CHAPTER 4

Fermentative Production of Folate in Skim Milk by *Lactococcus lactis* CM28

4.1. Introduction

Folate is a generic term for a number of folic acid derivatives that differ in their state of oxidation. The essential B group vitamin with a daily recommended intake of 200 - 400 µg has a major role as a cofactor in normal cellular functions and also in growth and development (Blount et al., 1997; Sybesma et al. (2003b)). To prevent the deficiency it is advised to increase the folate intake of populations to the recommended levels. However, with varying food habits even at individual level it may not be feasible for the whole population to achieve this by consumption of foods naturally rich in folate such as fruits and vegetables (De Jong et al., 2005).

The folate content in milk is low when compared to other folate rich foods (Konings et al., 2001) and pasteurization will result in further reduction of the folate titre. Fermented milk can contribute significantly to the daily recommended intake of folate (Alm, 1982) and the folate producing probiotics could aid in this fortification. The folate binding proteins present in milk can increase its stability and bioavailability and thus it is an ideal matrix for folate fortification (LeBlanc et al., 2007). However, in milk fermentation, majority of the bacteria are folate utilizers consequently decreasing the amount of folate (Lin & Young, 2000). Hence, only by judicial selection of a suitable starter culture or a consortium of folate producers it is possible to increase the dietary folate content. A number of LAB such as *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus plantarum* have been reported to produce folate (LeBlanc et al., 2007; Sybesma et al., 2003b). The folate pools in fermented milk are further influenced by the cultivation conditions, presence of folate precursors, prebiotic supplementation etc (Iyer et al., 2010; Tomar et al., 2009).

The chapter deals with fermentative production of folate by *Lactococcus lactis* CM28 in skim milk and optimization of the fermentation conditions to increase the total and bioavailable folate. The study also aimed to determine the storage stability of the fermented milk.

4.2. Materials and Methods

4.2.1. Bacterial Strains and Growth Conditions

L. lactis CM28 (NCBI Genbank accession number: KJ676682) was used for the present study. *Lb. casei* NCIM 2364 was used for microbiological assay for the quantification of folate. The cultivation conditions of the cultures were described in section 2.2.1.

4.2.2. Inoculum for Folate Fortification of Skim Milk

L. lactis CM28 was inoculated in FAA medium supplemented with nutrient solution (section 3.2.3) and incubated at 37 ºC for 18 h. Inoculum concentration of 1% (v/v) was used for the fortification studies.

4.2.3. Effect of Culture Conditions and Additives on Folate Production

Fermentation studies were carried out in 4% (w/v) skim milk medium. Optimum incubation time and temperature was determined by checking the levels of extracellular folate at various incubation times (6, 7, 8, 9, 10, 11 and 12 h) and temperature (30, 37 and 42 °C). The effect of various additives on folate production was studied individually and the optimized levels of each were incorporated in the subsequent experiments. The efficacy of the prebiotics (sorbitol, mannitol and fructooligosaccharides (FOS)) were analysed by their addition at varying concentrations of 0.2, 0.4, 0.6, 0.8 and 1% (w/v) into the skim milk medium.

Similarly, the effect of the two folate precursors (PABA and glutamate) was evaluated by supplementation at different concentrations (25, 50, 75 and 100 µmol/L). Thereafter, the effect of supplementation of reducing agents like sodium ascorbate, sodium thioglycholate and cysteine hydrochloride (0.2% w/v) at both static and agitating (100 rpm) conditions was evaluated.

4.2.4. Batch Fermentation in Bioreactors

Optimized conditions in the flask level were scaled up in Infors HT (Switzerland) parallel fermenter system (750 mL) with a working volume of 300 mL. The fermenter unit, 350x500x850 mm (width x diameter x height) was equipped with a pH probe, three way inlet, sampling port and DO probe. Agitation was done by a rushton type turbine and was controlled by coupling of the stirrer and control base driven by magnetic stirrer. The

influence of agitation was studied by varying the levels between 50 and 100 rpm. The temperature was maintained at 37 ºC by the aluminium heating block with integral cooling coil and the fermentation was continued till 9 h. Samples were aseptically collected at 7, 8 and 9 h and analysed for folate.

Based on the experimental results in the parallel fermenter, folate production was studied in 5 L stirred tank bioreactor (Biostat B-5; B. Braun Biotech-Sartorius, Germany) with a working volume of 3 L. The system was equipped with six-bladed rushton turbine impeller controlled by an external motor, for agitation. *L. lactis* CM28 grown in FAA medium was used as the inoculum for the modified skim milk production medium. The studies in parallel fermenter gave the highest folate titre at 8 h of incubation and hence, the fermentation was carried out at 37 °C for 8 h at an agitation speed of 50 rpm.

4.2.5. Folate Analysis

Intracellular and extracellular folate was extracted from the fermented milk and the total folate was quantified after enzymatic deconjugation of the samples by human plasma. Folate was quantified by microbiological assay using *Lactobacillus casei* NCIM 2364 as per the protocol described in section 2.2.2.a.

4.2.6. Storage Stability of the Fermented Milk

After fermentation, the skim milk was stored at 5 ºC for 15 days. Samples were collected at 5 days interval and analysed for folate, pH, lactic acid % in terms of titratable acidity (TA). The viable count of bacteria was also determined. TA is determined by titration of the fermented milk with 0.1N NaOH using phenolphthalein as the indicator of endpoint (2.2.2.c). For the viable count of bacteria, the samples were serially diluted in sterile saline and appropriate dilutions were plated onto M17 agar plates supplemented with 0.5% (w/v) glucose. The plates were then incubated at 37 ºC for 2 days. Colony forming units (CFU) were enumerated and the viable count was expressed in log CFU/ml.

All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (SD).

4.3. Results and Discussion

4.3.1. Effect of Culture Conditions and Additives on Folate Production

The extracellular folate production by *L. lactis* CM28 was highest (18.66 ± 0.36 µg/ L) at 8 h of fermentation (**Fig. 4.1**) when 4 % (w/v) skim milk medium supplemented with 6 µM glycine and 6 µM methionine was used. The initial folate content of the medium was 3.82 ± 1.3 µg/L.

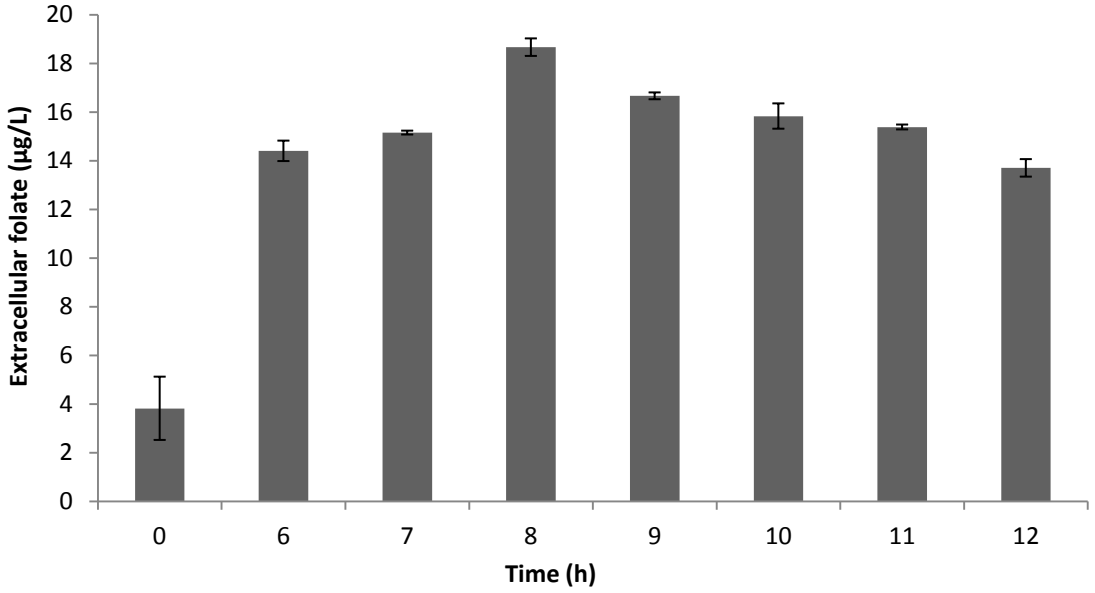

Fig. 4.1. Effect of incubation time on folate production by *L. lactis* CM28

There are previous reports where the folate production was highest between 6 to 8 h of fermentation (Lin & Young, 2000; Padalino et al., 2012). Further increase in incubation time resulted in decreased folate levels. The higher utilization of folate for the growth and metabolism of the bacteria might have lowered the folate content (Kundu & Deep, 2014). Sybesma et al. (2003b) have reported that there was a 10 fold increase in folate production when there was a 90% decrease in specific growth rate.

Temperature is found to have an influence on the activity of enzymes essential for the growth and metabolite production in the microorganisms. Hence incubation temperature is a significant parameter which should be optimized to improve the metabolite production. The most suitable temperature for the maximum folate production by the *Streptococcus thermophilus* NCIM 2904 and *Lactobacillus helveticus* NCIM 2733 was reported as 40 °C and 37 °C respectively by Kundu and Deep (2014). In the present study the incubation temperature of 37 ºC was optimum compared to 30 ºC and 42 ºC

(**Fig.4.2**). The folate production increased from 16.72 ± 0.56 µg/ L at 30 ºC to 18.59 ± 0.28 µg/ L at 37 ºC and dropped to 8.06 ± 0.6 µg/ L at 42 ºC.

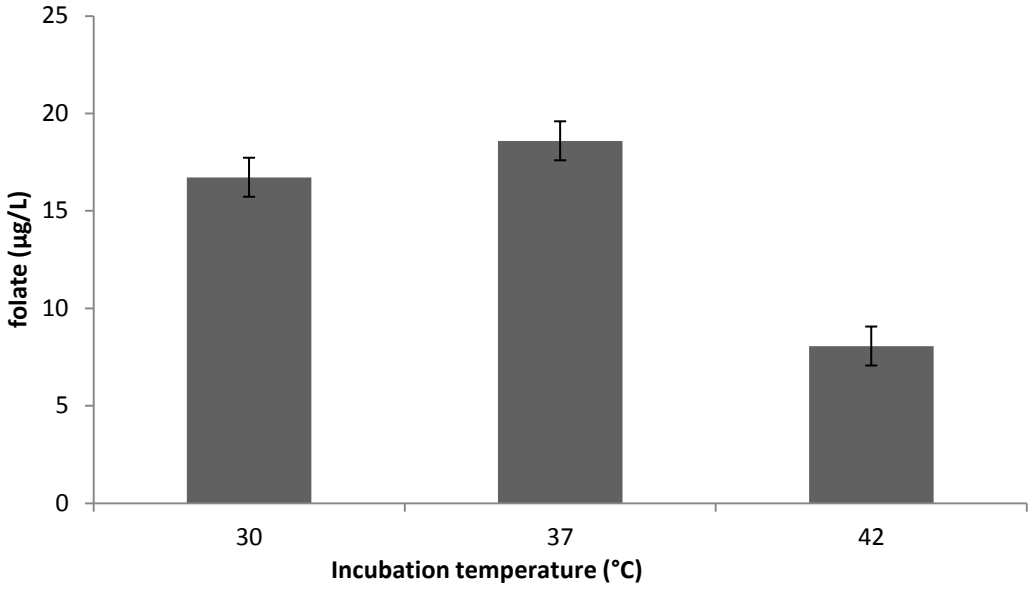

Fig. 4.2. Effect of incubation temperature on folate production by *L. lactis* CM28

Prebiotics are non digestible food ingredients that promote the growth and survival of the probiotics. In this study, the effect of sorbitol, mannitol and FOS at varying concentrations (0.2, 0.4, 0.6, 0.8 and 1% v/w) were checked. An increase in extracellular folate with increase in mannitol was observed up to a concentration of 0.6% (w/v) and further increase in mannitol concentration reduced the folate levels. Similarly, 0.2% (w/v) FOS and up to 0.4% (w/v) sorbitol enhanced the folate levels in fermented milk but the levels dropped beyond this concentration. The results are presented in **Fig. 4.3**.

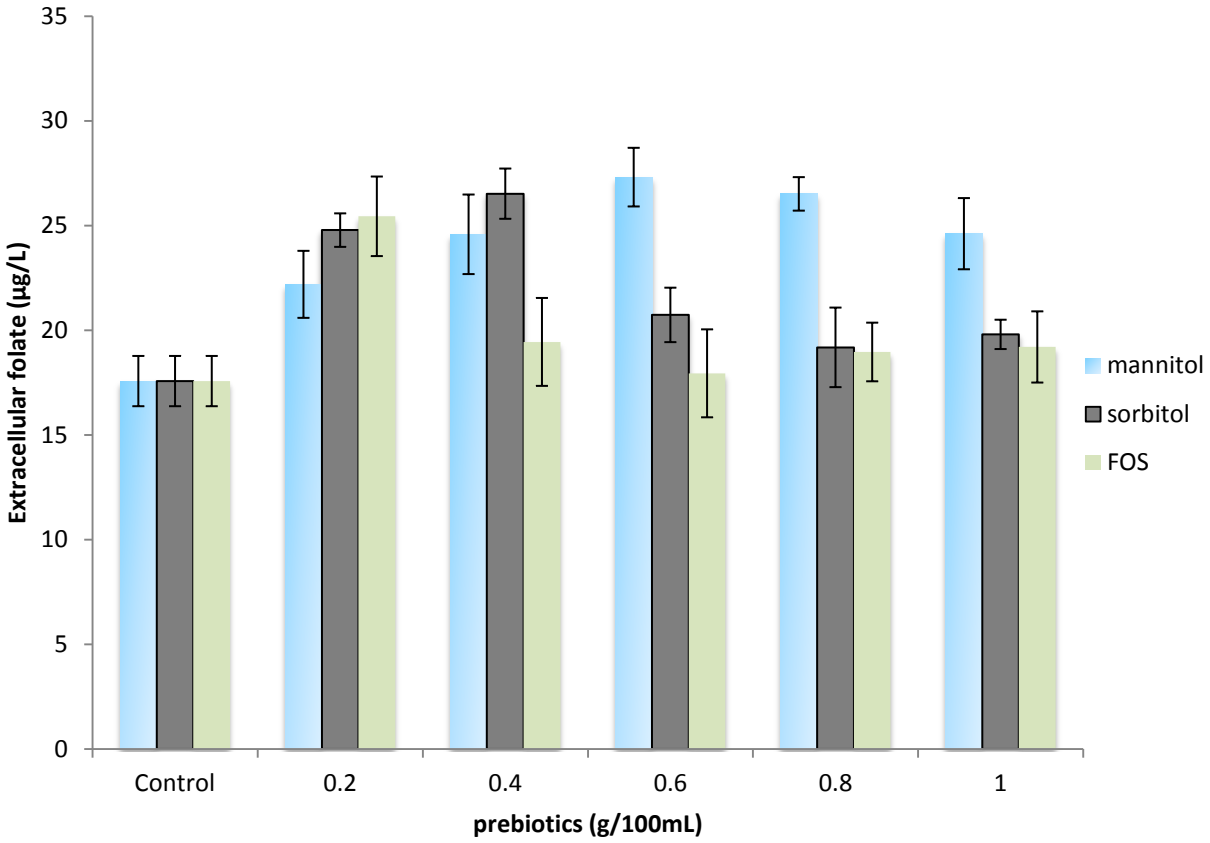

Fig. 4.3. Effect of prebiotics on folate production by *L. lactis* CM28

When compared to different concentrations of sorbitol and FOS, 0.6% (w/v) mannitol had a superior effect on extracellular folate levels which increased up to 27.31± 1.4 µg/ L. In contrary, Padalino et al. (2012) have reported that the use of prebiotics like FOS and galactooligosaccharides (GOS) in the medium had a little effect on the folate production level even though it stimulated the growth of the probiotic bacteria. In their study among the five LAB strains, an increase in folate production was observed in *Lactobacillus plantarum* with FOS supplementation and with the addition of GOS only *Streptococcus thermophilus* showed elevated folate production. In another study, the simulative effect of four prebiotics FOS, mannitol, maltodextrin and pectin on the growth of *Lactobacillus* strains was reported (Yeo & Liong, 2010). In their study, Pricope et al. (2010) showed the effect of sorbitol in reducing the lag period of Bifidobacteria. The synergistic effect of *Lactobacillus paracasei* and FOS combination on faecal microflora of weaned pigs was reported by Bomba et al. (2002). Hence, in general it can be

concluded that supplementation of prebiotics can stimulate the growth but the functional properties like enhanced folate production can be strain dependent.

Folate is synthesized from its precursors PABA, glutamate and GTP. PABA is obtained from the shikimate pathway and GTP is produced through the purine biosynthetic pathway. Folate can be synthesized by *L. lactis* even in the absence of PABA, indicating that *L. lactis* has the ability to synthesize PABA. However, the folate production by *L. lactis* was reported to be dependent on the concentration of PABA in the culture medium suggesting that PABA synthesis is a rate determining step in folate production (Sybesma et al., 2003b). In the present study the addition of PABA at100 µM/L increased the extracellular folate production to 31.93 ± 2.8 µg/ L (**Fig. 4.4**).

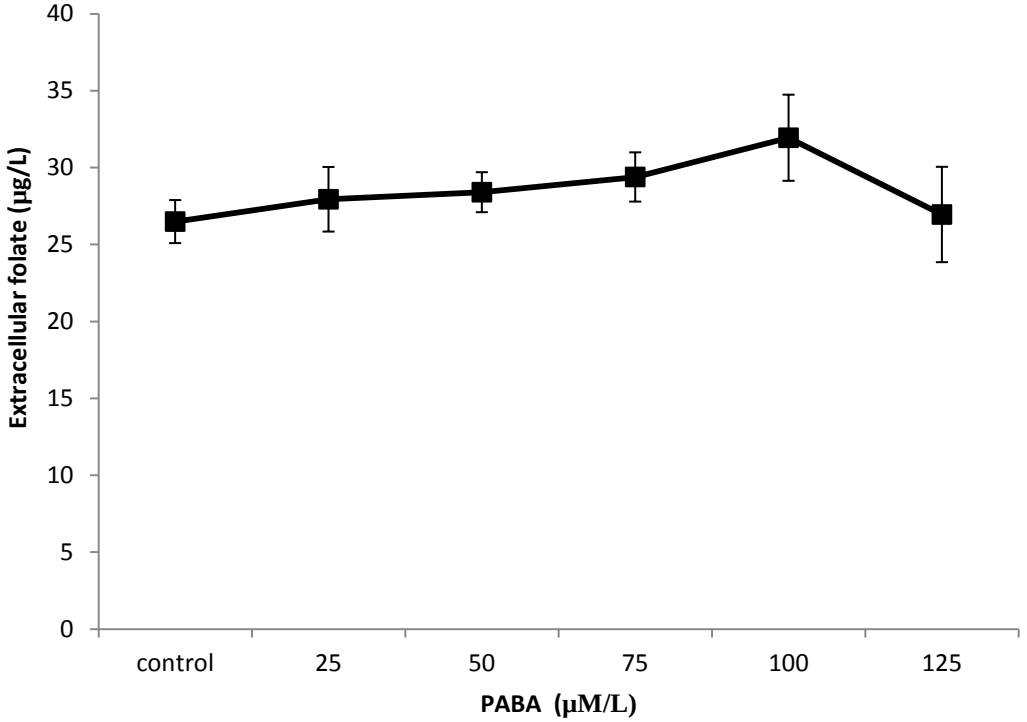

Fig. 4.4. Effect of PABA on folate production by *L. lactis* CM28 in skim milk

The elevated PABA levels in the medium could result in the inactivation of folylpolyglutamate synthase, the enzyme responsible for the elongation of the polyglutamate tail of the folate molecule thereby increasing the amount of secreted folate (Wegkamp et al., 2007). The intracellular retention of polyglutamyl folates is higher than the monoglutamyl folates. Further increase in folate was observed on the addition of glutamate (75 µM/L) to the PABA (100 µM/L) containing medium. The cumulative effect of PABA and glutamate resulted in 37.56± 1.8 µg/ L extracellular folate (**Fig. 4.5**).

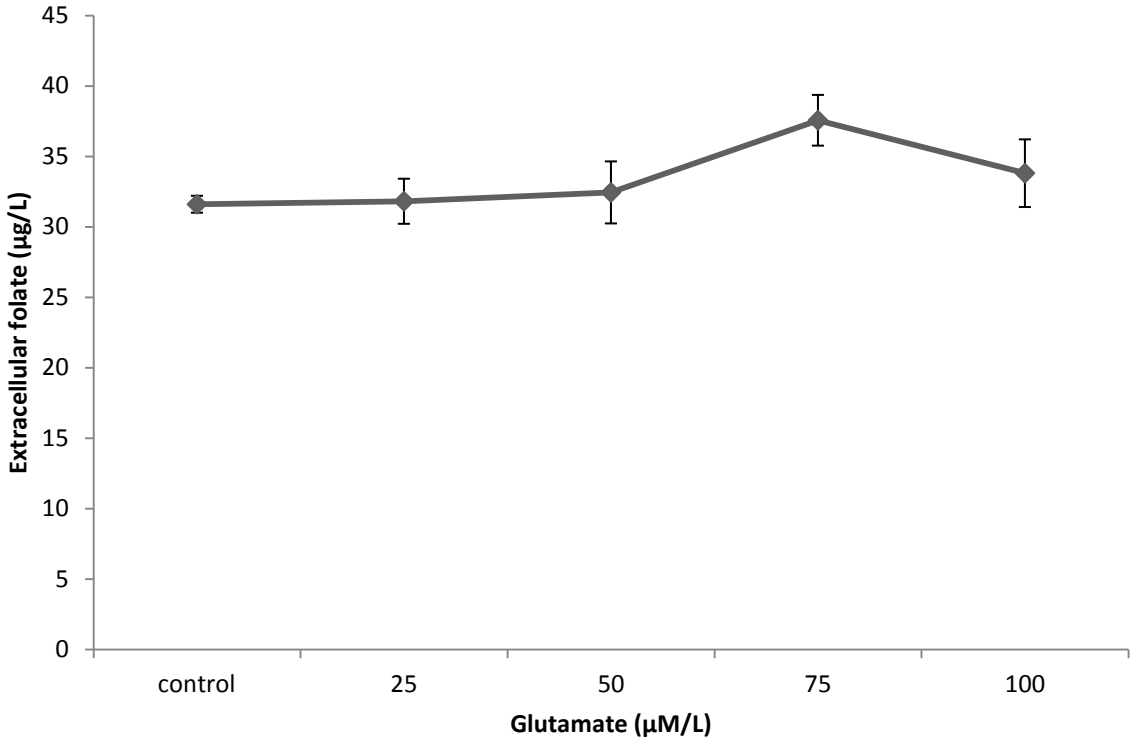

Fig. 4.5. Effect of glutamate on folate production by *L. lactis* CM28

Folate bioavailability is highly dependent on the physico-chemical properties of the folate forms, food ingredients present and stability of the food folate (Seyoum & Selhub, 1998). Folates are highly susceptible to oxidation by air, heat and the acid-peptic milieu of the stomach. Significant loss in activity occurs due to oxidation resulting in conversion to inactive forms such as p-aminobenzoyl glutamate (Forssén et al., 2000). Several studies have shown that reducing agents like sodium ascorbate can prevent the oxidative damage of folate and also result in the reversion of inactive forms to active folate. In our study, highest extracellular folate production of 61.02 ± 1.3µg/L was obtained when the medium was supplemented with 0.2% (w/v) sodium ascorbate. The addition of sodium thioglycholate also improved the extracellular folate content (46.05 ± 2.8 µg/L) when incubated at 37 ºC with an agitation of 100 rpm (**Fig. 4.6**). This increase could be due to the increased stability of the folate in presence of these antioxidants or the higher biomass achieved in microaerophilic environment provided by the reducing agent (Gangadharan & Nampoothiri, 2011).

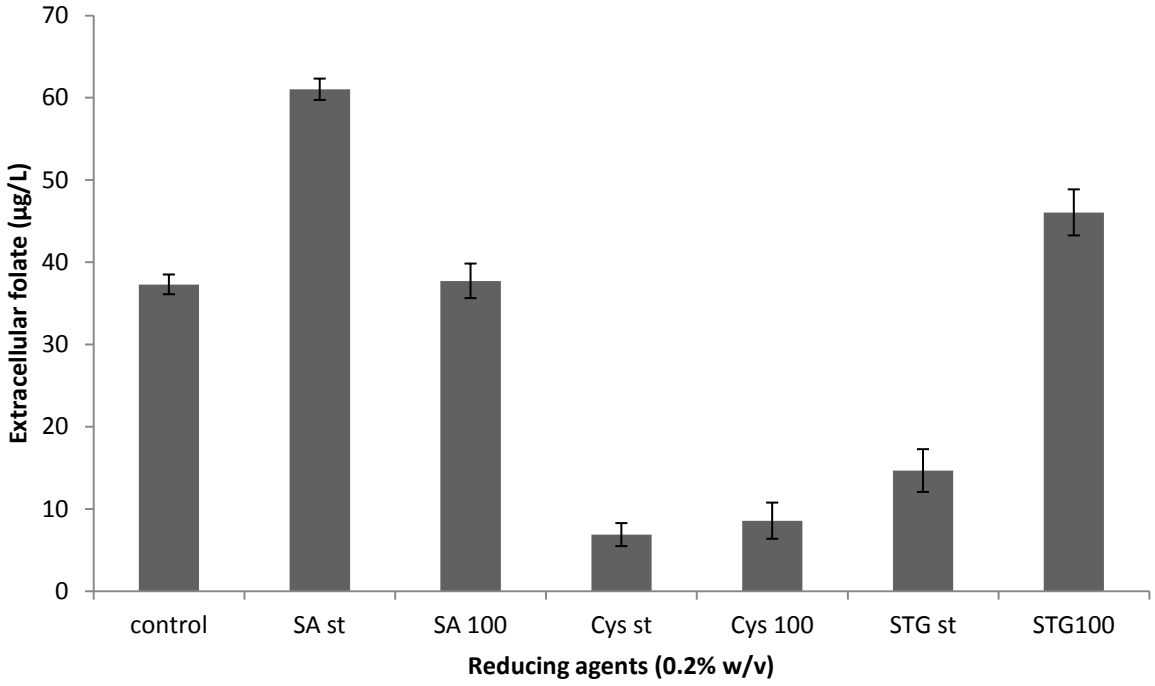

Fig. 4.6. Effect of reducing agents on folate production by *L. lactis* CM28

SA st – Sodium ascorbate (0.2% w/v) at static condition, SA 100 – Sodium ascorbate (0.2% w/v) with agitation (100 rpm), Cys st– Cysteine (0.2% w/v) at static condition, Cys 100 – Cysteine (0.2% w/v) with agitation (100 rpm), STG st – Sodium thioglycholate (0.2% w/v) at static condition, STG 100 – Sodium thioglycholate (0.2% w/v) with agitation (100rpm)

Thus, the optimized medium for folate production by *L. lactis* CM28 was skim milk (4% w/v) supplemented with glycine (6 µmol/L), methionine (6 µmol/L), mannitol (0.6% w/v), PABA (100 µmol/L), glutamate (75 µmol/L) and sodium ascorbate (0.2% w/v) with a fermentation period of 8 h at 37 ºC at static conditions. A fourfold increase in the extracellular folate (61.02 ± 1.3µg/L) was obtained after optimization which can be correlated to the bioaccessible folate.

The total folate (extra and intracellular) production was also determined under optimized conditions after deconjugation with human plasma. Extracellular folates will contain shorter polyglutamyl tails which make them more bioavailable than polyglutamyl folates. Monoglutamyl folates can be directly absorbed in the human gut whereas polyglutamyl folates require enzymatic deconjugation before intestinal absorption and metabolic utilization. The effective intake of folates is highly dependent on the degree of polyglutamylation (Gregory, 2001; Sybesma et al., 2003a). The major portion of folate produced by *L. lactis* is retained intracellularly (Sybesma et al., 2003b). In this study, after deconjugation the total folate detected was 129.53 ± 1.2 µg/L in 100 mL skim milk

medium. This indicated that approximately 53% of folate produced by the LAB strain *L. lactis* CM28 is in the polyglutamyl form and is retained intracellularly (**Table 4.1**). Pompei et al. (2007) demonstrated that intracellular accumulation of folate is highly strain dependent in Bifidobacteria.

Table 4.1. Folate levels in fermented skim milk under optimized conditions (8 h)

Folate accumulation	**Folate (µg/L)**
Extracellular folate	61.02 ± 1.3
Intracellular folate	68.51 ± 4.2
Total folate	129.53 ± 1.2

4.3.2. Batch Fermentations in Bioreactors

The optimized medium from the experiments performed in flask level was used to analyse the folate production by *L. lactis* CM28 in parallel fermenter. The experimental set up is shown in **Fig. 4.7**.

Fig. 4.7. Batch fermentation for folate production in parallel fermenter

Highest titre of 142.3 ± 1.6 µg/L folate was obtained after 8 h fermentation with an agitation of 50 rpm (**Fig. 4.8**). The uniform distribution of nutrients due to proper mixing at 50 rpm might have resulted in the slight increase in folate compared to static conditions where the folate titre was 138.02 ± 0.1 µg/L.

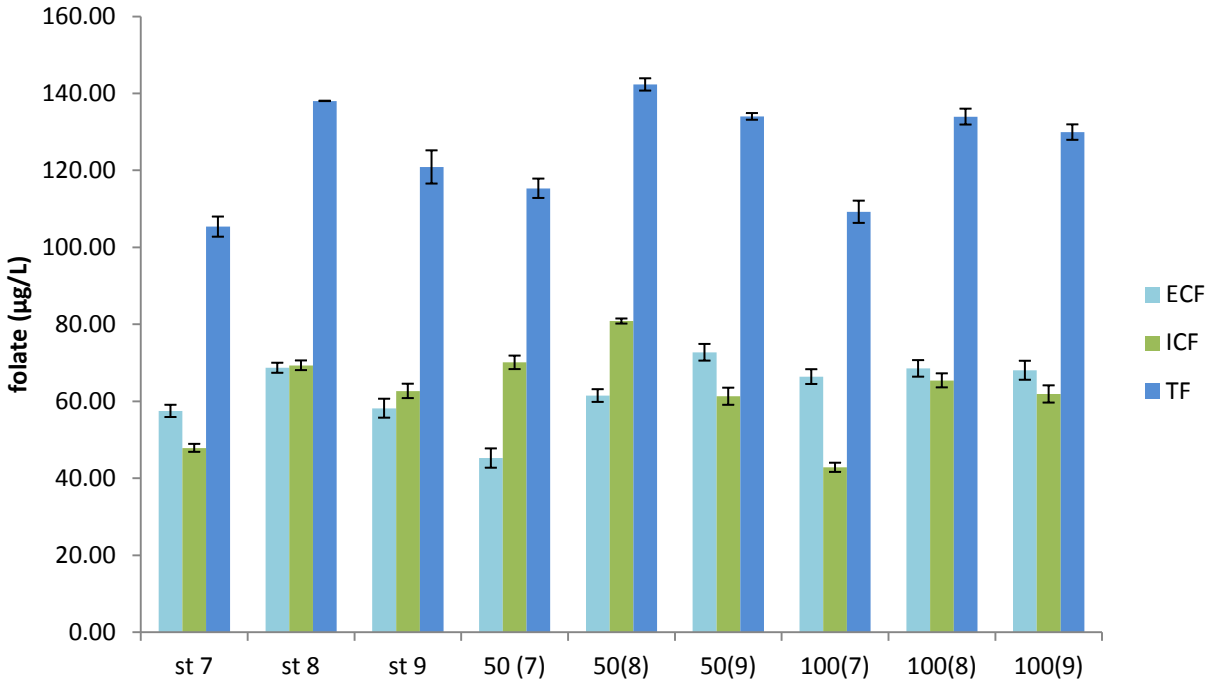

Fig. 4.8. Folate production in parallel fermenter

St 7 – static, 7 h; St 8 – static, 8 h; st 9 – static 9 h; 50 (7) – 50 rpm, 7 h; 50 (8) – 50 rpm, 8 h; 50 (9) – 50 rpm, 9 h; 100 (7) – 100 rpm, 7 h; 100 (8) – 100 rpm, 8 h; 100 (9) – 100 rpm, 9 h

The folate production studies were then carried out in 5 L fermenter with the optimized conditions in parallel fermenter and the experimental set up is shown in **Fig. 4.9**. The folate titre was 141.9 ± 4 µg/L after 8 h fermentation (37 ºC, 50 rpm). The results are presented in Fig**. 4.10**.

Fig. 4.9. Skim milk fermentation for folate production in 5 L fermenter

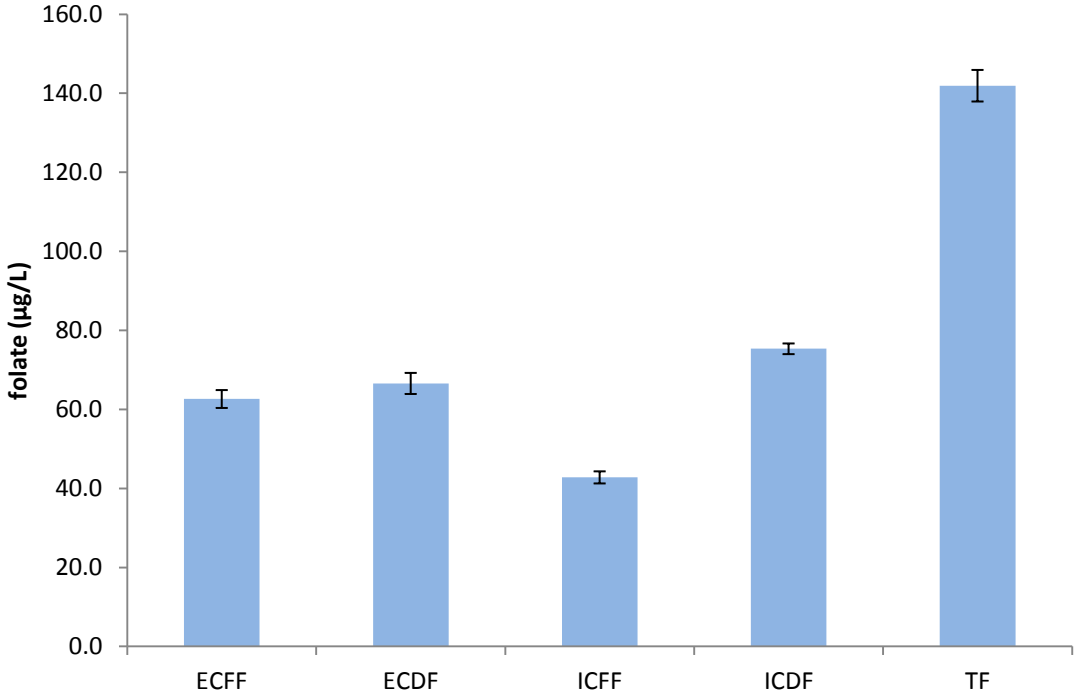

Fig. 4.10. Folate production in 5 L fermenter

ECFF – extracellular free folate, ECDF – extracellular deconjugated folate, ICFF – intracellular free folate, ICDF – intracellular deconjugated folate, TF – total folate

The effectiveness of scale up is demonstrated by the comparable folate levels in flask and bioreactor studies. Folate production of 54.53 µg/L by *Streptococcus thermophilus* BAA-250 in batch fermentation in 2 L stirred tank bioreactor (37 ºC, 150 rpm, pH 7) was reported by Mousavi et al. (2013). It was observed that pH had no

significant effect on intra- and extracellular folate production by *S. thermophilus* BAA-250. In contrast, Sybesma et al. (2003b) observed an increase in extracellular folate by *S. thermophilus* B119 at lower pH but pH did not affect the intracellular and extracellular distribution of folate in *L. lactis* MG1363. Folate production was therefore found to be dependent on the LAB strains, growth conditions and medium composition.

4.3.3. Storage Stability Studies of the Fermented Milk

Probiotics, upon ingestion should survive the acid milieu of the stomach and reach the intestine in high numbers, adhere to the intestinal walls and multiply to exert their health benefits on the host. According to the recommendation of the International Dairy Federation, the minimum number of probiotic cells at the time of consumption should be $\geq 10^7$ CFU/g (Ouwehand & Salminen, 1998). It is also important that the probiotic product retains its functionality throughout the storage period (Daneshi et al., 2013). The shelf life of yoghurt is usually about two weeks under refrigerated conditions. The folate levels, pH, TA and viable counts of the fermented milk were measured initially and then every five days for 15 days **(Table 4.2)**.

Table 4.2. Storage stability studies of the fermented skim milk

Storage period (days)	**1**	**5**	**10**	**15**
pH	4.74	4.53	4.42	4.42
TA (% lactic acid)	0.28 ±0.028	0.32±0.007	0.42±0.028	0.48±0.014
Viable count (log CFU/ml)	11.6±0.28	11.48±0.1	10.9± 0.11	10.78±0.07
Folate stability (%)	100.00	99.24±0.11	95.11±1.32	90.78±0.71

About 90% of the folate was retained in an active form after 15 days. The viable count of bacteria was 6 x 10^{10} CFU/ml (10.78 log CFU/ml) after 15 days which were sufficient to exert health benefits on the host. Less than one log unit reduction was there in the viable count of bacteria during the storage period. Over the storage time, it was noticed that the TA slightly increased and pH decreased. The post acidification of fermented milk on cold storage could be due to β galactosidase activity (Kailasapathy, 2006). This in turn might have resulted in slight decrease in the viability of the probiotic bacteria.

4.4. Conclusion

The results from the present study indicate that skim milk is a suitable medium for folate production by *L. lactis* CM28. Addition of folate precursors, prebiotics and sodium ascorbate at optimum levels enhanced the extracellular folate levels in fermented skim milk. Optimization of medium and culture conditions resulted in a fourfold increase in the readily available extracellular folate. The feasibility of scale up of the process up to 3 L was demonstrated in bioreactor. Also, the strain maintained an acceptable viability and about 90% of the produced folate was retained in an active form over the storage period of 15 days at 5 ºC. Since the strain *L. lactis* CM28 exhibited significant probiotic properties along with folate production, it could be a potential choice for probiotic preparations that could compensate folate deficiency.