillin-Am; chloramphenicol-C; ciprofloxacin-Cf; erythromycin-E; gentamicin-G; kanamycin-K; tetracycline-T; norfloxacin-N; Vancomycin- Va; streptomycin-S

S: sensitive i.e. inhibition >50%; MS: moderately sensitive i.e. inhibition 10–30%; R: resistant i.e. no inhibition.

Zone of inhibition calculated according to the table given by NCCLS.

4.4.5 Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity is a relevant characteristics of probiotics by which they can grow in and colonize the intestine and eliminating the toxicity of conjugated bile salts readily excreted from GI tract by deconjugating them in the duodenum (De Smet *et al.*, 1995). In our study, both strains were able to grow in the presence of bile salts even after 24 h of incubation. However, both the strains showed the hydrolase activity only with sodium salt of taurodeoxycholic acid (TCDA). *L. helveticus* showed no hydrolase activity with glycodeoxycholic acid (GDCA) as no precipitate was found around the colonies as indicated by the BSH test on MRS agar plates. Thus *L. helveticus* hydrolysed only the sodium salt of taurodeoxycholic acid (TCDA) and *S. thermophilus* had the ability to show hydrolase activity with both TCDA and GDCA (Table 4.7). BSH activity is desirable properties of a probiotic strain since it enhances the survival and persistence in the gastrointestinal tract however it had been observed that most frequently used probiotics genera *Lactobacillus* and *Bifidobacterium* did not have the ability to hydrolyse the conjugated bile salts (Takahashi and Morotomi, 1994; Ahn *et al.*, 2003).

Table 4.7: Bile salt hydrolase activity of the folate producer strains

Strains	Bile salt hydrolase activity	
	TCDA*	GDCA
S. thermophilus	+	+
L. helveticus	_	+

* TCDA-Sodium salt of taurodeoxycholic acid, GDCA- Glycodeoxycholic acid

4.4.**6 Cell surface hydrophobicity test**

Adherence of bacteria to intestinal epithelial cells is determined by the degree of hydrophobicity. Cell surface hydrophobicity reflects the physical and chemical characteristics of the cell surface. As microbial adhesion is a combined impact of long-range van der waal forces and electrostatic forces and various other short-range interactions, the strains adhering well to the hydrocarbons could be considered as positive probiotic candidate. Cell surface hydrophobicity may be influenced by the incubation time, growth conditions, and growth medium. In this study, both the strains were evaluated for their cell surface hydrophobicity towards hydrocarbons i.e. n-hexadecane and xylene. Both strains showed some extent of hydrophobicity with both the hydrocarbons (Table 4.8). As evident from the table, it has been observed that both *S. thermophilus* and *L. helveticus* have relatively more affinity towards the xylene than n-hexadecane. The percent hydrophobicity values observed with xylene were 19.2±0.42% and 5.6±1.5% for *S. thermophilus* and *L. helveticus* respectively while it was less in case of n-hexadecane, 15.95±0.9 and 3.35±0.2 respectively. The results were in accordance with that of reported by Iyer *et al.,* (2010) in which cell surface hydrophobicity of *S. thermophilus* with xylene and n-hexadecane was found in the range of 18.3 to 24.5%. Different strains may show variation in hydrophobicity with different solvents due to the fact the adhesion is dependent on both the origin of strains as well as surface properties (Morata *et al.*, 1998). Flint *et al.* also reported that hydrophobicity of *S. thermophilus* may vary from 24% to 98% depending on their source (Flint *et al.*, 1997).

Table 4.8: Cell Surface hydrophobicity activity of the folate producer strains

Strains	% hydrophobicity	
	n-hexadecane	xylene
S. thermophilus	15.95±0.9	19.2±0.42
L. helveticus	3.35±0.2	5.6±1.5

Streptococcus thermophilus showed better probiotic potential than *Lactobacillus helveticus* in terms of gastrointestinal viability, antimicrobial activity, antibiotic sensitivity, bile salt hydrolase activity and cell surface hydrophobicity. Thus, *S. thermophilus* was used as probiotic for the rest of the studies.

4.5 High Performance Liquid Chromatogram of folate produced by the *S. thermophilus*

For the validation of microbiological assay results, qualitative validation was done by performing HPLC assay. It has been observed that Retention time of standard folic acid was found to be 16.62 min (Figure 4.6). Retention time of uninoculated milk sample was observed as 2.559 min (Figure 4.7). The retention time of fermented milk produced by the *S. thermophilus* was found to be 16.728 min (Figure 4.8) which confirmed the presence of folate in the fermented milk

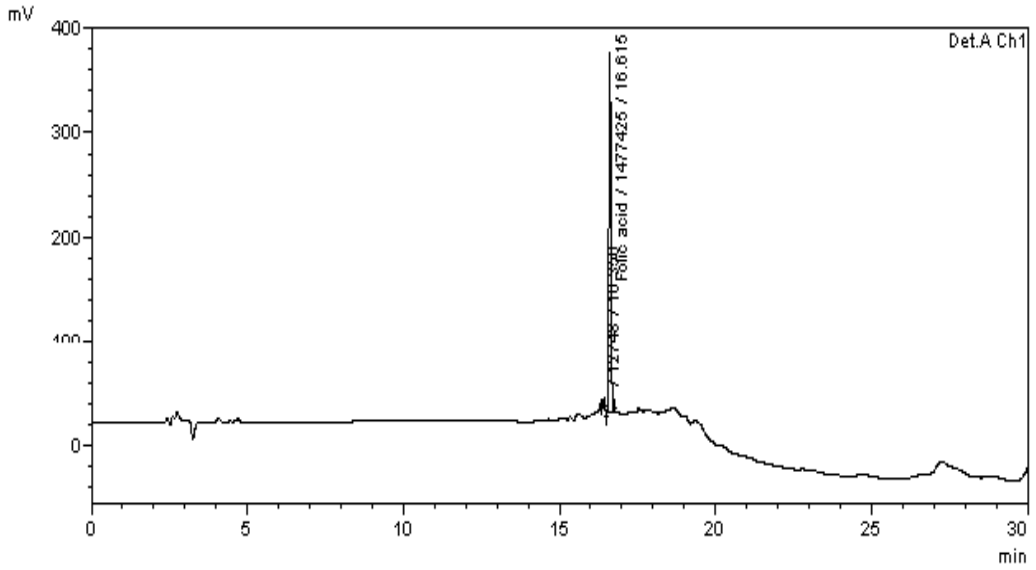


Fig 4.6: HPLC Chromatogram of Standard folic acid solution

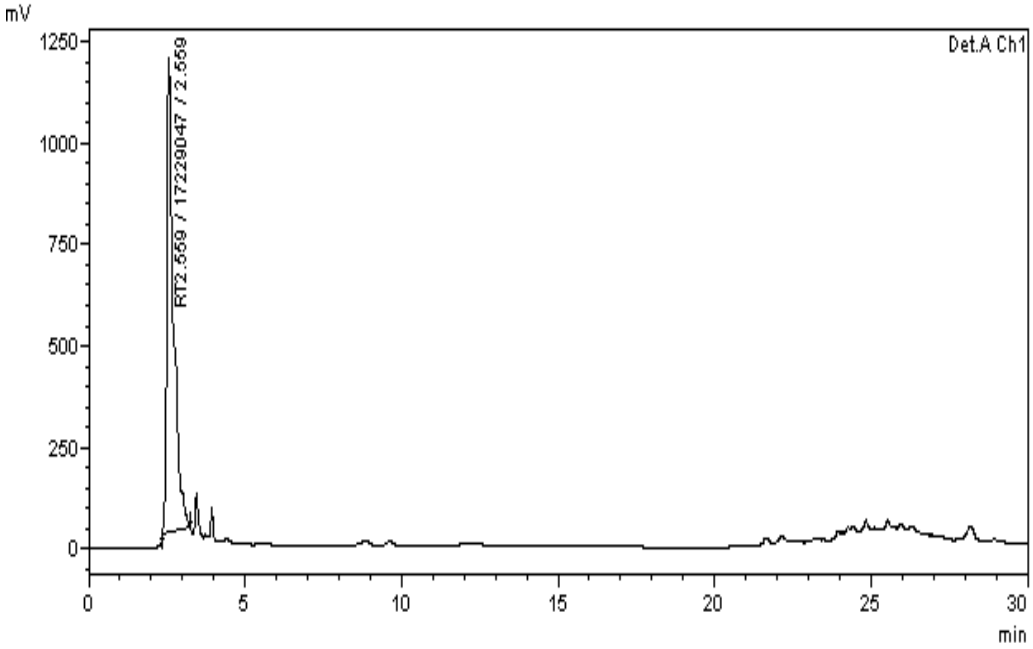


Fig 4.7: HPLC Chromatogram of Uninoculated milk sample

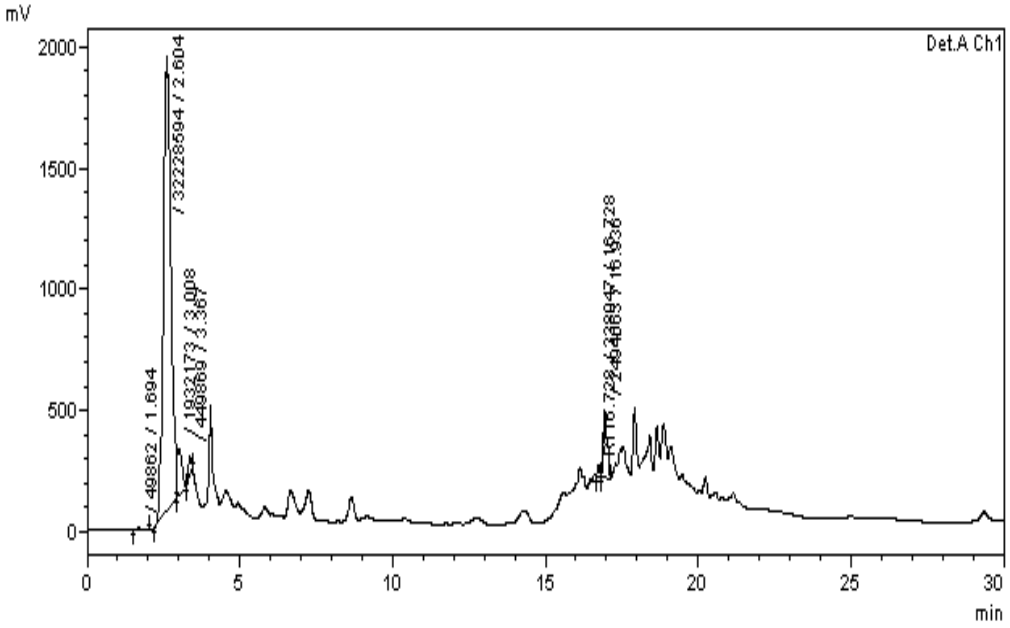


Fig 4.8: HPLC Chromatogram of fermented milk produced by the *S. thermophilus*

4.6 Optimization of process parameters for enhanced folate production by *Streptococcus thermophilus*

In order to evaluate the effect of various process parameters and media constituents affecting the growth of the producer strain and ultimately the production of folate, various experiments have been carried out using one-factor-at-a-time method (OFAT) i.e. varying one process parameter at a time and keeping other factors constant in shake flask culture.

4.6.1 Effect of temperature

In order to optimize the temperature for maximum folate production, *S. thermophilus* was allowed to grow in shake flask containing sterilized reconstituted skim milk medium at different temperatures in range of 33-45°C at static condition. Other cultural conditions were kept identical as according to the time course study of the folate producer strains in the reconstituted medium and production was found to be maximum at 6 h so the samples were taken out at 6 h to evaluate the folate concentration. The folate production data was shown in Figure 4.9.

As the data showed, production of folate was found to be increased with the rise in temperature upto 40°C, afterwards folate production decreases with increase in temperature. Therefore, 40°C incubation temperature was found to be optimum with maximum folate production and was used for further studies. The rise in temperature from 33°C to 40 °C has increased the folate production by 59.5%. Whereas, increasing the temperature from 40°C to 45°C has decreased the production of folate by 26.82%.

Temperature affects the activity of enzymes present in the microorganism essential for the growth as well as the metabolite production. As *S. thermophilus* is the thermophilic bacteria, its growth is most favored at the higher temperature (Deep & Kundu, 2014). Most of folate production studies have been done using different yogurt starter cultures or lactic acid bacteria in which the maximum folate production was noticed in the temperature range of 37-45°C (Holasova *et al.*, 2004; Mousavi *et al.*, 2013; Rao *et al.*, 1984).

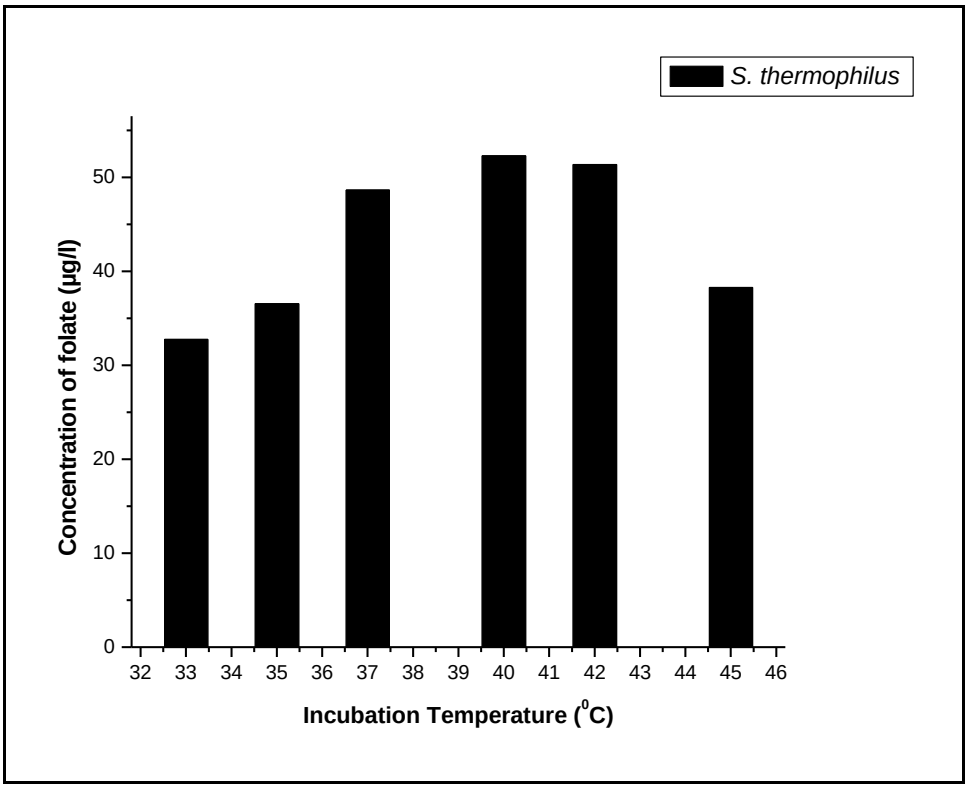


Fig. 4.9 Effect of temperature on folate production

4.6.2 Effect of pH on folate production

The effect of pH on folate production by *S. thermophilus* was studied in shake flask containing sterilized reconstituted skim milk medium by adjusting the pH of the medium in the range of 5.5-8 at 40°C. The observed data are shown in the graph (Figure 4.10).

It was noticed that the production of folate was increased to nearly 2.23 fold with increasing the pH of the medium from 5.5 to 6.5. Folate production was most favored by the *S. thermophilus* at pH 6.5 (49.45 µg/L). Further increase in pH resulted in continuous reduction in folate production and approached minimum concentration of 33.1 µg/L at pH 8.0.

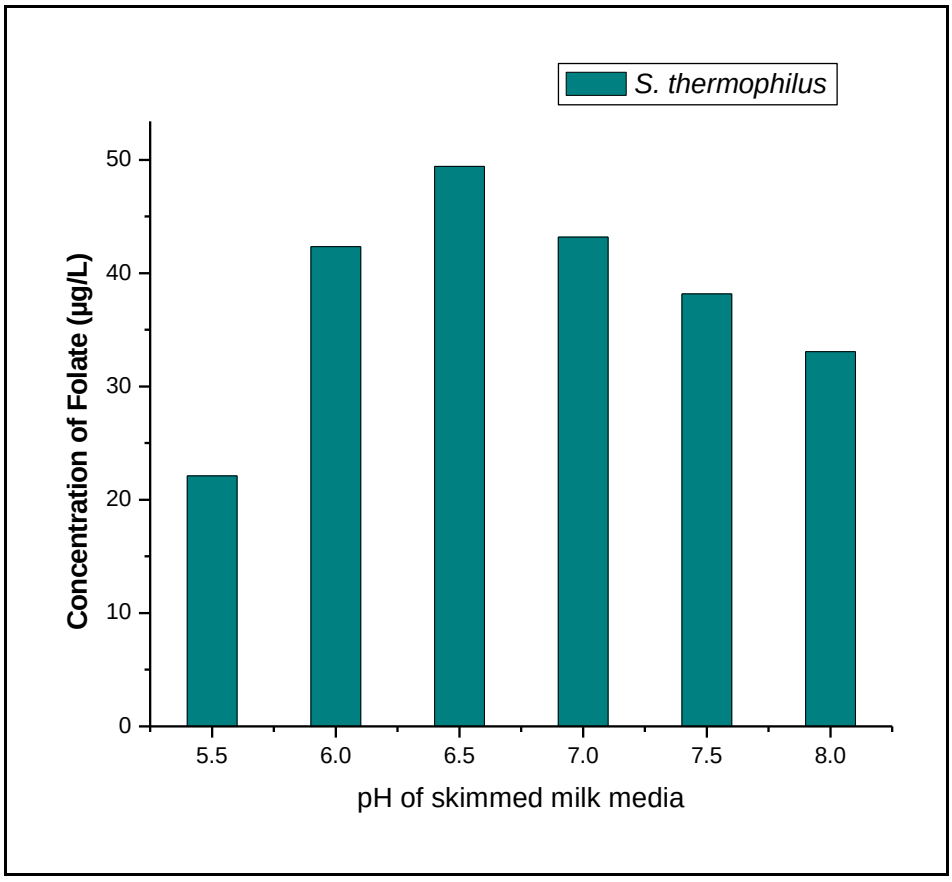


Fig. 4.10 Effect of pH on folate production

It may be due to the increased retention of the intracellular folate with the increase in pH which causes difficulty in the complete recovery of folate. Cell retention of folate may be dependent on the negative charge of the carboxyl group of the polyglutamyl folate. At low pH, folate is protonated and became electrical neutral so its transport across the membrane became very much easier (LeBlanc *et al.,* 2011). The observed data trend is in accordance with literature report of a study (Sybesma *et al.,* 2003). However pH 6.5 at which significant increase in the folate concentration occurred is generally the natural pH of the milk as it varies between the 6.5-6.7. So it may be considered as there is no need of the change the initial pH in case of milk medium.

4.6.3 Effect of inoculum age

To study the effect of inoculum age on the folate production, seed culture of different incubation time ranging from 6-24 h were prepared and allowed to grow in the reconstituted skimmed milk medium at 40°C. All the other physical parameters were kept identical as mentioned earlier. The Figure 4.11 shows the effect of different inoculum ages on folate production.

The maximum production of folate (54.2 µg/L) was achieved with the seed culture of 15 h age. As the age of inoculum increased from 15 h to 24 h, slight reduction in the folate production was observed. The maximum folate production using 15 h of seed culture was observed and was taken as optimum for further studies. This may be due to the growth profile of the microorganisms as this phase is the late exponential phase of the *S. thermophilus*.

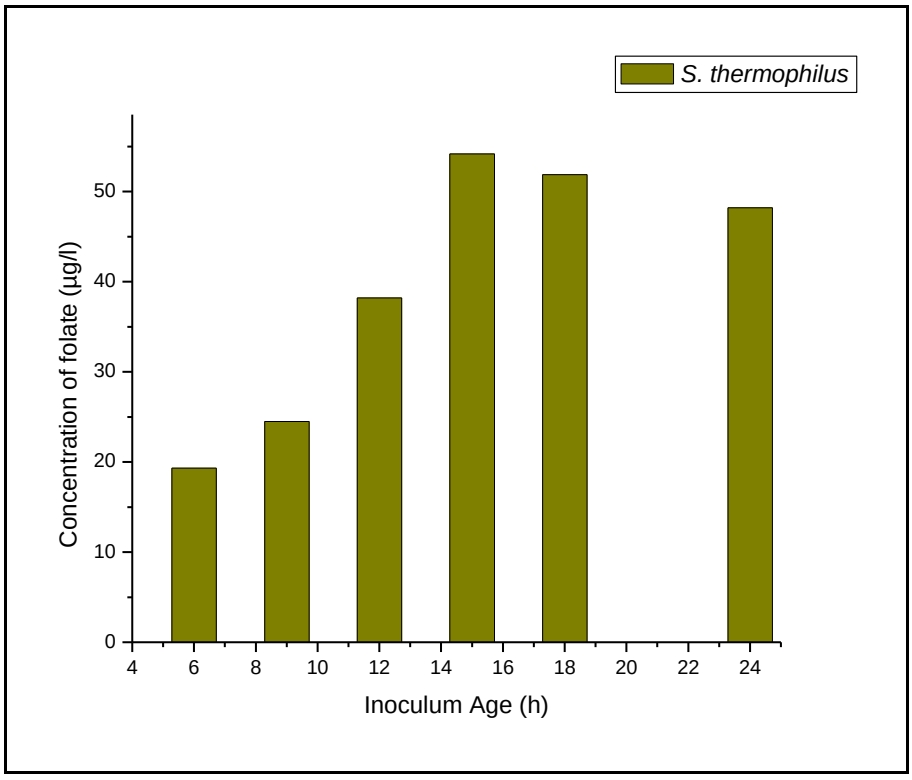


Fig. 4.11 Effect of inoculum age on folate production

4.6.4 Effect of inoculum volume

To evaluate the effect of inoculum volume on the folate production, different inoculum sizes of the seed medium (1-10% v/v) were inoculated to reconstituted skim milk medium. All the other physical parameters were kept identical as mentioned earlier. The Figure 4.12 shows the effect of different inoculums volumes on folate production.

The maximum production of folate (55.22 µg/L) was obtained with an inoculum size of 5% (v/v). A higher inoculum of 8-10% (v/v) was found to enhance the production of folate than if the lower inoculum size of 1-2% (v/v) was used. Therefore, high inoculum volumes do not necessarily give higher folate. But at 10% inoculum level, decline in folate level was observed as the increase in cell density which may hamper the folate production due to limited substrate. Therefore 5% v/v inoculum level was found to be optimum and was used for further studies.

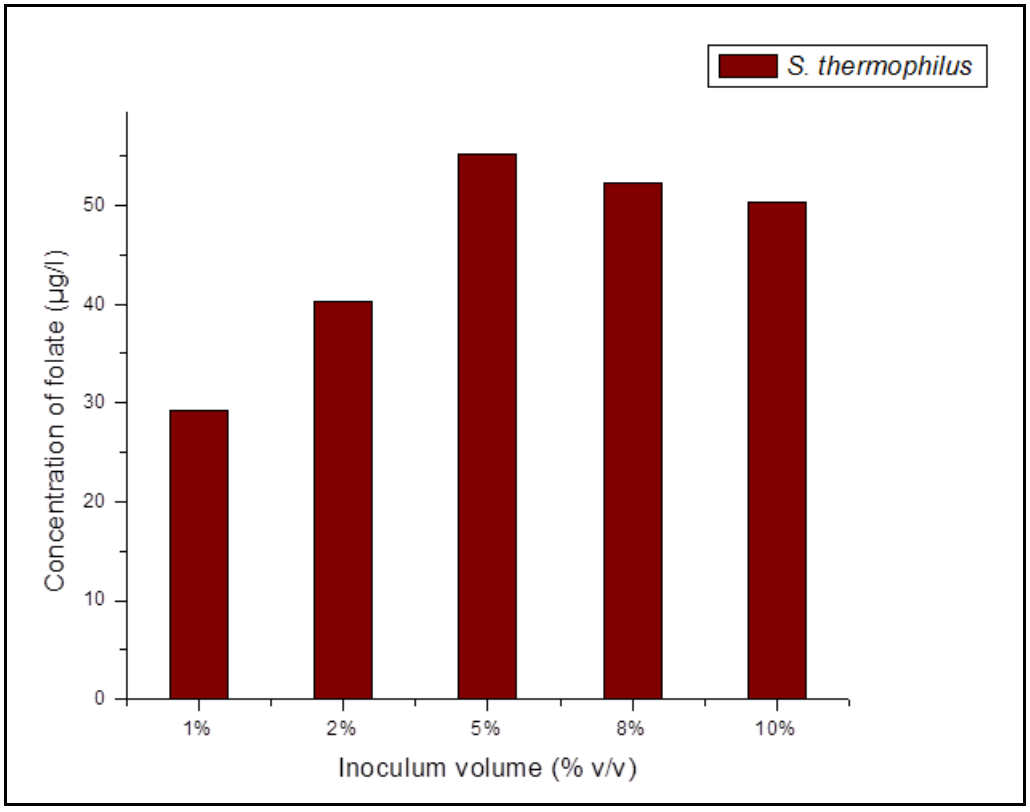


Fig. 4.12 Effect of inoculum volume on folate production

4.6.5 Effect of different carbon sources on folate production

The effect of different carbon source on folate production was studied after addition of external carbon sources in reconstituted skim milk such as glucose, fructose, galactose, lactose, sucrose and maltose (Carbon content was maintained at 4.0 g/L) independently. Control was also taken without addition of any external carbon source. The folate production was monitored after 6 h of incubation at 40°C, at static condition. From the Figure 4.13 it was observed that *S. thermophilus* was capable of utilizing these carbon sources at a variable extent thus affecting the folate production. Maximum folate production was observed with the lactose (63.78 µg/L) followed by glucose (57.56 µg/L), galactose (55.3 µg/L), fructose (53.34 µg/L), sucrose (52.8 µg/L), and maltose (51.64 µg/L).

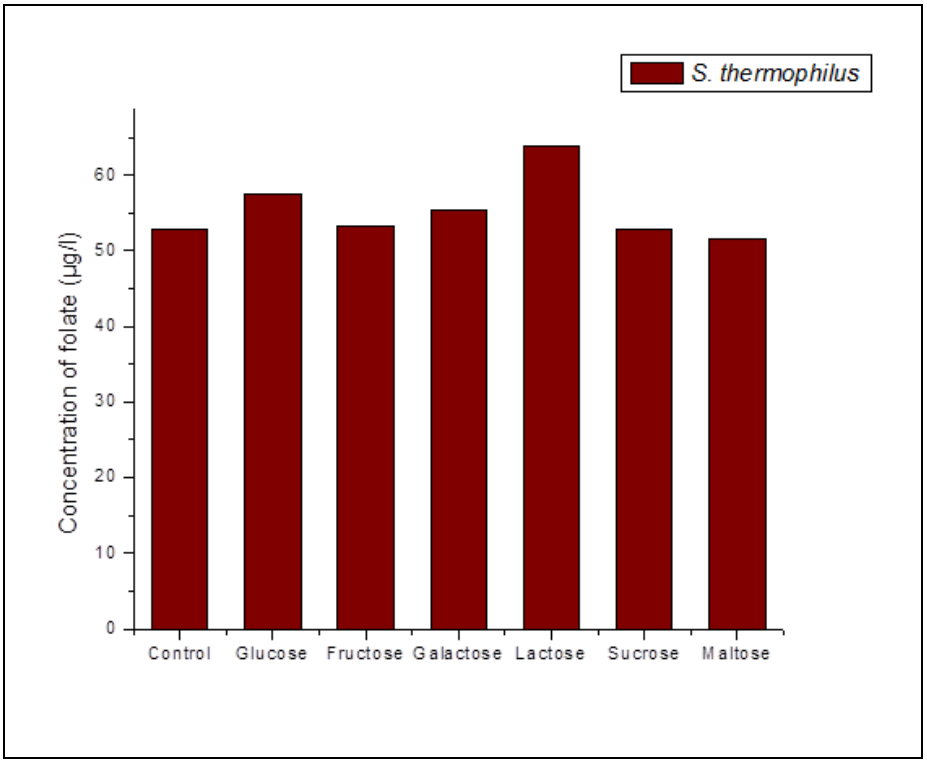


Fig. 4.13 Effect of different carbon sources on folate production

The reason for lactose giving maximum folate production might be due to the fact that lactose is the principal sugar, also present in milk, utilized by most of the yogurt starter cultures and thus metabolized easily by the *S. thermophilus* for the folate

production. This data observed in this study was somehow in accordance to the data reported in the study in which 2% lactose addition had resulted in the increase in folate production (Lin and Young, 2000).

4.6.6 Effect of Media additives on folate production

In order to study the influence of some inducers qualitatively, precursors like P-aminobenzoic acid and glutamate and prebiotics like sorbitol, mannitol, Fructooligosachharide and galactooligosachharides were used in different concentrations. PABA and Glutamate were added in the concentration of 50µM. Sorbitol and mannitol were added in the concentration of 0.4% w/v however fructooligosaccharides and galactooligosaccharides in the concentration of 10g/L. Prebiotics are the digestive polysaccharide which enhances the growth of the probiotics. These all the additives were added in the reconstituted skimmed milk medium. All others factors were kept identical as followed by the previous experiments. The folate production was monitored after 6 h incubation at 40°C at static condition.

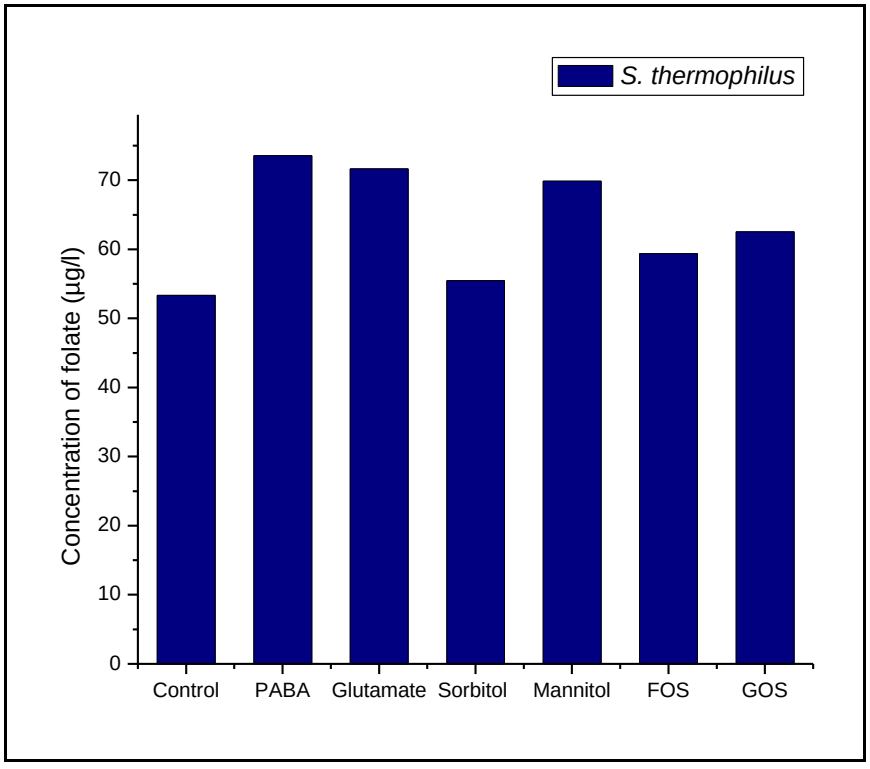


Fig. 4.14 Effect of different precursors and prebiotic addition on folate production

Figure 4.14 depicts PABA and glutamate addition in the production medium enhanced the folate production. PABA and glutamate addition resulted in the 37.96% and 34.36% increase in folate production respectively. This may be due to the role of PABA and glutamate as precursor which is essential for the folate production as folate is made up of PABA, glutamate and GTP subunits (Hugenschmidt *et al.*, 2011; Rao *et al.*, 1984). Prebiotics addition in the production medium showed the rise in folate production. However, only mannitol and GOS enhanced the folate production to a significant level as compared to other prebiotics. Prebiotics are mainly soluble oligosaccharides which have the potential to enhance the growth of probiotics thus enhanced folate production might be due to better growth of the culture. Further the individual optimum concentration of all these media additives for the maximum folate production were optimized by the Response Surface methodology.

After the preliminary method of optimization (OFAT), the results obtained for maximum folate production are tabulated as following:

Table 4.9: Results of optimization by OFAT method

Culture conditions and media additives	**Optimum Factors**
Carbon source	Lactose
Inoculum age	15 h
Inoculum level	5% (v/v)
pH	6.5
Incubation Temperature	40 °C
Incubation Time	6 h
Inducer	PABA, Glutamate, Mannitol, FOS, GOS

4.7 Optimization through statistical design and analysis

In order to optimize more precise conditions, the experimental design and statistical analysis were done by using statistical software Minitab version 15.1.0.0, USA. The media components optimized through OFAT method were screened for having impact on folate production using Plackett-Burman design (PBD) and determining the optimum level for screened components using central composite design (CCD) method.

4.7.1 Selection of significant variables by using Plackett-Burman design

The Plackett-Burman design (BBD) was applied to find out the significant medium components with respect to their effect on folate production. PBD was a fraction of two-level factorial design, in which each factor is investigated at two widely spaced levels, a high (+1) and a low (-1) level (Plackett and Burman, 1946). A total of seven variables considered for the experimental design were lactose, PABA, Glutamate, Sorbitol, Mannitol, FOS and GOS and their higher and lower levels are designed as given Table 4.10.

The responses from 12 individual experiments were utilized for generating regression coefficient values. The Plackett-Burman design is based on the first-order polynomial model-

$$Y = \beta_0 + \sum \beta_i X_i \qquad (i = 1 \ldots., k)$$

Where, Y denotes the response (Folate production), β_0 is model intercept and β_i is the factor estimates. X_i is the level of the i^{th} independent variable. From regression analysis, the variables showing P-values below 5% level ($P<0.05$) were considered to have greater impact on folate production and used further for central composite design (CCD).

Table 4.10 Media factors used for folate production by *Streptococcus thermophilus* with their higher and lower level using PBD

Factors codes	Factors	Lower level (-1)	Higher level (+1)
X_1	lactose	0.5 g/L	3.0 g/L
X_2	PABA	25 µM	100 µM
X_3	Glutamate	25 µM	100 µM
X_4	Sorbitol	0.2 %	1.0 %
X_5	Mannitol	0.2 %	1.0 %
X_6	FOS	5 g/L	15 g/L
X_7	GOS	5 g/L	15 g/L

By applying the empirical data recorded from each 12 sets of experiments to the BBD, the predicted values of folate production were obtained and shown in Table 4.11.

On the basis of analysis of variance (ANOVA) and values of coefficient for significance ($P<0.05$), four factors out of the seven, *viz.* lactose, PABA, Glutamate and mannitol were found to have significant effect on the folate production. The *P*-value was the probability of magnitude of a contrast coefficient due to random process variability**.**

Table 4.11 Experimental conditions designed by using PBD for folate production by *Streptococcus thermophilus* and their results

Run	Media components							Folate Production (µg/L)	
	Lactose (X_1)	PABA (X_2)	Glutamate (X_3)	Sorbitol (X_4)	Mannitol (X_5)	FOS (X_6)	GOS (X_7)	Observed value	Predicted value
1.	3.0	25	100	1.0	0.2	15	5	69.3	69.75
2.	0.5	25	100	1.0	1.0	5	15	67.20	68.55
3.	0.5	25	25	0.2	0.2	5	5.0 5	69.10	68.35
4.	0.5	100	100	0.2	1.0	5	1.0 5	71.50	70.92
5.	0.5	25	25	1.0	1.0	15	1.05	77.70	77.68
6.	3.0	25	100	0.2	0.2	5	15	70.20	70.18
7.	3.0	25	25	0.2	1.0	15	15	90.10	89.08
8.	3.0	100	25	1.0	0.2	5	5	82.70	83.02
9.	0.5	100	25	0.2	0.2	15	15	70.40	72.18
10.	0.5	100	100	1.0	0.2	15	15	64.40	62.62
11.	3.0	100	100	0.2	1.0	15	5	81.30	81.88
12.	3.0	100	25	1.0	1.0	5	15	93.10	92.78

4.7.2 Pareto chart

In order to see the relevance of each factor on the production of folate, a Pareto chart was prepared and shown in Figure 4.12. It is clear that lactose has significant effect on the folate production. The order of significance was as below:

Lactose > Glutamate > Mannitol > PABA

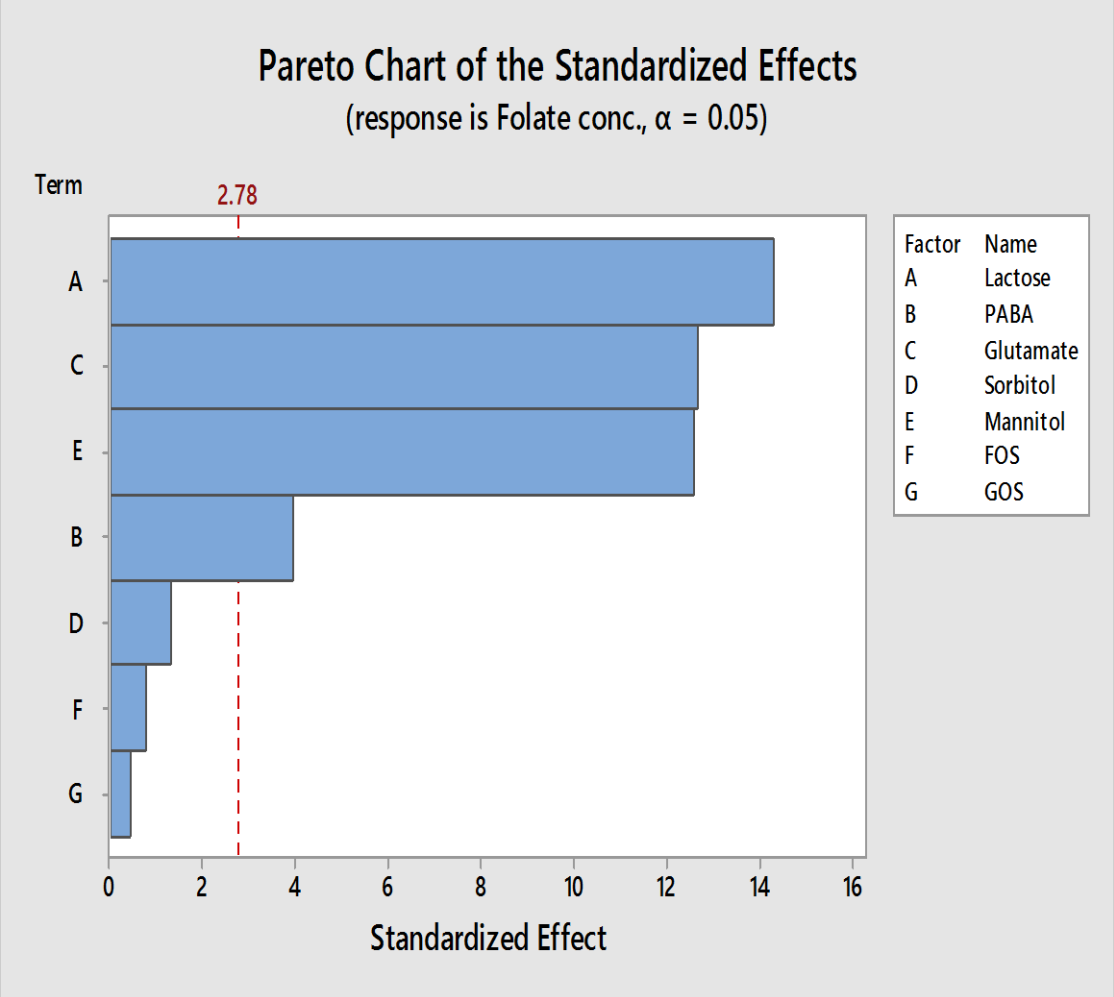


Fig. 4.15 Pareto chart on the basis of PBD showing significant factors for the folate production

The statistical analysis with effects, value of coefficients, standard error of coefficients, *t* and *P* values of the experimental design, generated by software has been shown in the Table 4.12. The significant and insignificant factors based on their *P*-value are indicated by single and double asterisk respectively in Table as well.

Table 4.12 Statistical analysis through PBD showing coefficients and effects for each factor on folate production

Factors	**Effect**	**Coefficient**	***t*-value**	***P*-value**
Constant		75.583	158.88	0.000
(X_1): Lactose	11.067	5.533	11.63	0.000*
(X_2): PABA	3.300	1.650	3.47	0.026*
(X_3): Glutamate	-9.867	-4.933	-10.37	0.000*
(X_4): Sorbitol	0.300	0.150	0.32	0.768**
(X_5): Mannitol	9.133	4.567	9.60	0.001*
(X_6): FOS	-0.100	-0.050	-0.11	0.921**
(X_7): GOS	0.633	0.317	0.67	0.542**

R^2=0.9886, Adj - R = 0.9687, Pred. R^2= 0.8976
* Significant at 95% confidence level ($P<0.05$)
** Insignificant at 95% confidence level ($P>0.05$)

From the data shown in Table 4.12, it is clear that Lactose, PABA, glutamate and Mannitol were significant (P<0.05). Based on coefficient values, the following regression equation was obtained:

Folate conc. = 65.70 + 4.427 Lactose + 0.0440 PABA - 0.1316 Glutamate + 0.38 Sorbitol + 11.42 Mannitol - 0.0100 FOS + 0.0633 GOS

From the Table 4.12 it is clear that three factors i.e. lactose, PABA, and mannitol among the four significant factors shows the positive value of effect, which means that the higher folate production can be achieved at higher concentration of these factors. On the other hand only glutamate shows negative value of effect, which means that glutamate facilitate higher folate production at its lower concentrations. A coefficient close to zero value means that a factor has little or no impact on desired output. The *t*-values were calculated by dividing each coefficient through its standard error. The goodness of the fit of the regression model was represented by coefficient of

determination (R^2). For a good statistic model, R^2 should be closure to one. In the presented model, R^2 was 0.9886, which indicated that the model is efficient enough to calculate the variability in folate production.

Folate production data obtained from PBD showed a broad range of variation i.e. 64.40-93.10 µg/L that showed the necessity of further optimization of media components. The insignificant variables (sorbitol, FOS and GOS) were not considered for further optimization experiments.

4.7.3 Optimization of concentration for selected medium components

Response surface methodology (RSM), an empirical combination of mathematical and statistical techniques, is a quite powerful tool for modeling, and optimizing the processes. The significant medium components selected through Plackett-Burman design technique were subjected to central composite design (CCD), a popular second-order experimental design for developing sequential experimentation and predicting the level of factors, to get an optimal response through regression analysis. The effect of four independent variables, *viz*., lactose, PABA, glutamate and Mannitol on the production of folate was studied at five different levels (-2, -1, 0, +1, and +2) (Table 4.13). A full factorial central composite design was performed to build a total of 31 experiments, having 2^4= 16 cube points plus 7 centre points and (4 X 2 = 8) axial points. The experimental design and statistical analysis of the data was done by using statistical software Minitab version 15.1.0.0, USA. The second-degree polynomial equation was used to calculate the relationship between the independent variables and the response. Considering all the linear terms, square terms and by linear interaction terms, the quadratic regression model can be illustrated as -

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j$$

Where, β_0 is the constant, n denotes the number of variables, β_i the slope or linear effect of the input variable X_i and β_{ii} the quadratic effect of input factor X_i and βij is the linear by linear interaction effect between the input variable X_i and X_j. The contour plots

were obtained for the determining the optimum levels of factor variables for maximum folate production.

Table 4.13 Media factors used for folate production with their five different levels using CCD

Factors codes	Factors	Pre-lower level (-2)	Lower level (-1)	Mid-level (0)	Higher level (+1)	Post-higher level (+2)
X_1	Lactose	1.0	1.5	2.0	2.5	3.0
X_2	PABA	25	50	75	100	125
X_3	Glutamate	20	40	60	80	100
X_5	Mannitol	0.2	0.4	0.6	0.8	1.0

All the experimental trials were performed based on the CCD (Table 4.14) in order to get the optimal concentration level of entire significant parameters for maximum folate production.

A second-order polynomial equation relating folate production with the independent factors, *viz*. lactose (X_1), PABA (X_2), Glutamate (X_3) and mannitol (X_5) is shown below:

Folate Conc. = -120.4 + 76.58 Lactose + 1.358 PABA + 1.234 Glutamate
+ 144.0 Mannitol - 14.53 Lactose*Lactose - 0.011393 PABA*PABA
-0.004020 Glutamate*Glutamate - 41.14 Mannitol*Mannitol + 0.1830 Lactose*PABA -
0.1450 Lactose*Glutamate - 35.63 Lactose*Mannitol - 0.001950 PABA*Glutamate
+ 0.1325 PABA*Mannitol - 0.450 Glutamate*Mannitol

Table 4.14 Effect of significant factors on folate production based on CCD criterion

Runs	Media concentration				Folate Production (µg/L)	
	Lactose (X_1)	PABA (X_2)	Glutamate (X_3)	Mannitol (X_5)	Observed value	Predicted value
1.	1.5	50	40	0.4	70.3	70.3542
2.	1.5	100	40	0.4	62.8	65.2875
3.	2.0	75	60	0.2	84.1	82.7583
4.	2.5	100	80	0.4	84.5	82.5542
5.	2.0	75	60	0.6	89.1	91.8571
6.	2.0	75	60	0.6	94.3	91.8571
7.	2.0	75	60	1.0	87.8	87.7917
8.	2.0	75	60	0.6	91.5	91.8571
9.	2.5	50	40	0.4	77.8	77.9042
10.	2.0	75	60	0.6	91.4	91.8571
11.	1.5	100	80	0.8	79.2	79.0208
12.	1.5	50	40	0.8	80.4	82.2708
13.	2.0	75	60	0.6	92.2	91.8571
14.	2.0	125	60	0.6	63.6	62.2583
15.	3.0	75	60	0.6	79.4	79.4250
16.	2.5	100	80	0.8	74.3	75.6708
17.	1.5	100	40	0.8	80.7	79.8542
18.	2.0	75	100	0.6	86.4	87.2417
19.	2.5	100	40	0.8	81.3	82.3042
20.	2.5	50	80	0.4	80.1	82.3708
21.	2.0	25	60	0.6	64.5	64.4917
22.	1.5	50	80	0.8	84.8	85.3375
23.	2.5	100	40	0.4	81.1	81.9875
24.	2.5	50	40	0.8	75.4	75.5708
25.	1.5	100	80	0.4	70.4	71.6542
26.	1.0	75	60	0.6	76.6	75.2250
27.	2.0	75	60	0.6	90.7	91.8571
28.	2.0	75	60	0.6	93.8	91.8571
29.	1.5	50	80	0.4	81.7	80.6208
30.	2.0	75	20	0.6	85.8	83.6083
31.	2.5	50	80	0.8	75.4	72.8375

4.7.4 Analysis of variance

The significance of model terms (linear, squared, and quadratic) is analyzed by performing the analysis of variance (ANOVA). The Table 4.15 shows regression coefficients for ANOVA.

Table 4.15 ANOVA study for folate production

Source	**Degree of freedom**	**Adjusted sum of square**	**Adjusted mean of square**	***F value***	***P value***
Regression	14	2194.15	156.72	39.91	0.000
Linear	4	40.56	10.14	2.58	0.077
Square	4	1707.90	426.98	108.73	0.000
Interaction	6	394.50	65.75	16.74	0.000
Residual Error	16	62.83	3.93	-	-
Lack-of-Fit	10	43.69	4.37	1.37	0.363
Pure Error	4	19.14	3.19	-	-
Total	30	2256.97			

The ANOVA table depicts that the R^2 value (multiple correlation coefficient) for the regression model was 0.9722 suggesting that 97.22% of variability in folate production is explained in the regression model. The *P*-value for "lack of fit test" (0.363) indicates the quadratic model adequately fits the data. The *P*-value of the coefficients for all linear as well as quadratic relationship suggests they have high significance in the folate ($P < 0.0001$).

4.7.5 Regression analysis for determining interaction between factors

In order to study the interaction between process variables, the regression analysis was carried out using Minitab software. The resulted data are shown in Table 4.16.

Table 4.16 Regression coefficients for response through RSM for folate production

Model term	Coefficient	Standard error coefficient	*t*-value	*P*-value
Constant	91.634	0.734	124.85	0.000
(X_1): Lactose	-0.037	0.549	-0.07	0.946
(X_2): PABA	-1.290	0.549	-2.35	0.032
(X_3): Glutamate	-1.880	0.859	-2.19	0.044
(X_5): Mannitol	-0.092	0.549	-0.17	0.870
($X_1.X_1$): Lactose x Lactose	-3.633	0.371	-9.80	0.000
($X_2.X_2$): PABA x PABA	-7.121	0.371	-19.22	0.000
($X_3.X_3$): Glutamate x glutamate	-2.513	0.579	-4.34	0.001
($X_5.X_5$): Mannitol x Mannitol	-1.646	0.371	-4.44	0.000
($X_1.X_2$): Lactose x PABA	2.288	0.495	4.62	0.000
($X_1.X_3$): Lactose x glutamate	-1.812	0.619	-2.93	0.010
($X_1.X_5$): Lactose x Mannitol	-3.563	0.495	-7.19	0.000
($X_2.X_3$): PABA x glutamate	-1.219	0.619	-1.97	0.067
($X_2.X_5$): PABA x Mannitol	0.662	0.495	1.34	0.200
($X_3.X_5$): Glutamate x Mannitol	-2.250	0.619	-3.63	0.002

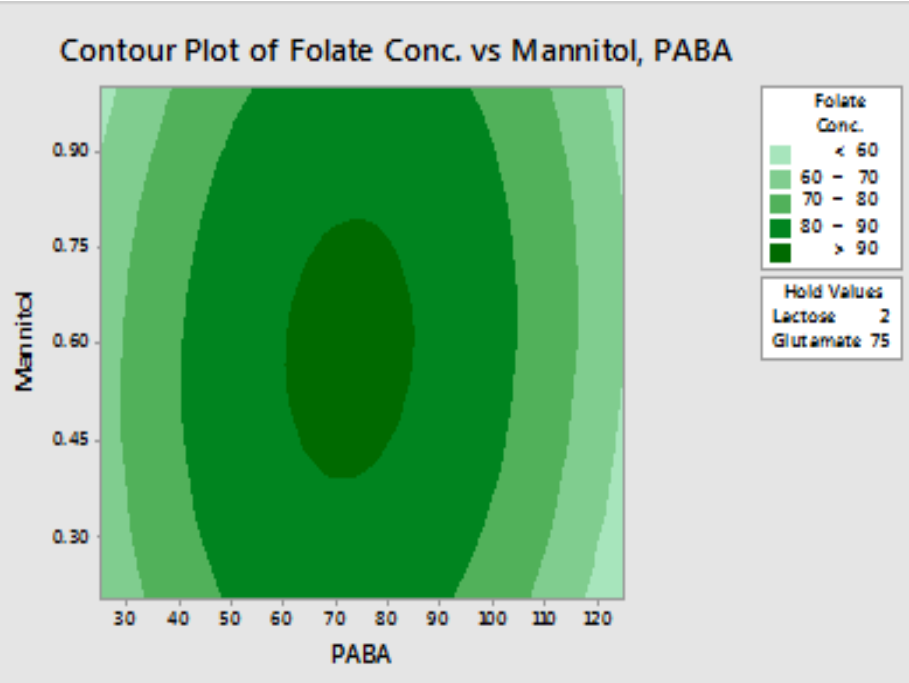
In the presented model, R^2 was 0.9722, which is closure to one, indicated that the model is accurate and well related for folate production.

4.7.6 Contour plots for analysis of interaction between parameters

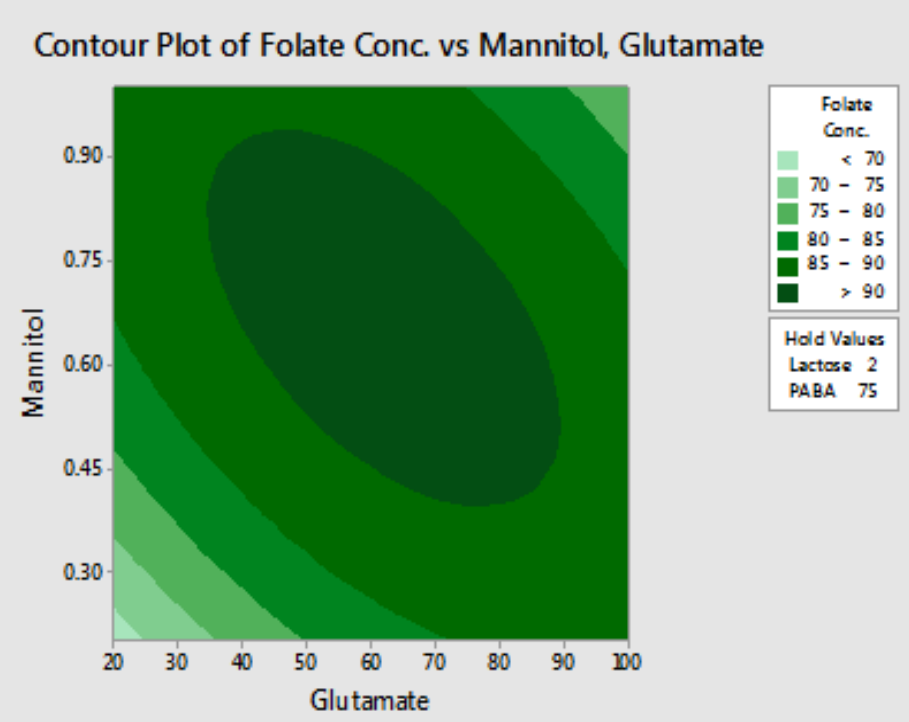
The two-dimensional contour plots were constructed to achieve the main goal of optimization of media constituents for maximum folate production by *S. thermophilus* (Figure 4.16 a-f). Each contour plot illustrates the effects of two parameters on the folate production, keeping other two parameters constant at their middle value. The predictive concentration of folate for a particular set of was numerically represented inside the plot. Further "crosshairs" tool of MINITAB 15 software can be utilized to explore the predictive response at any particular point. From the analysis of contour plots (figure

4.13a-f), the optimum combination of parameters for the maximum production of folate was found as follows: Lactose 1.96 %, PABA 74.49 µM, Glutamate 62.02 µM and Mannitol 0.67 g/l whereas, optimum folate production was found to be 92.12 µg/L.

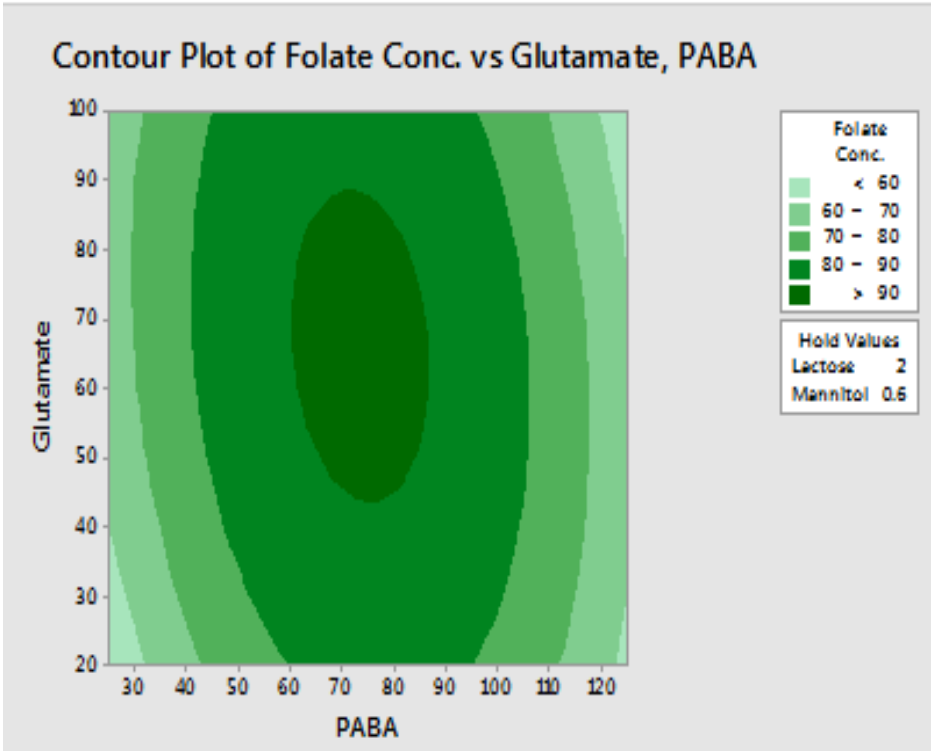
a)



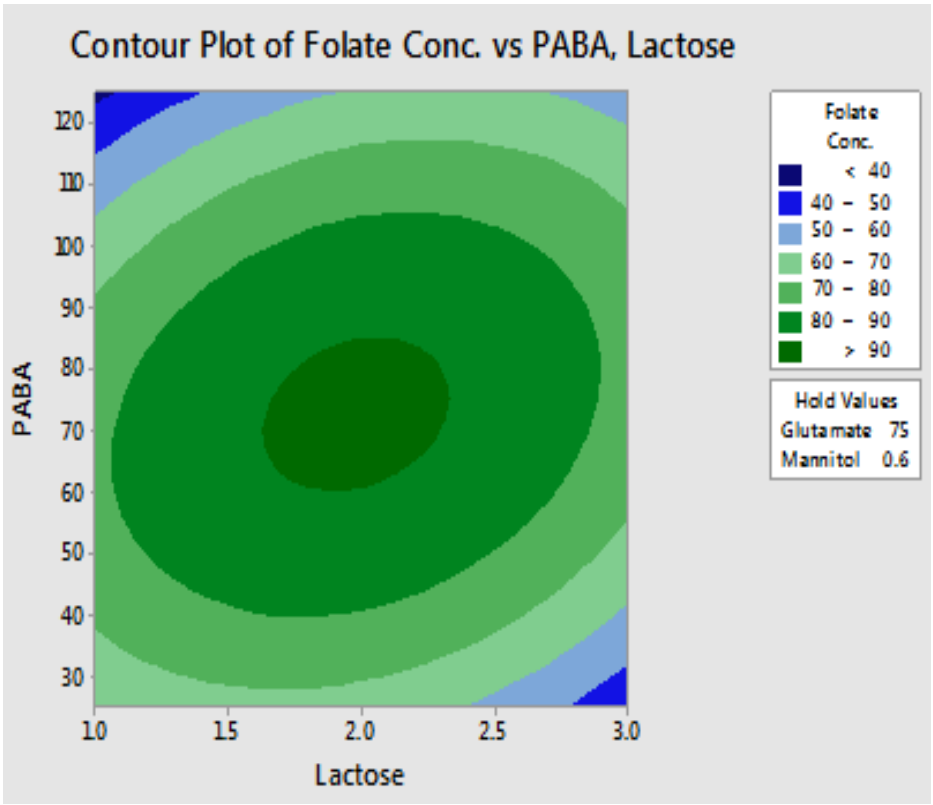
b)



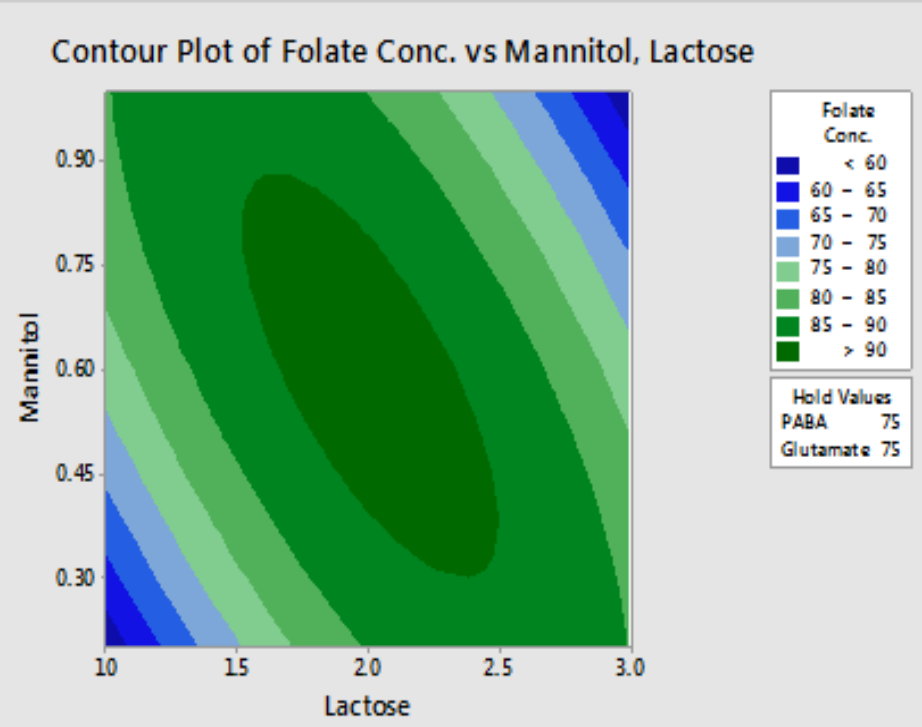
c)


Contour Plot of Folate Conc. vs Glutamate, PABA
Glutamate
PABA
Folate Conc.
< 60
60 – 70
70 – 80
80 – 90
> 90
Hold Values
Lactose 2
Mannitol 0.6

d)


Contour Plot of Folate Conc. vs PABA, Lactose
PABA
Lactose
Folate Conc.
< 40
40 – 50
50 – 60
60 – 70
70 – 80
80 – 90
> 90
Hold Values
Glutamate 75
Mannitol 0.6

e)



f)

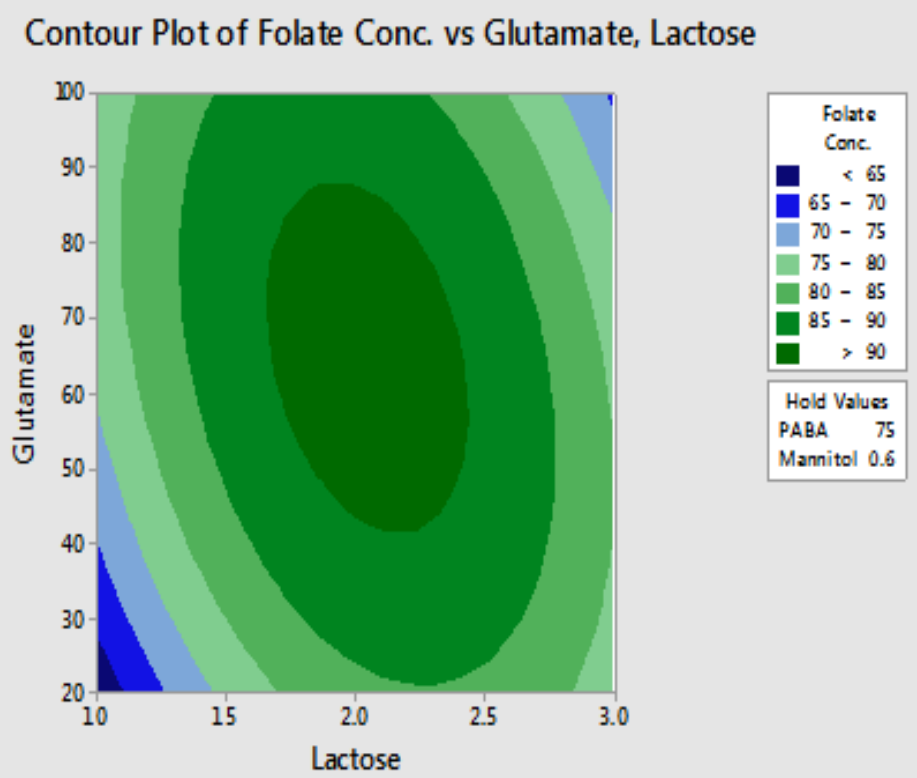


Fig. 4.16 Contour plot for folate production showing the synergistic effects of (a) Mannitol and PABA, (b) Mannitol and Glutamate, (c) Glutamate and PABA, (d) PABA and Lactose, (e) Mannitol and lactose, and (f) Gluatamte and lactose

4.7.7 Validation of Experimental designs

In order to validate the optimal results generated by RSM models, independent experiments were performed using the optimum levels of significant factors and at middle level of other media components at same physical conditions. Experimentation with RSM data, the observed folate production was 95.34 µg/L, which is in good accordance with model predicted value, 92.12 µg/L. Thus the final optimum cultural conditions and production media constituents for the further fermentation studies were as follows:

Table 4.17 Final optimum media constituents and cultural conditions for the maximum folate production in reconstituted skim milk medium

Media Additives and cultural conditions	**Optimum values**
Skim Milk	10 % (w/v)
Lactose	1.96% (w/v)
PABA	74.49 µM
Glutamate	62.02 µM
Mannitol	0.67 g/l
Incubation Time	6 h
Incubation temperature	40°C
pH	6.5
Inoculum volume	5.0 % (v/v)
Inoculum age	15 h

4.8 Folate production studies using Immobilized cells of *S. thermophilus* and comparative analysis with the free cells

Immobilization studies for folate production have been carried out in optimized production media i.e. Reconstituted skimmed milk medium supplemented with 1.96% of lactose, PABA 74.47 µM, Glutamate 62.02 µM and mannitol 0.67 g/L. Immobilized beads of *S. thermophilus* of approximately 2 mm size were inoculated in the production media and incubated for 30 h under static condition at 40 °C. For the immobilization of cells, entrapment within food grade porous matrix like alginate, starch, milk proteins, etc. is preferred because this method is most commonly used in dairy industry (Prevost & Divies, 1992; Doleyres *et al.*, 2002). It also provides a physical barrier to the immobilized probiotics microorganisms against the harsh gastrointestinal passage conditions thereby enhancing the number of viable cells reaching the small intestine for the consumer benefits. It also promotes the controlled release of the probiotics and passage of metabolites. Although in this study there is no *in vitro* survival study, still it has been observed in previous study that entrapment techniques of immobilization also improves the survival efficiency of the probiotic strain present in the fermented milk upon ingestion (Sultana *et al.*, 2000; Krasaekoopt *et al.*, 2006). It has been observed in figure 4.17 that fermentation time was extended from 6 to 12 h for the immobilized cells which may be due to the low mass transfer caused by the permeability barrier and low metabolic activity. Folate production by the immobilized cells was found to be 90.57 µg/L at 12 h which was even lesser than the folate production by free cells (98.65 µg/L) at 6 h. It has also been observed that immobilization of cells stabilizes the folate degradation ability on longer incubation time as it is very prominent in free cells. Thus it may be concluded that immobilization provides prolonged viability of the cells for the longer incubation time and storage. Various studies were there for the yogurt production and lactic acid production by the immobilized probiotic cells and their stability during the gastrointestinal tract either *in vitro* or *in vivo (*Kourkoutas *et al.*, 2005; Senthuran *et al.*, 1999*)*. For the study, calcium alginate is chosen preferably due to the wide acceptance as a material for the probiotics immobilization and due its nontoxic nature and relatively simple and cheap procedure.

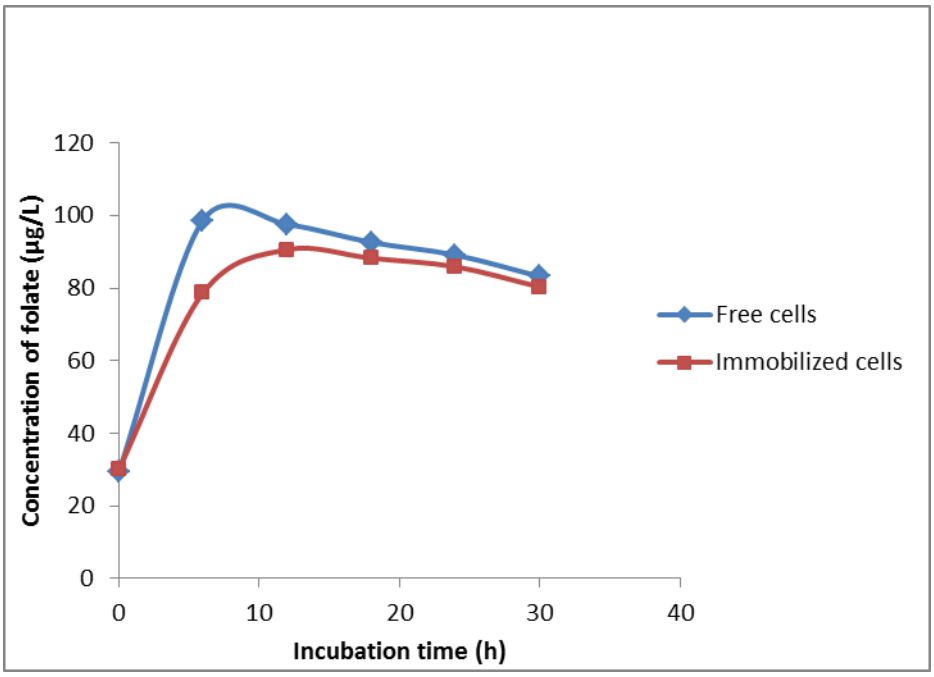


Fig. 4.17 Comparative profile of folate production by the free and immobilized cells of *S. thermophilus* in calcium alginate beads

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

The natural production media (reconstituted skim milk medium) was used for these studies of folate production by *S. thermophilus*, which is a probable potential probiotic strain. This whole process can also be referred as microbial fortification. Fortification is the process of adding some scientifically significant micronutrients to foodstuffs by various means without affecting the original constituents. Among the various methods, microbial fortification or microbial biofortification is the process of adding probiotics to the foodstuff so that it can exert the health benefits or it can produce the useful metabolite in the fermented foods. These kinds of food can exert the dual advantages as being probiotics and enhanced beneficial metabolite content. Thus the enhanced folate content in fermented milk by the probiotic strain i.e. *S. thermophilus* is an example of microbial biofortification of folate in fermented milk. After the extensive work of microbial biofortification in fermented milk, this study was further extended to some more food stuffs which are more likely to consume by most of all age groups without any restriction of choice and taste like cakes and fruit juices. In this section, microbial biofortification

using *S. thermophilus* was implemented in the fruit cake, orange juice and tomato juice to evaluate the folate content.

4.9.1 Microbial folate fortification in fruit cake

In the fruit cake pieces, *S. thermophilus* was allowed to grow and incubated for 24 h. It has been observed from the figure 4.18 that folate production by the *S. thermophilus* in fruit cake was continues to increase till 18 h after the concentration tends to decrease a bit. Maximum folate concentration was found to be enhanced by the 2.7 fold (22.62 µg/100g) than the initial level of folate in the fruit cake (8.38 µg/100g). However the texture became little bit harder which is not likely to be consumable in a favorable manner so the further observation was needed to check the folate level before the 18 h. it has been further checked that folate concentration was likely to be enhanced by 2.36 fold (19.78 µg/100g) till 6 h which is more significant to be taken under further consideration. So for the fruit cake fortification, optimum incubation time was chosen as 6 h till this time consistency and flavor of the cake did not affected very much so it may be gracefully and beneficially consumed by the person of all age groups. Further there is more scope to optimize the various parameters to optimize the maximum folate production in fruit cake without affecting its basic flavor, consistency and softness.

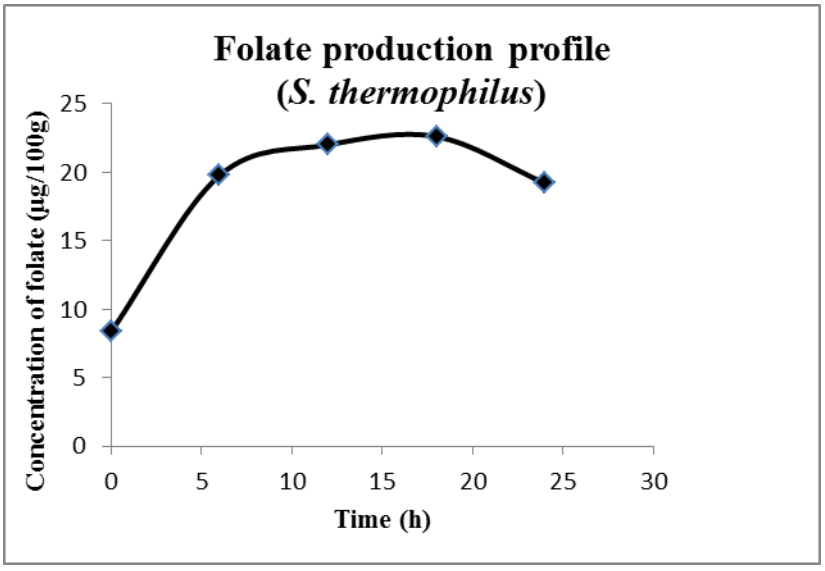


Fig. 4.18 Folate production profile in fruit cake by the *S. thermophilus*

4.9.2 Microbial folate fortification in Orange Juice

Although in the orange juice some folate content is available but that is not sufficient to meet the daily recommended intake in small quantity like a small cup. So in this study microbial biofortification was tried in the orange juice. Membrane sterilized orange juice was inoculated with *S. thermophilus* and folate content was measured. It has been observed that folate content was found to be maximum at 6 h i.e. 45.3 µg/100mL (Figure 4.19). Thus folate concentration was enhanced up to 2.35 fold of the initial value of folate in orange juice (19.23 µg/100mL) in 6 h. After that folate concentration showed the similar reduction in folate concentration profile as observed earlier in the fermented milk. It may be due to the behavior of yogurt starter culture and lactic acid bacteria as the folate consumers for the metabolic activities and further growth as well as folate producers. At 24 h of incubation time the folate concentration decreases by 13.48 % (39.13 µg/100mL). Similar kind of observation was reported with *L. lactis ssp. cremoris* which was inoculated in cucumber juice and melon juice. Folate content was increased to 60±1.9 ng/mL from 10±0.2 ng/mL in cucumber juice whereas increment was 26±1.6 ng/mL from 18±0.9 ng/mL in melon juice (Gangadharan & Nampoothiri, 2011).

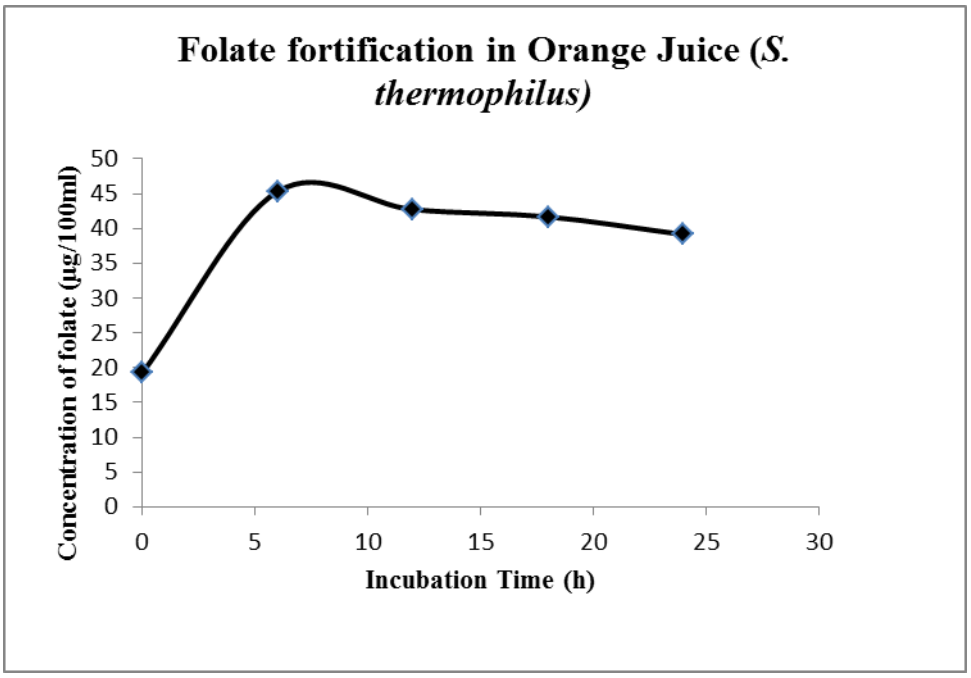


Fig. 4.19 Folate production profile in orange juice by the *S. thermophilus*

In the next phase of study, PABA and glutamate (50µM) which acts as precursors were also added in the orange juice and its effects on the folate production was studied. It has been observed that folate concentration was increased to 53.5 µg/100mL (2.78 fold) in 6 h on PABA addition. Glutamate addition also enhanced the folate level to 57.67 µg/100mL (2.99 fold) in 6 h (Figure 4.20). Combination of both PABA and glutamate addition increased the folate concentration to the maximum level of 64.8 µg/100mL (3.36 fold) than the initial folate level of orange juice 19.23 µg/100mL. It has also been noticed that reduction in folate concentration till 24 h is lesser in case of addition of PABA and glutamate addition than without any additives. Only 3.53% reduction in folate concentration was noticed on both PABA and glutamate addition at 24 h in comparison to 6 h. PABA and Glutamate (25 µmol/L) was also added as precursor in a study reported previously by Gangadharan & Nampoothiri, 2011. In this study, PABA and glutamate was added in cucumber and melon juice which is fermented by *L. lactis subsp. cremoris* which ultimately elevated the folate level of these juices. Folate level of cucumber juice did not show significant results on PABA and glutamate addition whereas folate content was enhanced to 36 ng/mL from the initial level of 10 ng/mL water melon juice.

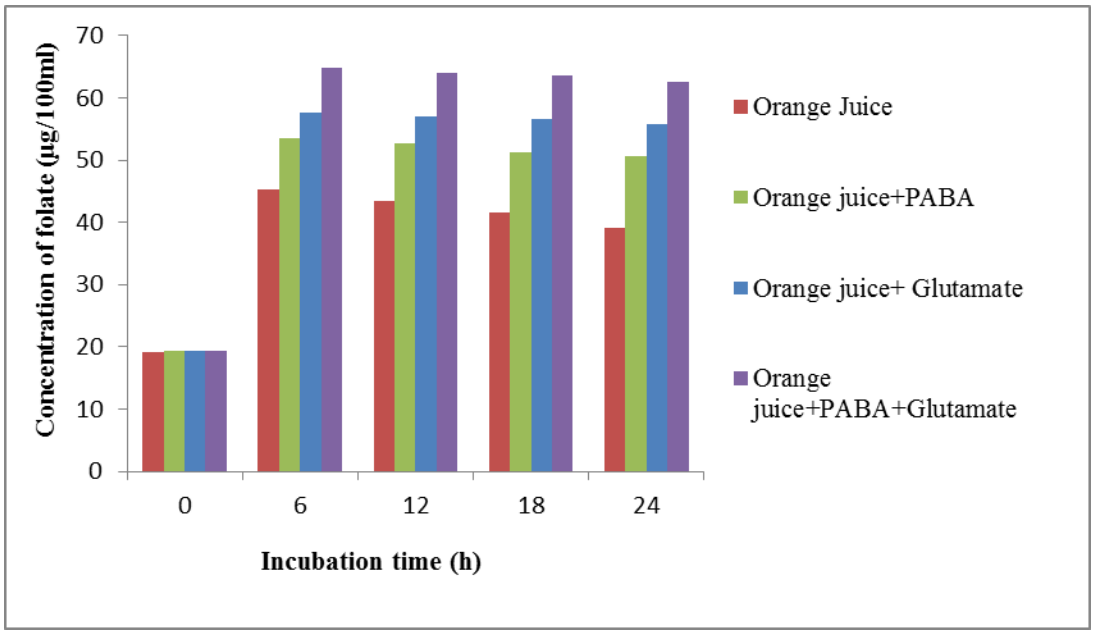


Fig. 4.20 Comparative folate production profile in orange juice by the *S. thermophilus* without any additive, with PABA and glutamate addition

4.9.3 Microbial folate fortification in Tomato Juice

Similarly in the tomato juice some folate content is available but that is not sufficient enough to take in even large quantity to meet the daily recommended intake. Like the orange juice, microbial biofortification was also tried in the tomato juice. Membrane sterilized tomato juice was inoculated with *S. thermophilus* and folate content was measured at different time interval. It has been noticed that folate content was enhanced to 33.56 µg/100mL than the initial folate level of 19.83 µg/100mL in 6 h (Figure 4.21). Thus folate concentration was increased by the 1.69 fold to the initial value. After that a decline trend was observed and folate concentration was reduced to the 25.32 µg/100mL at 24 h that is equivalent to 24.55 % reduction in folate content.

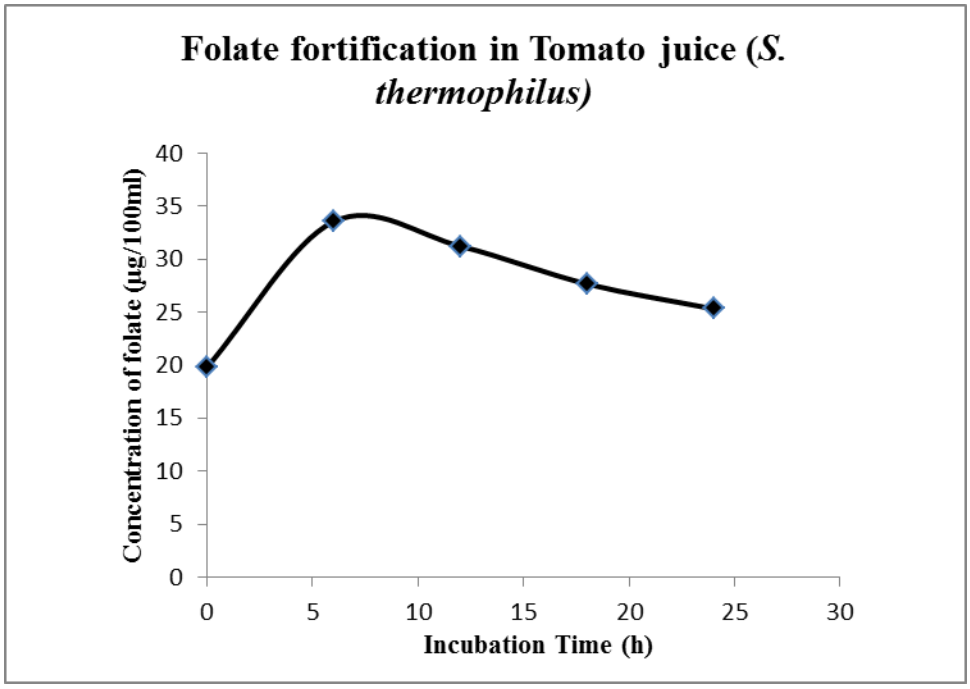


Fig. 4.21 Folate production profile in tomato juice by the *S. thermophilus*

In the next phase of study, PABA and glutamate (50µM) were also added in the tomato juice to evaluate its effect on folate production. It has been observed that folate concentration was increased to 42.39 µg/100mL (2.13 fold) on PABA addition and 42.86 µg/100mL (2.16 fold) on glutamate addition in 6 h. Simultaneous Glutamate and PABA addition increased the folate concentration to the maximum level of 43.7 µg/100mL (2.20

fold) than the initial folate level of tomato juice 19.83 µg/100mL at 6 h (Figure 4.22). Decline in folate concentration after 6 h is less on PABA and glutamate addition. However, it has been noticed that glutamate addition did not affect the folate production in tomato juice. Although it is not necessary those precursors exert same response in terms of folate production in different fermentation medium. It may also be said that higher or lower concentration than the 50 µM might exert better results for folate production in tomato juice.

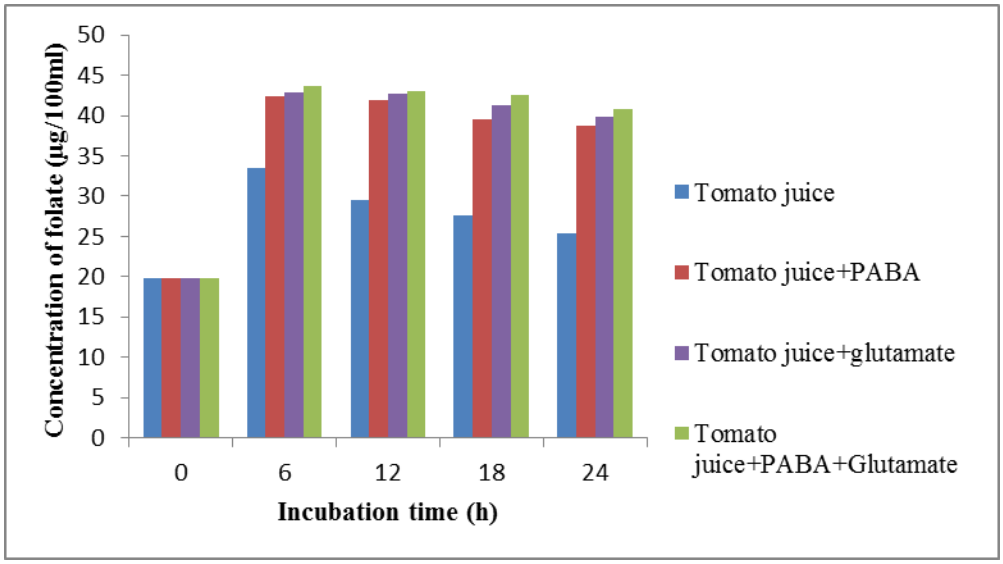


Fig. 4.22 Comparative folate production profile in tomato juice by the *S. thermophilus* without any additive, with PABA and glutamate addition

4.10 Efficacy and Stability studies of fortified food products

During the whole study folate production or microbial folate fortification was studied in the fermented milk, fruit cake, orange juice and tomato juice. All these folate rich probiotic fermented food stuffs can be consumed if prepared using all the safety criteria at all the level as such without any purification needed. For the market purpose, long term storage of these products is also an important issue to be discussed. So in this phase of study, efficacy and stability studies of these folate rich probiotic food stuffs has been carried out. Samples were collected after every week till fourth week and stored at low temperature until analysis was done. Folate content itself along with the viable cell count and pH are the important factors which are taken under consideration in this study

to evaluate the shelf life of the products. Viable cell count is important factor for the probiotic functional food as the microorganism should be live on long term storage at low temperature before ingestion and even after ingestion in the gastrointestinal tract. Viable cell count (log cfu/ml or log cfu/g) was calculated by the plating of serially diluted sample on the MRS agar plate at 37°C for 48 h (Daneshi *et al.*, 2013)

4.10.1 Efficacy and Stability studies of folate rich fermented milk

Folate rich fermented milk which is produced by the inoculation of free cells of *S. thermophilus* was evaluated at different levels to check the shelf life of the product when refrigerated at low temperature during the storage. From the table 4.18, it has been observed that folate content was almost stable till 28 days of storage. Folate content was declined by 10.22% (88.47 µg/L) than the initial value at 6 h just before the storage (98.55 µg/L). It is almost negligible as still it is good amount of folate present in fermented milk even after four weeks of storage. Viable cell count was sharply decreased to very less 4.34 log cfu/mL after 28 days of storage. In the probiotic food, minimum viable cell count should be present around the 7 log cfu/mL to exert its health benefits. After 14 days of storage, viable cell count remained only 7.37 log cfu/mL and after this it tends to decline continuously and become less than 7 log cfu/mL. pH of the product was initially found to be 5.6 which is continuously decreased a bit and remain 4.4 after 28 days. After the inspection of all the factors it may be concluded that this fermented milk can be stored for 14 days after that it is not suitable for consumption in terms of probiotics effects. Thus the shelf life of this fermented milk prepared by the free cells of *S. thermophilus* is 14 days.

Table 4.18 Efficacy and stability studies of fermented milk prepared by free cells of *S. thermophilus*

Days	**0**	**7**	**14**	**21**	**28**
Folate content (µg/L)	98.55	96.49	95.56	89.65	88.47
Viable cell count (Log cfu/mL)	10.49	8.56	7.37	5.26	4.34
pH	5.6	5.1	4.9	4.8	4.7

Folate rich fermented milk prepared by the immobilized cells of *S. thermophilus* was also checked for the shelf life in similar manner. It has been noticed that folate content was degraded up to 6.51 % (84.45 µg/L) from the initial value of 90.34 µg/L. It has been observed that folate content was almost stable till 28 days of storage (Table 4.19). Folate content produced by free cells was declined by 10.22% (88.47 µg/L) than the initial value at 6 h just before the storage (98.55 µg/L). Thus it is concluded that immobilized cells can increase the shelf life of fermented milk as folate degradation is very less in comparison to free cells. Viable cell count tends to decrease by 45.43% and become 6.34 log cfu/mL after 28 days of storage however log cfu/mL value was found to be more than 7 till the 21 days which is the basic requirement of a probiotic functional food. Thus the shelf life of the fermented milk prepared by the immobilized cells was considered as the 21 days. pH of the fermented milk was varies between 5.5 to 4.4.

Table 4.19 Efficacy and stability studies of fermented milk prepared by immobilized cells of *S. thermophilus*

Days	**0**	**7**	**14**	**21**	**28**
Folate content (µg/L)	90.34	89.67	89.23	87.34	84.45
Viable cell count (Log cfu/mL)	11.62	10.86	9.43	8.31	6.34
pH	5.5	5.3	4.9	4.6	4.4

4.10.2 Efficacy and Stability studies of folate fortified fruit cake

Folate rich fruit cake was also evaluated for the shelf life in the similar manner. From the table 4.20, it has been observed that folate content and viable cell count both were decreased abruptly even after 7 days of storage. Folate content was decreased almost 50% and remain only 10.28 µg/100g which was initially 21.23 µg/100g just after the sampling. Viable cell count remained only 5.37 log cfu/mL which means the cake can be stored only up to 7 days. pH was also drastically reduced from 5.6 to 3.6 which showed the acidic nature of the fruit cake till long storage. Besides all these efficacy and stability

parameters, the texture of the fruit cake also gets affected at low temperature. It loosened its soft texture and became hard and dried a lot. So this kind of fruit cake can only be stored for shorter duration less than a week or maximum one week. From this it may be concluded that microbial folate fortification in fruit cake was not that much successful effort.

Table 4.20 Efficacy and stability studies of folate fortified fruit cake prepared by *S. thermophilus*

Days	**0**	**7**	**14**	**21**	**28**
Folate content (µg/100g)	21.23	20.03	15.42	13.34	10.28
Viable cell count (Log cfu/mL)	8.23	7.45	5.37	3.26	2.34
pH	5.6	4.4	4.6	3.9	3.6

4.10.3 Efficacy and Stability studies of folate fortified orange juice with added PABA and glutamate

Folate fortified orange juice with added PABA and glutamate has been evaluated for the shelf life in terms of folate content, viable cell count and pH of the juice after some time of storage. From the table 4.21, it has been detected that folate content was almost stable till 28 days of storage. Initially the folate content was 64.32 µg/100mL which was reduced to 53.48 µg/100mL thus there is not a major degradation in folate content. Only the viable cell count decreased continuously and remains low 6.89 log cfu/mL at 21 days and became 4.67 log cfu/mL at 28 days. This just touches the boundary line of minimum viable cell count of probiotic functional food. pH of the juice is already low as juices are acidic in nature. However, it tends to almost stable and varies in the range of 3.1- 3.7. After the analysis of all the required studies, it may be concluded that orange juice with PABA and glutamate additives can be stored for maximum 21 days.

Table 4.21 Efficacy and stability studies of folate fortified orange juice with added PABA and glutamate by *S. thermophilus*

Days	**0**	**7**	**14**	**21**	**28**
Folate content (µg/100mL)	64.32	62.34	59.46	57.34	53.48
Viable cell count (Log cfu/mL)	9.35	8.92	7.29	6.89	4.67
pH	3.7	3.4	3.3	3.1	3.1

4.10.4 Efficacy and Stability studies of folate fortified tomato juice with added PABA and glutamate

Shelf life of folate fortified tomato juice with added PABA and glutamate has also been evaluated in the similar manner (Table 4.22). Folate content was found to be stable somehow till the four week of storage. Folate content was 43.28 µg/100mL just after the completion of incubation time which was declined by the 15.4% and reached to 36.6 µg/100mL. pH of the folate rich tomato juice was also quite stable during the storage and varies between 3.95-4.6 however the decline in viable cell count was observed till the 28 days and became very low as 3.12 log cfu/mL. Although decline till 28 days was prominent but it was somehow alright after the 21 days of storage i.e. 6.56 log cfu/mL. Finally, it may be concluded that tomato juice supplemented with PABA and glutamate had the shelf life of maximum 21 days.

Table 4.22 Efficacy and stability studies of folate fortified tomato juice with added PABA and glutamate by *S. thermophilus*

Days	**0**	**7**	**14**	**21**	**28**
Folate content (µg/100mL)	43.28	42.9	40.67	37.45	36.6
Viable cell count (Log cfu/mL)	8.67	8.23	7.22	6.56	3.12
pH	4.6	4.58	4.2	4.11	3.95

4.11 Contribution of the Folate fortified food products in the % Daily Value

Percent Daily value is the percentage of any nutrient provided in the one serving of food with respect to the daily need of that nutrient. This is generally mentioned on the nutrition fact label of the food. If it refers as 25 percent for folate, it means that one serving will provide the 25 percent of the folate you need every day. In this section % DV of all the folate fortified products of this work was calculated by considering the daily recommended intake of folate for an adult i.e. 400 µg/day. Table 4.23 showed the % DV of all the fortified food products by this we can have the approximate idea of how much folate we will consume each day in one serving of these folate fortified food products.

Table 4.23 Efficacy and stability studies of folate fortified tomato juice with added PABA and glutamate by *S. thermophilus*

Food Products	**Before Fortification**		**After Fortification**	
	Folate level	**% Daily value**	**Folate level**	**% Daily value**
Fermented milk- free cells	6.26 µg/cup	1.5%	22.55 µg/cup	5.6%
Fermented milk- immobilized cells	6.26 µg/cup	1.5%	21.42 µg/cup	5.35%
Fruit cake	8.38 µg/100g	2.1%	21.78 µg/100g	5.44%
Orange juice supplemeted with PABA & Glutamate	46.91 µg/cup	11.72%	153.30 µg/cup	38.32%
Tomato juice supplemeted with PABA & Glutamate	45.49 µg/cup	11.37%	103.38 µg/cup	25.84%

Serving Size- 1 Cup= 236.58 mL
%DV is calculate by considering 400 µg/day

From the table 4.22, it has been observed that maximum percent daily value was found in orange juice supplemented with PABA and glutamate that is 38.32%. It means consumption of one serving of this orange juice daily will fulfill the 38.32% of daily recommended intake of folate. However least % DV was shown by the fruit cake thus it has least significant effect on daily consumption. Fermented milk prepared by the free cells have greater % DV than the immobilized cells however percentage of gastrointestinal survivability may be higher for immobilized cells. Tomato juice also showed the significant impact on the % daily value as it contributed the 25.84% of daily recommended intake in one serving of juice.

Thus these folate fortified probiotic rich fermented foods should be incorporated in our daily diets to meet the daily recommended intake of folate which ultimately benefit the human health in a great way.