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Comparative Studies on Folate Production and Parameter Optimization in Fermented Milk from Yoghurt Starter Culture

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Abstract

Among the several causes reported for neural tube defects in newborns and cardiovascular diseases, deficiency of folate is one of the important reasons to be considered. Folate, water soluble vitamin, plays significant role in metabolic reactions. Humans cannot synthesize folate itself hence supplements and natural food sources are advisable to consume. Supplements have severe side effects on long term usage. Although milk is a poor soucrce of folate however folate producing microorganisms can enhance the folate content in the fermented milk. In this study, yogurt starter culture *Streptococcus thermophilus* NCIM No. 2904 and *Lactobacillus helveticus* NCIM No. 2733 were selected. Both the cultures were grown in MRS media and production was checked in reconstituted nonfat dry milk medium. Folate production was analysed by the TLC method and the modified ninhydrin assay which is not previously used on food products. Both cultures produced the folate in the fermented milk however *S. thermophilus* was found to be the best producer. After the production, optimization of culture condition such as temperature, pH, age of inoculum, inoculum volume and carbon source i.e. lactose concentration was carried out to obtain the enhanced folate content. *S. thermophilus* and *L. helveticus* gave the maximum production at 40°C and 37°C respectively and optimum pH was found to be 6.5 for both the cultures. Optimum age of inoculum was observed as the 15 hr while 5% inoculum volume was found to be optimum. Lactose concentration i.e. carbon source was also optimized and 2% lactose was found to be the optimum for the maximum folate production.

Keywords: Neural Tube Defects, Folate, Modified ninhydrin assay

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Introduction

It has been reported for several years that neural tube defects in newborns (Yates et. al., 1998, Van Der Put et. al., 2001, Barkai et. al., 2003), cardiovascular diseases (Loria et. al., 2000, Bazzano et. al., 2002, Wald et. al., 2002) and certain forms of cancer such as breast cancer, cervical cancer (Choi et. al., 2002, Leahy et. al., 2005, Giovannucci 2002, Fang et. al., 1996) and certain extent of Alzheimer's disease (Wang et. al., 2001, Shea et. al., 2002) occur due to the deficiency of folate. Folate consumption is essential for human body because it cannot be synthesized in the body. It acts as cofactors for carbon transfer reactions such as nucleotides and amino acids synthesis. Folate deficiency, water soluble B vitamin also known as B9, pteroyl-L-glutamic acid and folacid, hampers the DNA synthesis and cell division mainly in the rapidly dividing cells such as haematopoitic cells leads to the megaloblastic anaemia (Lynn et. al., 2006).

There is growing need to enhance the folate content of food items by microbial fortification as the minimum requirement for folate for an adult is approximately 50 µg/day while a recommended intake mentioned by the WHO is approximately 200 µg day (Debree et al., 1997) and 400-800 µg/day for pregnant and childbearing age women (Crittenden et. al., 2003). Natural available food sources of folate should be incorporated as 4-6 servings to fulfill the necessity as containing limited amount of folate but it cannot be afforded due to certain limitations like the availability of food sources, financial crisis for each individual mainly in developing countries as well as in developed countries (Konings et. al., 2001, O'Brien et. al., 2001). Supplements are the alternatives however synthesized mainly via the costly chemical process and having severe side effects. Microbial food fortification eliminates the need of the downstream processing and food can be consumed as it is. Already this is reported in case of milk that is converted into the fermented milk i.e. yoghurt by the probiotics microorganisms and it is highly beneficial than the milk itself (Lin & Young, 2000).

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The objective of this study was to investigate the folate production from two different yoghurt starter cultures and optimization of their fermentation conditions for the folate enrichment in milk. Milk is the natural source of folate but quantity is very less. Milk was chosen for the study because of the stability of folate on long term storage due to the presence of folate binding proteins and the availability of milk worldwide across the developed as well as developing countries. Milk is easily accessible and consumable by each and every age group in population. Folate production from some species of lactic acid bacteria is reported but very little information is available for the yogurt starter culture (Lin et. al., 2000, Rao et. al., 1984).

Materials and methods

Procurement of the Bacterial cultures and Chemicals

Yoghurt starter cultures *Streptococcus thermophilus* NCIM 2904 and *Lactobacillus helveticus* NCIM 2733 were obtained from National Collection of Industrial Microorganisms, culture collection of the National culture Laboratory, Pune. All strains were grown on MRS media and incubated at 37°C. Subculturing of all the strains was done serially at least three times prior to use for production to minimize the lag phase of the culture. MRS media was prepared using media composition as follows: Peptone- 10.0 g/l, Yeast Extract- 5.0 g/l, Beef Extract- 10.0 g/l, Dextrose- 20.0 g/l, Ammonium Citrate- 2.0 g/l, Sodium Acetate- 5.0 g/l, Magnesium Sulphate- 0.1 g/l, Manganese Sulphate- 0.05 g/l, Dipotassium Phosphate- 2.0 g/l. Peptone and dextrose were purchased from the Merck and rest of the chemicals and folate standard were purchased from the Himedia. Human Plasma for the extraction of folate from the fermented milk was purchased from Sigma.

Culture conditions and Growth kinetics studies of the microorganisms

All the procured strains were initially maintained in the semistab prepared with MRS media at 37°C for 24 hr and stored at 4°C±1°C and these stock cultures were continuously transferred in fresh MRS semistab in every 3-4 weeks. Subculturing of all the strains was done serially at least three times prior to use for production to minimize the lag phase of the culture.

Growth characteristics of the microorganisms were studied in sterilized reconstituted nonfat milk medium (10%) for 28 hrs at 37°C. Samples were collected initially and at 2 hr interval for 28 hours and dry cell mass were determined. Growth profile was plotted between the dry cell mass x (gm/l) vs time t (h). For the determination of specific growth rate, log x (dry cell mass) vs t (Time) graph was plotted.

Folate production in reconstituted nonfat milk medium

Sterile reconstituted non fat milk medium (10%) was prepared. For folate production, Milk was inoculated with 5% inoculum from the seed culture of *S. thermophilus* and *L. helveticus* prepared in sterile reconstituted non fat milk (10%). Milk media inoculated with the cultures were incubated at 37°C at static condition. Samples were collected at 0 hr, 2 hr, 4 hr, 6 hr, 18 hr, 24 hr and 30 hrs and stored at 4°C until extraction. The estimation of folate was done afterwards by modified ninhydrin assay (Rao et.al., 1977).

Extraction of folate from fermented milk

Six ml sample of fermented milk was taken and 10 ml of extraction buffer (0.1 M phosphate buffer containing 0.5% ascorbic acid) was added into sample. This mixture was kept in boiling water bath for 15 min and centrifuged at 4000 rpm for 10 min. 3 ml of supernatant was taken and 0.4 ml of human plasma was added into supernatant. Mixture was incubated at 37^0C for 1 hr under continuous rotation condition. Reaction was stopped by placing the samples in boiling water bath for 5 min. Extract were centrifuged at 27000 rpm for 10 min (Lin et. al., 2000). Supernatant was filtered off and filtrate was kept for analysis by modified ninhydrin assay.

Qualitative Estimation of folate by Thin Layer Chromatography

For the confirmation of folate production, thin layer chromatography was performed by the method described by the Cimpoiu et. al. For this, solvent mixture of Butanol, Acetic acid and Water in the ratio 6:2:2 was used (Cimpoiu et. al., 2007).

Modified Ninhydrin Assay for the folate Estimation

Modified ninhydrin test used by the Rao et. al. in 1977 was furthur modified a little bit for the analysis of folate in food products rather than pharmaceutical preparation. (Rao et. al.,1977). In this method, folic acid is reduced to 2,4,5-triamino-6-hydroxy-pyrimidine (TAHP) which reacts with the ninhydrin to form the stable purple complex. This method was little bit modified as follows. For this, 0.4 ml of purified sample and standard folic acid (20µg/l) was taken in a test tube and diluted upto 4 ml with distilled water. 1 ml of 3N HCl and 0.25 gm of Zn dust was added into the sample. This mixture was kept under continuous agitation for 10 min and then filtered. Filterate was taken and its pH was maintained at 7.5 with the help of the 1N NaOH. 1 ml

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of ninhydrin reagent (0.35 gm ninhydrin in 50% ethanol) was added into the filterate and kept in boiling water bath for 5-7 min. Absorbance of the mixture was taken at 555 nm. On the basis of absorbance of standard folic acid soultion, folate concentration in the fermented milk sample was calculated.

Optimization of Incubation temperature and pH for the folate Production

For this, experiments were carried out at different incubation temperatures (30°C, 35°C, 37°C, 40°C, 42°C and 45°C) keeping other factors constant. Fermentation was carried out for 6 h for *S. thermophilus* and 18 h for *L. helveticus* respectively in all the optimization studies as the folate concentration declined afterwards. To study the effect of the pH, milk media of different initial pH of 5.5, 6, 6.5, 7, 7.5 and 8 were prepared, inoculated and incubated at appropriate temperature. For each run, Samples were collected and subjected to extraction and analysis by the method described above.

Optimization of inoculum age and inoculum level for the folate production

For each culture, inoculum (6 h, 9 h, 12 h, 15 h, 18 h and 24 h) for each run was prepared seperately under identical conditions. Prepared inoculums of varied age were finally inoculated in the production medium i.e. sterilized reconstituted milk medium (10%). Again the production studies were carried out with different inoculum level (1%, 2%, 5% and 10% v/v). All other culture conditions were kept constant.

Optimization of carbon source i.e. lactose concentration for the folate production

Lactic acid bacteria utilises the lactose as the carbon source for their growth as well as for the metabolite production so the optimization of lactose concentration was carried out to get the maximum folate production. Principal sugar present in milk is lactose however additional lactose is added in the milk medium. For this, different concentration of lactose (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) was added in the sterilized reconstituted milk media (10%) and inoculated.

Results and discussion

Growth curves of the microorganisms

S. thermophilus and *L. helveticus* grew well in reconstituted nonfat dry milk media (10%) at 37^{0}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 1. Specific growth rate was found to be 0.32 h^{-1} for *S. thermophilus* and 0.29 h^{-1} for *L. helveticus.*

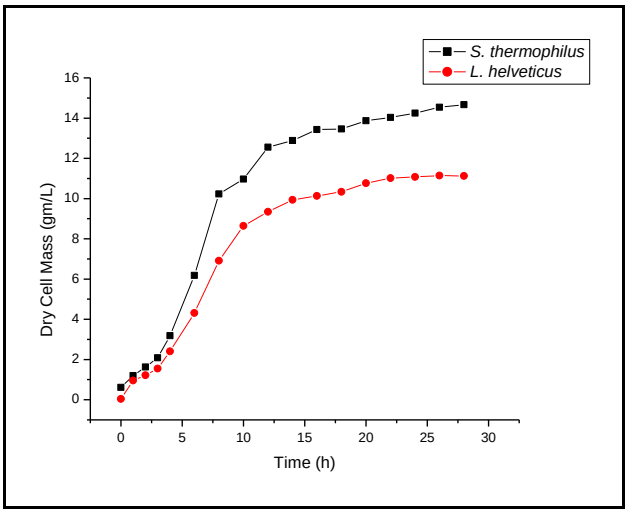

Figure 1: Growth profile of the yoghurt starter cultures S. thermophilus and L. helveticus

Folate Production by the *S. thermophilus* and *L. helveticus*

Thin layer chromatography was performed for the qualitative estimation of folate produced and R_f value was calculated. It was observed from the R_f values that both the *S. thermophilus* and *L. helveticus* produced the folate in the fermented milk (data not shown). Furthur, folate concentration in fermented milk was calculated by the standard curve obtained with the various concentration of standard folic acid. As illustrated from time course curve of folate (figure 2), it is clear that folate is produced by both the strains. Folate production by *S. thermophilus* was found to be maximum (47 µg/l) at 6 h however in *L. helveticus*, maximum production (42 µg/l) was observed at 18 h. Folate concentration increased to 1.8 fold by the *S. thermophilus* in 6 h however 1.6 fold increase was observed by the *L. helveticus* in 18 h. Afterwards folate concentration declined in both the cases may be due to the reason reported in various studies that the folate synthesizing bacteria also consumed folate for the growth and metabolism as folate in involved in the synthesis of nucleotides. Thus, this decreased level may be due to the higher utilization of folate for the cell division and growth (Lin et al, 2000, Gangadharan et al, 2011).

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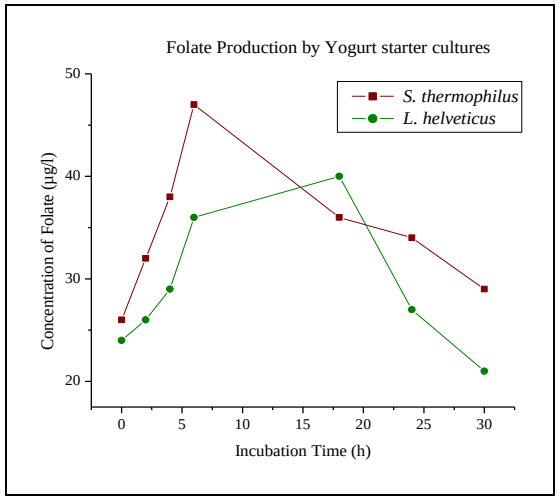

Figure 2: Folate production in reconstituted nonfat milk medium (10%) by S. thermophilus and L. helveticus

Incubation temperature, initial pH and folate production

Incubation temperature is the most significant parameter among the physical parameter which should be optimized to enhance the metabolite production. 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 favoured at the higher temperature. It was observed from the figure 3 that 40°C and 37°C were found to be the most suitable temperature for the maximum folate production by the *S. thermophilus* and *L. helveticus* respectively. *S. thermophilus* enhanced the folate content to 51µg/l in 6 h and *L. helveticus* raised the folate to 45µg/l in 18 h. Therefore, in all subsequent studies 40°C and 37°C were used as the incubation temperatures respectively for the *S. thermophilus* and *L. helveticus.*

Initial pH of the reconstituted milk medium was varied from the 5.5 to 8.5. It showed that maximum folate production was obtained at the initial pH of 6.5 afterwards concentration decreased (Figure 4). 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). This result is in agreement with the results shown in 2001 by the Sysbema et. al. However pH 6.5 at which significant increase in the folate concentration occured 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 as maximum production was observed at pH 6.5.

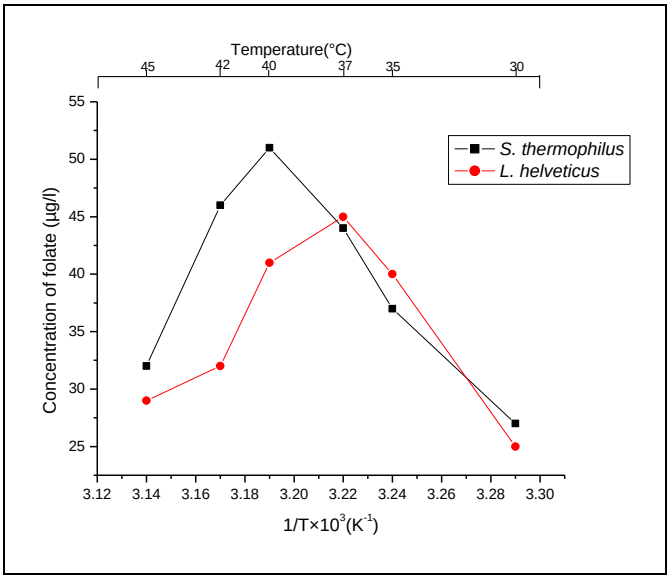

Figure 3: Effect of incubation temperature of the fermentation on folate production

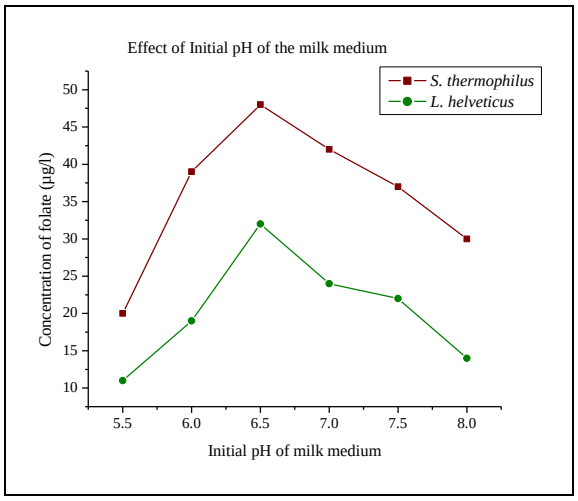

Figure 4: Folate production at different initial pH of the production medium

Optimization of the inoculum age and inoculum volume

Experiments were performed with inoculum of different age (6 h, 9 h, 12 h, 15 h, 18 h and 24 h) at optimum temperature. It was found that culture age of 15 h showed the highest folate production in both *S. thermophilus* and *L. helveticus*. Growth profiles of

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both the microorganisms were found to be almost similar and 15 h is observed as the exponential phase of the culture. Folate concentration was increased to 53µg/l and 49µg/l respectively by *S. thermophilus* and *L. helveticus* as shown in Figure 5. Again, experiments were carried out to find out the optimum inoculum level (1-10% v/v) for the maximum folate production. In both the cases, appreciable increase was observed with the 5% v/v inoculum level (Figure 6). Folate concentration was found to be 58µg/l and 51µg/l respectively by *S. thermophilus* and *L. helveticus* with 5% inoculum volume. 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.

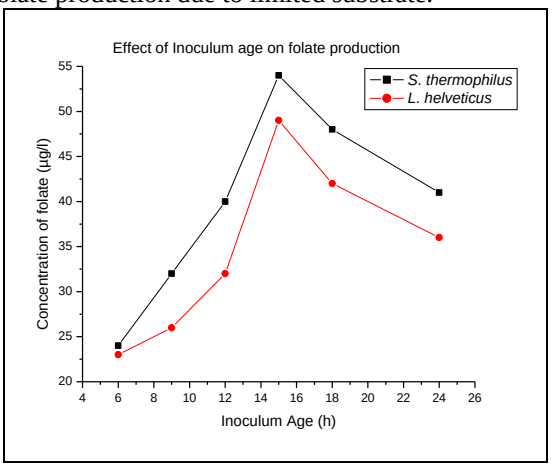

Figure 5: Effect of the age of the inoculum on the folate production

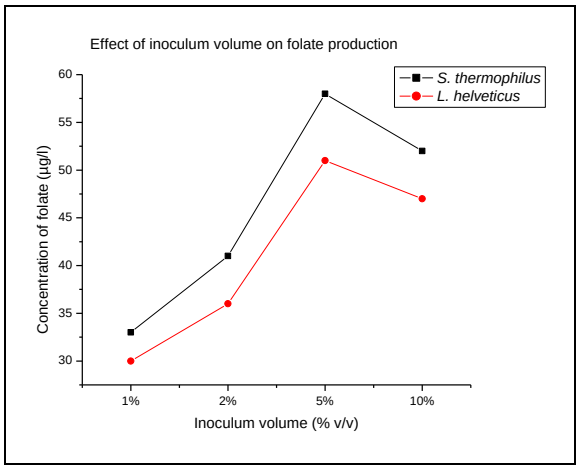

Figure 6: Effect of inoculum volume on folate production

Optimization of the Lactose Concentration

Lactose is the basic carbon source utilised by the lactic acid bacteria from which lactic acid can be synthesized. From the figure 7, it was observed that lactose acts as an enhancer for the folate synthesis in the fermented milk. Addition of 2% lactose was found to be optimum for the maximum folate concentration as furthur increase of lactose concentration resulted in negligible increase in the folate production. Folate concentration was increased to 3.1 fold by the *S. thermophilus* (81µg/l) and 2.65 fold (69 µg/l) by *L. helveticus* on the addition of 2% lactose.

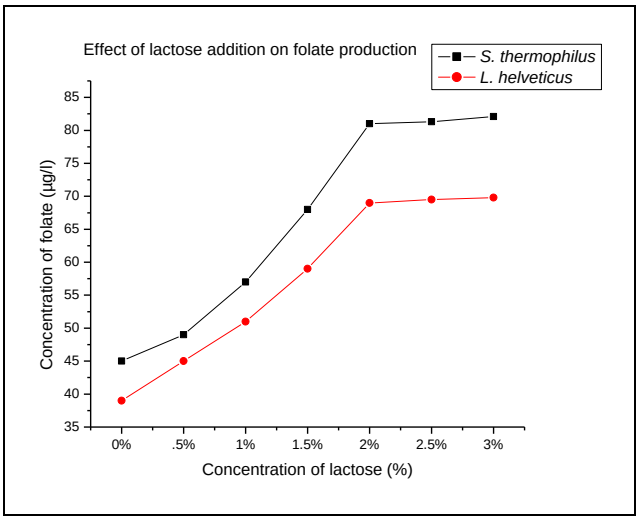

Figure 7: Effect of addition of lactose on folate production

Folate production in optimized medium

After the optimization of the fermentation condition by the one factor at one time method, we get the optimum culture conditions to get the maximum folate production. Now the reconstituted nonfat milk medium having optimum culture conditions was prepared and inoculated (table1). *S. thermophilus* enhanced the folate content upto 18 h rather than 6 h under the combined optimum conditions and *L.helveticus* produce the folate upto the 24 hr. It may be due to the additive effect of the all the optimum culture conditions. From this study it can be concluded that the media with the optimized culture conditions resulted in the enhanced production of folate in both the yogurt stater culture.

Table 1: list of parameters optimized by the classical method for folate production by S. thermophilus and L. helveticus

Optimum Culture Conditions	***S. thermophilus***	***L. helveticus***
Incubation Temperature (°C)	40	37

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Initial pH of the media	6.5	6.5
Inoculum Age (h)	15	15
Inoculum Volume (%v/v)	5%	5%
Lactose Concentration (%)	2%	2%

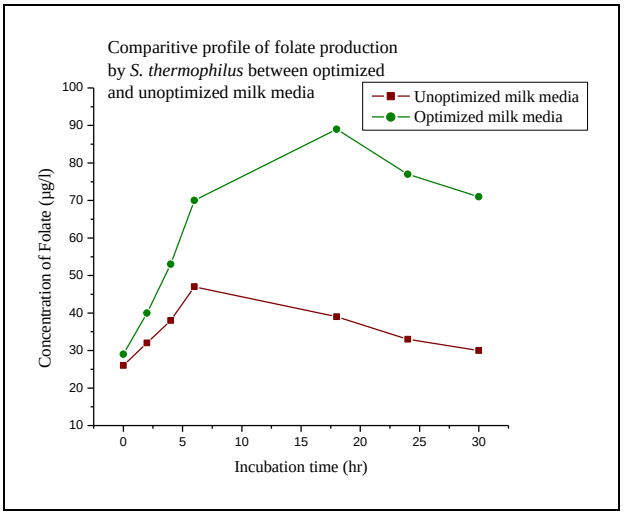

Fig 8: Comparision of folate production by S. thermophilus in optimized and unoptimized medium

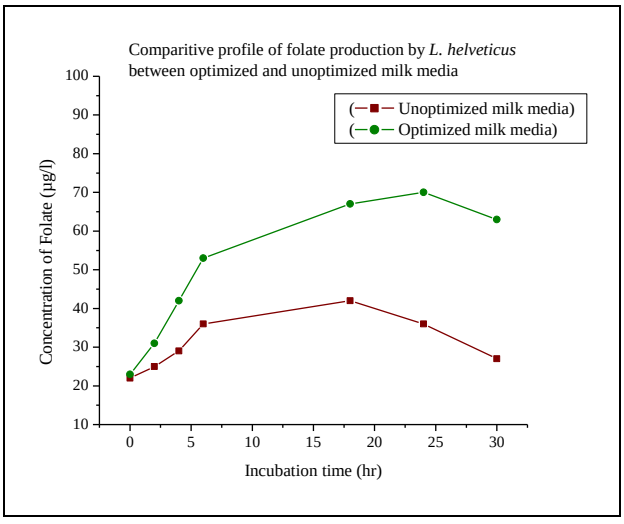

Fig 9: Comparision of folate production by L. helveticus in optimized and unoptimized medium

Conclusion

Fermented dairy products are suitable source of folate for the completion of the daily recommended intake (RDI) for the most of the population. Strain selection is the most important parameter to optimize the maximum folate enrichment in milk and dairy products. These results indicate that both the microorganisms *S. thermophilus* and *L. helveticus* have the potential to enhance the folic acid content of milk whereas *S. thermophilus* was found to be the best producer for enrichment of the folate content in milk. Milk is chosen for the study due to the presence of folate binding proteins in the milk which are responsible for bioavailability during consumption as well as the stability of the folate produced. It was found that initially *S. thermophilus* and *L. helveticus* produced maximum folate only upto 6 h and 18 h respectively however after optimization of culture conditions folate content produced upto 18 h and 24 h. Decline in folate content after some time may be due to increased acidity and suppression of growth of the culture. Whereas another important issue with the yogurt starter culture is that it produces the folic acid as well as consumes the folic acid itself also for the growth and metabolism.

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