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Effect of various Nutritive Substrate on the Growth of Lactic Acid Bacteria

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ABSTRACT: Dahi is an important fermented milk product having heterogeneous lactic microflora which play a major role in human diet. Dahi samples of 5 each were collected from houses and local markets of Bengaluru. That showed total lactic bacterial viable count ranging from 5.36 to 7.04. Among 72 lactic isolates obtained. lactobacilli predominated (38) followed by leuconostoc (18); lactococci (9) and streptococci (7). The lactic cultures that set the milk with higher direct microscopic counts in sterile skim milk were *Leuconostoc* sp. Leu6: *L. lactis* ssp. *lactis* Lc1*: L. fermentum* Lb8*: S. thermophilus* St3 especially at the ratio of 0.15 %: 0.3 %: 0.5%: 1 %. The growth of mixed dahi culture on sterile black gram dhal with 1% skim milk powder, 10% tomato juice and 70% moisture as solid substrate medium showed 9.42 log_{10}cfu/g at 24 hrs of incubation.

Keywords: Lactic acid bacteria, Dahi, Fermentation, Skim milk powder and Black gram.

INTRODUCTION

Microorganisms are ubiquitous in nature. Based on their usefulness they have been grouped into beneficial and harmful. Beneficial bacteria are useful in the preparation of fermented foods. Lactic acid bacteria (LAB) are one among beneficial bacteria that are Gram positive, facultative anaerobes, acid tolerant and are widely used as starters in the manufacture of various fermented foods such as dahi, yoghurt, batter, sauerkraut etc.

India ranks first in milk production with 221.10 million tonnes during the year 2021. The percentage of fliud milk conversion is 46 percent and remaining is utilized as milk products. Fermentation is one method of preserving the milk. Beneficial bacteria are used to bring about controlled fermentation and they include lactic acid bacteria such as lactococci, streptococci, pediococci, leuconostoc and lactobacilli. About 7.1% of milk produced in India is used in the preparation of dahi.

Dahi is a fermented milk product made from cow milk or buffalo milk or combination of the two, where part of lactose has been converted into lactic acid by lactic acid bacteria. Dahi has the same nutritional value as that of milk from which it is made and easily digestible than milk, as 60% of lactose is fermented and protein partially hydrolyzed (Awan and Rahman 2002).

Meshref *et al*. (2007) conducted the growth study of *Bifidobacterium infantis* and *B. angulatum* by inoculation into the milk and incubated at 37°C for 16 hrs. The bacterial counts were conducted at the beginning and every 4 hours and upto 16 hrs, the viable counts ranged from 8.12 to 8.56 log_{10}cfu/ml.

Lactococcus lactis ssp. *lactis, L euconostocmesentriodes* ssp. *mesentroides, Lactobacillus viridescens* when inoculated individualy to sterile skim milk at their optimum growth temperature, curdled the milk at 24 hrs of incubation. At every 6 hrs, the samples drawn showed peak in the DMC at 24 hrs of incubation that ranged between 8.13 to 8.31 log_{10}cells/ml (Pradeep, 2007).

Schaffner and Benchat (1986) experimented on suitability of aqueous plant seed extract to serve as substrates for fermentation by lactic acid bacteria. The bacteria investigated were *Lactobacillus heleveticus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus* and *Streptococcu thermophilus.* The aqueous extracts of cowpea, peanut, soybean and sorghum were used as substrates for nutritionally superior sour tasting product containing 20 – 25 grams lactic acid/kg cereal. Sensory qualities improved and shelf life were improved due to protective action of lactic acid.

Yoghurt like product was prepared by fermentation of rice with lactic acid bacteria *Lactobacillus plantarum*, *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* singly or in various combinations. The product inoculated with all the three bacteria was superior to other products (Shin, 1989).

Chickpea and blackgram dhal, very well supported the growth of *Lactobacillus acidophilus* with counts reaching upto 9.32 log_{10}cfu/g with addition of skim milk powder (Vanisri, 1995).

Solid state fermentation is defined as growth of microorganisms on solid materials with out the presence of free liquid (Lopez and Henry 1998).

Prabha (1999) obtained maximum viable count of 9.10 log_{10}cfu/g when fermented dried (2°C for 20 hours) black gram dhal using *Bifidobacterium* spp was used as substrate with 1 : 0.8% of moisture, 1% of skim milk powder and $MnS0_4$ of 0.005%. .

Kabede *et al.* (2007) studied effects of temperature (7°, 15°, 25° & 37°C) on the growth of lactic acid bacteria and yeasts in 'Sethemi', South African naturally fermented milk. Lactic acid bacterial counts decreased rapidly at 37°C, from 2nd day to 5th day, while the numbers remained stable at 25°C. pH of the milk was decreased with higher temperature than at 7°C and 15°C of incubation. The temperature of 25°C was found to be ideal for producing fermented milk with high lactic acid bacterial counts, low pH and a visually acceptable coagulum.

Lactobacillus strains (2) and *Lactococcus* strain (1) were selected to study their growth and lactic acid production. The *Lactobacillus* strains, both isolated from fermented milk product 'Tapai', produced higher amounts of cells and lactic acid from glucose as compared to the *Lactococcus* strain isolated from fresh goat's milk (Ahmad *et al*., 2007).

Zhao *et al*. (2008) developed novel, SSF method, using a netting bag bioreactor for probiotic cultures. A high yield of biomass (1.2×10^{11} cfu/g dry substrate) was obtained using this method. The netting bag method was more cost-effective as well as time and space saving. The material cost was low. Thus, it was suggested that netting bag SSF can be applied widely to produce probiotic bacteria in developing areas.

Dieghri-Hocine *et al*. (2007) studied that Chick pea based medium appeared specially efficient for the growth of plant isolate of *Lactobacillus plantarum* with 1.28 log_{10}cfu/g higher than that obtained on MRS medium.

The functions of lactic acid bacteria include production of lactic acid to impart a distinctive fresh and acidic aroma, contribution towards production of flavour for the fermented products, mild proteolytic and lipolytic changes which impart to the body and texture of the products and finally prevents the growth of spoilage organisms by acid production and by production of bacteriocins by certain lactic acid bacteria.

In the production of various fermented products, the use of starter cultures occupy the center stage. The quality of the finished product is therefore dependent on the activity and performance of starters used. Starters produced by conventional methods such as liquid culture, freeze dried, frozen culture, concentrated cultures etc. have been used by industry with various drawbacks. Continuous attempts are being made to improve the methodology for the production of starters for industrial application with various nutritive substrate

MATERIALS AND METHODS

A. Maintenance of Lactic acid bacteria

Stock lactic acid cultures were maintained in a yeast glucose agar stabs (0.75% of agar) and subcultured once in 21 days. Working lactic cultures were maintained in a yeast glucose broth and sterile skim milk, which were stored in refrigerator and subcultured once in a week.

B. Isolation of Lactic acid bacteria

Domestic and market samples of curd were collected in a sterile sample bottle. Sample of 11g were weighed separately under aseptic condition on to the sterile aluminum foil and transferred to 99ml of diluent bottle and mixed by rotating by placing on working bench to get 10^{-1} dilution and using this, required dilutions were prepared. Appropriate dilutions were transferred to four sets of Petri plates. To the first set of Petri plate sterile, molten Neutral Red Chalk Lactose Agar (NRCLA) maintained at 50°C was added, to the second set of Petri plates, Yeast Glucose Agar (YGA) was added, to the third set of Petri plate, Sucrose Agar (SA) was added, to the fourth set of Petri plate, Rogosa Agar (RA) was added, mixed thoroughly and allowed to solidify. First dilution was lab pasteurized (63°C/30min), cooled to room temperature and required dilutions were prepared using sterile pipettes, 1ml quantity of required dilutions were transferred to label Petri plates and sterile molten YGA maintained at 50°C was poured, mixed thoroughly and incubated at 37^0C. Neutral Red Chalk Lactose Agar, Sucrose Agar plates were incubated in anaerobic jar at 30°C for 24 - 48hours. Yeast Glucose Agar, Rogosa Agar plates were incubated in anaerobic jar at 37°C for 24 - 48hours. After incubation pink coloured colonies in NRCLA plates, all the colonies in YGA plates and glistening colonies on Sucrose Agar, all the colonies on Rogosa Agar were counted. The counts were expressed as colony forming units per gram (cfu/g) by multiplying average count with dilution factor and then converted into log_{10} cfu/g of sample. The colonies were selected based on Harrison's disc. The isolates were purified by streaking on YGA for 3 times and purified isolates were maintained in Yeast glucose broth and stabs containing 0.75% of agar.

C. Screening of isolates based on their activity in sterile skim milk

All the isolates were inoculated at 2% level in 10 ml of sterile skim milk tubes. Isolates were selected based on setting time, Direct Microscopic Count and titratable acidity.

D. Growth study of selected isolates in sterile skim milk and on solid substrate

Selected isolates were inoculated into sterile skim milk and black gram dhal as solid substrate with additives 1% skim milk powder, 10% of tomato juice and 70% moisture. The setting time, Direct Microscopic Count and titratable acidity in terms of % lactic acid was determined for every 6 hrs up to 48 hrs in sterile skim milk and only Direct Microscopic Count in solid state fermented cultures.

RESULTS AND DISCUSSION

A. Activity of lactic acid bacterial isolates in sterile skim milk

L. lactis ssp. *lactis* Lc1 & Lc2 and *L. lactis* ssp. *cremoris* Lc8 curdled the milk at 24 hrs of incubation at

30°C (Table 1) and DMC ranged from 8.08 to 8.31 log_{10}/g and acidity ranged between 0.35 to 0.44% lactic acid. Other isolates showed curdling of milk at 30hrs of incubation.

The isolates (6 numbers) of *Streptococcus thermophilus* St1 – St6 curdled the milk at 18 hrs of incubation with DMC ranged from 8.21 to 8.69 log_{10}/g and titratable acidity was determined between 0.41 to 0.69% lactic acid (Table 2).

All the leuconostoc isolates irrespective of their identity set the milk at 18hrs. The DMC observed from 8.20 to 8.90 log_{10}/g with acidity of 0.29 to 0.47 % lactic acid. Among the isolates Leu1 and Leu6 showed highest DMC (Table 3).

All the isolates of the lactobacilli (25 numbers) irrespective of their identity as *L. fermentum, L. hilgardii* and *L. acidophilus* set the milk at 18 hrs of incubation at 37°C with DMC ranging between 7.97 to 8.91 log_{10}/g and acidity produced by the isolates ranged between 0.32 to 0.96 % lactic acid (Table 4). *L. fermentum* Lb8 and Lb35 showed highest DMC of 8.77 and 8.91 log_{10}/g respectively.

B. Growth study of selected lactic acid bacterial isolates in sterile skim milk

The selected isolates of lactic acid bacteria were inoculated into sterile skim milk and incubated at optimum temperature (30°C or 37°C depending on the isolates), for every 6 hrs the sample were drawn up to 48 hrs. The setting time of milk DMC and titratable acidity were recorded.

The DMC increased with simultaneous increase in titratable acidity up to 24 hrs of incubation at 30°C. DMC in case of Lc1 ranged between 6.01 to 8.43 log_{10} /g with acidity of 0.16 to 0.36. While DMC of Lc2 ranged between 6.13 to 8.03 log_{10}/g with acidity of 0.16 to 0.33% lactic acid (Fig. 1). The statistical analysis indicated significant difference between *L. lactis ssp. lactis* Lc1 and Lc2 with respect to DMC.

S. thermophilus isolates (St1, St3) were grown in the sterile skim milk and curdled the milk at 18 hrs of incubation at 37°C. The DMC of St1 ranged between 6.02 to 8.49 log_{10}u/ml while DMC of St3 ranged from 6.04 to 8.76 log_{10}/g (Fig. 2). Initial titratable acidity was 0.16 and reached 0.30 in case of St1 and 0.41 by St3 at 24 hrs of incubation. The statistical analysis indicated significant difference between *S. thermophilus* St1 and St3 with respect to DMC.

Skim milk was curdled by *Leuconostoc lactis* (Leu1) and *Leuconostoc*sp.leu6 at 18 hrs when incubated at 30°C. The DMC increased with simultaneous increase in titratable acidity from 0.16 to 0.30 during 0 to 18 hrs of incubation in both the isolates. The DMC ranged between 6.04 and 8.21 log_{10}/ml at 0 to 24 hrs of incubation and 6.02 to 8.57 log_{10}/g (Fig. 3) respectively at 0 to 18 hrs of incubation in case of leu1 and leu6 respectively. The statistical analysis indicated significant difference in DMC with respect to *Leuconostoc lactis* Leu1 and *Leuconostoc* sp. Leu6.

L. fermentum (Lb8, Lb35) when grown in sterile skim milk curdled the milk at 18 hrs at 37°C with DMC ranging between 6.03 to 8.52 log_{10}/g and 6.02 to 8.46 log_{10}/g respectively. Both the isolates increased acidity from initial 0.16% to 0.63% and 0.59% in case of Lb8 and Lb35 respectively (Fig. 4). The statistical analysis indicated significant difference between *L. fermentum* Lb8 and Lb35 with respect to DMC.

C. Growth study of lactic acid bacterial isolates on solid substrate

Blackgram dhal was used as edible solid substrate to carry out solid state fermentation along with additives like skim milk powder (1%), tomato juice (10%) and moisture of 70%. The medium was sterilized and inoculated with the selected isolates and incubated at their optimum temperature either at 30°C or 37°C depending on culture. At every 6 hrs interval and upto 48 hrs the samples were drawn and DMC was determined.

L. lactisssplactis when grown on black gram dhal showed DMC ranging from 6.03 to 9.46 log_{10}/g and *L. lactis* ssp *lactis* (Lc1)showed highest count of 9.46 log_{10}/g at 24 hrs of incubation at 30°C. *S. thermophilus* St1, St3 showed DMC of 6.01 to 9.28 and 6.04 to 9.45 log_{10}/g respectively from 0 to 24 hrs of incubation at 37°C.

Leuconostoc lactis (Leu1) and *Leuconostoc* sp. (Leu6) showed DMC of 9.10 and 9.38 log_{10}/g respectively at 24 hrs of incubation at 30°C. *L. fermentum* (Lb8 and Lb35) showed highest DMC of 9.29 and 9.24 log_{10}/g at 24 hrs of incubation at 37°C respectively (Table 5).

D. Setting time of Dahi culture at different temperature

As dahi culture is heterogeneous in nature, based on DMC of dahi samples procured, the lactic cultures were combined at 0.15%, 0.35%, 0.5%,1%, of *Leuconostoc lactis, Lactococcus lactis* ssp. *lactis, Lactobacillus fermentum* and *Streptococcus thermophilus* respectively and inoculated into sterile whole milk for their performance at different temperatures such as 20°C, 22°C, 25°C, 27°C and 30°C. At 30°C, Set I (*Leuconostoc* sp. Leu6: *Lactococcus lactis ssp lactis* Lc1: *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3) culture curdled the milk at 6 hrs only while Set II (*Leuconostoc lactis* Leu1: *Lactococcus lactis* ssp *lactis* Lc2 : *Lactobacillus fermentum* Lb35: *Streptococcus thermophilus* St1) took 8 hrs, hence set I dahi culture was used in further studies (Table 6).

E. Growth study of mixed Dahi culture in sterile skim milk

Mixed Dahi culture was prepared by combination of *Leuconostoc* sp. Leu6: *Lactococcus lactis* ssp *lactis* Lc1: *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3 at the ratio of 0.15 %: 0.3 %: 0.5%: 1%. The growth of mixed Dahi culture was observed by inoculating to sterile skim milk and incubated at 30^0C for 0 to 48 hrs. The setting time was noticed at 18 hrs but peak DMC was noticed at 24 hrs of incubation i.e., 8.68 log_{10}/g with acidity of 0.65% LA. The statistical analysis indicated significant difference in the growth of mixed dahi culture at different hours of incubation (Table 7).

Table 1: Activity of lactococcal isolates in sterile skim milk.

Isolate No.	Identity	DMC (log_{10}/g)	Setting time (hrs)	Titratable Acidity (% LA)
Lc1	*L. lactis* ssp. lactis	**8.31**	**24**	**0.44**
Lc2	*L. lactis* ssp. lactis	**8.20**		**0.39**
Lc8	*L. lactis* ssp. cremoris	8.08		0.35

Note: Incubation temperature was at 30°C.

Table 2: Activity of *Streptococcus thermophilus* isolates in sterile skim milk.

Isolate No.	DMC (log_{10}/g)	Setting time (hrs)	Acidity (% LA)
St1	**8.67**	**18**	**0.67**
St2	8.40		0.48
St3	**8.69**		**0.69**
St4	8.21		0.41
St5	8.50		0.55
St6	8.52		0.58

Note: Incubation temperature was at 37°C.

Table 3: Activity of leuconostoc isolates in sterile skim milk.

Isolate No.	Identity	DMC (log_{10}/g)	Setting time (hrs)	Acidity (%LA)
Leu_1	*L. lactis* (4 numbers)	**8.76**	**18**	**0.41**
Leu_2		8.20		0.37
Leu_3		8.21		0.30
Leu_4		8.20		0.31
Leu_5	Unidentified (14 numbers)	8.57		0.39
Leu_6		**8.90**		**0.47**
Leu_7		8.30		0.36
Leu_8		8.24		0.30
Leu_9		8.25		0.29
Leu_{10}		8.32		0.30
Leu_{11}		8.40		0.36
Leu_{12}		8.46		0.33
Leu_{13}		8.28		0.29
Leu_{14}		8.30		0.32
Leu_{15}		8.39		0.37
Leu_{16}		8.48		0.38
Leu_{17}		8.35		0.39

Note: Incubation temperature was at 30°C.

Table 4: Activity of lactobacilli isolates in sterile skim milk.

Isolate No.	Identity	Setting time (hrs)	DMC (log_{10}/g)	Acidity (% LA)
Lb7	*L. fermentum*	18	8.34	0.72
Lb8			**8.77**	**0.92**
Lb9			8.10	0.50
Lb10			8.60	0.56
Lb18			8.56	0.70
Lb25			8.53	0.89
Lb26			8.67	0.81
Lb27			8.76	0.90
Lb28			8.34	0.71
Lb34			8.54	0.78
Lb35			**8.91**	**0.96**
Lb36			8.43	0.64
Lb37			8.52	0.75
Lb38			8.26	0.50
Lb39			8.65	0.81
Lb13	*L. hilgardii*		7.97	0.40
Lb14			8.41	0.81
Lb15			8.30	0.76
Lb16			8.20	0.35
Lb17			8.22	0.33
Lb19	*L. acidophilus*		8.09	0.32
Lb20			8.32	0.50
Lb21			8.49	0.69
Lb22			8.28	0.57
Lb23			8.26	0.52

Note: Incubation temperature was at 37°C.
L. lactis ssp. *lactis* (Lc1 and Lc2) when grown in skim milk

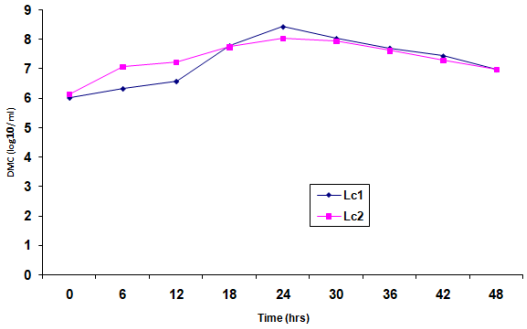

Fig. 1. Growth study of *Lactococcus lactis* ssp. *lactis*, Lc1 and Lc2 in sterile skim milk.

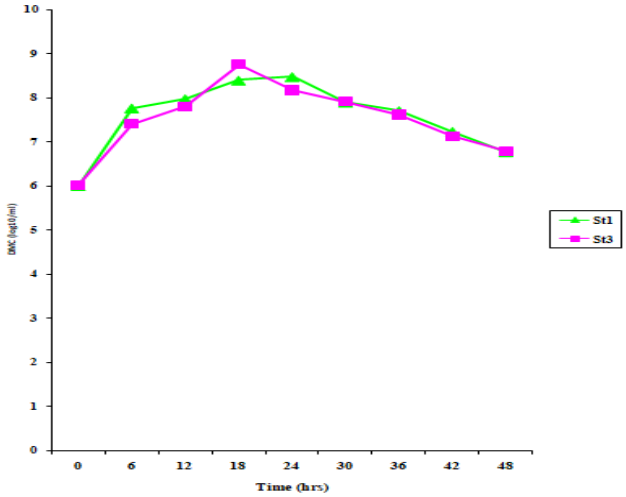

Fig. 2. Growth study of *Streptococcus themophilus* , St1 and St3 in sterile skim milk.

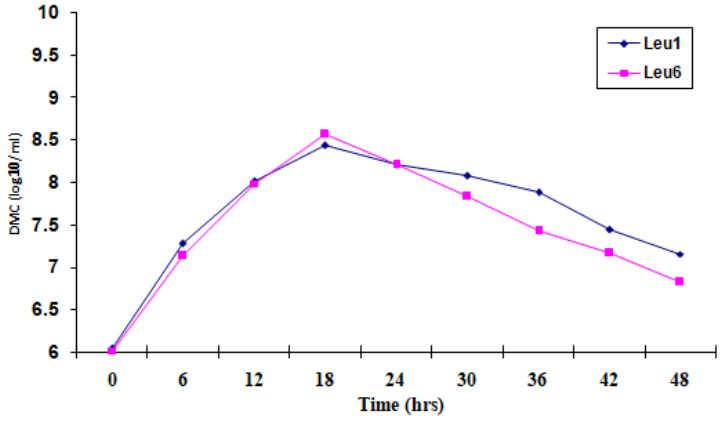

Fig. 3. Growth study of *Leuconostoc lactis*, Leu1 and unidentified isolate Leu6 in sterile skim milk.

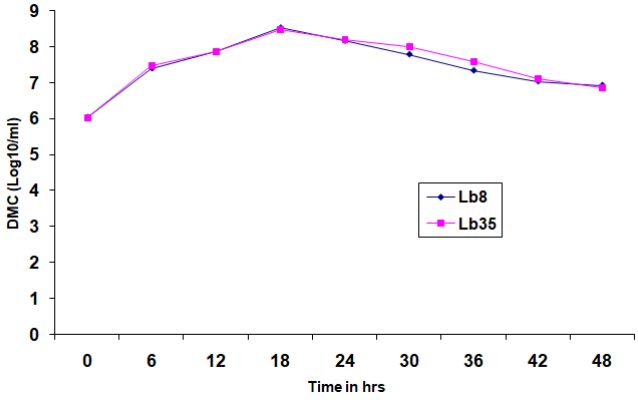

Fig. 4. Growth study of *Lactibacillus fermentum*, Lb8 and Lb35 in sterile skim milk.

Table 5: Growth studies of isolates on solid substrate medium.

Isolate	Incubation period in hrs.								
	0	6	12	18	24	30	36	42	48
	DMC (log_{10}/g)								
L. lactis ssp. *lactis* (Lc1)	6.03	7.14	8.24	8.80	**9.46**	8.44	8.08	7.66	7.01
L. lactis ssp. *Lactis* (Lc2)	6.01	7.53	8.36	8.64	**9.34**	8.59	8.19	7.82	6.92
S. thermophilus (St1)	6.01	7.47	8.00	8.83	**9.28**	8.54	8.29	7.93	7.36
S. thermophilus (St3)	6.04	7.23	7.82	8.60	**9.45**	8.50	8.12	7.86	7.29
L. lactis (Leu1)	6.03	7.37	7.92	8.88	**9.10**	8.45	8.05	7.66	7.19
Unidentified sp. (Leu6)	6.01	7.39	8.24	8.93	**9.38**	8.64	8.96	7.67	7.05
L. fermentum (Lb8)	6.04	7.82	8.19	8.82	**9.29**	8.88	8.44	8.02	7.69
L. fermentum (Lb35)	6.02	7.96	8.50	8.71	**9.24**	8.77	8.12	7.89	7.42

Note: -Solid substrate consists of Black gram dhal, skim milk powder (1%), tomato juice (10%) and moisture (70%)
-Growth study was carried out at every 6hr of interval for up to 48 hrs.
-Isolates in solid substrate medium were incubated at their optimum Temperature

Table 6: Setting time of mixed culture at different temperature.

Temperature of incubation (0°C)	Dahi culture	
	Lc1+St3+Leu6+Lb8 Set I	Lc2+St1+Leu1+Lb35 Set II
	(Setting time in hrs)	
20	24	36
22	8	12
25	8	12
27	8	12
30	6	8

Note: Combination was done at the ratio of 0.15 %: 0.3 %: 0.5%: 1 % = *Leuconostoc lactis*: *Lactococcus lactis* ssp *lactis*: *Lactobacillus fermentum*: *Streptococcus thermophilus*

Table 7: Growth study of Dahi Culture in sterile skim milk at 30°C.

Time (hrs)	DMC (log_{10}cfu/g)	Acidity (%LA)
0	6.04	0.19
6	7.40	0.31
12	7.81	0.42
18	8.37	o.50
24	**8.68**	**0.65**
30	8.47	0.60
36	8.27	0.60
42	7.90	0.55
48	7.72	0.55
CD ≥ 0.05	**0.09**	-

Note: Dahi culture-Isolates were combined in the ratio of 0.15 % : 0.3 % : 0.5% : 1 % =*Leuconostoc* sp. Leu6: *Lactococcus lactis* ssp *lactis* Lc1 : *fermentum* Lb8: *Streptococcus thermophilus* St3

CONCLUSIONS

Mixed Dahi culture was inoculated to sterile skim milk and incubated at 30°C to study the growth at every 6 hrs of interval upto 48 hrs. higher DMC of 8,68 log with 0.65% lactic acid was observed 24 hrs of incubation. Mixed dahi culture was grown on sterile black gram dhal with 1% of skim milk powder, 10% of tomato juice and 70% moisture as solid substrate medium at 30°C for 48 hrs. DMC was highest at 24 hrs of incubation accounting for 9.42 log10/g. Incubation period of 24 hrs was considered ideal for the growth of mixed dahi culture in order to obtain good biomass.

FUTURE SCOPE

We can produce the Solid state fermented (SSF) Dahi culture or DVS Dahi culture for the preparation of dahi product.

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