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Technology for the production of low alcoholic self carbonated fermented beverage

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Abstract: A pure yeast isolate from whey beverage, phenotypically characterized and D1/D2 domain of 26S rRNA and Internal Transcribed Spacer (ITS) region sequenced, was identified as *Clavispora lusitaniae*. A technology to produce low alcoholic self carbonated beverage from carrot-lemon juice with this yeast was developed. It is a reliable, controllable, reproducible technology, and especially safeguards the interest of horticulturists during seasonal glut of the fruits.The physicochemical characteristics of freshly prepared fermented carrot-lemon beverage (3:1) TSS 16ºB, pH 3.7, acidity 0.36%, brix acid ratio 44.44, ethanol 0.4% (w/v), CO_2 0.9 bar and viable cell count was $1x10^7$cfu/ml. The physico-chemical changes recorded after storage for three months at refrigerated temperature showed TSS 13°B, pH 3.3, Brix acid ratio 26.53, acidity 0.49%, ethanol 0.8% (w/v), CO_2 1.5 (bar), and viable cell count (cfu/ml) was $9.5x10^8$ cfu/ml. Naturally produced CO_2 during fermentation adds effervescence, sparkle, tangy taste to the beverage. CO_2 has antimicrobial properties and shelf life of beverage is three months.

Key words: beverage, low alcoholic, self carbonated, yeast

Introduction

India stands second in the world for production of fruits and vegetables owing to the remarkable diversity of its geographical conditions. The country produces about 50 million tonnes of fruits per year but only 2% of this goes for processing, while over 25% is spoiled due to improper handling and storage which results in quantitative and qualitatively losses (flavour, texture, nutritional value and safety) (Singh and Goswami, 2006).Consumers like carrot juice because of its high nutritive value, fiber, carbohydrates, vitamin A derived from its high α carotene (β ε-carotene), β-carotene content, colour, aromatic compounds and refreshing characteristics (Desobry *et al.*,1998).A major problem for processing carrot is color loss and requires double pasteurization (Czepa and Hofmann 2004). Lemon fruits are astringent with harsh flavour (limonin and naringin) and are not palatable for direct consumption, but due to its nutritional and therapeutic values it offers enormous potential for processing. It contain ascorbic acid (39mg/100g), carbohydrates (11.1g/100g) and minerals (K-270mg, Ca-70mg & P-10mg/100g) (Gopalan *et al.*, 2002).Fermentation of blended carrot and lemon juice can provide health beverage with refreshing thirst quenching, medicinal and therapeutic properties. The fermented beverage retains nutrients, and additionally CO_2 so produced is anti microbial and adds tangy taste, fizz and sparkle to the beverage. Carrot and Lemon are available for a short span of time in a year and result in seasonal glut. To make them available throughout the year in the form of beverage, the present study is proposed with objective to develop a reliable, controllable, reproducible technology for the production of low alcoholic self carbonated beverage with shelf-life of three months.

Material and Methods

Physiological and biochemical characterization

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°C, 30°C, 35°C, 37°C and 42°C, growth in 50% and 60%

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D-glucose medium, urea hydrolysis and 0.01% and 0.1% cycloheximide sensitivity (Van der Walt and Yarrow ,1984).

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 (Sambrook *et al*., 2002). 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 analyzed for finding the closest homology for the microbe. The MEGA 4.0 package (Tamura *et al*., 2007) was used for all analyses.

Screening of yeast isolate for potential of fermentation of fruit juices

It was carried out by inoculating the yeast isolate 84 in lemon juice (procured from the Deptt. of Horticulture, Punjab Agricultural University, Ludhiana).Lemon juice (10%) was boiled, brix adjusted to 16°B with boiled and cooled sucrose solution. A loopful of 24 h old yeast culture was transferred to 100 ml fruit juice in 250 ml Erlenmeyer flask and incubated at 20±5°C for 24 hrs to achieve concentration 107-108 cells/ml. Studies on fermentation potential of yeast isolate in fruit juice was done in one litre glass bottles each containing 750 ml juice (16°B), inoculated @ 0.5% v/v and incubated at 20±5°C. The bottles were analyzed for alcohol (% v/v) and carbon dioxide (bar) production after 36hrs at 20±5°C temperature. Culture of Yeast was maintained at 4°C in Glucose Yeast extract agar (GYE) slants and sub cultured fortnightly.

Vegetables and fruits

Carrot var. PC was procured from the Department of Vegetable crops, PAU, Ludhiana. Lemon var. Baramasi were obtained from Department of Horticulture, PAU, Ludhiana.

Extraction of juices

Healthy fruits and vegetables were washed with chlorinated water and peeled. Carrot juice was extracted aseptically using Electronic juicer (INALSA). Lemon were washed and cut into two halves, deseeded and squeezed with electrical lemon squeezer to extract juice. Extracted juice was filtered through muslin cloth.

Preparation of sugar solution

The granulated sucrose procured from local market of Ludhiana city, was boiled in equal water (500g/100litre) for 5 minutes and then cooled to room temperature to prepare sugar solution.

Physico-chemical analysis of carrot and lemon juice

Physico-chemical analysis of carrot and lemon juice was done (TSS (˚B), pH, acidity (%), Brix-acid ratio, Juice recovery per kg). The Carrot and Lemon juices were mixed in the ratio of 3:1, 1:1, 1:3. Blended juice was diluted in the ratio 1:2 with water. Diluted juice was pasteurized at 82°C for 15 secs, cooled and brix adjusted to 16°B by adding sugar solution followed by inoculation of yeast @ 0.5% (v/v). It was incubated for 36 hrs at 20±5 °C. The beverage was refrigerated for 24 hrs, siphoned, bottled and stored in refrigerated conditions.

Chemical analysis

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 (AOAC ,1980). Percent total soluble solids (%TSS) determined by using Erma hand refractometer of 0-32ºB (UNICO). Total sugars estimated by phenol-sulphuric acid method of Dubois *et al.*, (1956) using glucose as standard. Reducing sugars estimated by the method of Miller (1959). Ascorbic acid was determined by titrametric method using 2, 6-dichlorophenol indophenol dye AOVC (1996). 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. Sensory evaluation of beverage was carried out using nine-point hedonic scale (Amerine *et al.*, 1965).Statistical analysis was done by using GSTATO4 and CPCS1 software developed by Maths, Statistics and Physics Department, PAU, Ludhiana.

Results and Discussion

Physiological ,biochemical and molecular characterization

Preliminary identification was attempted using classical techniques involving physiological and biochemical tests. After three days of growth in GYE broth at 25^{0}C, 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, DL-lactate, 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 42^{O}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) 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).

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

Yeast isolate *Clavispora lusitaniae* showed the potential to produce low alcohol and carbonation in lemon juice with carbonation of 0.9 bars and ethanol concentration of 0.4 % (v/v) .So the beverage was prepared using *Clavispora lusitaniae* under standardized condition. Markides (1986) reported that yeasts ferment the sugar to alcohol and producing CO_2 as the by-product having the bottle pressure of about 500-600 KPa (5-6 atmospheres) at 10°C; after the completion of secondary fermentation and for each 100 KPa of pressure rise, approximately 4g/l of sugar was required.

Technology for preparation of low-alcoholic self carbonated beverage under optimized conditions of fermentation

Low alcoholic self carbonated beverage was prepared from carrot and lemon juice blend under optimized conditions of inoculum concentration (0.5%), incubation temp (20±5°C), incubation time (36hrs) and TSS (16°B). Low alcoholic self carbonated beverage is fresh, safe, more stable minimally processed, free from additives contaminants, adulterants and harmful pathogenic bacteria.

Preparation of low alcoholic self carbonated beverage from carrot and lemon blend

The acceptability of beverages is very much dependent on its physico-chemical and organoleptic properties like color, appearance, texture, and aroma. There were not significant changes in physico-

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**											*

chemical characteristics that impart flavour and aroma to the beverages during pasteurization and storage. The stability of fruit-based beverages is also influenced by the type of fruit juice used in their formulation (Deak *et al.,* 1986).

Physico-chemical composition of carrot juice

The physico-chemical composition of Carrot juice was evaluated on the basis of chemical analysis. The physico-chemical characteristics of PC-34 carrot variety was TSS 8.5, % titrable acidity 0.25, pH 7.1, Brix-acid ratio 34, total sugars 2.4 %, reducing sugars 1.97 %, Total carotenoids 204 mg/100ml and juice yield 48.4%.

Physico-chemical composition of lemon juice

The flavour of citrus fruit is a function of relative levels of soluble solids, titrable acidity and presence of various aromatic and bitter principles. The physico-chemical composition of lemon juice was TSS 8.0, % titrable acidity 4.77, pH 2.4, Brix-acid ratio 1.67, total sugars 5.67 %, reducing sugars 3.36 %, Ascorbic acid 38.05 mg/100ml and juice yield 48.4%.

Standardization of carrot-lemon beverage for shelf life study

The beverage has been rated as liked very much during sensory evaluation due to its effervescence, improved tangy taste, color, appearance, texture, and aroma as well as enriched with the nutrients and typical flavour of the fruits. As compared to fruit juices the formulation of low alcoholic self carbonated beverage offers more variety of flavour, nutrients, long shelf life and other physiological benefits with greater margin of safety in a fermented drink. The fermentation conditions and technology is simple and can be adopted at small and pilot scale. Carrot-lemon (3:1, 1:1, 1:3) beverages were analyzed by Panelists for sensory scores till period of three months (Table 2). Blended carrot beverage is pale yellow in color and not red as expected by consumer because of settling of red pigment. No significant difference in color, appearance, texture, and aroma was recorded. But overall acceptability and taste showed significant difference. Blended beverage from carrot-lemon (3:1) scored highest for taste (7.6) and overall acceptability (7.7).

Shelf-life studies

Shelf-life of low alcoholic self carbonated carrot-lemon (3:1) blended beverage stored at refrigerated temperature was studied and evaluated fortnightly

Table 2. Effect of blending on sensory scores* of low alcoholic self carbonated beverage

Sensory attributes	A	B	C	F-ratio	CD at 5%
Color	7.6±0.89	7.4±0.89	7.3±0.67	0.17^{NS}	NS
Appearance	7.4±0.55	7.2±0.84	6.8±0.84	0.82^{NS}	NS
Texture	7.4±0.84	7.2±0.45	7.0±0.71	0.40^{NS}	NS
Taste	7.6±0.55	6.4±0.55	6.5±0.50	7.82	6.83
Aroma	7.6±0.89	7.6±0.89	6.8±0.75	1.47^{NS}	NS
Overall Acceptability	7.7±0.37	7.0±0.71	6.1±0.31	12.91	6.95

* mean value of five replicates,NS-Non-significant

Carrot- PC-34
Lemon- Baramasi
A- Carrot: Lemon (3 :1)
B- Carrot: Lemon (1 :1)
C- Carrot: Lemon (1 :3)
NS- Non-significant

Table 3. Effect of storage on physico-chemical characteristic of low alcoholic self carbonated carrot: lemon (3:1) beverage

Carrot: Lemon (3:1)	Fresh	15d	30d	45d	60d	75d	90d	F-ratio	CD at 5%
TSS (°B)	16.00	16.00	15.45	15.10	14.10	14.50	13.00	329.11	0.2
pH	3.70	3.70	3.60	3.50	3.50	3.40	3.30	-	NS
Acidity (%)	0.36	0.36	0.36	0.45	0.48	0.48	0.49	-	NS
Brix acid ratio	44.44	44.44	42.91	33.55	29.37	29.08	26.53	1180.09	0.77
Alcohol (w/v)	0.40	0.50	0.60	0.60	0.65	0.70	0.80	-	NS
CO_2	0.9	0.90	0.90	1.20	1.20	1.20	1.50	-	NS
Total plate count (cfu/ml)	$1.0x10^7$	$2.2x10^7$	$3.0x10^7$	$3.5x10^7$	$1.3x10^8$	$6.5x10^8$	$9.5x10^8$	17.27	.35E+09

for biochemical and microbiological qualities.

Effect of fermentation on physicochemical properties of carrot:lemon beverage (3:1)

The results of carrot:lemon beverage (3:1) (Table 3) show significant decrease in brix from 16.0 to 13.0 and Brix acid ratio from 44.44 to 26.53. The pH of the beverage decreased from 3.7 to 3.3 and acidity increased from 0.36 to 0.49 during fermentation. The decrease in pH and increase in acidity was non-significant. This is due to buffering action of juices. These results are in accordance with Aruna et al. (1992) who observed that during storage, total soluble solid, and pH decreased while acidity increased. Acceptability was good even after three months of storage. Babajide et al. (2002) also reported decrease in pH and increase in acidity during storage of non-alcoholic beverage made from millet grain. Ezeronye (2005) observed decrease in Brix from 200B to 60B during fermentation. Ilamaran and Amutha, (2007) reported gradual decrease in BAR content of carbonated banana beverage during storage. The alcohol after 15 days was 0.50% v/v and gradually increased to 0.65% v/v after 60 days and reached up to 0.80% v/v after 90 days. Higher alcohols like propanol, butanol, isopropanol and acetaldehyde and ethyl acetate was absent in beverage till 90 days of storage. The CO2 pressure of fresh beverage was 0.90 bar that increased to 1.5 bar at the end of 90 days. Viable cell count increased from 1.0x107-9.5x108 cfu/ml. Kumar (1996) found that carbonated pure mandarin juice beverage at 100 psi pressure of carbonation, the best, similarly low alcoholic self carbonated beverage from carrot:lemon (3:1) has been adjudged the best with highest sensory quality characteristics and shelf life of three months.

Conclusion

The alarming wastage associated with carrot and lemon coupled with its low level of industrial utilization in the developing countries calls for a great concern. The nutritional and therapeutic value of lemon and carrot can be tapped by processing them into value added fermented product (low alcoholic self carbonated beverage) with retention of nutritional properties, highest sensory qualities and shelf life of three months.

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