Journal of Applied Biology & Biotechnology Vol. 5 (04), pp. 066-075, July-August, 2017
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2017.50410

Production, characterization and optimization of fermented tomato and carrot juices by using *Lysinibacillus sphaericus* isolate

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ARTICLE INFO

Article history:
Received on: 03/03/2017
Accepted on: 11/06/2017
Available online: 14/08/2017

Key words:
Tomato and Carrot juices, *Lysinibacillus sphaericus*, Fermentation, Response surface methodology, Antimicrobial activity.

ABSTRACT

Fermentation of tomato and carrot juices was carried out with a native isolate of *Lysinibacillus sphaericus*. Initial screening showed that total phenolics, antioxidants and titratable acidity were higher in both the fermented juices than respective un-fermented controls. Better fermentation characteristics were found in fermented tomato juice at 37ºC (pH 5.8, TA% 0.38 and cell viability of 7.8 x 10^7 CFU/mL) than those of fermented carrot juice (pH 5.5, TA% 0.30 and cell viability of 6.9 x 10^7 CFU/mL). Hence, fermentation of tomato juice was optimized by Central Composite Design using temperature, pH, incubation time and sucrose concentrations as critical parameters. The optimized conditions for fermentation of tomato juice were determined to be pH 6.2, temperature of 37ºC, inoculum size of 7.58 log CFU/mL, sucrose concentration of 10% and fermentation for 24 h. The antimicrobial activity of the fermented tomato juice against Bacillus cereus MTCC 7190 was better (inhibition zone of 10 mm) than the fermented carrot juice (8 mm). The cell *viability of L. sphericus* at 4ºC for 6 weeks was better in tomato juice (0.9 x 10^6) than in the carrot juice (0.6 x10^6 CFU/mL). Fermented juices exhibited good sensory attributes and could possibly be used as probiotic drinks.

1. INTRODUCTION

The interest in probiotic products has been increasing during the last two decades due to the health awareness of consumers [1]. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. Present industrial probiotic foods are mainly dairy products, which may represent inconveniences due to their lactose and cholesterol content [3]. However, there is a real interest in the development of fruit juice based functional beverages with probiotics, because they have taste profiles that are appealing to all age groups and also they act as healthy and refreshing foods [4, 5]. Probiotic microorganisms are reported to have many health-supporting effects. They are useful in prevention of infections in intestine, colon cancer and avoid constipation. Probiotcs have good characteristics like resistance to acid, bile, attachment to epithelial cells and colonization in the stomach [6]. Vegetable juices are also considered as ideal media for cultivating probiotic microorganisms, because they do not contain any dairy allergens that might prevent usage by certain kind of the population [7]. Regular consumption of tomatoes has been associated with a reduced risk of various types of cancer [8] and heart deseases [9]. Positive effects are mainly attributed to the presence of antioxidants, especially carotenoids, flavonoids, lycopene and beta-carotene. Carrot juice contains (for 100 g): carbohydrates 9.6 g, sugars 4.7 g, dietary fiber 2.8 g, protein 0.93 g, fat 0.24 g, vitamin A, C, B1, B2, B3, B6 and E. Carrot is rich source of carotenes, antioxidants and polyacetylenes (especially falcarinol), have the ability to inhibit growth of colon cancer cells [10]. In the present study tomatoes and carrots are used for the production of fermented juices. Response surface methodology (RSM) is a useful statistical technique which has been applied in the research involved in complex variable processes. It uses multiple regression and correlation analyses as tools to assess the effects of two or more independent factors on the dependent variables. Its principal advantage is the reduced number of experimental runs required to generate sufficient information for a statistically acceptable result [11]. It is also an efficient tool for the evaluation of the simultaneous effect of some important technological and microbiological parameters on the acidification rate and the growth of starter bacteria during the fermentation process of yoghurt and it has been successfully used to determine and optimize the rheological properties and gelation kinetics of yoghurt [12].

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Lysinibacillus is commonly found in soil, plants and animals. The genome of *Lysinibacillus sphaericus* was the first strain in the genus *Lysinibacillus* which is taxonomically classified on the basis of cell wall and peptidoglycan. The antimicrobial potential of *Lysinibacillus* has been reported and its bacteriocin used as food preservative to combat against food-borne bacterial and fungal pathogens [13].

The main aim of the present study was to characterize the *Lys. sphaericus* isolate and to optimize its growth in tomato and carrot juices. Other aims include phyto-chemical analyses of the fermented juices and their antagonistic activity as well as determining the shelf-life of the viability of *Lys. sphaericus* isolate in tomato and carrot juices during storage at 4°C.

2. MATERIALS AND METHODS

2.1 Source of Microorganism

Lysinibacillus sphaericus isolate [14] was collected from Microbial Biochemistry Laboratory, Department of Biochemistry, Sri Venkateswara University, Tirupati.

2.2 Biochemical characterization

To determine the ability of the isolate to grow in NaCl, MRS tubes containing 2, 4, 6, 8 and 12% NaCl were inoculated with a loopful of actively growing 24 h old culture of *Lys. sphaericus* and incubated at 37 °C for 72 h. To determine the optimum growth temperature, the culture was inoculated in 10 mL of sterile MRS broth tubes and was incubated at 20, 25, 30, 35, 37, 40 and 45°C for 24 - 48 h. To optimize the pH for growth, the pH of the MRS broth was adjusted from 9.2 to 9.8, individually, and a loopful of 24 h old culture was added to each tube and incubated at 37°C for 72 h.

2.3 Acid and bile tolerance test

Resistance to pH 3 is often used *in vitro* assay to determine the possible resistance to stomach pH. Because the foods stay in the stomach for 3 h, this period of time limit was taken into account [15]. For this purpose, actively growing culture (18 h) was used. Cells were harvested by centrifugation for 10 min at 8000 rpm and 4°C.

The cell pellets were washed once in phosphate buffer saline (PBS at pH 7.2) and were resuspended in PBS (pH 3) and incubated at 37 °C. Viable microorganisms were enumerated after the incubation for 0, 1, 2 and 3 h by following pour plate technique.

The plates were incubated at 37°C under anaerobic conditions for 48 h and the viable counts were enumerated. Bile salt tolerance is potentially a probiotic property of LAB cultures. In this experiment, MRS broth containing 0.3% of bile salt (taurocholic acid) was inoculated with *Lys. sphaericus* and incubated at 37°C for 24 h. The control comprised of MRS broth without bile salt. Bacterial growth was monitored by measuring optical density at 600 nm after overnight incubation.

2.4 Preparation of vegetable juices

Fresh carrots (*Daucus carota* L) and tomatoes (*Solanam lycopersicum* L) were purchased from a local vegetable market in Tirupati, India and were stored in a box at room temperature for further maturation (5 - 7 days). The matured vegetables were washed with tap water to remove soil and other impurities, air-dried at room temperature and treated with steam for 3 to 4 min for easy peeling. Juice was prepared from homogenized skinless slices of tomatoes and carrots, separately, by using a laboratory grinder and filtered through a muslin cloth to get a clear juice.

2.5 Inoculum preparation

The isolated culture was maintained in MRS agar stabs as pure cultures. It was grown in two successive MRS broth cultures at 37 ºC for 24 h. The activated culture was again inoculated into MRS broth incubated at 37ºC for 24 h and this was used as the inoculum.

2.6 Fermentation of tomato and carrot juices

The tomato and carrot juices (100 mL) were taken in 250 mL Erlenmeyer flasks, individually and autoclaved for 15 min at 121°C. The flasks were inoculated with actively growing cells of *Lys. sphaericus* (10^9 CFU/mL) and incubated at 37 °C for 72 h.

2.7 Optimization using central composite design (CCD)

Fermentation process was optimized using response surface methodology (RSM) protocol [16]. The effect of pH (X_1), temperature (X_2), time (X_3) and sucrose (X_4) on cell viability (Y_1), biomass (Y_2), and acidity (Y_3) of *Lys. sphaericus* in vegetable juices was studied through central composite design according to response surface methodology using Design-Expert version 9 (Stat-Ease Inc., Minneapolis, MN, USA) software. The range and the levels of the variables investigated in this CCD study are given in the Table 4. A 2^4-factorial CCD, with six replications at the centre points (n_0=6) leading to a total number of 30 experiments were employed (Table 5 and 6) for the optimization of the fermentation conditions. The second degree polynomials equations were used with the statistical package to approximate the response of the dependent process variable. Variance determined for each factor was divided into linear, quadratic and interactive components and were represented using the second order polynomial function as follows.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4$$

Where Y is the predicted response, X_1, X_2, X_3 and X_4 were independent variables, b_0 is the offset term, b_1, b_2, b_3 and b_4 were linear effects, b_{11}, b_{22}, b_{33} and b_{44} were squared effects and b_{12}, b_{13},b_{14}, b_{23},b_{24} and b_{34} were interaction terms. The significance of all terms in the polynomial functions were assessed statistically using F-value at a probability (P) of 0.001, 0.01 or 0.05. The three-dimensional (3D) plots were generated by keeping one variable constant at the centre point and varying the other variables within the experimental range. Optimized values of four independent

variables for maximum activities were determined using a numerical optimization package of Design-Expert version 9.

2.8 Chemical characterization

The pH of fermented carrot and tomato juice was measured with a pH meter. Total acidity (TA), is expressed as percent oxalic acid, and was determined by titrating the vegetable juice samples with 0.1 N NaOH to pH 8.2, using phenolphthalein indicator. Total soluble solids (TSS) are determined using a hand Refractometer (0-30) (Erma, Japan) in terms of °Bx (°Brix).

2.9 Biomass and cell viability determination

The cell concentration was determined by measuring the optical density of the cell suspension. Samples of the fermented juice were adequately diluted in water and the absorbance was read at 590 nm. The number of viable cells was determined as CFU. Serial decimal dilutions of each sample were plated in triplicate onto MRS agar and incubated at 37°C for 72 h. The colonies as viable count was determined and the results were expressed as log CFU/mL.

2.10 Antimicrobial activity of fermented juices

The agar-well-diffusion method was used to determine the antimicrobial property of the fermented vegetable juices. A 24 h culture of the pathogenic strains (*Escherichia coli* MTCC 40, *Bacillus cereus* MTCC 6840, *B. cereus* MTCC 7190) were grown individually in Luria Broth (LB) medium and the cell suspension was spread over the surface of Muller-Hilton agar plates using sterile spreaders. The plates were allowed to dry and a sterile well borer of 5 mm diameter was used to cut uniform wells in the agar. Each well was filled with 100 µL of cell-free fermented juice of tomato or carrot. After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition (ZOI) around the well. Results were considered positive if the diameter of the ZOI was greater than 1 mm [17].

2.11 Sample preparation and sugar extraction

Vegetable juices were treated individually with heat-stable α-amylase, protease, and amylo glucosidase in order to hydrolyze starch and proteins. HPLC analysis of carbohydrates was carried out by the procedure described earlier [18]. The chromatographic system consisted of a Shimadzu Chromatograph (Model LC6A) equipped with the system controller, SCL6A, RID-10A RI detector, an integrator C-R3A chromate pack and stainless steel LC-NH2 25 cm X 4.6 mm column preceded by a Supel-guard column containing LC-NH_2 packing (Supelcosil, 5 µm particle size). The sample extract (10 µL) was injected into the HPLC column. Isocratic separation of carbohydrate fractions was accomplished, with a mobile phase consisting of acetonitrile: water (80:20 v/v) at a flow rate of 1 mL per min. The HPLC was calibrated by injecting 10 µL of a mixture of standard sugars (fructose, glucose, sucrose, maltose and lactose, all from Fluca Chemicals, USA) and the concentration of each sugar ranging from 4.5 to 9.6 mg/mL. The sugars present in the vegetable juices were identified by referring to standards.

2.12 Estimation of total phenolic content (TPC)

The TPC was determined by using Folin-Ciocalteu method as described by Singleston and Rossi [19]. Briefly, 0.5 mL of appropriately diluted juice samples or standard solutions of gallic acid was pipetted in to test tube, along with 5 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent, and the mixture was allowed to react for 3 min. For color development 1 mL of 20% Na_2CO_3 solution was added, mixed well and then left to stand for 1 h at 37°C. Absorbance against prepared reagent blank was measured at 750 nm using a Spectrophotometer and the TPC content was expressed in mg GAE/100 mL.

2.13 DPPH (1,1-diphenyl, 2-picrylhydrazyl) radical scavenging activity

A stock solution was prepared by dissolving 14 mg of DPPH in100 mL methanol and then stored at -20°C [20]. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1±0.02 units at 517 nm using a Spectrophotometer. Different volumes of various vegetable juices (50-200 µL) were allowed to react with DPPH solution (final volume 4 mL) and were shaken vigorously and allowed to stand for 30 min in dark at room temperature. Methanol was used as a blank. BHT (butylated hydroxyl toluene) was used as a standard [21]. A control sample with no added vegetable juice was also analyzed and radical-scavenging activity (% inhibition) was calculated using the following equation. DPPH free radical scavenging activity (%) = [(A control - A sample)/A control] × 100 (A = absorbance at 517 nm).

2.14 Effect of cold storage on cell viability in fermented tomato and carrot juice

After 72 h of fermentation, the samples of tomato and carrot juice were subjected for storage studies in order to evaluate cell viability [22]. Fermented juice samples (200 mL) were dispensed aseptically into 250 mL amber sterile glass bottles. The bottles were closed with screw caps and stored at 4°C for 6 weeks. Biomass, pH and viable cell counts were expressed as colony forming units per mL (CFU/mL) were determined and recorded prior to cold storage, and at weekly intervals for 6 weeks during storage.

2.15 Sensory evaluation

The sensory characteristics of the vegetable juices were evaluated according to Dias *et al*. [23] with a 20-membered panel. The preferences for taste, acidity, mouth feel, aroma, flavour, color and overall acceptability were determined by 9-point Hedonic scale. Randomized refrigerated (10°C) samples (50 mL) were served in clear tulip- shaped glasses coded with a random 3-digit code. The mean intensity scores of all the attributes were calculated and plotted.

2.16 Statistical analysis

All the experiments were carried out in triplicate and the mean value and standard deviation were presented. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS, version 16.0.

3. RESULTS AND DISCUSSION

3.1 Isolation and characterization of the strain

In the present study the *Lys. spharecius* was used for fermentation of tomato and carrot juices in order to characterize its potential as probiotic bacterium. The isolated *Lys. spharecius* was rod-shaped, Gram-positive, catalase negative and produced ammonia from arginine hydrolysis, which is the main characteristic feature, as observed with all *Lactobacilli,* and the results are presented in Table 1.

Table 1: Characteristic features of *Lys. sphaericus* isolate.

Parameter	Observation
Cell morphology	Rod shaped
Grams staining	+
Growth at temperature 10 - 45ºC	+
Growth at pH range pH 3.0 - 9.6	+
Growth at NaCl concentration 1 - 10%	+
Catalase	-
Arginine hydrolysis	+
Fermentation of carbohydrates	+
Acid tolerance	+
Bile salt tolerance	+

The culture could grow at different temperatures (20 to 45ºC), pH (2.0 to 9.8) and NaCl concentrations (2 to 12%) and in the presence of bile salt. It was found to be resistant at a low pH for 4 h. It showed better survival after treatment of cells in the presence of pepsin (pH 2.0) for 1 h, and the viability decreased significantly beyond 3 h. The survival of the strain at pH 6.4 - 6.7 was also tested using 0.3% taurocholic acid. The results showed that 10^5 CFU/mL survived even after 14 h at 37°C, and then significantly decreased up to 24 h. A 1 and 2% bile salt hydrolase (BSH) activity for 3 h with a viable count of 10^4 - 10^3 CFU/mL were reported earlier [15]. The above results support that the strain is tolerant to both environments, *i.e.,* acidic conditions (pH 2.0 - 3.0) and in the presence of bile (Table 1).

3.2 Fermentation of tomato and carrot juices

Two vegetable juices prepared using tomatoes and carrots independently were selected for fermentation, and tomato juice was found to have higher viable count of 7.8 log CFU/mL with a final pH of 5.8 and TA% (0.38) than carrot juice which had a viable count of 6.9 log CFU/mL with a final pH of 5.5 and TA% (0.30) (Table 2). Hence, tomato was selected for further fermentation and for optimization of the product preparation. These results are similar to an earlier report on the use of vegetable and fruit juices for fermentation by four lactic acid bacteria (*Lb. acidiphillus* LA39, *Lb. plantarum* C3, *Lb. casei* A4 and *Lb. delbrueckii* D7) [3] and tomato juice has been well recognized as one of the healthy beverages [24].

Physico-chemical properties like TSS, pH, TA, and cell viability of fermented tomato and carrot juices are presented in Table 2. Total soluble solids (TSS) were still higher in tomato juice than that of carrot juice at 24 h. of fermentation. However, titratable acidity and cell viability were increased in up to 24 h and later decreased, whereas, the pH gradually decreased from 6.8 to 4.1 during fermentation of 72 h. An increase in TA% from 0.12 to 0.38 was noticed within 24 h. Fermentation improved the utilization of sugars as indicated by decrease in their contents as the fermentation time increased from 24 to 72 h (Table 2). These results corroborate with the probiotication of mixed water melon and tomato juice [25].

Sugars levels were gradually decreased during fermentation period of 72 h in tomato and carrot juices as compared to control juices (Figure 1). The biomass and cell viability increased gradually during first 24 h fermentation by rapidly consuming the substrates (sugars) present in the tomato and carrot juices and liberated their products into the medium. Later slowly decreased due to, depletion of nutrients and reduction in pH (Figure 2). These results corroborate with the probiotication of tomato juice by Yoon *et al.* [26].

The DPPH radical scavenging activity gradually increased in fermented tomato and carrot juices as the fermentation period increased, and it was less in unfermented juices (Figure 3). Fermented tomato and carrot juices showed more antioxidant activity than control juices. This observation is in agreement with an earlier report [27]. The total TPC content increased significantly in both fermented tomato and carrot juices during 72 h of fermentation (Figure 4). When compared to unfermented juices (310 mg/GAE/100 mL), fermented tomato and carrot juices had higher levels of TPC (392 mg/GAE/100 mL). In tomato juice, TPC enhanced gradually with increase in fermentation period, and these results are in agreement with an earlier report on probiotication of mango and sapota juices using *Lb. plantarum* [21].

The antimicrobial activity of the both fermented tomato and carrot juices was evaluated against *E. coli* MTCC 40, *B. cereus* MTCC 6840 and *B. cereus* MTCC7190 as compared to the Ampicillin control and their inhibition zone sizes are presented in (Table 3). Fermented tomato juice efficiently inhibited the growth of *B. cereus* followed by *E. coli*, with a maximum zone of inhibition of 10 and 9.1 mm, respectively, whereas, fermented carrot juice showed an inhibition zone of 8.5 and 8.2 mm against *B. cereus* and *E. coli*, respectively.

The fermented tomato and carrot juices were stored in refrigerator for six weeks at 4°C in order to study the shelf-life of the cell viability. Viability of *Lys. sphaericus* in the juices during the storage period was analyzed weekly and the results are presented in Table 4. The cell count was reduced to below initial level (2.5 log CFU/mL) in tomato and carrot juices after six weeks of storage at 4°C.

Table 2: Physico-chemical analyses of tomato and carrot juices fermented with *Lys. Sphaericus.*

Type of juice	Time (h)	TSS (Brix)	Titratable acidity (%)	pH	Cell viability (CFU/ mL)
FTJ	12	19±0.8	0.28±0.08	6.6±0.04	$6.5\pm0.6 \times 10^{7}$
	24	17±1.0	0.38±0.03	5.8±0.04	$7.8\pm0.9 \times 10^{7}$
	48	13±0.8	0.31±0.02	4.7±0.08	$7.1\pm0.5 \times 10^{6}$
	72	10±0.8	0.21±0.08	4.1±0.08	$5.5\pm0.3 \times 10^{6}$
FCJ	12	18±0.47	0.22±0.016	6.5±0.04	$6.2\pm0.6 \times 10^{8}$
	24	16±0.81	0.36±0.08	5.5±0.04	$6.9\pm0.9 \times 10^{7}$
	48	14±0.47	0.30±0.004	4.3±0.04	$6.6\pm0.5 \times 10^{6}$
	72	11±0.81	0.20±0.08	3.8±0.04	$6.0\pm0.3 \times 10^{6}$

FTJ (Fermented tomato juice), FCJ (Fermented carrot juice).

Table 3: Antimicrobial activity of fermented tomato and carrot juice.

Sample	Zone of inhibition (mm)		
	E. coli **MTCC 40**	***B. cereus*** **MTCC 6840**	***B. cereus*** **MTCC 7190**
FTJ (A)	9.1±0.1	8.2±0.2	10 ±0.1
FCJ (B)	8.2±0.2	7.8±0.1	8.5±0.3
Ampicillin (C)	13.77+1.74	16.23+0.86	18.73+0.27

Table 4: Viability of *Lys. sphaericus* stored at 4°C over a period of 6 weeks.

Storage (weeks)	Cell viability of *Lys. sphaericus* at 4°C (CFU/mL)		
	FTJ	FCJ	FTJ + FCJ
0	$4.5\pm0.6 \times 10^{8}$	$4.2\pm0.4 \times 10^{9}$	$4.0\pm0.8 \times 10^{8}$
1	$4.0\pm0.9 \times 10^{7}$	$3.9\pm0.2 \times 10^{6}$	$3.7\pm0.6 \times 10^{6}$
2	$3.8\pm0.5 \times 10^{6}$	$3.4\pm3.4 \times 10^{6}$	$3.2\pm0.7 \times 10^{6}$
3	$3.2\pm0.3 \times 10^{6}$	$3.0\pm3.4 \times 10^{6}$	$2.8\pm0.3 \times 10^{6}$
4	$2.5\pm0.4 \times 10^{6}$	$2.2\pm0.3 \times 10^{6}$	$1.9\pm0.6 \times 10^{6}$
5	$1.7\pm0.2 \times 10^{6}$	$1.4\pm0.3 \times 10^{6}$	$1.1\pm0.6 \times 10^{6}$
6	$0.9\pm0.2 \times 10$	$0.6\pm0.2 \times 10$	$0.3\pm0.2 \times 10$

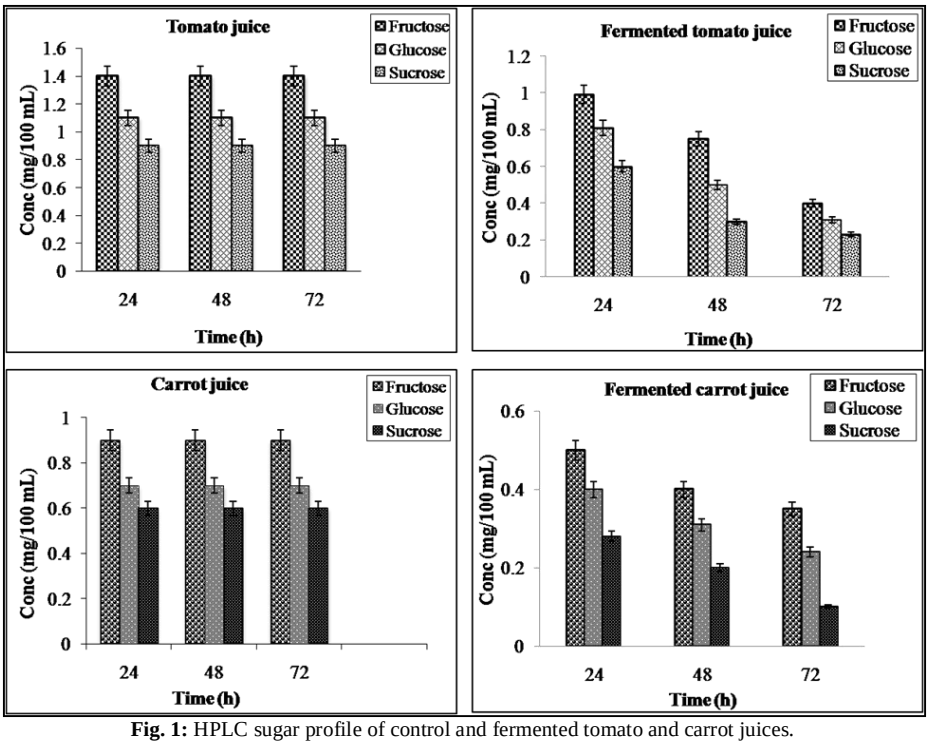

Fig. 1: HPLC sugar profile of control and fermented tomato and carrot juices.

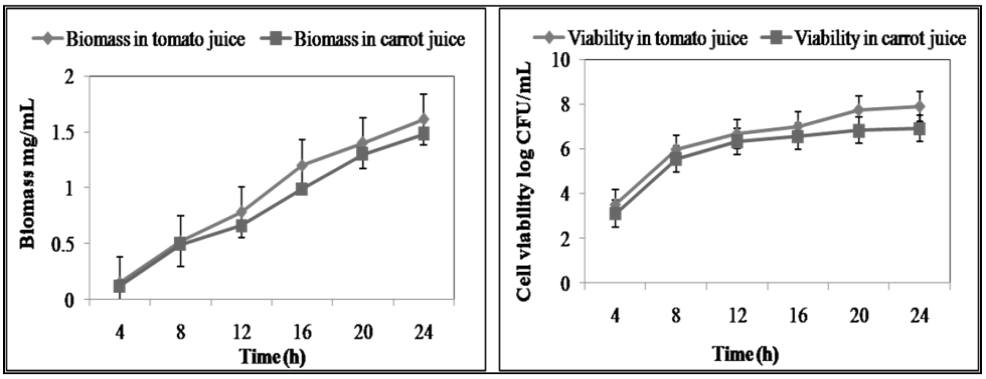

Fig. 2: Cell viability and biomass in fermented tomato and carrot juices.

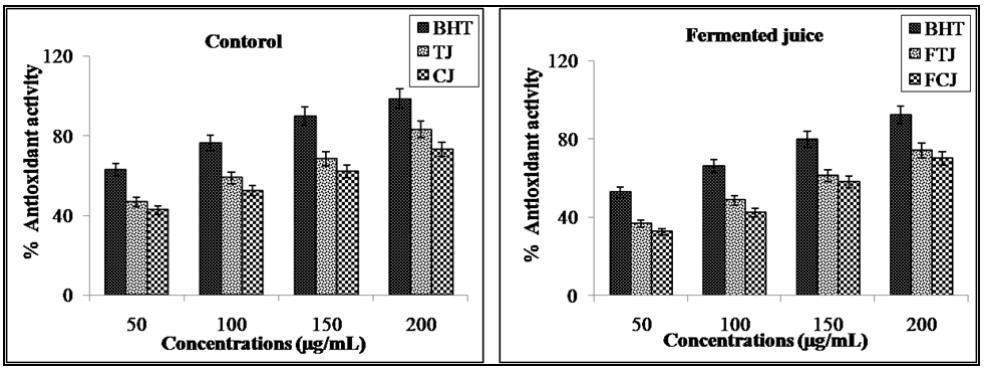

Fig. 3: DPPH scavenging activity of fermented juices.
(BHT- Butylated hydroxyl toluene, FTJ - Fermented tomato juice, FCJ - Fermented carrot juice).

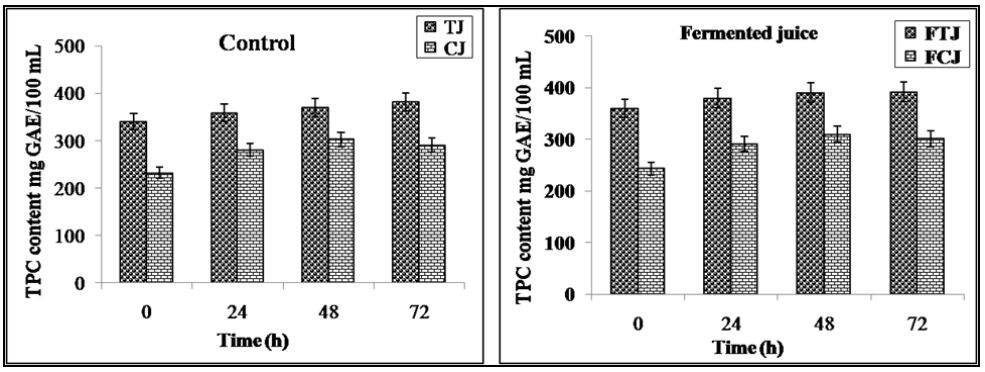

Fig. 4: Total phenolic content in control and fermented tomato and carrot juices.

3.3 Optimization of tomato juice production using RSM

Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess [28]. The influence of pH, temperature, time and sucrose on acidity, cell viability, and biomass was investigated using RSM. For each run, the experimental responses along with predicted responses were calculated from the regression equation (Eq. 2, 3, 4) and the actual and predicted values of variables were presented in Table 5. The effect of each factor and its interaction was analyzed using the analysis of variance (ANOVA) and X^2 - test as appropriate to the experimental design used. Calculated regression equation for the optimization of fermentation conditions showed that the cell viability (Y1, CFU/mL), biomass (Y2, O.D) and acidity (Y3, %) are functions of the pH (X_1), temperature (X_2, ºC), time (X_3, min) and sucrose (X_4, %). By applying multiple regression analysis on the experimental model data, the following second order polynomial equation is found to represent the cell viability, biomass and acidity effectively.

Cell viability (CFU) $Y_1 = 30.1+4.7X_1+1.0X_2+0.08X_3+1.5.X_4–0.51X_1^2–0.01X_2^2–4.16X_3^2–0.09X_4^2+5.63X_1X_2+1.59X_1X_3 + 0.03X_1X_4–8.39X_2X_3+ 3.0X_2X_4+6.55X_3X_4$ (2)

Biomass (O.D) $Y2 = 3.18+0.46X_1+0.10X_2+7.42X_3+0.18.X_4–0.05X_1^2–1.43X_2^2–5.92X_3^2–9.83X_4^2+3.37X_1X_2+5.06X_1X_3+2.25X_1X_4 –6.94X_2X_3 + 1.85X_2X_4+4.62X_3X_4$ (3)

Acidity (%) $Y3 = 8.76+0.16X_1+0.07X_2+4.72X_3+0.28.X_4–0.07X_1^2–1.13X_2^2–6.42X_3^2–6.43X_4^2+3.25X_1X_2+5.72X_1X_3+2.31X_1X_4–5.81X_2X_3+1.63X_2X_4+4.62X_3X_4$ (4)

The predicted levels of cell viability, biomass and acidity in fermented vegetable juices using the above equations are given in Table 6, along with experimental data. The usefulness of the present model could be checked with the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2) was calculated as 0.912 for cell viability, 0.918 for biomass and 0.920 for acidity, indicating that the statistical model can explain above 90% of the variability in the response, showed that models for each response variable were well fitted to explain the relationships among the variables and only about 10% of the total variation could not be attributed to the independent variables. Normally, a regression model with R^2 value more than 0.90 is considered as a high correlation [29].

Here, the value of R for all three response variables, for cell viability=0.830, biomass=0.837 and for acidity=0.846 were higher than 0.80, indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. Besides the relationship between the predicted and actual experimental values (Figure 5 a, b and c) that plotted points cluster around the diagonal line, indicating better fitness of the model.

Table 5: Actual and coded values of the variables in Central Composite Design.

Factor	Name	Low actual	Middle Actual	High actual	Low coded	Middle coded	High coded
X_1	pH	3.1	5.2	6.8	-1	0	1
X_2	Temperature (ºC)	20	37	42	-1	0	1
X_3	Time (h)	0	24	72	-1	0	1
X_4	Sucrose (%)	5	9	14	-1	0	1
Response	Name	Units	Obs[a]	Min.	Max.	Mean	Std.Dev.
Y_1	Cell viability	CFU	30	1.1	9.1	4.9	2.80
Y_2	Biomass	OD	30	0.04	0.86	0.42	0.28
Y_3	Acidity	%	30	0.27	0.49	0.37	0.07

Obs[a] = Observed runs.

Table 6: Central Composite Design expérimental design matrix.

S. No	A	B	C	D	Cell viability (CFU/mL)	Biomass (O.D at 590 nm)	Acidity (%)
1	-1	-1	-1	1	1.30 (2.18)	0.15 (0.21)	0.45 (0.43)
2	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
3	-1	1	-1	1	1.20 (2.32)	0.07 (0.17)	0.47 (0.45)
4	1	1	-1	-1	1.80 (2.45)	0.10 (0.16)	0.48 (0.46)
5	0	0	0	0	9.10 (8.76)	0.86 (0.83)	0.27 (0.29)
6	0	0	0	-2	1.10 (0.81)	0.05 (0.02)	0.49 (0.49)
7	1	1	1	-1	4.20 (4.62)	0.38 (0.44)	0.34 (0.34)
8	1	1	-1	1	2.00 (2.97)	0.10 (0.17)	0.48 (0.46)
9	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
10	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
11	1	-1	-1	-1	1.70 (2.62)	0.08 (0.15)	0.47 (0.45)
12	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
13	0	0	-2	0	6.20 (4.07)	0.46 (0.26)	0.33 (0.38)
14	-1	1	-1	-1	2.60 (2.98)	0.19 (0.23)	0.42 (0.41)
15	-1	-1	1	1	4.90 (5.07)	0.41 (0.44)	0.36 (0.35)
16	-1	-1	-1	-1	2.70 (3.17)	0.19 (0.24)	0.43 (0.42)
17	0	-2	0	0	8.20 (7.06)	0.79 (0.66)	0.31 (0.35)
18	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
19	-1	-1	1	-1	5.30 (5.64)	0.41 (0.45)	0.37 (0.37)
20	1	-1	1	1	5.20 (6.12)	0.49 (0.56)	0.32 (0.30)
21	-1	1	1	-1	4.90 (4.72)	0.44 (0.38)	0.33 (0.35)
22	0	2	0	0	7.30 (6.31)	0.65 (0.58)	0.34 (0.36)
23	2	0	0	0	3.40 (1.99)	0.28 (0.13)	0.39 (0.44)
24	1	-1	1	-1	5.80 (5.51)	0.51 (0.49)	0.35 (0.34)
25	-1	1	1	1	4.10 (4.49)	0.31 (0.35)	0.37 (0.36)
26	0	0	0	0	8.70 (8.76)	0.82 (0.83)	0.29 (0.29)
27	1	-1	-1	1	1.80 (2.80)	0.04 (0.19)	0.48 (0.43)
28	1	1	1	1	5.20 (5.56)	0.45 (0.48)	0.34 (0.32)
29	-2	0	0	0	2.20 (1.48)	0.15 (0.09)	0.45 (0.45)
30	0	0	0	2	2.60 (0.76)	0.21 (0.03)	0.43 (0.48)

Std: Standard run order.

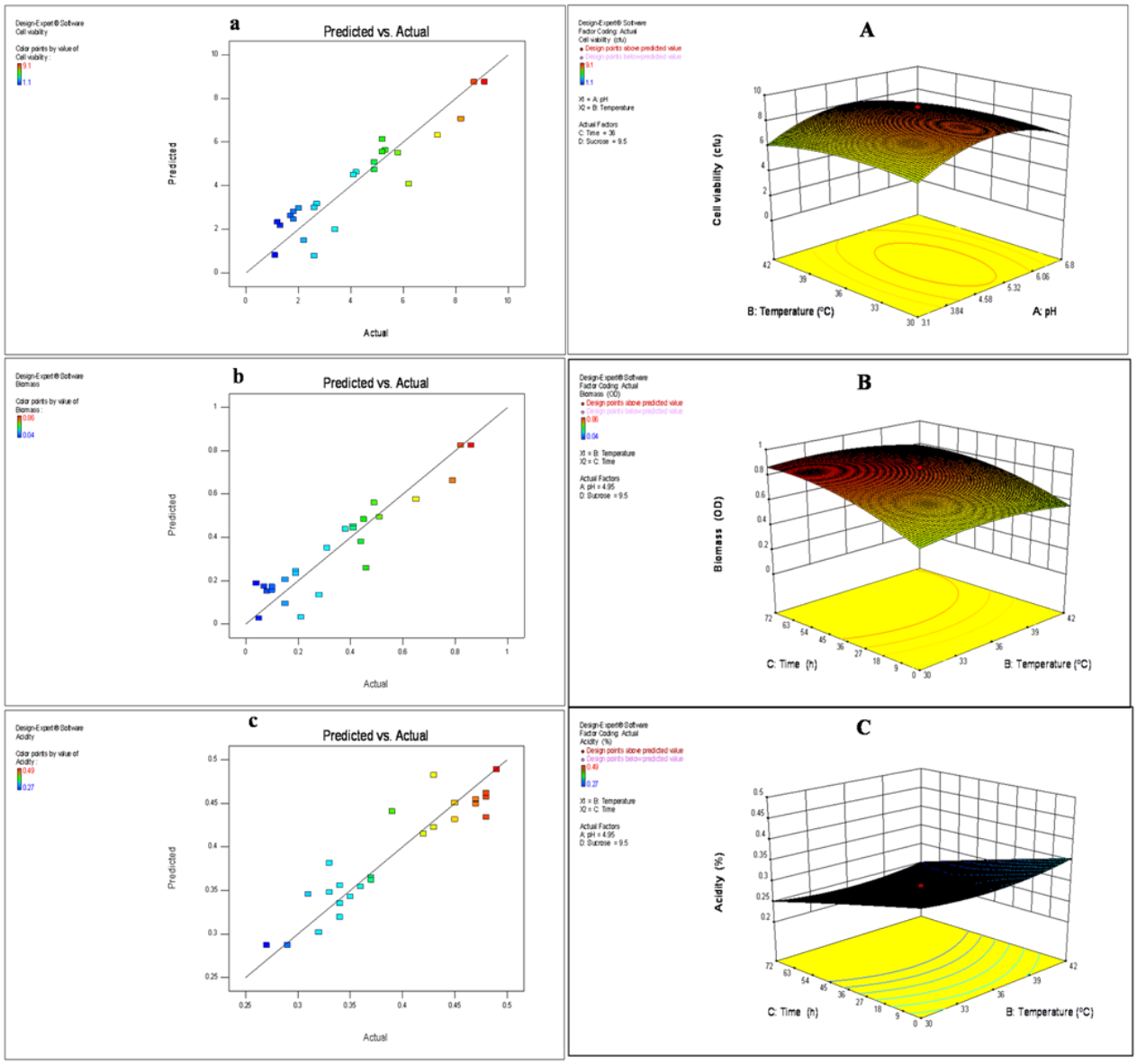

Fig. 5: (a) Predicted and actual levels of pH and temperature on cell viability; (A) 3D graph represents effect on cell viability; (b) Predicted and actual levels of temperature and time on biomass; (B) 3D graph represents effect on biomass; (c) Predicted and actual levels of pH and time on acidity; (C) 3D graph represents effect on acidity.

From the ANOVA in Table 7, it can be seen that time (58 h) and sucrose (10%) had strongest influence on cell viability and biomass yield, and pH showed significant influence on acidity. At higher temperature (42°C) there was a decrease in the cell viability. Table 7 shows the F test and the corresponding P value along with the parameter estimate. For cell viability, the model terms X_1 and X_2 were significant with a probability of 99%. There is a significant interaction in between model terms X_1 and X_2 indicates the positive effect of these variables on increase in cell viability in tomato juice. Regarding biomass yield, model terms X_3, and X_3^2 are significant with a probability of 99% and X_3 is significant with a probability of 95%. The significant interaction between the X_3 and X_4 process variables will influence on the biomass yield in tomato juice. Considering acidity, the significant probability of 95% is with the model term X_1. 99% significant probability was shown by the model term X_3^2 (Table 7). The growth of *Lys. sphaericus* in the first hour of fermentation resulted in increased biomass, and the adaptation of the microorganism was very fast in both tomato and carrot juices because an increase on cell viability was observed since the beginning of fermentation. There was an increase of 3 log cycle of viable cell counting of *Lys. sphaericus* during 12 h of fermentation at 37°C. At this point, the culture reached the logarithmic growth phase (5.5-7.8 log CFU/mL).

Besides the microbiological safety, the pH of the medium can influence the viability of probiotic microorganisms. This finding corroborates well with the earlier report of Shah [30], who attributed the decline of pH and the accumulation of organic acids as the main reasons for viability loss of probiotic microorganisms.

Table 7: ANOVA of Central Composite Design.

		F-value			P-value		
Source	**Df**	**Y_1**	**Y_2**	**Y_3**	**Y_1**	**Y_2**	**Y_3**
Model	14	11.13	12.42	9.86	< 0.0001	< 0.0001	< 0.0001
X_1	1	0.30	0.19	0.15	0.0052*	0.0111*	0.0044*
X_2	1	0.63	0.88	0.15	0.0086*	0.0185*	0.0193*
X_3	1	20.48	22.63	42.27	< 0.0001	< 0.0001	< 0.0001
X_4	1	2.800	5.221	0.066	< 0.0001	< 0.0001	< 0.0001
X_1X_2	1	4.667	0.018	0.099	0.6666	0.3062	0.1473
X_1X_3	1	0.13	1.43	3.00	0.3384	0.2776	0.5543
X_1X_4	1	1.03	0.44	0.89	0.3543	0.4259	0.3868
X_2X_3	1	0.39	0.28	0.099	0.3991	0.2197	0.6179
X_2X_4	1	0.079	0.031	0.62	0.3933	0.4586	0.2827
X_3X_4	1	0.13	0.070	0.40	0.0088	0.0085	0.1061
X_1^2	1	63.91	68.79	43.30	0.0005	0.0005	0.0061
X_2^2	1	5.56	5.79	6.95	0.6236	0.341	0.41
X_3^2	1	3.56	7.58	0.031	0.0001	0.0001	0.0018
X_4^2	1	82.37	86.21	67.91	0.9906	0.6476	0.4518
Residual	20.09						
Lack of fit	19.95						
Pure error	0.14						
Cor total	20.09						

Y1 - Cell viability, Y2 - Biomass, Y3 – Acidity.

Recent report has shown, however, that *Lys. sphaericus* possessed the property of bacteriocin production, which can be used as stable and safe food preservative [13]. Hence, the isolated culture was used to ferment tomato and carrot juices which would perhaps impart the same property.

Response surface graphs showed the effect of four fermentation variables on cell viability, biomass and acidity in the tomato juice. The results indicated that the cell viability and biomass response surfaces had a maximum point with acidity at limiting point. Although, response surface models were useful in indicating the direction in which the variables have to be changed in order to maximize the cell viability, biomass and minimize the acidity. The highest level of cell viability was achieved at lower pH (3.1) and temperature at 32°C (Figure 5A) and there is considerable interaction occurred between these two variables. The response surface plot of biomass indicated that maximum yield of biomass in fermented tomato juice was attained at 32°C with longer fermentation time (72 h) (Figure 5B). The low acidity in fermented tomato juice response showed and the results were obtained at lower pH (3.1) for fermentation with higher fermentation time (Figure 5C). Under the fermentation conditions with pH (3.1), temperature (32°C), incubation time (58 h) and sucrose (10%) the cell viability, biomass and acidity were 9.1, 0.86 and 0.27, respectively. Maintenance of the viability of the probiotic culture during storage is a fundamental condition for the beneficial effects after its ingestion. David and Shah [22] reported that the viability of probiotic microorganisms during storage of food products depends on the factors like, the availability of nutrients, growth promoters and inhibitors. In this study the optimal fermentation time was found in log phase in a short time (12 h). Stopping the fermentation at this point, the microorganisms still have enough nutrients for their maintenance during refrigerated storage. The exposure of the juice to high temperatures was also short, reducing the risk of the deterioration of nutritional compounds such as vitamins. In this study, no evidence of juice contamination was observed after fermentation or after storage period.

3.4 Sensorial Evaluation

Sensory evaluation results indicated good sensory scores for fermented tomato and carrot juices (Figure 6). The fermented juices had better acceptance among the consumers than control juices. Only a marginal difference was noticed between the sensory scores of fermented and control juices. The taste, acidity, mouth feel, aroma, flavour, color and overall acceptance were changed in fermented juices. The results are in agreement with the earlier report of sensory evaluation of probioticated mango and sapota juices using *Lb. plantarum* [21].

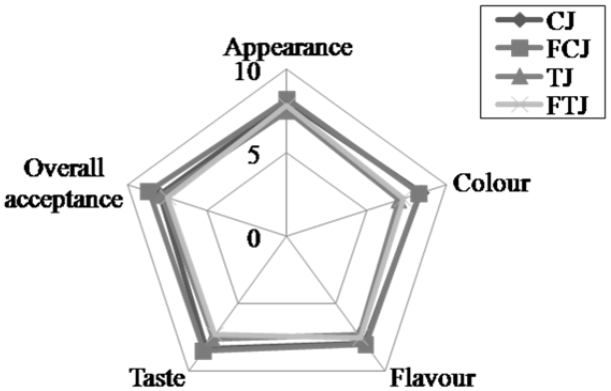

Fig. 6: Sensory evaluation of control and fermented juices CJ (Carrot juice), FCJ (Fermented carrot juice), TJ (Tomato juice), FTJ (Fermented tomato juice

4. CONCLUSION

In this study *Lys. sphaericus*, an isolate from large intestinal tract of fresh water fish, was used for the fermentation of tomato and carrot juices, and the conditions were optimized for

tomato juice fermentation by using RSM. Good cell viability in both tomato and carrot juices was noticed up to 6 weeks during the cold storage at 4°C and no contamination was detected. The fermented juices had a good acceptance in sensory evaluation. Hence, the fermented tomato and carrot juices can be explored as alternatives to dairy based probiotics to get health benefits, especially in lactose intolerance persons.

5. ACKNOWLEDGEMENT

We are thankful to Dr. S. C. Basappa, former Deputy Director, Central Food Technological Research Institute (CFTRI), Mysuru, for his encouragement and critical comments on this manuscript.

Financial support and sponsorship: Nil.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Seelam NS, Akula H, Katike U, Obulam VSR. Production, characterization and optimization of fermented tomato and carrot juices by using Lysinibacillus sphaericus isolate. J App Biol Biotech. 2017; 5 (04): 066-075.