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Viability of *Lactobacillus bulgaricus* as Yoghurt Culture Under Different Preservation Methods

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ABSTRACT

In present study, *Lactobacillus bulgaricus* (yoghurt starter culture) was isolated from indigenous sources and preserved by three different methods namely on agar slopes, under oil and in liquid form conditions using MRS medium. Best method of preservation was suggested on the basis of viability, morphology and Gram's staining ability of culture during storage of two months. Viability checks were made at 0, 15, 30, 45 and 60 days of storage. Under oil preservation method was found to be the best method for maintenance and preservation of starter culture.

Key Words: *Lactobacillus bulgaricus*; Yoghurt; Culture; Preservation

INTRODUCTION

Yoghurt is a fermented milk product and is most popular in South Asia. It is more nutritive as compared to milk in terms of vitamins content, digestibility and as a source of calcium and phosphorus (Foissy, 1983). Tamime and Robinson (1985) suggested that yoghurt starter culture is consisted of two symbiotically growing bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophillus* in the ratio of 1:1. In Pakistan, starter cultures for the production of yoghurt are normally imported from other countries which are preserved in the form of liquid, spray dried, freeze dried and in frozen form. These imported cultures result in added costs to the end product. Keeping in view the existing situation, the project was planned to isolate mono starter culture from local sources and subsequently to preserve it for further use in yoghurt production under available facilities at laboratory scale.

MATERIALS AND METHODS

Procurement of samples. Six samples (10 mL each) of commercial curd were collected in sterilized screw-capped bottles (sterilized at 171°C for 30 min in hot-air-oven) from different areas of Faisalabad. The samples were brought to the Department of Food Technology, University of Agriculture Faisalabad and stored immediately under refrigeration conditions for further processing.

Isolation of *Lactobacillus* spp. Isolation of the particular microorganisms was done according to the method given by Harrigan and McCance (1976) and Holt *et al.* (1994). Curd samples were diluted to 1:10 in sterilized normal saline solution and 1mL volume from each dilution was inoculated onto the surface of Milk agar (MA) and Nutrient agar (NA) and incubated at 37°C for 24 h. The growth thus obtained was checked for morphological and cultural characteristics.

Morphological examination of culture. Morphological and cultural examination was carried out by using Gram's staining method as described by Awan and Rahman (2002).

Purification of isolates. All the colonies from Milk agar, showing frequent rods, pair or chain forming pattern and Gram positive character were separately studied and later on purified on Acetate agar (AA) and Rogosa agar (RA) by applying streak plate techniques as described by Awan and Rahman (2002). Growth obtained after 24 h at 37°C was then examined for morphological and cultural characteristics. Pure colonies of these cultural isolates were finally transferred onto, de Man, Rogosa and Sharpe agar (MRS agar) plates and broth. Incubation was made at 37°C for 24 h and growth thus obtained was again examined for morphological and cultural characteristics following the procedure mentioned by Harrigan and McCance (1976) and Cappuccino and Sherman (1996).

Identification of pure culture. Pure culture thus isolated in the above process was identified up to species level on the basis of their growth at different temperatures and sugar fermentation tests as recommended by Harrigan and McCance (1976). Biochemical tests like indole test, methyl red test, voges proskauer test, citrate utilization test and catalase test were also performed for identification of culture isolates according to the method described by Awan and Rahman (2002).

Preservation of pure culture. Pure culture of *Lactobacillus bulgaricus* was preserved by three different methods as described by Harrigan and McCance (1976). Briefly, (A) On Agar Slopes Methods: Pure culture of *Lactobacillus* spp. was preserved on MRS agar slopes after taking a growth of 48 h at 37°C at ordinary refrigeration temperature, (B) Under Oil Methods: Pure culture was grown on agar slants and then completely covered with sterile liquid paraffin sterilized at 160°C for 1-2 h in hot-air-oven and placed under refrigeration conditions, and (C) In Liquid Form Conditions: Growth of pure culture was taken in MRS

broth and then stored for two months at refrigeration temperature i.e. 4°C.

Viability if culture. Viability, morphology and Gram's staining ability of the cultures was estimated as described by Awan and Rahman (2002) at 0, 15, 30, 45 and 60 days of storage.

Statistical analysis. The data obtained were statistically analyzed as described by Steel *et al.* (1996).

RESULTS AND DISCUSSION

Isolation of microorganisms. Three different types of colonies were observed on Nutrient agar and Milk agar plates (Table I). Most of the colonies were white, irregular, circular and pinpoints. The cultural and morphological characteristics were further resolved on the basis of microscopic examination. Majority of microorganisms were amongst Gram's positive (G +ve) rods and cocci shaped bacteria. Similar findings have also been reported by Masud *et al.* (1991) and Amoroso *et al.* (1992). S_1 and S_2 were found to have maximum numbers of G +ve indicating *Lactobacillus* spp. The growth from two samples S_1 and S_2 was then specially transferred on to the surface of Rogosa agar and Acetate agar plates for more specifications. Exuberant growth (Table II) of *Lactobacillus* spp. was obtained on Acetate agar as compared to Rogosa agar plates showing the suitability of Acetate agar for isolation of *Lactobacillus* spp. as also recommended by Harrigan and McCance (1976) and Cappuccino and Sherman (1996).

Purification of isolates. The colonies on Acetate agar for S_1 showing G +ve chain forming long rods were further purified onto the surface of MRS agar plates and in MRS broth. Single types of colonies were obtained after 24 h incubation at 37°C. Colonies found on MRS agar plates were white in color, convex in shape, small in size, pinpoint, smooth and 1- 2.2 mm in diameter; whereas, there was turbidity, sedimentation and small white suspensions in MRS broth (Table III). MRS medium was found to be the most suitable selective medium for *Lactobacillus* spp. as also confirmed by Harrigan and McCance (1976). Results of sugar fermentation tests, growth at different temperatures and that of biochemical tests for culture isolates (from selected S_1) are shown in Tables IV and V. On the basis of these results it was confirmed that the culture isolated (from S_1) was a pure culture of *Lactobacillus bulgaricus*.

Preservation of pure culture. After isolation and purification, the pure culture (S_1) was preserved by three different methods in order to find out the best method of preservation. For this viable count, morphology and Gram's staining ability of culture were considered as basic parameters.

Table I. Cultural and morphological characters of microorganisms isolated from yoghurt collected from different areas of Faisalabad on general and selective media

Yoghurt samples	NA plates (colonies)	Morphology	M.A. Plates (colonies)	Morphology
S_1	White, irregular, big circular	G+ve, Rods, curved shape, round	White, Pin point	G + ve rod, Chains, G+ve cocci, G+ve clusters.
S_2	White, irregular	G+ve rods, Spherical, Curved shape	White, circular	G+ve rod, Chains, G+ve cocci
S_3	White, circular	G+ve, G–ve, Rods, round, coccobacilli	White, dark yellow	G+ve rod, Chains, G+ve, G-ve, cocci
S_4	-	-	-	-
S_5	White, small	G+ve, G-ve, Round, coccobacilli	Whit big circular	G +ve, G –ve, cocci
S_6	-	-	-	-

N.A. = Nutrient agar, M.A. = Milk agar

Table II. Cultural and morphological characteristics observed on selective and differential media with special reference to G+ve chain forming bacteria isolated form yoghurt samples

Yoghurt sample No.	Differential medium rogosa agar plates (colonies)	Morphology	Selective medium acetate Agar Plates (colonies)	Morphology
S_1	White,sharp, smooth,Irregular,	G+ve, rods, chains	White, sharp, circular, pinpoints,	G+ve, long rods, chains
S_2	White,irregular, small, circular,	G +ve, rods, cocci,Chain	White, irregular, sharp,	G +ve, short rods, cocci

Table III. Cultural and Morphological characteristics of G +ve chain forming bacteria on MRS agar medium and in MRS broth

Yoghurt sample No.	MRS Agar plates (colonies)	Morphology	MRS-broth (colonies)	Morphology
S_1	White,circular, 1-2.2 mm	G+ve rods, chains,single	Turbidity, sedimentation, white suspensions	G + ve, long rods, chains

Table IV. Results indicating sugar fermentation tests recorded for the culture isolates from yoghurt sample (S_1)

Sugars	Acid production	Gas production
Lactose	+	-
Sucrose	-	-
Maltose	-	-

+ = Acid production, Gas production, – =No acid production, No gas production

Table V. Results indicating biochemical tests recorded for the culture isolates from yoghurt samples (S_1)

Experimental procedure	Observation	Results
Indole production	Layer not red	-ve
Methyl red test	Bright red color	+ve
Voges prospkauer test	Pink color	+ve
Citrate utilization	Green Color	- ve
Catalase activity	No bubbling	-ve
Growth at 15°C	No growth	-ve
Growth at 45°C	Growth observed	+ve

Viable count. It is evident from the results (Table VI) that the original figure of viable count $9.9x10^6$ in method A and $4.9x10^6$ in method C had decreased to $3.5x10^5$ and $2.5x10^5$, respectively after 60 days of storage. As far as method of preservation under oil (B) is concerned, it gave the desired trend and the figure for viable count almost remained same i.e., $9.5x10^6$ at 0 days and $9.0x10^6$ at 60 days of storage, respectively.

Table VI. Viable counts (number/mL) determined during storage of culture isolated from yoghurt (S_1) preserved by different methods

Methods	0	15	30	45	60	Mean
A	$9.9x10^6$	$9.6x10^6$	$4.9x10^6$	$4.2x10^5$	$3.5x10^5$	$5.034x10^6$
B	$9.5x10^6$	$9.3x10^6$	$9.3x10^6$	$9.0x10^6$	$9.0x10^6$	$9.22x10^6$
C	$4.9x10^6$	$4.5x10^6$	$3.2x10^6$	$3.0x10^5$	$2.8x10^5$	$2.636x10^6$
Mean	$8.10x10^6$	$7.8x10^6$	$5.8x10^6$	$3.24x10^6$	$3.21x10^6$	

Results are expressed in means of three observations; A= On agar slopes method; B= Under oil method; C = In liquid form condition

Morphology and Gram's staining ability. It was observed that there was no change in morphology and Gram's staining ability of culture after 60 days of storage in either case. After 60 days, all the bacteria were G +ve and parallel rods. It is obvious from results that method of preservation under paraffin oil exhibited the best method of preservation for *Lactobacillus bulgaricus*. Although on agar slopes and in liquid form methods gave good preservation for two months of storage time but they were found to be secondary categorically. The results obtained in these studies are in line with those of Standbury and Whitaker (1990) who stated that preservation of cultures on agar slopes may be extended to 1 year if the slopes are covered with sterile mineral oil. The similar results were also concluded by Greig *et al.* (1970) who observed that freeze-drying is much better than the preservation in nutrient broth. Silva *et al.* (1983) also worked on the preservation of *Lactobacillus bulgaricus*. Although, these all above values were reduced after 60 days but the effect of days was found to be non-significant when statistically observed. Mode of preservation and methods showed significant effect on the preservation of the starter culture (Table VII).

Table VII. Analysis of variance for viability of culture (s_1) observed during its storage

S.O.V.	D.F.	S.S.	M.S.	F.value
Days	4	67.222	16.805	3.3061^{NS}
Methods	2	111.037	55.518	10.9220^{**}
Error	8	40.665	5.083	
Total	14	218.924		

**= Highly significant

LSD test for culture's viability

A	B	C
5.034	9.220	2.636
B	A	B

CONCLUSIONS

It is concluded that preservation under paraffin oil method was the best method of preservation. Therefore, this method can be employed for culture preservations at laboratory scale.

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