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Studies on the preparation of low alcoholic naturally carbonated blended beverage from guava and lemon

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Abstract

A pure yeast isolate *Clavispora lusitaniae* from whey beverage, phenotypically and molecularly characterized, was used to develop a reliable, controllable and reproducible technology for preparation of low alcoholic naturally carbonated beverage from guava var. *Allahabad Safeda, Lucknow-49, Punjab Pink* and its blends with lemon var. *Baramasi* under optimized fermentation conditions. The specific growth rate (h^{-1}) and generation time (h) of yeast in guava: lemon (1:1) beverage was 0.35 and 1.93 respectively. The physicochemical and microbiological characteristics of *Allahabad Safeda-Baramasi* (1:1) beverage were juice 12.5%, TSS 12.1^{0}B, pH 2.6, acidity 0.51%, ascorbic acid 9.02 mg/100ml, alcohol (%v/v) 0.85,CO_2 1.55 bar and total plate count 15x10^9 cfu/ml ,*Lucknow-49- Baramasi* (1:1) beverage were juice 12.5%, TSS 11.9^{0}B, pH 2.6, acidity 0.52%, ascorbic acid 8.4 mg/100ml, alcohol (%v/v) 0.82,CO_2 1.50 bar and total plate count 25x10^9 cfu/ml and *Punjab Pink- Baramasi* were juice 12.5%, TSS 12.1^{0}B, pH 2.6, acidity 0.50%, ascorbic acid 10.2 mg/100ml, alcohol (%v/v) 0.81,CO_2 1.50 bar and total plate count 85x10^8 cfu/ml with a shelf life of 90 days under refrigerated conditions (4^{0}C). The beverage scored 'liked very much' to 'liked moderatly' due to naturally produced CO_2 during fermentation that added effervescence, sparkle and tangy taste to the beverage. The optimized temperature and potassium metabisulphite concentration to inhibit the left over yeast culture was standardized as 55^{0}C for 5 min and 700ppm respectively, but organoleptically heat treated beverage was superior than potassium metabisulphite treated beverage.

Key words: Low alcoholic, naturally carbonated, *Clavispora lusitaniae*, fermentation, guava, lemon, blended, shelf life, physicochemical

Introduction

The agro climatic suitability coupled with abundance of natural resource endowment, equips India with a unique comparative edge in the cultivation of variety of horticultural crops[14]. But the slump side is that enormous production and its potentiality is marred by colossal wastage, very low level of processing and non-availability of good post-harvest infrastructure[17]. The post harvest loss of fresh fruits and vegetables are estimated to be 20- 30 %. Currently, most of the perishable fruits are lost during their journey through the agri food chain, microbial spoilage, physiological decay, water loss, mechanical damage, packaging or due to transportation[3]. The perishable nature of ripened fruits poses serious public health problem through contamination by moulds. Tropical fruit beverages have become important in recent years due to overall increase in natural fruit juice consumption as an alternative to the traditional caffeine- containing beverages such as coffee, tea or carbonated soft drinks[16]. By incorporating tropical fruits into fruit- juice blends, the scientists are able to exploit their exotic flavors without adding artificial flavors. This is especially true with highly aromatic fruits such as guava and lemon.

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Fruits like amla, lemon and pineapple because of high acidity and astringent taste are not palatable for direct consumption. To make them palatable and available throughout the year in the form of beverage, a reliable, controllable and reproducible technology has been developed for production of a low alcoholic naturally carbonated beverage with retention of all the nutrients of the fruit. Compared to fruit juices the formulations of low alcoholic naturally carbonated fruit beverage offers more variety of flavors nutrients long shelf life and other physiological benefits with a greater margin of safety in drink with a lower inherent cost. Guava(*Psidium guajava* L.) (Family Myrtaceae) known as Apple of Tropics is one of the exotic fruits prized for its very pleasant, sub acid and aromatic flesh. The fruit contains high concentration of vitamin A (200- 400 IU), ascorbic acid (88.2- 250.8 mg/100g), lycopene (45.3µg/ g FW), total sugars (10- 15.3%), reducing sugars (2.05- 6.08%), acidity (10- 15.3%), pectins (0.62%) and phenols (170- 345 GAE/ g FW)[16]. It is rich source of many important minerals such as phosphorus (23- 37 mg/100g), calcium (14- 30 mg/ 100g) and iron (0.6- 1.4 mg/100g) and dietary fiber (12.72g/100g). The antioxidants like polyphenols and ascorbic acid reduce incidence of degenerative diseases e.g. arthritis, cancer, heart disease, brain dysfunction, retard aging and involved in metabolism of fat[13]. The nutritive value of lemon lies in its high contents of acidity, ascorbic acids minerals, flavonoids and phenolics. Its nutrients, vitamins and flavonoids help to prevent unwanted damage to cell membranes and other structures of the body by neutralizing free radicals[30]. The production of low alcoholic naturally carbonated fermented blended beverage from guava and lemon is considered as promising method of utilization of fruit during seasonal glut and making the fruit available in the form of beverage throughout the year.

Material and Methods

Physiological and biochemical characterization of yeast

Feta cheese was prepared by inoculating starter mesophilic culture (CHOOZIT 230, Bulk cultures, Danisco, Germany) containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* and thermophilic yoghurt culture (YO-MIX 532, Bulk cultures, Danisco, Germany) containing *Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus.* The whey so obtained was used for beverage making. A total of ten morphologically identical yeast colonies were screened, isolated from whey beverage which on streak purification revealed one distinct colony type, initially designated as 84. Identification of the yeast isolate determined on the basis of biochemical activities included fermentation of sugars, assimilation of carbon compounds, growth on vitamin free medium, growth at 25℃, 30℃, 35℃, 37℃ and 420C, growth in 50% and 60% D-glucose medium, urea hydrolysis and 0.01% and 0.1% cycloheximide sensitivity[29].

Molecular characterization

The yeast isolate 84 was further identified phenotypically and by sequencing based on partial ITS2 region of the rDNA sequence. Genomic DNA was isolated from pure culture[25]. Using consensus primers, D1/D2 domain of 26S rRNA, ITS-1 and 2 region fragment (0.4Kb) was amplified using high fidelity Taq polymerase (Fermentas,USA). The PCR products were directly sequenced. Sequence data was aligned and analysed for finding the closest homology for the microbe. The MEGA 4.0 package[28] was used for all analysis.

Growth kinetics of yeast in Guava juice during fermentation

A loopful of 24 hrs old actively growing yeast culture (procured from Deptt. of Microbiology, PAU, Ludhiana) was inoculated in 500 ml Erlenmeyer flasks containing 250 ml Glucose Yeast Extract (GYE) broth. It was incubated at 30±5°C for 24 hrs to prepare as starter culture. From the primary inoculum, 0.5% v/v (10^5-10^6cfu/ml) was inoculated in pasteurized, diluted and blended guava –lemon (1:1) juice with 15° B. Growth curve was obtained in terms of viable cell count ($\log_{10}$ no. of cells per ml).

Fruits

a) Guava

Guava var. *Allahabad Safeda, Lucknow-49, Punjab Pink* were procured from Department of Horticulture, PAU, Ludhiana and PAU Regional Fruit Research Station, Bahadurgarh, Patiala.

b) Lemon

Lemon var. *Baramasi* was procured from the Department of Horticulture, PAU, Ludhiana.

Extraction of juice

Guava

Healthy even sized fruits were selected. These were washed, calyx removed and then cut into small pieces. Juice was extracted (screw type juice extractor) by pressing fruit by rotating against the fixed sieve or by blanching. Extracted juice was filtered through muslin cloth and pasteurized at 82^0C for 15 seconds.

Lemon

Fruits were washed and cut into two halves, deseeded and squeezed with lemon squeezer to extract juice.

Preparation of sugar solution

Granulated sucrose was procured from local market of Ludhiana. The sugar solution was prepared by boiling (500g) granulated sucrose in one litre of water for 10 min and then allowed to cool at room temperature and stored aseptically in glass bottles.

Physico-chemical analysis of guava and lemon juice

The physico-chemical analysis, TSS, pH, acidity, Brix acid ratio, total sugars, reducing sugars, ascorbic acid, total phenols and juice yield of guava and lemon juice was done. The guava and lemon juices were mixed in the ratio 1:1, 1:2 and 2:1. Diluted juice was pasteurized (82^0C for 15 s),

cooled and brix adjusted to 15ºB by adding sugar solution and palatable acidity varying from 0.32-0.40% followed by inoculation of yeast at 0.5 per cent (v/v). It was incubated for 36 hrs at 30±2ºC. The beverage was refrigerated for 24 hrs, siphoned, bottled and stored in refrigerated conditions.

Chemical, microbioogical and organoleptic analysis

Percent total soluble solids (%TSS) in beverage was determined using Erma hand refractometer of 0-32°B (UNICO make). The pH of the juice was determined using a digital pH meter (Electronic Corporarion of India Ltd., Hyderabad, type 101). Total acidity expressed as % anhydrous citric acid by titration against standardized 0.1N NaOH[3] .Total sugars estimated by phenol-sulphuric acid method of Dubois *et al*[9] using glucose as standard. Reducing sugars estimated by the method of Miller[19].Ascorbic acid was determined by titrametric method using 2, 6-dichlorophenol indophenol dye AOVC[4],total phenols were estimated by method of Malik and Singh[18]. Percent Alcohol (v/v) in beverage was estimated by the Spectrophotometric method.Higher alcohols,aldehyde and ethyl acetate in beverage were estimated by GC Headspace Injection,TR wax Column, Detection by FID. Carbon dioxide volumes in beverage bottles were determined by Zahm and Nagel piercing device. Total yeast count was enumerated on Glucose Yeast Extract agar (GYE) by serial plate dilution method. Sensory evaluation of beverage was carried out using nine-point hedonic scale[1].Statistical analysis was done by using GSTATO4 and CPCS1 software developed by Maths, Statistics and Physics Department, PAU, Ludhiana.

Evaluation of inhibitory effects of heat treatment and potassium metabisulphite treatment on the organoleptic properties of non-alcoholic naturally carbonated fermented blended guava: lemon beverage (1:1)

Heat treatment

The guava: lemon (1:1) blended beverage after one week of storage was thermally treated at various temperatures (45^0C, 50^0C, 55^0C, 60^0C, 65^0C and 70^0C) for 2 and 5 minutes.

Potassium metabisulphite treatment

Varying concentrations (100ppm, 300ppm, 500ppm, 700ppm and1000ppm) of potassium metabisulphite were used for chemically clarifying the blended beverage after one week of storage. The treated beverages were stored at room temperature for organoleptic evaluation.

Result and Discussions

Physiological, biochemical and molecular characterization of yeast

Preliminary identification was attempted using classical techniques involving physiological and biochemical tests. After three days of growth in GYE broth at 250C, cells of yeast isolate 84 was mostly elliptical (5.1 x 6.5µ m and 5.3 x 6.7µm). The colony morphology of the isolate on solid media exhibited viscous texture with off-white colouration and matt appearances, the shape of the colonies were considerably distinct. After four weeks on GYE agar, 84 colonies were off-white, butyrous, dull, waxy, and had convex to umbonate elevations. The results of the carbon assimilation and the fermentation tests showed that the yeast isolate 84 was able to ferment D-glucose, D-xylose and raffinose while assimilate D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, sucrose, maltose, Alpha,alpha- Trehalose, alpha-D-Glucoside, melezitose, glycerol, ribotol, D-glucitol, D-mannitol, D-glucono-1,5- lactone, 2-keto-D-gluconate, D-gluconate, DLlactate, succinate, citrate and ethanol. Isolate 84 had an identical physiological and biochemical profile to *Debaromyces hansenii* except that 84 were unable to metabolize soluble starch, ethylamine, L-lysine, and cadaverine. Similarly, isolate 84 was able to grow at temperatures up to 42OC; in high osmotic pressure conditions (50 % glucose); exhibited a negative starch test; was resistant to 1000 ppm cycloheximide; and was not able to grow in vitamin-free media. On the basis of physiological, biochemical, nucleotide homology and phylogenetic analysis (Table 1, Fig 1 and Plate 1) the isolate 84 was detected to be *Clavispora lusitaniae* and was deposited in GenBank of NCBI under accession Number: EF221824. Nearest homologous genus and species of isolate 84 was found to be *Candida flosculorum* (Accession No. EF137918).

Studies on growth kinetics of yeast *Clavispora lusitaniae* in blended guava: lemon (1:1) juice during fermentation

The growth curve of yeast *Clavispora lusitaniae* in blended guava-lemon (1:1) juice with juice per cent 12.5%, pH 3.0, and total soluble solids 15°B at temperature 30±2°C for 72 hrs under aerobic conditions with respect to viable cell count ($\log_{10}$ no. of cell per ml)(Fig 2). It was found to show normal pattern with first a short lag period of 3 hrs followed by exponential growth upto 27 hrs as indicated by sharp increase in viable cell count ($\log_{10}$ no of cells per ml) from 5.25 to 8.97 followed by a stationary phase of 9 hrs where the viable cell count remained almost constant. Sener *et al*[26] stated the short lag phase in yeast growth may be the result of the pre-adapted state of the cells used as inoculums. He also reported that when all the sugar was used up and the ethanol concentration rose to the maximum level, the yeast growth stopped and the cells entered the stationary phase.

The ethanol accumulation in fermenters inhibits specific growth rate, cell viability and substrate consumption. Viable cell count started decreasing from 36-72 hrs showing the death phase. The acceleration of yeast death may be due to inadequate supply of nitrogenous substances, vitamins, concentration of dissolved oxygen and insoluble solids[22].

The growth of yeast in terms of increase in cell number with time can be characterized by specific growth rate or the generation time. The specific growth rate (h^{-1}) and generation time (h) with respect to viable cell count ($\log_{10}$ no of cell per ml) were calculated as 0.35 and 1.93

respectively. These results are in accordance with Deak[7] who reported that the yeast growth can be characterized with a growth rate (h^{-1}) and generation time (h) in the range of 0.17-0.35 and 2-4, respectively.

Physicochemical characteristics of three guava var. *Allahabad Safeda, Lucknow-49, Punjab Pink* and lemon var. Baramasi

The acceptability and higher sensory score of beverages is very much dependent on its physicochemical properties including appearance, flavor, acidity and TSS. There may be changes in the physicochemical characteristics and loss of some compounds that impart flavor and aroma to the beverages during pasteurization and storage[12]. The physicochemical composition of guava pulp evaluated on the basis of chemical analysis var. *Allahabad Safeda* were TSS 10.47°B, titrable acidity 0.38 per cent, pH 4.1, Brix acid ratio 27.55, total sugars 6.93 per cent, reducing sugars 3.84 per cent, ascorbic acid 193.7mg/100g, total phenols 314mg/100g and pulp yield 56.75 per cent. The physicochemical characteristics of guava var. *Lucknow-49* were TSS 10.56°B, titrable acidity 0.43 per cent, pH 4.0, Brix acid ratio 27.55, total sugars 5.57 per cent, reducing sugars 3.36 per cent, ascorbic acid 215 mg/100g, total phenols 278 mg/100g and pulp yield 71.42 per cent. and the physicochemical characteristics of guava var. *Punjab Pink* were TSS 10.73°B, titrable acidity 0.41 per cent, pH 4.1, Brix acid ratio 26.17, total sugars 6.40 per cent, reducing sugars 3.57 per cent, ascorbic acid 184.08 mg/100g, total phenols 253 mg/100g and pulp yield 66.66 per cent. The physicochemical characteristics of lemon var. *Baramasi* were TSS 8.0, titrable acidity 4.77 per cent, pH 2.4, Brix acid ratio 1.67, total sugars 5.67 per cent, reducing sugars 3.36 per cent, ascorbic acid 40.0 mg/100ml and juice yield 28.0 per cent

Jain and Nema[11] reported that the various physicochemical parameters in five different guava cultivars like pulp yield, TSS, pH, acidity and ascorbic acid ranged between 54.0-54.8 per cent, 11.8-12.8^{0}B, 3.57-3.98, 0.38-0.48 per cent and 165.41-261.0 mg/100g pulp respectively. The key parameters like TSS, titrable acidity and brix acid ratio determine the final sensory quality attributes like appearance, color, aroma, taste, bouquet, body, flavor, astringency and overall acceptability of the beverage**.** Owing to their rich antioxidant properties and flavor guava and lemon are ideal for preparation of ready-to-serve beverages.

Standardization of blending ratio of guava and lemon on the basis of sensory evaluation

The trials for development of low alcoholic naturally carbonated fermented beverage from different blends of guava and lemon with improved sensory scores and nutritional quality were carried out. Lemon was selected for blending with guava due to its lower pH and high titrable acidity which provides optimum fermentation conditions to yeast and gives appealing aroma, good texture and mouthfeel to the beverage. The trial ratios of guava and lemon, 1:2, 1:1 and 2:1 (v/v) (Table 2), were analyzed by panelists for appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability to select the most preferred one for the shelf life study. The blended beverages varied significantly with respect to the sensory attributes. Blended guava-lemon (1:2) scored highest for appearance (7.3), taste (7.4), color (7.7) and astringency (7.6) while guava-lemon (1:1) was rated highest for aroma (7.6), bouquet (7.55), body (7.4), flavor (8.0) and overall acceptability (7.57). Hence the blending ratio 1:1 was selected for the microbiological preparation of low alcoholic naturally carbonated blended beverage from guava and lemon.

Pandove[22] investigated the effect of blending on the basis of sensory evaluation and established 1:1 ratio of carrot and amla juice as the best treatment with improved texture, taste and overall acceptability for the preparation of low alcoholic self carbonated beverages from carrot and its blends.

Shelf-life studies

Shelf life of low alcoholic naturally carbonated fermented blended guava: lemon (1:1) beverage stored at refrigerated temperature (4^0C) was studied for a period of 90 days evaluated fortnightly for physicochemical and microbiological evaluation.

Effect of storage time on microbiological and physicochemical properties of low alcoholic naturally carbonated fermented guava: lemon (1:1) blended beverage

The results of blended *Allahabad Safeda- Baramasi* beverage (Table 3) shows significant decrease in brix from 15.0°B to 12.1°B and brix acid ratio decreased from 83.33 to 23.72 at the end of 90 days. The pH decreased from 2.9 to 2.6 and acidity increased from 0.18 percent to 0.51 percent at the end of 90 days. The decrease in pH and the increase in acidity are attributed to the production of CO_2 that forms weak acid on dissolution. The percentage decrease in total sugars and reducing sugars was 20.08 percent and 32.12 percent at the end of 90 days. Burdurlu *et al* [6]reported 17 – 85 per cent degradation of vitamin C in citrus juice concentrates during storage at different temperatures. Ocloo and Ayernor[21] reported decrease in pH values with increased total acidity with concomitant increase in yeast growth and alcohol contents of the fermenting sugars syrup. The decrease in soluble solid contents and reducing sugar content was also observed due to disappearance of carbohydrates in the fermenting medium and rapid multiplication of yeast cells. There was a significant decrease in the ascorbic acid content from 27.8 mg/100ml to 9.02 mg/100ml at the end of 90 days. The percentage decrease in total phenols was 24.30 percent during fermentation. The percentage decrease in total phenols is less than that of ascorbic acid because ascorbic acid can inhibit browning reactions by reducing the quinones back to the original phenol compounds. In the presence of oxygen or metal ions phenols can readily

convert to quinones. The alcohol after 15 days was 0.26 percent v/v and gradually reached up to 0.85 percent v/v after 90 days. The CO_2 pressure of 0.74 bar starts after 15 days and increased to 1.18 bars after 60 days and reached up to 1.55 bars at the end of 90 days of storage. Viable cell count increased from 35 x 10^8 to 15 x 10^9 during storage.

The results of blended beverage *Lucknow-49-Baramasi* (Table 4) shows a significant decrease in Brix from 15.0°B to 11.9°B and the brix acid ratio from 78.9 to 22.8. The pH decreased from 2.8 to 2.6 and acidity gradually increased from 0.19 percent to 0.52 percent at the end of 90 days. Sirohi *et al*[27] and Naik *et al*[20] also observed an increase in acidity with decrease in pH during storage of whey based mango herbal pudina beverage and whey based watermelon beverage, respectively. The total sugars and reducing sugars decreased to 10.50 percent and 4.82 percent,respectively at the end of 90 days. The percentage decrease in ascorbic acid and total phenols was 67.31 percent and 26.94 percent respectively during 90 days of storage. The ascorbic acid decreased from 25.7 mg/100ml to 8.4 mg/100ml while total phenols decreased from 28.5 mg/100ml to 20.82 mg/100ml at the end of 90 days. Polydera *et al*[24] also reported also 50% ascorbic acid loss in conventionally pasteurized orange juice at the end of 40 days of storage at 5°C. Degradation of vitamin C depends upon various factors such as oxygen, heat, light, storage condition and type of container. It is also very much dependent on pH i.e. the juice with higher pH are much less susceptible to browning[23].The alcohol formation begins at the end of 15 days (0.27 percent v/v) and reached up to 0.82 percent v/v after 90 days. The CO_2 pressure of 0.79 bars started after 30 days and reached to 1.50 bars after 90 days. The viable cell count increased from 41 x 10^6 to 25 x 10^9 cfu/ml.

The results of blended *Punjab Pink-Baramasi* beverage (Table 5) in show significant decrease in brix from 15.0°B to 12.1°B and the brix acid ratio from 78.94 to 24.2 during fermentation. The pH decreased from 3.0 to 2.6 and the acidity increased from 0.19 percent to 0.50 percent at the end of 90 days. During the 45 days storage of whey guava beverage, decrease in pH and increase in acidity with significant loss of ascorbic acid was observed[8]. The total sugars decreased from 13.18 percent to 10.68 percent after 90 days. The percentage decrease in reducing sugars was 32.56 percent after 90 days. The percentage decrease in ascorbic acid content was 62.08 percent and total phenols were 29.09 percent. Vitamin C is known to be thermo labile and equally susceptible to oxidation on exposure to atmospheric oxygen. It is converted to oxidized form known as dehydroascorbic acid[10]. Kabasakalis *et al*[15]reported loss of ascorbic acid from different commercial fruit juices stored in closed containers for a period of four months at room temperature ranged between 29 and 41%. The alcohol formation begins at the end of 15 days (0.28% v/v) and reached to 0.87 percent after 90 days. The CO_2 pressure of 0.84 bars started after 15 days and increased to 1.27 bar after 60 days and after 90 days reached upto 1.49 bars. Arora[5] reported increase in alcohol per cent, CO_2 pressure and viable cell count to 0.86 per cent, 1.5 bar and $3.3x10^8$,respectively during 90 days of storage of non-alcoholic naturally carbonated lemon beverage.

Evaluation of inhibitory effects of heat treatment and potassium metabisulphite treatment on the organoleptic properties of low alcoholic naturally carbonated fermented guava: lemon (1:1) blended beverage

The heat and potassium metabisulphite treated low alcoholic naturally carbonated fermented blended beverage was microbiologically analyzed and evaluated organoleptically. Temperature exerts profound effect on all aspects of growth, metabolism and survival of yeast. The range of growth temperature of microorganisms is characterized by cardinal (minimum, optimum and maximum) temperatures. However, the temperature limits and range for growth of yeasts vary with species. The cardinal temperature 10-40^{0}C influences the growth, chemical composition, substrate intake and enzymatic activity of yeast. The blended beverage was clarified at different temperatures 45^{0}C, 50^{0}C, 55^{0}C, 60^{0}C, 65^{0}C and 70^{0}C for 2 and 5 mins. The blended beverage was chemically treated with varying concentrations of potassium metabisulphite (100ppm, 300ppm, 500ppm, 700ppm amd 900ppm) for 1 hour.

The optimized temperature and potassium metabisulphite concentration to kill the left over viable yeast cells for clarification was standardized as 55^{o}C for 5 min and 700ppm for 1 hour, respectively (Table 7) without imparting bitter taste to the beverage. These results are in accordance with Arora[5]. The mean scores of the various sensory attributes varied non-significantly in both the treated beverages (Table 6). The heat treated beverage was best rated for appearance, color and bouquet. Organoleptically heat treated beverage was superior than potassium metabisulphite treated beverage.

Conclusion

This technology can redress the problems of horticulturalist by minimizing the post harvest losses, avoid fruit glut in the market, and efficiently utilization of astringent, highly nutritive fruits in the form low alcoholic naturally carbonated beverage with retention of nutrients and nutraceutical properties of fruits for a period of three months.

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TABLE 1: Percentage homology of yeast isolate 84 based on nucleotide sequence

SL. No.	ISOLATES	PERCENTAGE HOMOLGY'										-- --
		1	2	3	4	5	6	7	8	9	10	11
1	84	*	100	100	100	98	99	95	96	82	99	77
2	EF221824		*	100	100	98	99	95	96	*82*	99	77
3	EF568047			*	100	98	99	95	96	82	99	77
4	EF568024				*	98	99	95	96	82	99	77
5	AYI74102					*	98	95	96	82	98	77
6	A Y 493434						*	94	95	81	98	77
7	EU568925							*	93	80	98	76
8	A Y321464								*	80	96	77
9	EF137918									*	81	78
10	A Y321465										*	77
11	EF060724											*

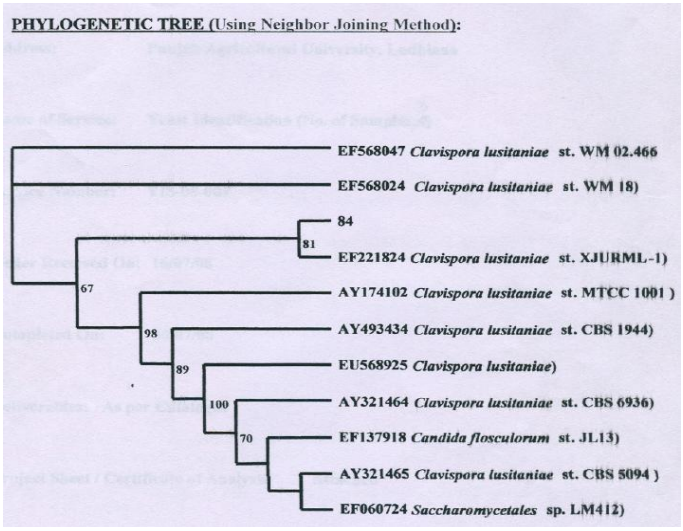
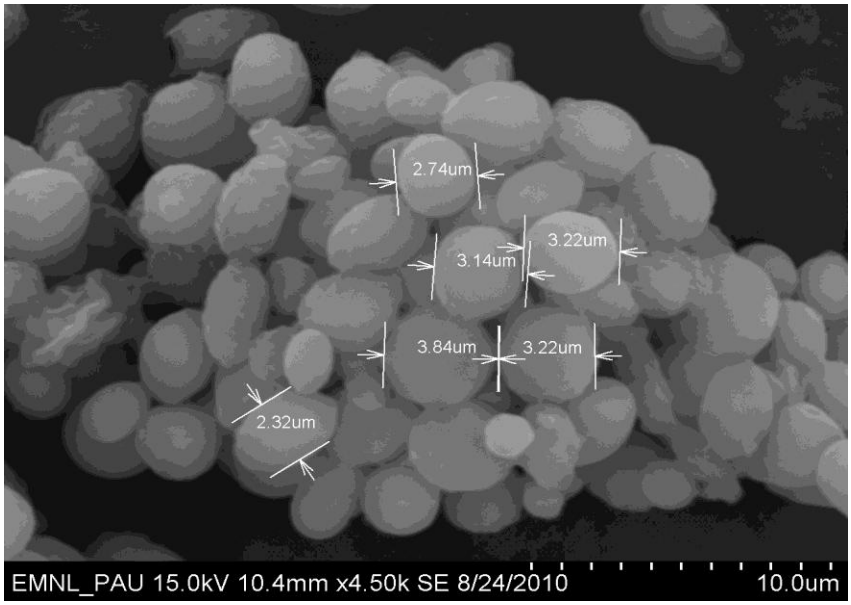


Figure 1. Phylogenetic tree of yeast isolate (84) (using neighbor joining method)



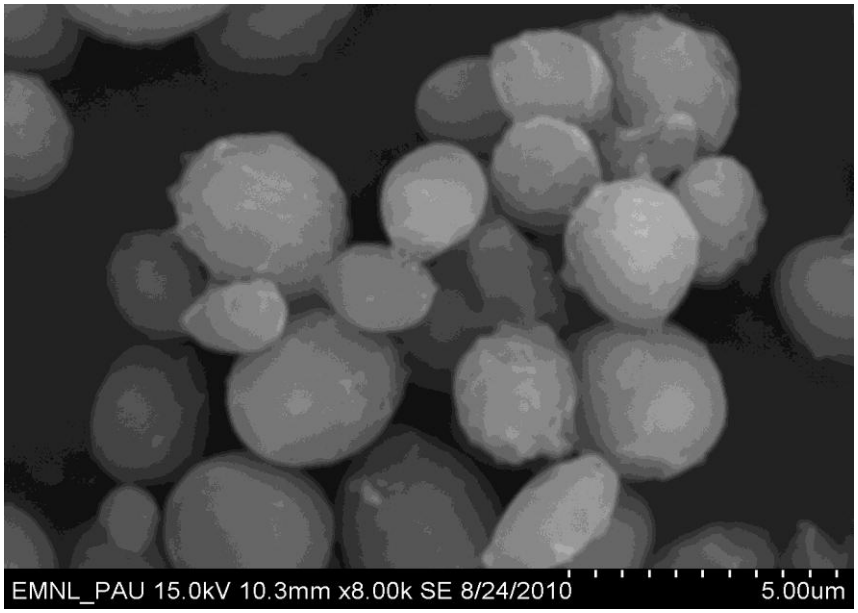


Plate 1 : Scanning Electron Micrographs of yeast *Clavispora lusitaniae*(84)

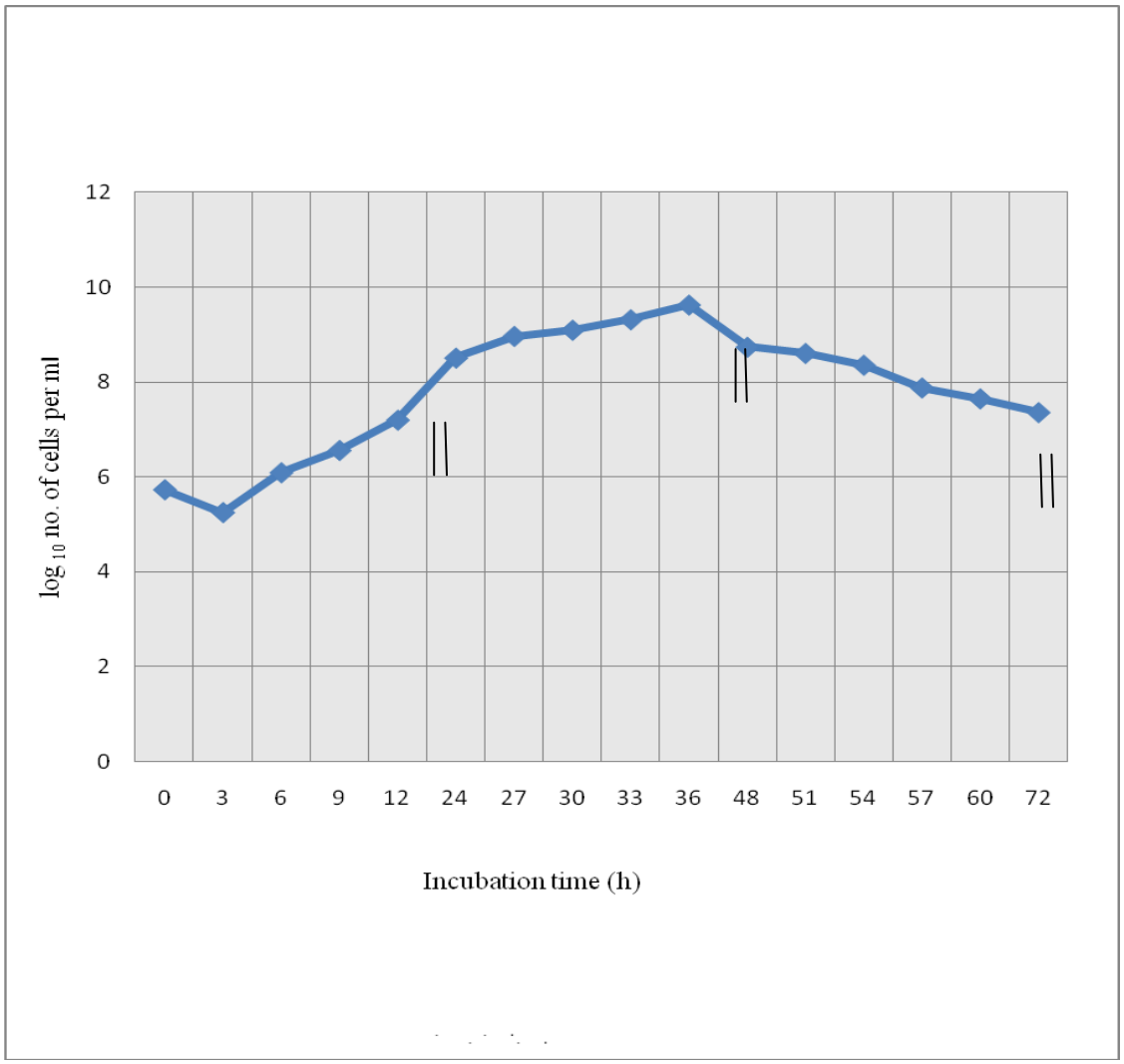


FIGURE 2.Growth Kinetics of *Clavispora lusitaniae* in blended guava: lemon (1:1) juice during fermentation

TABLE 2.Standardization of blending ratio of guava and lemon on the basis of sensory evaluation

Sensory attributes	Guava: lemon (1:2) (v/v)	Guava: lemon 1:1 (v/v)	Guava: lemon (2:1) (v/v)	CD 5%
Appearance	7.3	7.2	7.0	0.048
Taste	7.4	7.1	6.8	0.071
Color	7.7	7.5	7.0	0.037
Aroma	7.4	7.6	7.1	0.041
Bouquet	7.5	7.55	7.4	0.043
Body	7.2	7.4	7.0	0.041
Flavor	7.5	8.0	7.2	0.041
Astringency	7.6	7.5	7.2	0.044
Overall acceptability	7.45	7.57	7.08	0.036

*mean value of three replicates

TABLE 3.Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Baramasi* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS ºB	15	14.8	14.3	13.8	13.2	12.7	12.1	0.04
Acidity %	0.18	0.22	0.26	0.39	0.45	0.48	0.51	0.008
Ph	2.9	2.9	2.8	2.7	2.7	2.7	2.6	0.018
Brix-acid ratio	83.33	67.27	55.0	35.38	29.33	26.45	23.72	0.66
Total sugars (%)	13.64	13.12	12.71	12.25	11.89	11.36	10.9	0.039
Reducing sugars (%)	8.56	8.07	7.82	7.54	6.94	6.23	5.81	0.035
Ascorbic acid (mg/100ml)	27.8	24.6	20.9	17.2	14.4	11.7	9.02	0.04
Total phenols (mg/100ml)	31.6	29.8	28.6	27.3	26.6	25.0	23.92	0.038
Alcohol (%,v/v)	-	0.26	0.34	0.46	0.65	0.77	0.85	0.003
CO_2 (bar)	-	0.74	0.86	1.05	1.18	1.44	1.55	0.0043
Total yeast count (cfu/ml)	35×10^6	58×10^7	92×10^7	43×10^8	24×10^8	87×10^8	15×10^9	-

% Juice in beverage – 12.5%
Storage temp - 4±2℃
*Multiple Regression Equation
$Y = 2.20 – 0.15X_1 + 0.99X_2$
Where
Y = per cent alcohol
X_1= ºBrix
X_2= per cent acidity
**$R^2 = 0.95$

TABLE 4.Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented blended *Lucknow-49– Baramasi* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS°B	15	14.7	14.3	13.7	13.1	12.4	11.9	0.06
Acidity %	0.19	0.21	0.24	0.35	0.41	0.48	0.52	0.004
pH	2.8	2.8	2.7	2.7	2.6	2.6	2.6	0.024
Brix-acid ratio	78.9	70.0	59.6	39.14	31.9	25.8	22.8	0.69
Total sugars (%)	13.45	13.06	12.69	12.11	11.83	11.27	10.5	0.049
Reducing sugars (%)	7.45	6.98	6.47	6.05	5.63	5.21	4.82	0.032
Ascorbic acid (mg/100ml)	25.7	21.2	18.6	15.1	11.9`	10.3	8.4	0.22
Total phenols (mg/100ml)	28.5	26.3	24.9	23.8	24.5	21.7	20.82	0.318
Alcohol (%,v/v)	-	0.27	0.35	0.49	0.61	0.75	0.82	0.0014
CO_2 (bar)	-	0.79	0.86	1.13	1.23	1.42	1.50	0.0018
Total yeast count (cfu/ml)	41×10^6	63×10^7	22×10^8	68×10^8	51×10^8	97×10^8	25×10^9	-

% Juice in beverage – 12.5%
Storage temp - 4±2°C
*Multiple Regression Equation
$Y = 4.94 – 0.31X_1 – 0.63X_2$
Where
Y = per cent alcohol
X_1= °Brix
X_2= per cent acidity
**$R^2 = 0.94$

TABLE 5.Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Baramasi* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS ºB	15.0	14.5	14.1	13.6	13.2	12.7	12.1	0.04
Acidity %	0.19	0.23	0.29	0.35	0.42	0.47	0.50	0.005
Ph	3.0	2.9	2.9	2.8	2.7	2.7	2.6	0.02
Brix-acid ratio	78.94	63.04	48.62	38.85	31.42	27.02	24.2	0.59
Total sugars (%)	13.18	12.76	12.32	11.83	11.29	10.91	10.68	0.017
Reducing sugars (%)	7.83	7.25	6.96	6.64	6.11	5.76	5.28	0.017
Ascorbic acid (mg/100ml)	26.9	22.7	19.1	16.5	13.8	11.3	10.2	0.18
Total phenols (mg/100ml)	26.4	24.1	22.8	21.5	20.7	19.5	18.72	0.038
Alcohol (%,v/v)	-	0.28	0.37	0.53	0.62	0.77	0.81	0.19
CO_2 (bar)	-	0.84	0.91	1.12	1.27	1.42	1.49	0.004
Total yeast count (cfu/ml)	38×10^6	56×10^7	88×10^7	29×10^8	64×10^8	58×10^8	85×10^8	-

% Juice in beverage – 12.5%
Storage temp - 4±2ºC
*Multiple Regression Equation
$Y = 1.47 – 0.11X_1 + 1.43X_2$
Where
Y = per cent alcohol
X_1= ºBrix
X_2= per cent acidity
**$R^2 = 0.9$

TABLE 6.Effect of clarification treatments on sensory attributes of non-alcoholic naturally carbonated fermented guava: lemon (1:1) blended beverage

Sensory attributes	Heat treatment*	Potassium metabisulphite**	CD (5%)
Appearance	7.25	7.0	NS
Taste	7.0	6.5	NS
Color	7.25	7.0	NS
Aroma	7.0	6.0	NS
Bouquet	7.25	6.5	1.28
Body	7.0	6.5	NS
Flavor	6.5	6.0	NS
Astringency	6.5	6.5	NS
Overall acceptability	7.0	6.0	NS

❖ Mean of three replicates
* Treatment at 55^0C for 5 min
** Treatment at 700ppm for 1 hr

TABLE 7.Standardization of heat inhibiting temperature and potassium metabisulphite concentration of left over viable yeast cells

Heat treatment	Temperature (^{o}C)											
	45		50		55		60		65		70	
	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min
Yeast growth	growth occured		growth occured		growth occured	No growth	No growth		No growth		No growth	
Potassium metabisulphite treatment (1hr)	Concentration (ppm)											
	100		300		500		700				900	
Yeast growth	Growth occured		Growth occured		Growth occured		No growth				No growth	