Preparation of Non-Alcoholic Naturally Carbonated Beverage Using Yeast Isolate from Whey Beverage

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Abstract

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Four pure yeast isolates from feta cheese whey beverage were phenotypically characterised and D1/D2 domain of 26S rRNA and Internal Transcribed Spacer (ITS) region were sequenced. These isolates were identified as *Clavispora lusitaniae* (84), *Candida* sp. YS12A (86), *Clavispora lusitaniae* (B82), and *Candida hyderabadensis* (S82). The fermentation potentials of all yeast isolates were determined in plum, amla, lemon, guava, kinnow, and pineapple, and *Clavispora lusitaniae* (84) was reported as the best yeast for carrying out fermentation with CO_2 levels of 1.5 bar. Using *Clavispora lusitaniae,* a reliable, controllable, simple, and reproducible technology from astringent fruits has been developed for the production of non-alcoholic naturally carbonated beverage with improved tangy taste, appearance, aroma, extended shelf life, and retention of all the nutrients. This yeast on inoculation @ 0.5% in astringent in amla juice (13%), TSS adjusted to 16.0°B, and fermentetion at 20 ± 5°C for 36 h produces a new non-alcoholic naturally carbonated beverage. The physicochemical parameters of freshly prepared beverage juice 13%, pH 3.0, TSS 16.0°B, acidity 0.38%, Brix acid ratio 42.10, ascorbic acid 120.0 mg/100 ml. The physicochemical parameters did not change significantly during storage. The volatile components like propanol, butanol, acetaldehyde, methanol, ethyl acetate, and isopropanol were found to be absent while the percentage of ethanol was 1.16% after three months of storage. Shelf life of the beverage is three months under refrigerated conditions (4°C).

Keywords: feta cheese; D1/D2; 26S rRNA; *Clavispora lusitaniae*; fermentation; fruit

A variety of fruits and dairy products offer a special ecological niche that selects for the activity and occurrence of specific yeast species. Yeasts are detected in high numbers in dairy products reflecting a good adaptation to a substrate rich in proteins, lipids, sugars, and organic acids. A wide distribution is a consequence of proteolytic and lipolytic activities, as well as the ability to ferment/assimilate lactose and to utilise citric, lactic, and succinic acids. In addition, yeasts are able to grow in substrates with a high salt concentration, low temperatures, low pH, and low water activity. Due to their inherent trait of adapting to complex substrates, yeasts play either beneficial (e.g. in ripening processes) or detrimental (spoilage organisms, inhibitors of the growth of starter cultures) roles in the dairy production (Jakobsen & Narvhus 1996). As part of the microbial community, together with bacteria, yeasts contribute to the sensory characteristics of kefir, koumiss, and different cheese varieties influencing the biosynthesis of aromatic compounds (Viljoen 2001). Yeasts are frequently found in the microflora of a number of cheese varieties, especially soft-mould,

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smear, and brine-ripened cheeses wherein they contribute to a flavour development. The main yeasts include *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and various *Candida* spp. The bacteria include various species of *Lactobacillus*, *Leuconostoc*, *Micrococcus*, and *Brevibacterium.*

Feta cheese is the most popular traditional Greek cheese made from sheep's milk, goats' milk, or a mixture of both. The characteristics of each cheese type are determined by the influence of various factors, among which the type of the starter culture plays one of the most important roles. In smear surface-ripened cheeses moulds, yeasts and bacteria are present in large numbers on the surface of the cheese and play a significant role in determining the final characteristics and attributes of the cheeses. Yeasts such as *Candida* spp., *Cryptococcus* spp., *Debaryomyces* spp., *Geotrichum candidum* (*Galactomyces geotrichum*), *Rhodotorula* spp., *Saccharomyces* spp., and *Yarrowia lipolytica* are often and variably isolated from the smear surface-ripened cheeses (Corsetti *et al.* 2001).

Fruits like amla, lemon, and pineapple, because of their high acidity and astringent taste, are not palatable for direct consumption, but due to their excellent nutritional and therapeutic values offer enormous potentiality for processing into value added products. Compared to fruit juices, the formulation of naturally carbonated beverage offers more variety of flavours, nutrients, long shelf life, and other physiological benefits with a greater margin of safety in a drink with a lower inherent cost. The Indian gooseberry (*Emblica officinalis* L), also called as 'amla' or 'Aonla', is one of the tropical fruits belonging to the family Euphorbiaceae, with high contents of ascorbic acid (600 mg/100 g), free radical scavenger choline (256 mg/100 g), and polyphenols which impart characteristic acidic and astringent taste to the fruit. According to the Recommended Dietary Allowances (RDA), adequate intakes of ascorbic acid and choline, 90 mg/day and 550 mg/day for males and 75 mg/day and 425 mg/day for females, respectively, are recommended. The fermented beverage retains nutrients, and the additional CO_2 so produced is antimicrobial and adds tangy taste, fizz, and sparkle to the beverage. Amla is available for a short span of time in a year it has shelf life of 6 days to 7 days and results in seasonal glut. The alarming wastage associated with the amla coupled with its low level of industrial utilisation in the developing countries calls for a great concern. To make it available throughout the year in the form of beverage, the present study is proposed with the objective to develop a reliable, controllable, reproducible technology for the production of low alcoholic self carbonated beverage with shelf-life of three months using yeast isolate from whey beverage.

MATERIAL AND METHODS

Isolation, physiological and biochemical characterisation of yeast isolates. Feta cheese was prepared by inoculating the starter mesophilic culture (Choozit 230, Bulk cultures; Danisco, Niebüll, Germany) containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, and thermophilic yoghurt culture (YO-MIX 532, Bulk cultures; Danisco, Niebüll, Germany) containing *Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus.* The whey so obtained was used for beverage making. A total of four morphologically different yeast colonies were screened, isolated from the whey beverage which, on streak purification, revealed one distinct colony type, initially designated as 84, 86, B82, and S82. The identification of the yeast isolate determined on the basis of biochemical activities included the fermentation of sugars, assimilation of carbon compounds, growth on vitamin free medium, growth at 25, 30, 35, 37, and 42°C, growth in 50% and 60% D-glucose medium, urea hydrolysis, and 0.01% and 0.1% cycloheximide (van der Walt & Yarrow 1984).

Molecular characterisation of yeast. The yeast isolates were further identified by sequencing based on partial ITS2 region of the rDNA sequence. Genomic DNA was isolated from the pure culture (Sambrook *et al.* 2001). Using consensus primers, D1/D2 domain of 26S rRNA, ITS-1, and two region fragment (0.4 kb) was amplified using high fidelity Taq polymerase (Fermentas, Glen Burnie, USA). PCR was performed with a 50-µl reaction mixture containing the following (per reaction): 25 ng of genomic DNA; 1× *Taq* buffer (50mM KCl, 10mM Tris-HCl [pH 8.4], 1.5mM $MgCl_2$); a 0.25mM (concentration of each deoxynucleoside triphosphate); a 2µM concentration of each primer; 2 U of *Taq* polymerase (Fermentas, Glen Burnie, USA). The amplifications were performed in a thermo cycler (Biometra GmbH, Goettingen,

Germany), and the PCR program was as follows: 95°C for 2 min and 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min 30 s followed by a 10-min final extension at 72°C. The PCR product was cloned in pTZ57R/T vector (Fermentas, Glen Burnie, USA) as per manufacturer's instructions and plasmid DNA was bi-directionally sequenced using the forward, reverse, and internal primers. The sequence data was aligned and analysed for finding the closest homology for the microbe. The MEGA 4.0 package (Tamura *et al.* 2007) was used for all analyses.

Chemical analysis. Percentages of total soluble solids (%TSS) in juice and beverage were determined by using Erma hand refractometer of 0–32°B (Erma, Tokyo, Japan). pH of the juice was determined using a digital pH meter (ECIL, Hyderabad, type 101; Electronic Corporarion of India Ltd., Hyderbad, India). Total acidity expressed as citric acid was estimated following the procedure of AOAC (1999). Brix-acid ratio was calculated through dividing TSS value by total acidity of the juice and carbonated beverage. Total sugars were estimated by phenol-sulphuric acid method of Dubois *et al.* (1956) using glucose as standard. Reducing sugars were estimated by the method of Miller (1959). The titration method using 2,6-dichlorophenol indophenol dye was used to estimate ascorbic acid (AOVC 1996). Carbon Dioxide volumes in beverage bottles were determined by Zahm and Nagel piercing device (CO_2 tester, Zahm and Nagel Co., Inc., Holland, New York, USA). Total yeast count was enumerated on GYE agar by serial plate dilution method. The volatile components like propanol, butanol, acetaldehyde, methanol, ethyl acetate, isopropanol, and ethanol were analysed by HPLC with GC Headspace Injection, TR Wax column, and detected by FID.

Screening of yeast isolates for fermentation potential of fruit juices. Screening was carried out by inoculating the yeast isolates 84, 86, B82, and S82 into different fruit juices of plum, amla, lemon, kinnow (procured from the Department of Horticulture, Punjab Agricultural University, Ludhiana, Punjab, India), and pineapple procured from the local market of Ludhiana, Punjab, India. The study on the fermentation potential of yeast isolates in different fruit juices was done in one litre glass bottles each containing 750 ml of juice (brix adjusted to 16°B), inoculated @ 0.5% (v/v) and incubated at 30 ± 5°C.

Preparation of non-alcoholic naturally carbonated Amla beverage. The physico-chemical analysis (pH, % acidity, TSS, Brix acid ratio and juice yield) of fresh amla juice was performed. The raw amla juice was diluted with water (13% juice) and sugar solution was added to achieve the required TSS 16.0°B, % acidity (0.32–0.40), and brix acid ratio (40:1). Diluted juice was pasteurised at 82°C for 15 s, cooled, and brix was adjusted to 16°B by adding sugar solution followed by the culture @ 0.5% (v/v). It was incubated at 20 ± 5°C for 36 hours. The beverage was refrigerated for 24 h, siphoned, bottled, and stored in refrigerated conditions.

Statistical analysis. Statistical analysis was done by using GSTATO4 and CPCS1 software.

RESULTS AND DISCUSSION

Yeast isolation

From the primary yeast culture isolated from whey beverage, thirty colonies with identical morphological appearance were selected for further purification on Glucose Yeast Extract (GYE) agar, OGYE (Oxytetracycline Glucose Yeast Extract) agar, and Sabouraud agar at 37°C. A total of ten morphologically identical colonies were selected which on streak purification revealed four distinct colony types, initially designated as 84, 86, B82, and S82. Three yeast isolates 84, B82, S82 were isolated from the whey beverage while the fourth isolate 86 was isolated from the smear of surface-ripened Feta-cheese.

Yeast identification and characterisation

The results of the carbon assimilation and the fermentation tests showed that 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, α-trehalose, alpha-D-glucoside, melezitose, glycerol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, DL-lactate, succinate, citrate, and ethanol. Isolate 84 had an identical physiological and biochemical profile to *Debaromyces hansenii* except that 84 were unable to metabolise soluble starch, ethylamine, L-lysine, and cadaverine. Similarly, isolate 84 was

Table 1. Percentage homology of yeast isolate (84) based on nucleotide sequence

SL. No.	Isolates	Percentage homology										
		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	AY174102					*	98	95	96	82	98	77
6	AY493434						*	94	95	81	98	77
7	EU568925							*	93	80	98	76
8	AY321464								*	80	96	77
9	EF137918									*	81	78
10	AY321465										*	77
11	EF060724											*

able to grow at temperatures of up to 42°C; 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, Figure 1a) isolate 84 was detected to be *Clavispora lusitaniae* and was deposited in GenBank of NCBI under accession No. EF221824. The nearest homologous genus and species of isolate 84 was found to be *Candida flosculorum* (accession No. EF137918).

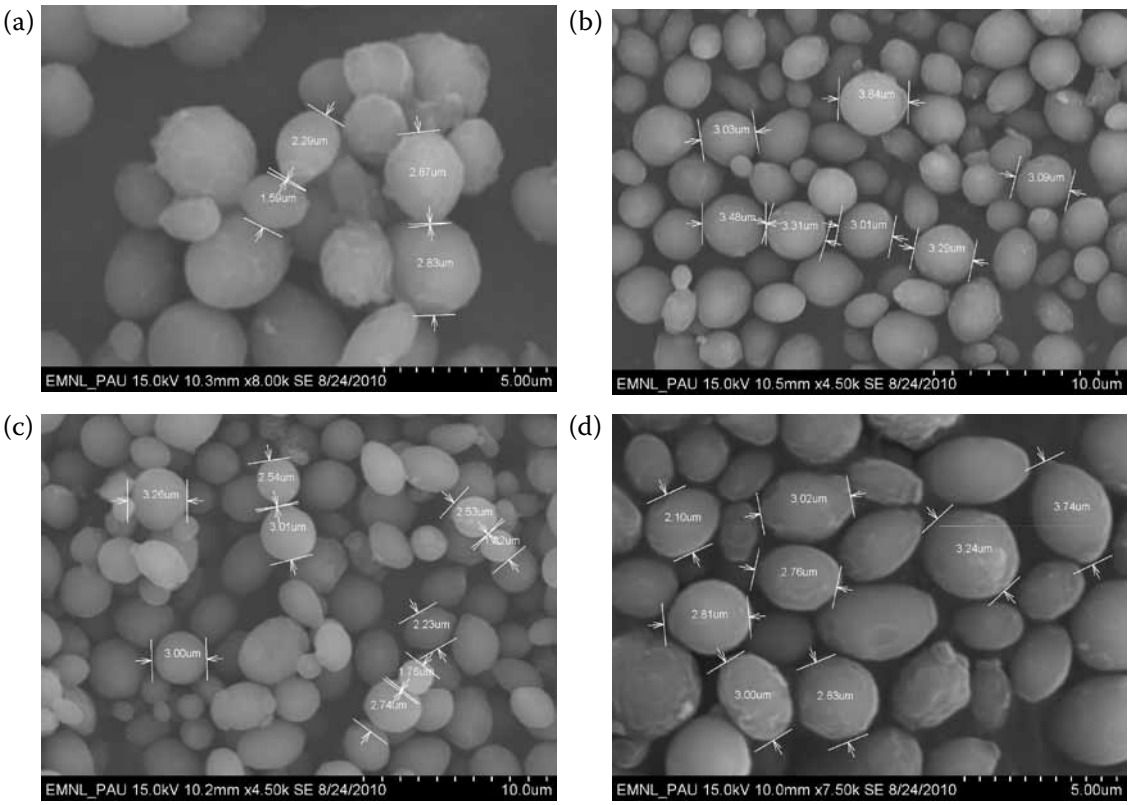

Figure 1. Electron micrographs of yeast isolates: (a) *Clavispora lusitaniae* (84), (b) *Candida* sp. YS12A (86), (c) *Clavispora lusitaniae* (B82), (d) *Candida hyderabadensis* (S82)

Table 2. Percentage homology of yeast isolate (86) based on nucleotide sequence

SL. No.	Isolates	Percentage homology										
		1	2	3	4	5	6	7	8	9	10	11
1	86	*	98	98	98	100	99	99	99	99	99	99
2	DQ857760		*	98	97	97	98	98	98	98	98	98
3	EF060428			*	98	98	99	99	99	99	99	99
4	EFI95357				*	99	98	98	98	98	98	98
5	ABl09231					*	98	98	98	98	98	98
6	EU564206						*	100	100	100	100	100
7	EU564205							*	100	100	100	100
8	EU564202								*	100	100	100
9	EU552501									*	100	100
10	AY391843										*	100
11	AY939798											*

Isolate 86 was able to ferment D-glucose and raffinose while assimilate D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, sucrose, maltose, Alpha, α-trehalose, α-D-glucoside, melezitose, glycerol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, DL-lactate, succinate, citrate, and ethanol. Isolate 86 had an identical physiological and biochemical profile to *Debaromyces hansenii* except that 86 was unable to metabolise soluble starch, ethylamine, L-lysine, and cadaverine. Similarly, isolate 86 was able to grow at temperatures of up to 42°C, 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 nucleotide homology and phylogenetic analysis (Table 2, Figure 1b) isolate 86 was detected to be *Candida* sp. YS12A. The nearest homologous genus and species of isolate 86 was found to be *Saccharomycetales* sp. LM46.

Isolate B82 was able to ferment D-glucose, D-xylose, and raffinose while assimilate D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, D-arabinose, sucrose, maltose, Alpha, α-trehalose, α-D-glucoside, melezitose, glycerol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, DL-lactate, succinate, citrate, and ethanol. Similarly, isolate S82 was able to grow at temperatures of up to 42°C; in high osmotic pressure conditions (50% glucose); exhibited a negative starch test; was

Table 3. Percentage homology of yeast isolate (B82) based on nucleotide sequence

SL. No.	Isolates	Percentage homology										
		1	2	3	4	5	6	7	8	9	10	11
1	B82	*	100	100	100	100	99	97	97	82	80	80
2	EF568047		*	100	100	100	99	97	97	82	80	80
3	EF568024			*	100	100	99	97	97	82	80	80
4	EF221824				*	100	99	97	97	82	80	80
5	AFI72262					*	99	97	97	82	80	80
6	AY 493434						*	97	97	81	79	79
7	AY321469							*	100	80	80	80
8	AY321465								*	80	80	80
9	EF192222									*	98	95
10	AY500373										*	96
11	EF0607768											*

Table 4. Percentage homology of yeast isolate (S82) based on nucleotide sequence:

SL. No.	Isolates	Percentage homology										
		1	2	3	4	5	6	7	8	9	10	11
1	S82	*	98	98	98	98	99	99	99	99	99	99
2	AM180949		*	97	97	97	98	98	98	98	98	98
3	EF060428			*	98	98	99	99	99	99	99	99
4	FM172983				*	100	99	99	99	99	99	99
5	AY391846					*	99	99	99	99	99	99
6	EU564200						*	100	100	100	100	100
7	AY700134							*	100	100	100	100
8	EU564193								*	100	100	100
9	AY391843									*	100	100
10	FM172980										*	100
11	EU564206											*

resistant to 1000 ppm cycloheximide; and was not able to grow in vitamin-free media. On the basis of nucleotide homology and phylogenetic analysis (Table 3, Figure 1b) isolate B82 was detected to be *Clavispora lusitaniae* and was deposited in GenBank of NCBI under accession No. EF568047. Morphological and cultural tests performed on B82 also yielded results analogous to those reported for *Candida intermedia*. The nearest homologous genus and species of isolate B82 was found to be *Candida intermedia.*

Isolate S82 was able to ferment D-glucose, D-xylose, and raffinose while assimilate D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, D-arabinose, sucrose, maltose, Alpha, α-trehalose, Me α-D-glucoside, melezitose, glycerol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, DL-lactate, succinate, citrate, and ethanol. Similarly, isolate S82 was able to grow at temperatures of up to 42°C; 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 nucleotide homology and phylogenetic analysis (Table 4, Figure 1d) isolate S82 was detected to be *Candida hyderabadensis* and was deposited in GenBank of NCBI under accession No. AM180949.

Table 5. Screening of yeast isolates for fermentation on different fruit juices for preparing non-alcoholic naturally carbonated beverage

Fruit Juice	Fementation by yeast isolates			
	Clavispora lusitaniae EF221824	*Candida* sp.YS12A DQ857760	*Clavispora lusitaniae* EF568047	*Candida hyderabadensis* AM180949
Plum	+++	++	++	+
Amla	+++	++	+	+
Lemon	++	+	–	–
Guava	+++	++	++	+
Kinnow	+++	+	+	–
Pineapple	+++	++	++	+

Initial sugar concentration – 16°B; incubation period – 36 h aerobically; incubation temperature – 30°C; +++ good fermentation (0.7% alcohol and 1.5 bar CO_2); ++ fair fermentation (0.4% alcohol and 0.9 bar CO_2); – no fermentation

The nearest homologous genus and species of isolate S82 was found to be *Saccharomycetales.*

Screening of yeast isolates for fermentation on different juices for preparation of non-alcoholic naturally carbonated beverage

Four yeast isolates, i.e. 84, 86, B82, and S82 identified as *Clavispora lusitaniae*, *Candida* sp. YS12A, *Clavispora lusitaniae*, and *Candida hyderabadensis*, respectively, were used for carrying out fermentation on different juices (Table 5). The yeast isolate *Candida* sp. YS12A (86) showed fair fermentation in plum, amla, guava, and pineapple juices whereas the isolate *Clavispora lusitaniae* (B82) showed fair fermentation in plum, guava, and pineapple juices with carbonation of 0.9 bar and alcohol concentration of 0.4% (v/v). The yeast isolate *Clavispora lusitaniae* (84) was more active and carried out vigorous fermentation in plum, amla, guava, kinnow, and pineapple juices as compared to *Candida* sp. YS12A (86), *Clavispora lusitaniae* (B82), and *Candida hyderabadensis* (S82), and was studied as the best yeast for carrying out fermentation with CO_2 level of 1.5 bar and was closely followed by 0.7% alcohol production in all fruit juices. CO_2 so produced is anti microbial and adds tangy taste, fizz, and sparkle to the beverage. In naturally carbonated beverages, pH, sugar concentration, optimum temperature, and CO_2 are the main factors that influence the yeast growth. Markides (1986) reported that yeasts ferment the sugar to alcohol and produce CO_2 as a by-product creating the bottle pressure of about 500–600 KPa (5–6 atmospheres) at 10°C; after the completion of the secondary fermentation and for each 100 KPa of pressure rise, approximately 4 g/l of sugar was required.

Technology for preparation of non alcoholic naturally carbonated beverage under optimised conditions of fermentation

A non alcoholic naturally carbonated beverage was prepared from amla juice under optimised conditions of inoculum concentration (0.5%), incubation temperature (20 ± 5°C), incubation time (36 h), and TSS (16 B), and using best yeast isolate *Clavispora lusitaniae* (84). The non alcoholic naturally carbonated beverage obtained is fresh, safe, stable, more natural, minimally processed, free from additives, contaminants, adulterants, and harmful pathogenic bacteria.

Table 6. Physicochemical parameters of amla juice

Parameters	Amla
pH	2.6
TSS (°B)	9.0
Acidity (%)	3.20
Brix-acid ratio	2.81
Total sugars (%)	2.18
Reducing sugars (%)	2.0
Ascorbic acid (mg/100 ml)	204.0
Juice yield (%)	44.0

Preparation of non alcoholic naturally carbonated beverage from Amla

The acceptability of beverages is very much dependent on their physico-chemical properties. There were not significant changes in physico-chemical characteristics that imparted flavour and aroma to the beverages during pasteurisation and storage. The stability of fruit-based beverages is also influenced by the type of fruit juice used in their formulation.

Physico-chemical composition of Amla juice

The physico-chemical characteristics of Amla cv. Francis was TSS 9.0°B, % titrable acidity 3.20, pH 2.6, Brix-acid ratio 2.81, total sugars 2.18%, reducing sugars 2.0%, ascorbic acid 204 mg/100 ml, and juice yield 44.0% (Table 6).

Shelf-life studies

Shelf-life of non alcoholic naturally carbonated amla beverage stored at refrigerated temperature was studied and evaluated fortnightly for organoleptic, biochemical, and microbiological qualities.

Effect of fermentation on physicochemical properties of Amla beverage

The results obtained with Amla beverage after 90 days (Table 7) showed a significant decrease in

Table 7. Effect of storage on physicochemcal parameters of Amla beverage

Parameters	Fresh	15 days	30 days	45 days	60 days	75 days	90 days	CD (5%)
pH	3.0	3.0	3.1	3.1	2.9	2.9	3.0	NS
TSS (°B)	16.0	15.50	15.0	15.0	14.0	13.50	13.0	0.303
Acidity (%)	0.38	0.38	0.44	0.48	0.51	0.54	0.57	0.003
Brix-acid ratio	42.10	40.78	34.09	31.25	27.45	25.0	22.80	0.046
Total sugars (%)	14.56	14.08	13.20	13.12	12.44	11.88	11.38	0.050
Reducing sugars (%)	9.96	9.53	8.77	8.32	7.06	6.58	5.75	0.047
Ascorbic acid (mg/100 ml)	120.0	110.0	108.0	105.0	102.5	100.0	90.0	0.396
Ethanol (%)	absent	0.15	0.29	0.57	0.73	1.09	1.16	ns
Propanol (%)	absent	absent	absent	absent	absent	absent	absent	ns
Butanol (%)	absent	absent	absent	absent	absent	absent	absent	ns
Acetaldehyde (%)	absent	absent	absent	absent	absent	absent	absent	ns
Methanol (%)	absent	absent	absent	absent	absent	absent	absent	ns
Ethyl acetate (%)	absent	absent	absent	absent	absent	absent	absent	ns
Isopropanol	absent	absent	absent	absent	absent	absent	absent	ns
CO_2 (bar)	–	0.90	0.90	1.20	1.20	1.50	1.50	ns
Total Plate count (CFU/ml)	–	4.5×10^6	5.8×10^7	6.4×10^7	7.8×10^7	2.3×10^8	3.8×10^8	–

Juice 13%; storage temperature 4°C; ns – non significant

brix from 16.0 to 13.0 and Brix acid ratio from 42.10 to 22.80. The pH remained 3.0 due to the buffering action of juices and acidity increased from 0.38 to 0.57 after fermentation during a storage period of 90 days under refrigerated conditions (4°C). The percentage decrease in total sugars with a storage period of 90 days was 21.84% as it decreased from 14.56% to 13.20% after 30 days and gradually decreased to 11.38% at the end of 90 days, while percentage decrease in reducing sugars was 42.26% as it decreased gradually from 9.96% to 5.75% after 90 days (Table 7). The ethanol production gradually increased from 0.15% to 1.16% after 90 days. The volatile components like propanol, butanol, acetaldehyde, methanol, ethyl acetate, and isopropanol were found to be absent from the beverage till 90 days (Table 7). The CO_2 pressure started to show after 15 days and reached up to 1.50 bar after 90 days. During fermentation, CO_2, alcohol, and glycerol produced were proportional to the amount of the sugar fermented. The yeast strain produced a large amount of glycerol at the expense of ethanol representing an advantageous alternative for the development of beverages with low ethanol contents versus physical processes which alter the organoleptic properties of the final product. Eglinton *et al. (*2002) reported glycerol to be a major fermentation product of *Saccharomyces cerevisiae* that contributes to the sensory character of wine. Ascorbic acid content was reduced from the initial concentration 120 mg/100 ml to 108 mg/100 ml after 30 days and decreased significantly to 90.0 mg/100 ml after 90 days ($P \leq 0.05$). Ascorbic acid (vitamin C) is an important antioxidant that protects against cancers, heart disease, and stress. It is part of the cellular chemistry that provides energy, is involved in collagen synthesis, bone and teeth calcification as well as in building cartilage, joints, skin, and blood vessels (Champe & Harvey 1994). It helps in the absorption of dietary iron by keeping it in the reduced form (ferrous form). Heating or drying of fresh fruits usually leads to the destruction of most of ascorbic acid originally present. Amla is an exception among fruits as it contains substances and high acidity which partially protect ascorbic acid from the destruction on heating or drying. The decrease of ascorbic acid in beverage during storage results from oxidation of ascorbic acid by ascorbic acid oxidase due to a combined effect of oxygen and light (Table 7). Alwazeer *et al.* (2003) demonstrated that factors contributing to the stability of pasteurised orange juice are redox potential (Eh 240–360 mV) and reducing condi-

tions (Eh –180 mV) which are necessary to stabilise colour and ascorbic acid during storage.

CONCLUSION

The technology presented here can redress the problems of horticulturalists by minimising the post-harvest losses, avoid fruit and vegetable glut in the market, and ensure efficient utilisation of astringent, highly nutritive fruits in the form of non alcoholic naturally carbonated beverage with the retention of nutrients and maintaining nutraceutical properties of fruits for a period of three months.

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