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Performance of dried powder of solid stated fermented (SSF) lactic cultures in milk

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Abstract
The present investigation was intended to perform the dried powder of solid state fermented lactic cultures in milk by making different fermented products like dahi. yoghurt and acidophilus milk. The dried powder of SSFdahi culture was inoculated at 0.5, 1.0 and 1.5 per cent levels into heated milk, incubation at 30^0C and then determined the milk setting time, DMC and titratable acidity. At all the inoculum levels, the milk setting time was 5 h only but DMC and titratable acidity results were varied. Dried powder of SSF dahi culture at 1 % inoculum, showed better with setting time of 5 h, DMC count of 8.60 log $_{10}$ cells /ml, 0.71 % LA and 8.47 score for overall acceptability in sensory evaluation. Dahi prepared using 1% inoculum of liquid milk culture took 6 h to set milk with DMC of 8.03 log $_{10}$ cells /ml and acidity of 0.70%LA and score of 8.12 for overall acceptability that indicated lower cell counts in liquid inoculum compared to SSF dahi inoculum. Yoghurt prepared with 0.5%, 1% and 1.5% of dried powder of SSF yoghurt culture took 3:30 h to set the milk at 42^0C. Yoghurt prepared using 0.5% SSF culture was better compared to 1 and 1.5% inoculum, with DMC of 8.61 log $_{10}$ cells /ml 0.79%LA and overall acceptability score of 8.41 respectively. Short set yoghurt prepared using 2% of liquid milk culture took 5:30 h to set with DMC of 8.12 log $_{10}$ cells /ml and acidity of 0.50 LA and score of 7.91 for overall acceptability that indicated lower cell counts in liquid culture compared to SSF dahi culture and probiotic product, acidophilus milk prepared with 2%, 2.5% and 3% of dried powder of SSF acidophilus culture curdled the milk at 9:30 h at 37^0C. Acidophilus milk prepared using 3% SSF culture was best compared to 2 and 2.5% inoculum, with DMC of 8.91 log, 0.67% lactic acid and overall acceptability score of 8.50 respectively. Acidophilus milk prepared using 3% of liquid milk culture took 11 h to curdle with DMC of 8.23 log $_{10}$ cells /ml and acidity of 0.61LA and score of 8.00 for overall acceptability that indicated lower cell counts in liquid milk inoculum compared to SSF acidophilus inoculum.

Keywords: solid state fermentation (SSF), dahi culture, yoghurt culture and probiotic culture

Introduction
Milk is a fluid lacteal secretion obtained from the female of all mammals. Milk is a complete food, almost unique as a balanced source for most human dietary needs since it contains both energy supplying nutrients in the form of lactose and milk fat and the body-building nutrients such as milk protein, casein and minerals like calcium, phosphorus are part and parcel of milk. The keeping quality of milk is enhanced by heat treatment such as pasteurization, boiling and ultra-high temperature treatment. Milk is also utilized to manufacture variety of milk products to increase its shelf life and to preserve the nutrients (Fox *et al.,* 2000) [4]. The observation that milk turns sour when kept at room temperature was exploited to minimize spoilage and then led to the production of countless forms of fermented products by deliberate addition of starter cultures (Wouters *et al.,* 2002) [14]. Out of total milk produced 7% is utilized for the manufacture of fermented milk products.

Archaeological evidence has indicated that the process of fermentation in foods was discovered accidentally thousands of years ago. However, over time, it has soon become apparent that many fermented foods had longer storage lives and improved nutritional values compared to their unfermented equivalents, making this form of food processing a popular technique (Farnworth, 2004) [3]. Fermentation is one of the important methods of increasing the shelf life of milk. Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as lactic acid bacteria and yeast.

Based on the substrate, fermentation may be submerged fermentation (SmF) in liquid media or Solid State Fermentation (SSF) on solid substrates like nutritive (dhal, rice) or inert substrates (paddy husk). Solid state fermentation technique has been widely used in preparation of fermented foods, enzymes, organic acids, polysaccharides, biomass of lactic acid bacteria, colours and flavours that involve the controlled growth of microorganisms on solid substrates in the absence of free moisture,

so as to obtain large number of viable cells in concentrated form (Bhargav *et al.*, 2008) [1].

Edible substrates such as dhal are used as solid substrates to grow the cells to maximum level up to 10^{10} cells per gram due to more surface area for growth. In conventional liquid culture, the cell growth is limited maximum up to 10^8 cells per millilitre, due to acidic pool during the growth of cells in the presence of free moisture (Koyani and Rajput, 2015) [8].

Lactic acid bacteria (LAB) are a group of microaerophilic, Gram positive, non-spore forming bacteria that ferment lactose to produce primarily lactic acid. LAB include a variety of industrially important genera such as *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*. LAB are known commonly as starter bacteria. They are naturally present in raw food material such as milk, vegetables and also in the human gastro-intestinal tract (probiotics), playing an important role as starter cultures for fermentation in dairy and food industries.

Probiotics like *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* are live microorganisms thought to be beneficial to the host organism when ingested through food. On the contrary, the term prebiotic generally refers to a food component like carrot juice, chicory root, tomato juice and many others that can be hydrolyzed only by selective microflora termed probiotics in colon of gastro-intestinal tract, activating them to manifest potentially beneficial effects on the host (Hussain *et al.*, 2016) [6]. Hence, in this study attempt has been made to perform dried powder SSF lactic cultures in heat treated milk for the production of fermented products.

Materials and Methods

Lactic acid bacterial cultures

The dahi culture consisting of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *Lactis* bv. *diacetylactis*, yoghurt culture that included *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* as probiotic culture, which had been maintained in sterile Yeast glucose chalk litmus milk (YGCLM) in the department of Dairy Microbiology, Dairy science college, KVAFSU, Hebbal, Bengaluru-24 were used in this study.

Collection and Screening of various dhals for aerobic spore counts

Dhal of 8 various types, commonly available in local market of Bengaluru such as raw bengal gram dhal, roasted bengal gram dhal, black gram dhal, green gram dhal, hyacinth dhal (avarae bele), masoor dhal, red gram dhal, soya bean dhal were purchased, cleaned to remove stones and unwanted plant materials and stored in a self-sealing polythene pouches. To determine the extent of spores present in dhal, they were subjected to aerobic spore counts by plating method as per Harrigan (1998) [5].

Various sporicidal treatments given to black gram dhal to use as solid substrate

Various treatments like dry heat treatment such as dry frying (5 min), exposure to microwave (1 min) and exposure to 100^0 C for 1 h in hot air oven and wet heat treatments like hydration of dhal for 30 min, 12 h and 24 h, 0.01% and 0.05% treatment with hydrogen peroxide and tantalization (steaming for 3 successive days) were given to dhal to reduce aerobic spore count and after treatment analysed the treated dhal for aerobic spore count as mentioned in Harrigan (1998) [5].

Supplementation of treated black gram dhal for the growth of lactic cultures

Best sporicidal treatment to completely destroy the aerobic spore was selected and then supplemented with skim milk powder, ash guard juice, carrot juice and tomato juice.

Preparation of ash guard juice, carrot juice and tomato juice

Ash guard, carrot and tomato were obtained freshly from local market. The edible portions were obtained, washed with potable water, grated, steamed for 15 min and mashed in clean, dry, mixer. The obtained puree was filtered through muslin cloth. After filtration the juices of ash guard, carrot and tomato were collected separately in a sterile conical flask.

Final supplementation to black gram dhal

The aerobic spore free black gram dhal was supplemented with each of SMP, ash guard juice, carrot juice and tomato juice at 0.5, 1, 1.5 and 2% level. The moisture maintained was 1:0.8 level, including volume of water in juices.

Growth study of SSF cultures on supplemented black gram dhal

The maximum growth period required for good biomass of lactic culture on supplemented black gram dhal was determined at optimum growth temperature for 48 h. At every 6 h interval, aseptically drawn samples of SSF dahi, yoghurt and acidophilus cultures were subjected for viability determination.

Determining the viability of lactic culture grown on supplemented black gram dhal

SSF cultures of dahi, yoghurt and acidophilus drawn at different growth periods were aseptically transferred to sterile pestle and mortar, triturated with sterile 99 ml of phosphate buffer separately, required dilutions were prepared and plated using yeast glucose agar for total lactic count. The plates were incubated at 30^0C for dahi culture and while in case of yoghurt and acidophilus cultures at 37^0C. The viable lactic counts were expressed as $\log_{10}$ cfu/g.

Preservation of SSF lactic culture

In order to reduce the moisture content and increase shelf life, various drying methods such as air drying, freeze drying and fluid bed drying were followed for the SSF lactic cultures such as dahi, yoghurt and acidophilus grown on black gram dhal.

Air Drying of SSF lactic cultures

The SSF lactic cultures were dried at 20^0C, 25^0C and 30^0C for a period of 20 h in BOD incubator set to that temperatures. Before and after drying, moisture content and viability of lactic cultures on fermented black gram dhal were determined.

Drying of SSF lactic cultures using Freeze drier

The SSF cultures were dried using freeze dryer at temperature of -42^0C with vacuum of 0.01 mm mercury for a period of 8 h. The freeze dried SSF lactic cultures were subjected to determined moisture content and viable counts.

Drying of SSF lactic cultures using fluid bed drier

SSF fermented lactic culture was transferred to the sterile fluid bed drier and dried at ambient temperature (25 ± 1^0C) for

periods of 1, 1.5 and 2 h and the samples were aseptically drawn to determine the moisture and viable lactic counts.

Preparation of powder of dried SSF lactic cultures

The best dried SSF lactic cultures grown on supplemented black gram dhal, based on less retained moisture and higher viable count were made into powder using sterile dry mixer. Powdered SSF lactic cultures were transferred to labelled self-sealing polythene pouches.

Performance of dried powder of SSF lactic cultures in heat treated milk

The activity of dried powder of SSF lactic cultures in heat treated (90^0C /10 min) milk was studied by inoculating at 0.5, 1 and 1.5% level for dahi and yoghurt while for acidophilus the level of inoculum was 2, 2.5 and 3%. After the inoculation, the dahi culture was incubated at 30^0C, yoghurt culture at 42^0C and acidophilus at 37^0C till curdling was noticed. Curdling time, DMC, titratable acidity, coliforms, aerobic spore count, yeast and mold counts were determined, along with sensory evaluation.

Activity of dried powder of SSF cultures in milk

Preparation of dahi, yoghurt and acidophilus milk from dried SSF lactic culture

Whole milk was taken and standardized to 3.5% fat and 8.5% solids not fat which was homogenized at 65^0C/two stages (2500 PSI and 500 PSI). After homogenization, heat treated to 90^0C/10 min, cooled to incubation temperature and inoculated with dried powder of SSF dahi, yoghurt and acidophilus cultures separately at 1%, 0.5% and 3% respectively in duplicate. One set was used for analysis and another set was given for sensory evaluation. The inoculated milk was incubated at 30^0C, 42^0C and 37^0C for dahi, yoghurt and acidophilus milk, respectively.

Curdling time

Before keeping milk for incubation the initial time was noted and after setting the milk again time was recorded. The difference between initial time and finale time was considering on curdling time.

Direct microscopic count (DMC)

DMC was determined as per the standard procedure of Harrigan (1998) [5]. Samples to be analyzed were mixed well and diluted to 1:10 by using physiological saline as diluent. Sample of 0.01 ml (10 µl) was transferred using a micro pipette onto a marked slide of $1cm^2$ and smear was prepared by spreading evenly with in the marked area. The smear was fixed using ethanol for 2 min, de-fattened using xylene for 2 min and stained using borax methylene blue for 5 min and rinsed with tap water. Then the smear was observed under the oil immersion objective and were counted in each of the field. Microscopic factor had to be determined using stage micrometre for the final computation to formula to obtain DMC. The diameter of microscopic field was measured and then calculated area using πr^2. The obtained value was the numbers of microscopic fields of obtained diameter with in 1sq.cm of smear. The average number of organisms per field obtained was multiplied by microscopic factor, dilution factor and 100 (conversion from 0.01 to 1) expressed as $\log_{10}$ per g.

Determination of titratable acidity

Titratable acidity for the curdled milk was determined as per the standard procedure shown on (IS: SP: 18, part XI, 1981) [7]. Acidity of dahi, yoghurt and acidophilus milk was determined by taking exactly 10 g of well mixed sample in a conical flask to which 10 ml of boiled, cooled water was added and mixed thoroughly. Phenolphthalein indicator of 5 drops was added and titrated against standardized N/10 sodium hydroxide (NaOH) and the rundown of NaOH was multiplied by 0.09 and expressed as per cent Lactic acid.

$$\% \text{ Lactic Acid (\% LA)} = \frac{9 \text{ x } 0.1 \text{ x Titer value}}{\text{Weight of sample}}$$

Sensory analysis

Dahi, yoghurt and acidophilus milk prepared separately were served to panel of judges for sensory evaluation such as colour and appearance, body and texture, flavour and overall acceptability. The scores given by panel of judges were then statistically analyzed.

Statistical analysis

The data was analyzed using R software [R. version 3.1.3 (2015-3-09), copyright © 2015, R foundation] for statistical computing both one way and two way Completely Randomed Design (CRD) which is the most appropriate for the study. Data on the respective variables were collected for three replication for each of these treatments. ANOVA tables were prepared to analyse the data and where the F value is significant the critical difference was calculated and used to identify where significant differences existed and was indicated in the table use superscripts. The formula for the critical difference (CD) is

$$CD = \frac{\sqrt{2 \times MSS\,(E)}}{R}\ t\alpha$$

Where, MSS (E) = Mean Sum of squares of the error
r = number of replications
$t\alpha$ = table t value of the α level of significance

Results and Discussion

Performance of dried powder of SSF dahi culture in milk

Performance of dried powder of SSF of lactic culture was observed, after inoculation into heated milk (90^0C/10 min) at different levels and incubation. Curdling time, DMC and titratable acidity along with that sensory score were also obtained to fix proper inoculum level of dried SSF lactic cultures. Milk culture was used as control for dried SSF culture performance.

It was found that among 0.5, 1 and 1.5% of inoculum for dahi preparation using dried powder SSF culture, 1% when inoculated to heated milk and incubated at 30^0C, curdled milk at 5:00 h with DMC of 8.60 $\log_{10}$ cells/g and titratable acidity of 0.71% LA (Table 1).

The sensory score was found to be 8.55, 8.50, 8.08 and 8.47 for colour and appearance, body and texture, flavour and overall acceptability. Compared to 0.5% and 1.5% inoculum of SSF dahi cultures, significant difference was not seen among parameters such as curdling time, DMC and titratable acidity. But 1% inoculum level of SSF dahi culture showed significant difference in body and texture and flavour with respect to dahi prepared by using liquid or milk dahi culture considered as control. Increase in DMC was maximum at 1% inoculum compared to 0.5 and 1.5% inoculum. Hence among the inoculums levels, 1% SSF dahi culture was considered in further studies with respect to the activity and sensory score.

A similar study by Deepa (2011) [2], who inoculated 0.5% SSF dahi culture into sterile skim milk, which took 6 h to curdle at 300C with acidity of 0.7% LA, log DMC of 8.51 and sensory score of 8 for over all acceptability with respect to 9 point hedonic scale.

Madhusudan (2016) [9] inoculated 1% liquid milk culture into sterile milk, which took 11 h to curdle at 30^0C with acidity of 0.55% LA, log DMC of 7.89 and sensory score of 8.50 for over all acceptability with respect to 9 point hedonic scale.

Table 1: Performance of dried powder of SSF dahi culture in milk

Type of culture	Activity in milk			Sensory scores (9 point hedonic scale)			
	Curdling time (h)	DMC (log_{10} cells/g)	Titratable acidity (% LA)	Colour and Appearance	Body and texture	Flavour	Overall Acceptability
Milk culture at 1% (V/V)	6:00	8.03[a]	0.70[a]	8.16[a]	8.22[a]	7.91[a]	8.12[a]
SSF cultures (W/V)							
0.5%	5:00	8.45[a]	0.68[a]	8.38[a]	8.44[b]	7.91[a]	8.08[a]
1%		8.60[b]	0.71[a]	8.55[b]	8.50[b]	8.08[b]	8.47[a]
1.5%		8.50[a]	0.69[a]	8.11[a]	8.41[a]	8.02[a]	8.06[a]
CD ($p \leq 0.05$)		0.47	0.03	0.35	0.21	0.15	0.41

Note:
- The results were average of three trials (n = 3).
- Same superscript show non-significance while different indicate statistically significant difference ($p \leq 0.05$).
- The milk culture had 6 log counts while SSF dahi culture possessed 8.33 log counts in the inoculum.

Performance of dried powder of SSF yoghurt culture in milk

Performance of dried powder SSF of yoghurt culture was observed by inoculating into heated milk (90^0C/10 min) at different levels and incubating at 42^0C. Curdling time, DMC and titratable acidity along with that sensory scores were also obtained to find an ideal inoculum level of dried SSF culture. Milk culture was used as control to compare with dried SSF culture performance.

All the three inoculum levels 0.5, 1 and 1.5% for yoghurt preparation using dried powder SSF culture curdled milk at 3:30 h with nearly same cell count and titratable acidity. Out of the three, 0.5% level of SSF culture as inoculum was used as optimum for short set yoghurt preparation with slightly more DMC of 8.61 log_{10} cells/g compared with 1% and 1.5% SSF culture (Table 2). The sensory score for short set yoghurt prepared using 0.5% inoculum of SSF culture was found to be 8.33, 8.25, 8.21 and 8.41 for colour and appearance, body and texture, flavour and overall acceptability. Except body and texture of yoghurt, none of the other sensory parameters, showed significant difference statistically.

Vanisri (1995) [13] used freeze dried SSF culture on supplemented black gram dhal at 1% to prepared short set yoghurt which took 4 h to curdled with acidity of 0.77% LA with total viable count of 8.53.

Inoculated 1% SSF yoghurt culture on supplemented paddy husk into sterile skim milk, which took 4 h to curdle at 42^0C with acidity of 0.60% LA, log DMC of 9.60 (Ramachandra, 1999) [11].

Ramachandra (2016) [12] inoculated 1% liquid culture of *Streptococcus thermophilus* ST6 into sterile skim milk, which took 6 h to curdle at 42^0C with acidity of 0.54% LA, log DMC of 7.20 and sensory score of 8.00 for over all acceptability with respect to 9.00 point hedonic scale.

Table 2: Performance of dried powder of SSF yoghurt culture in milk

Type of culture	Activity in milk			Sensory scores (9 point hedonic scale)			
	Curdling time (h)	DMC (log_{10} cells/g)	Titratable acidity (% LA)	Colour and Appearance	Body and texture	Flavour	Overall Acceptability
Milk culture at 2% (V/V)	5:30	8.12[a]	0.50[a]	8.20[a]	8.06[a]	8.11[a]	7.91[a]
SSF culture (W/V)							
0.5%	3:30	8.61[a]	0.79[a]	8.33[a]	8.25[a]	8.21[a]	8.41[a]
1%		8.51[a]	0.75[a]	8.30[a]	8.22[ab]	8.16[a]	8.20[a]
1.5%		8.33[a]	0.71[a]	8.12[a]	8.30[abc]	8.06[a]	8.00[a]
CD($p \leq 0.05$)		0.51	0.23	0.19	0.18	0.16	0.50

Note:
- The results were average of three trials (n = 3).
- Same superscript show non-significance while different indicate statistically significant difference ($p \leq 0.05$).
- The milk culture had 6 log counts while in case of SSF yoghurt culture contained 8.85 log counts in the inoculum.

Performance of dried powder of SSF *Lactobacillus acidophilus* in milk

Dried powder of SSF of acidophilus when inoculated into heated milk (90^0C/10 min) at 2, 2.5 and 3% levels and incubated at 37^0C, 3% inoculum curdle at 9:30 h with DMC of 8.91 log_{10} cells/g and titratable acidity of 0.67% LA (Table 3).

The sensory score was found to be 8.38, 8.44, 8.27 and 8.50 for colour and appearance, body and texture, flavour and overall acceptability (Table 10). Colour, body and texture of acidophilus milk showed significance statistically but other sensory profiles, curdling time, DMC and titratable acidity did not show any significance for acidophilus milk prepared using 3% SSF inoculum. Based on slightly higher DMC and titratable acidity of acidophilus milk prepared using 3% inoculum of SSF compared to acidophilus milk prepared using 2.0 and 2.5% inoculum, 3% inoculum was optimized for the acidophilus milk preparation.

Vanisri (1995) [13] inoculated at 1% of freeze dried SSF acidophilus culture to sterile skim milk, that curdled milk at

18 h at 37^{0}C with acidity of 0.50% LA and viable log count of 9.50. The curdling time enhancement may be due to freeze drying which took more time to adopt.

An identical study by Prabha (1999) [10] who reported that air dried SSF culture of B*ifidobacterium longum* PF1 when inoculated at1% level into sterile skim milk, curdling time was 20 h with acidity of 0.60% LA when compared to inoculated plain skim milk (2%) which took 48 h to curdle (0.4% LA).

Lactobacillus acidophilus 111 air dried SSF culture on paddy husk, inoculated at 1% took 26 h to curdle at 370C with acidity of 0.4% LA (Ramachandra, 1999) [11].

Ramachandra (2016) [12] inoculated 1% liquid culture of probiotic *Lactobacillus rhamnosus* LB3 into sterile skim milk, which took 24 h to curdle at 370C with acidity of 0.62% LA, log DMC of 7.79 and sensory score of 8.20 for over all acceptability with respect to 9.00 point hedonic scale.

Table 3: Performance of dried powder of SSF *Lactobacillus acidophilus* in milk

Type of culture	Activity in milk			Sensory scores (9 point hedonic scale)			
	Curdling time (h)	DMC (log_{10} cells/g)	Titratable acidity (% LA)	Colour and Appearance	Body and texture	Flavour	Overall Acceptability
Milk culture at 3% (V/V)	11:00	8.23^a	0.61^a	8.50^b	8.20^a	8.10^a	8.00^a
SSF cultures (W/V)							
2%	9:30	8.42^a	0.59^a	8.30^b	8.19^a	8.20^a	8.11^a
2.5%		8.74^c	0.63^a	8.31^a	8.24^b	8.26^a	8.15^a
3%		8.91^d	0.67^a	8.38^{bc}	8.44^b	8.27^a	8.50^a
CD ($p \leq 0.05$)		0.50	0.07	0.16	0.23	0.22	0.51

Note:

- The results were average of three trials (n = 3).
- Same superscript show non-significance while different indicate statistically significant difference ($p \leq 0.05$).
- The milk culture had 6 log counts while in case of SSF acidophilus contained 9.50 log counts in the inoculum.

Conclusion

The fluid bed dried SSF cultures were evaluated for the performance in the sterile milk for various activity parameters. The dried powder of SSF dahi, yoghurt and acidophilus culture were inoculated into the heated whole milk (90^{0}C /10 min) at 0.5, 1 and 1.5%. Incubation was done at 30^{0}C for dahi; 42^{0}C for yoghurt and 37^{0}Cfor acidophilus cultures respectively. The performance of these cultures were analysed by the curdling time, DMC, titratable acidity as activity test along with sensory evaluation.

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