

Original Research Article

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Production of Biomass of Lactic Acid Bacteria Using Optimized Solid Substrate in Self-Technique

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ABSTRACT

The present investigation was intended to optimize the solid substrate for the production of biomass of lactic acid bacteria using various dhals such as raw bengal gram dhal, roasted bengal gram dhal, black gram dhal, green gram dhal, hyacinth dhal (Avaraebele), masoor dhal, red gram dhal, soya bean dhals for growing Lactic Acid Bacteria using the solid state fermentation technique after screening for bacterial spores. The screened black gram dhal was selected which found to have lowest spore count of $1.52 \log_{10}$ cfu/g then were exposed to hot air oven at 100°C for 1 h and sterilization at 121°C for 30 min for complete destruction of spores. The black gram dhal was added with various supplements each of skim milk powder, ash guard juice, carrot juice and tomato juice as sources of growth factors at the rate of 0.5, 1, 1.5 and 2%. The cultures selected for biomass production were dhali culture, yoghurt culture and acidophilus cultures added at 1% level of inoculum. The optimum growth was obtained when supplements were added each at 1% concentration with maximum viable counts for dahi culture was $8.41 \log_{10}$ cfu/g while yoghurt culture of $8.98 \log_{10}$ cfu/g and acidophilus culture of $9.47 \log_{10}$ cfu/g.

Keywords

Black gram dhal, Supplements, Lactic acid bacteria, Biomass.

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Introduction

Food is an inevitable human need for optimum physical and mental functioning which collectively termed as health. Overall health, depends on the nutritional quality, safety and balance of the food consumed. Spoilage of foods mainly is due to physical damage, chemical reaction or through biological agents like microorganisms including bacteria, fungi and also may be worms as well as insects. Based on the water activity, foods are classified into perishable, semi perishable and non-perishable ones.

Milk is considered as universal food, as it has most of the nutrients required for a human being as well as a good medium for the

growth of microorganisms which enter from various sources. Fermentation of milk preserves its nutrients for long time and fermentation is made possible by inoculating the heat treated milk with lactic acid bacteria. Fermented milk products have their own importance in the human diet by extending potential therapeutic benefits to the consumers. People have the concept of using fermented milk products as they are safe for consumption due to fermentation end products of the lactic cultures.

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).

Biomass production of lactic acid bacteria is important to serve as active inoculum to produce fermented milk products. Lactic acid bacteria can be grown in milk or whey as liquid media or may be on solid substrate like dhal or bran.

Edible substrates such as dhals 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).

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*. Probiotics like *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* are live microorganisms thought to be beneficial to the host organism when ingested through food.

Hence, in this study attempt has been made to grow lactic cultures on suitable edible solid substrate with proper supplementation for the biomass production.

Materials and Methods

Lactic acid bacterial cultures

The dahi culture consisting of *Lactococcus lactis* sp. *lactis* and *Lactococcus lactis* sp. *lactis* bv. *diacetylactis*, yoghurt culture that included *Streptococcus thermophiles* and *Lactobacillus delbrueckii* sp. *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

Dhals 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 (avaraebele), 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 dhals, they were subjected to aerobic spore counts by plating method as per Harrigan (1998).

Various sporicidal treatments 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°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 tyndallization (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).

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

Best sporicidal treatment to completely destroy 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.

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 Randomized Design (CRD) which is the most appropriate for the study. Data on the response variables were collected for three replications for each of these treatments. ANOVA tables were prepared to analyse the data and where the F value was 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_{α} = table t value of the α level of significance

Results and Discussion

Screening of various dhals for aerobic spore count

Various locally available dhals were used as solid substrates for fermentation using lactic cultures as they provide more surface area for the growth of lactic acid bacteria and also a good source of proteins, calcium and iron. They imbibe water during soaking and also do not sag as well as agglomerate maintaining their grainy texture. Previous studies carried out in the same laboratory, also had shown use of dhal as good solid substrate for growth of lactic cultures. These dhals contain low moisture and hence major contaminant being aerobic spores. The aerobic spores of bacteria which enter through soil and air are most resistant for heat treatments and their initial load also has significant effect on the survivability. Preliminary requirement was to screen dhals for their aerobic spore counts.

The dhal samples of 8 numbers were obtained from local market, Bengaluru such as raw bengal gram dhal, roasted bengal gram dhal, black gram dhal, green gram dhal, hyacinth dhal, masoor dhal, red gram dhal and soya bean dhal, cleaned and stored in polythene pouches. Various dhals were subjected to enumeration of aerobic spore count by subjecting to heat treatment at 80°C for 10 min

with help of 2% Nutrient agar medium. The aerobic spore counts was less in black gram dhal (1.52 log₁₀ cfu/g) followed by masoor dhal (2.78 log₁₀ cfu/g), green gram dhal (2.98 log₁₀ cfu/g), raw bengal gram dhal (3.47 log₁₀ cfu/g), roasted Bengal gram dhal (3.54 log₁₀ cfu/g), hyacinath dhal (3.69 log₁₀ cfu/g), red gram dhal (4.15 log₁₀ cfu/g) and soya bean dhal (5.05 log₁₀ cfu/g). Predominance of aerobic spore counts was less in case of black gram dhal compared to other dhals may be due to unknown antimicrobial agent. Hence black gram dhal was selected as an edible solid substrate in the further studies (Table 1 and Fig. 1).

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

In order to completely destroy the spores, various methods were adopted in the present study. Black gram dhal was subjected for dry and wet treatments and finally autoclaved at 121⁰C for 30 min to destroy the aerobic spores completely.

Dry treatments given for black gram dhal included dry frying (5 min), exposure to microwave (1 min), exposure to 100⁰C for 30 min and exposure to 100⁰C for 1 h. These treatments reduced aerobic spore in the range 0 to 2.59 log₁₀ cfu/g in black gram dhal.

Among wet heat treatments for black gram dhal like hydration, hydrogen peroxide treatment and tyndallisation, the viable aerobic spore count was between 1.30 and 3.11 log₁₀ cfu/g (Table 2 and Fig. 2). Several treatments like dry frying, hydration increased the spore counts, indicating the ability to survive and resist the final heating at 121⁰C for 30 min. Even steaming for 3 successive days termed tyndallisation as well as the addition of hydrogen peroxide did not show complete destruction of spores. Exposure of dhal in hot air oven at 100⁰C for 1 h and then

sterilization at 121⁰C for 30 min resulted in complete destruction of aerobic spore. Hot air might have sensitized the spores and latter wet heat at 121⁰C for 30 min, helped to kill the remaining spores or vegetative cells. Hence, the same method was followed in further study.

Literature regarding sporicidal treatment to dhal is very scanty. Nakauma *et al.*, (2004) applied radiations combined with a traditional heat approach (85–95⁰C), to inactivate *Alicyclobacillus acidoterrestris* spores in dextrin powder by 1.5 log at 1.0 kGy/y for 1 h. Bevilacqua *et al.*, (2008) also reported reduction of *Alicyclobacillus acidoterrestris* in asparagus cream, containing olive oil from 2 log at 900 - 2450 MHz for 5–7 min.

Somavat *et al.*, (2012) had found ohmic treatments at frequencies of 60 Hz and 10 kHz showed a general trend of accelerated spore inactivation compared to conventional heating at 121⁰C to 130⁰C. They hypothesized that vibration of polar dipicolinic acid molecules and spore proteins to electric fields at high temperature conditions may result in the accelerated inactivation.

Effect of different levels of supplementation to black gram dhal on the growth of lactic cultures

In order to get maximum growth, black gram dhal was added with various supplements each of skim milk powder (milk source), ash guard juice (dietary fiber), carrot juice (pantathine) and tomato juice (manganese sulphate) as sources of growth factors or prebiotic for lactic and probiotic cultures at the rate of 0.5, 1, 1.5 and 2%. Out of 0.5 to 2% supplementation to black gram dhal for enhanced growth of dahi, yoghurt and acidophilus, 0.5% showed lower viable counts of 8.05, 8.39 and 8.15 log₁₀ cfu/g respectively (Fig. 3).

Table.1 Screening of various dhals for aerobic spore count

Name of dhal	Botanical nomenclature	Aerobic spore count (log ₁₀ cfu/g)
Bengal gram dhal		
a) Raw	<i>Cicer aritinum</i> L	3.47 ^a
b) Roasted		3.54 ^a
Black gram dhal	<i>Vigna mungo</i>	1.52 ^b
Green gram dhal	<i>Vigna radiata</i>	2.98 ^a
Hyacinath dhal (Avaraebele)	<i>Lablab purpureus</i>	3.69 ^a
Masoor dhal	<i>Lens culinaris</i>	2.78 ^a
Red gram dhal	<i>Cajanus cajan</i>	4.15 ^a
Soya bean dhal	<i>Glycine max</i>	5.05 ^a
CD (p ≤ 0.05)		1.73

Note

The results were average of three trials.

Same superscript indicates non-significance while different, indicates statistically significant difference at p ≤ 0.05.

First dilution heated to 80⁰C for 10 min for selection of aerobic spores.

Sterile 2% Nutrient agar was used for plating with incubation at 37⁰C for 24–48 h.

Table.2 Various sporicidal treatments to black gram dhal to use as solid substrate

Type of treatment	Surviving aerobic spores (log ₁₀ cfu/g)
I Dry treatments	
a) Dry frying (5 min)	2.59 ^a
b) Exposure to microwave (1 min)	1.84 ^a
c) Exposure to 100 ⁰ C for 30 min	1.45 ^b
d) Exposure to 100 ⁰ C for 1 h	0.00 ^b
II Wet treatments	
a) Hydration	
i. 30 min.	2.51 ^a
ii. 12 h.	3.11 ^a
iii. 24 h.	1.30 ^b
b) Hydrogen peroxide after hydration	
i. 0.01%	1.95 ^a
ii. 0.05%	1.69 ^a
c) Tyndallisation after hydration (steamed for 3 successive days)	1.31 ^b
CD (p ≤ 0.05)	
1.05	

Note

The results were average of three trials.

Same superscript indicates non-significance while different, indicates statistically significant difference at p ≤ 0.05.

After each treatment mentioned above, black gram dhal was autoclaved at 121⁰C for 30 min.

Table.3 Effect of different levels of supplementation to black gram dhal on the growth of lactic cultures

Culture	Parameter	Percentage of supplements added to black gram dhal each (W/V) Skim milk powder + Ash guard juice + Carrot juice + Tomato juice				CD (p ≤ 0.05)
		0.5%	1%	1.5%	2%	
		Viable counts (log ₁₀ cfu/g)				
Dahi	Control (No supplements)	6.24 ^a	6.24 ^a	6.24 ^a	6.24 ^a	0.02
	With supplements	8.05 ^a	8.41 ^a	7.75 ^a	7.29 ^a	1.13
Yoghurt	Control	6.53 ^a	6.53 ^a	6.53 ^a	6.53 ^a	0.02
	With supplements	8.39 ^a	8.98 ^a	7.80 ^a	7.30 ^a	1.96
<i>Lactobacillus acidophilus</i>	Control	6.00 ^a	6.00 ^a	6.00 ^a	6.00 ^a	0.02
	With supplements	8.15 ^a	9.47 ^a	7.98 ^a	7.79 ^a	1.70

Note:

The results were average of three trials.

Same superscript shows non-significance while different indicates statistically significant difference at p ≤ 0.05.

Moisture 1:0.8 were added to black gram dhal (Moisture of 0.8 includes volume of incorporated juices)

The inoculum was added at 1% with incubation of 30⁰C for dahi and 37⁰C for both yoghurt and acidophilus culture

Fig.1 Screening of various dhals for aerobic spore count

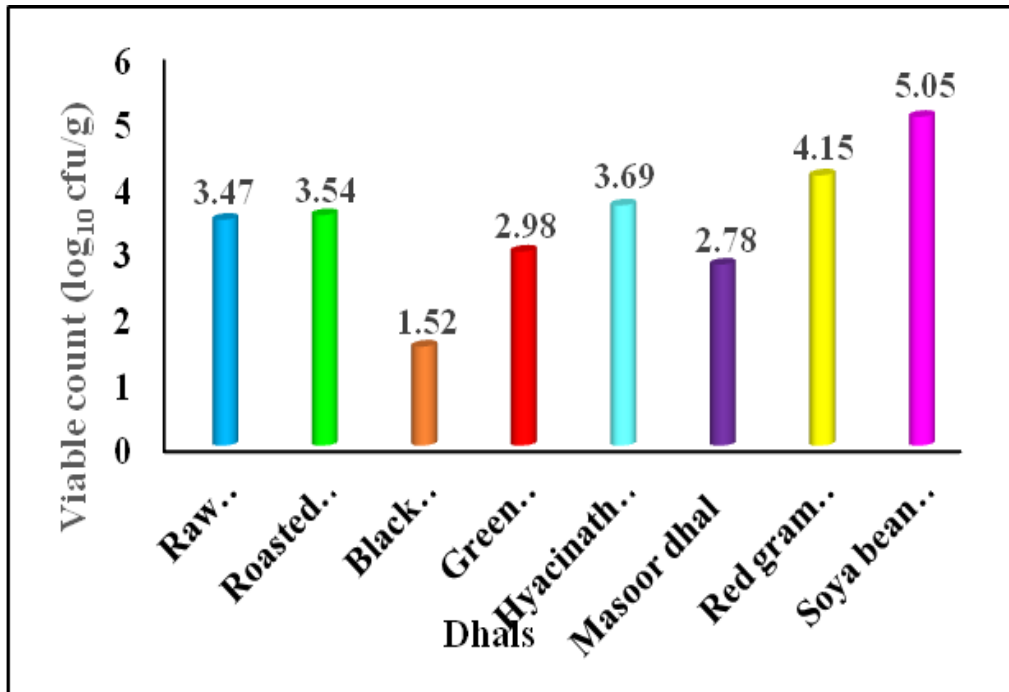


Fig.2 Various sporicidal treatment to black gram dhal to use as solid substrate.

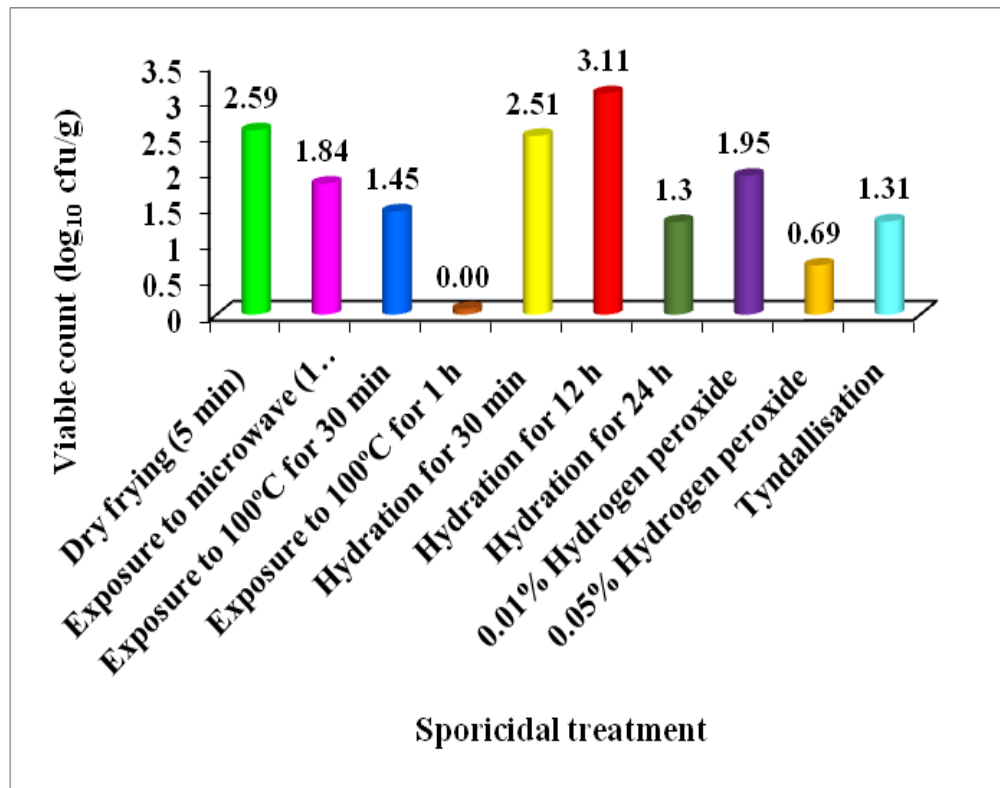
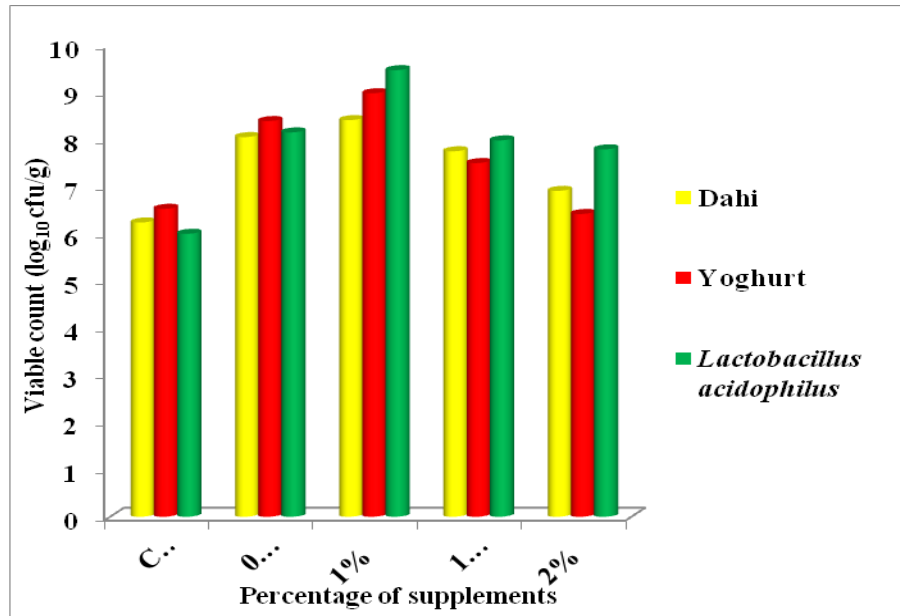


Fig.3 Effect of different levels of supplementation to black gram dhal on the growth of lactic cultures



The trend was almost similar at 1.5 and 2% supplementation, where in the viable counts obtained on black gram dhal for dahi, yoghurt and acidophilus were lower accounting for 7.75, 7.80, 7.98 log₁₀ cfu/g and 7.20, 7.30, 7.79 log₁₀ cfu/g respectively. Optimum growth was obtained when supplements were added each at 1% concentration with log counts of 8.41 for dahi, 8.98 for yoghurt and 9.47 log₁₀ cfu/g for acidophilus cultures (Table 3). Supplementation of 0.5% may be lesser for the growth of lactic cultures, higher per cent reduced counts which may be attributed to more lactic acid production that affected viability of lactic culture. Biomass increase among lactic cultures significantly varied with respect to per cent of incorporation of each supplement of SMP, ash guard juice, carrot juice and tomato juice at $p \leq 0.05$.

On par with the present study, Vanisri (1995) supplemented black gram dhal having (1:1) moisture, with 10% skim milk powder that supported the growth of *Lactobacillus acidophilus* culture from 5.7 to 9.7 log₁₀

cfu/g. But addition of skim milk powder in the present study was 1%. Harrigan (1998) replaced manganese sulphate with tomato juice indicating that tomato juice may be rich in manganese sulphate in Gibson semi solid medium.

The stimulatory ability of MnSO₄ at 0.05% in MRS broth was observed with respect to the growth of *Bifidobacterium longum* PF1 from 6 to 8.8 log₁₀ cfu/g. Ash guard, carrot and tomato juice when incorporated into skim milk (18% TS), inoculated with *Bifidobacterium longum* PF1, took 18 h to curdle at 37°C compared to plain skim milk (48 h to curdle) indicating the prebiotic nature of natural vegetable juices (Prabha, 1999).

Ramachandra *et al.*, (2008) reported that paddy husk lacking in the nutrients was incorporated with various supplements such as peptone (1%) as nitrogen source, yeast extract (1%) as vitamin source, skim milk powder (1%) for adaptation to milk, MnSO₄ (0.05%) as mineral source, black gram dhal (1%) as protein source, tween 40 (1%) as

surfactant that enhanced the growth of *Lactobacillus acidophilus* 111 at 37⁰C for 48 h from 6 log to 10 log₁₀cfu/g compared to non-supplemented paddy husk medium.

Dahi culture was grown on black gram dhal containing 70% moisture supplemented with 1% skim milk powder and 10% tomato juice at 30⁰C for 24 h where in DMC attained was 9.42 log₁₀ cell/g (Deepa, 2011).

Various dhals as solid substrates were used for growing biomass of lactic acid bacterial cultures after screening for bacterial spores. Among dhals tested, black gram dhal showed lowest spore count. Then black gram dhal treated with sporicidal treatment such as Hot air oven at 100⁰C for 1 h and sterilization at 121⁰C for 30 min completely destroyed the spores. In order to get maximum growth, black gram dhal was added with various supplements each of skim milk powder, ash guard juice, carrot juice and tomato juice as sources of growth factors or prebiotic for lactic and probiotic cultures at the rate of 0.5, 1, 1.5 and 2%. The optimum growth was obtained when supplements were added each at 1% concentration with maximum viable counts of dahi (8.41), yoghurt (8.98) and acidophilus (9.47 log₁₀ cfu/g).

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