Chapter 5 SUMMARY AND CONCLUSIONS

Folate (folic acid), is a water soluble vitamin which has wide importance for the human nutrition. Deficiency of folic acid in the body lead to the occurrence of diseases like anemia, cardio vascular disease and neural tube defects in the newborns. Folic acid has significant importance for the growth and metabolism of human as involved in the DNA replication, repair and methylation reactions. Folate is needed mainly for the cell division of rapidly proliferating cells such as leukocytes and monocytes. Human cannot synthesize folate itself so dietary consumption is essential. Daily recommended intake for an adult varies between 200-400 µg per day depending on the environmental condition, however, for pregnant women a double dose is essential for the proper development of the fetus. Some natural food sources are available but that is not sufficient to complete the daily recommended value. Some folate supplements are also available in the market but cause severe side effects on long term consumption hence we cannot take it for lifelong. However there is growing need to produce the folate to combat the deficiency despite the availability of food sources and supplements. From some time major attention is given on the microbial production rather than the chemical synthesis. Most of the lactic acid bacteria were used since centuries for the microbial production of B vitamins. Although strain selection is the important parameter for folate production to be considered as some lactic acid bacteria not only synthesizes folate but also consume it for their own growth and metabolism more or less after a definite time period. Current trend is growing towards the natural folate production using probiotic strains in natural fermentation medium so that the fermented food product can be consumed as it is. This procedure is also advantageous as it eliminated the need of the purification and cost of downstream processing.

Total ten microbial strains viz. *Streptococcus thermophilus* NCIM 2904, *Streptococcus thermophilus* NCIM 2412, *Streptococcus lactis* NCIM 2114, *Streptococcus lactis* NCIM 2180, *Lactobacillus helveticus* NCIM 2733, *Lactobacillus delbrueckii subsp. Bulgaricus* NCIM 2025, *Lactobacillus acidophilus* NCIM 2902, *Lactobacillus acidophilus* NCIM 2909, *Lactobacillus bulgaricus* NCIM 2056 and *Lactococcus lactis*

subsp. lactis MTCC 3041 were procured from National Collection of Industrial Microorganisms (NCIM), Pune and Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. All the microbial strains were checked for the ability of folate production. They were initially maintained and subcultured in the MRS medium and incubated at 37°C for 24 h and then they were stored at 4°C until the production studies were carried out.

Preliminary screening of folate producers was done on the basis of growth ability in folic acid assay medium. Folic acid assay medium contained all the necessary media constituents except the folic acid for the growth of lactic acid bacteria. Thus only the microbial strains having folate production ability can grow in the assay medium. Microorganisms showing growth more than or equal to 0.5 OD at 600nm corresponds to probable folate producers. Among the ten strains, eight strains showed the folate production ability as they showed their growth absorbance in the range of 0.578-0.823. Further the secondary screening was done by checking the folate production abilities in both MRS and skimmed milk medium. Almost all the procured strains showed more or less extent of folate production in the MRS and Reconstituted skim milk medium. However, *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 found to be second highest producer showing the folate production of 24.13 µg/L and 28.9 µg/L respectively in MRS medium and reconstituted skimmed milk medium as compared to other tested microorganisms. From the results of both the preliminary 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 for folate production. Hence, these two microorganisms were used for further production studies. Extraction of folate was done using human plasma and quantification was done by Microbiological assay using *Lactobacillus casei* NCIM No. 2364 as an indicator microorganism throughout the studies.

Time course profile indicated that both the strains showed maximum folate production in MRS media at 18 h however *S. thermophilus* showed maximum folate concentration in skimmed milk medium at 6 h and *L. helveticus* showed maximum folate

production in 18 h. After the comparative analysis of individual strains in both the medium, only reconstituted skimmed milk medium was chosen as the production medium for the rest of the studies. Reconstituted skim milk medium as production medium also add beneficial advantage due to the presence of the several folate binding proteins in milk which made folate easily bioavailable and more stable.

For the production in natural fermentation medium, microorganism should possess probiotic potential. So, the probiotics efficiency had been evaluated on the basis of WHO guidelines of both the folate producer strains. Tolerance to gastric acidity and bile salts, antimicrobial activity against the potential pathogenic microorganisms, antibiotic sensitivity, bile salt hydrolase activity and cell surface hydrophobicity are the main *in vitro* methods given in the guidelines for the probiotics evaluation efficiency. Based on the results of all these tests, *S. thermophilus* showed the better probiotic potential than *L. helveticus*, so it was used for all the further studies.

Optimization studies for cultural conditions and media additives had been done initially by One factor at a time (OFAT) method. The optimum temperature and pH were examined to be 40°C and 6.5, while the suitable inoculum size was 5% (v/v) and suitable age of inoculum was 15 h. Lactose served as best carbon sources for folate production, whereas PABA and glutamate (precursor) and sorbitol, mannitol, FOS and GOS (prebiotics) addition resulted in increased folate concentration. After the manual optimization, Statistical optimization by RSM was done to screen the significant factors and their optimal concentration for the enhanced folate production. The optimized media constituents were: Skimmed milk-10%w/v, Lactose-1.96%, PABA- 74.49µM, Glutamate-62.02µM, Mannitol-0.67 g/L, Incubation Time- 6 h, Temperature-40°C, pH-6.5, Inoculum size-5.0%, Inoculum age- 15 h. Optimized medium, obtained through RSM software have shown the 1.90 fold increase in comparison to unoptimized medium with maximum predicted folate production of 92.12 µg/l. On validation of optimized media additives and culture conditions, folate production was enhanced to 1.97 fold (95.34 µg/l). The results showed that application of Response Surface Methodology gives enhanced folate concentration than OFAT method.

Further the production studies were carried out using immobilized cells of *S. thermophilus* in calcium alginate beads in optimized milk medium. Most widely used immobilization technology in dairy industry is cell entrapment using food grade porous matrices such as alginate, carragenan, gellan, agarose and the like. Cell entrapment within calcium alginate beads was the employed method in this work due to the wide acceptability of calcium alginate beads in the dairy industry for making probiotic beverages due to its nontoxic nature. It has been observed that fermentation time was extended from 6 to 12 h for the immobilized cells which may be due to 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. Although folate production was found to be lesser in case of immobilized cells however immobilized cells showed promising possibility for the use in the making of probiotic beverages due to the increase in the gastrointestinal survivability.

After the extensive work of folate fortification in milk, further folate fortification studies were performed on some other food matrices. Fruit cake, orange juice and tomato juice were chosen. Folate concentration was enhanced to 2.7 fold at 18 h i.e. 22.62 µg/100g in fruit cake although texture got somehow affected till this time. Folate concentration was found to be 21.78 µg/100g (2.6 fold increase) even in 6 h. So the optimum incubation time is chosen as 6 h for fruit cake fortification. Folate fortification is also possible in orange juice by *S. thermophilus*. Folate enrichment in orange juice was found to be maximum 45.23 µg/100ml (2.35 fold) at 6 h then concentration decreased till 24 h. Folate content was enhanced upto 2.78 fold (53.5 µg/100ml) on PABA addition and 3.36 fold (64.8 µg/100ml) on PABA along with Glutamate addition. Folate fortification in tomato juice also showed positive results. Folate fortification in tomato juice was found to be maximum 33.56 µg/100ml (1.69 fold) at 6 h. Folate content was enhanced upto 2.13 fold (42.39 µg/100ml) on PABA addition and 2.20 fold (43.7 µg/100ml) on PABA along with Glutamate addition than the tomato juice without any additives (19.83 µg/100l). It has been observed that glutamate addition in tomato juice did not affect the folate content markedly than the PABA addition. It has been observed that tomato juice contains slightly more folate than orange juice but production was found to be higher in orange juice. It may be due to higher sugar content of orange juice.

Efficacy and stability studies of folate fortified food products was carried out to check the shelf life of the products. For this folate fortified samples were withdrawn at 6 h of incubation time and stored for four weeks at 4°C. After that folate concentration, pH and viable cell count was measured. Viable cell count should be minimum 7 log cfu/g to exert health benefits in the probiotics enriched food products to be consumed. Folate concentration is almost stable till 28 days however viable cell count decreases abruptly mainly in milk fermented with free cells. It was found that fermented milk with free cells and immobilized cells can be stored upto 14 days and 21 days respectively. Folate content decreased abruptly till 28 days in fruit cake. Viable cell count remained low after 14 days of storage as well as texture also get affected at low temperature so this kind of fruit cake can be stored only for short duration of around one week. Folate content is almost stable in both orange juice and tomato juice with PABA and glutamate upto 28 days of storage only the viable count decreases continuously. In the fourth week of storage viable cell count became too low to be used as probiotic food. So the shelf life of both orange juice and tomato juice could be as maximum 21 days. Still there is a lot of scope of work to be done in the field of microbial fortification in cake as well as in several other food products.

Therefore, it is concluded that folate levels in yogurts and fermented milks can be increased by the proper selection of the folate-producing microbial strains and optimization of their cultivation conditions along with the media additives. Besides individual strain, the food industries can also use the mixture of folate producing strains as part of their starter cultures after extensive research to produce fermented products with prominent levels of this essential vitamin. Such products would provide economic benefits to food manufacturers since increased level of ‘natural folate’ concentration would be an important value added effect without increasing production costs. Consumers would obviously get benefit from such folate rich products since they could increase their folate intakes by consuming these fermented products that essentially is a part of their normal life style. Microbial fermentation also offered advantage as a cheaper process, less time consuming and less labor intensive. But there are still more chances of improvements which can increase folic acid production.

There still remain several technical and economical considerations which require resolution. However, the results establish for the folate production in fermented reconstituted skim milk and fermentative fortification in fruit juices by different processing strategies will be a useful concept, which is likely to be used with considerable advantage. These techniques/methods do exhibit considerable potential and further studies are needed to fully exploit this potential.