



Development of low alcoholic naturally carbonated fermented debittered beverage from grapefruit (*Citrus paradisi*)

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Abstract: A pure yeast *Clavispora lusitanae*, isolated from whey beverage, phenotypically characterized and molecularly characterized by sequencing of D1/D2 domain of 26S rRNA and Internal Transcribed Spacer (ITS) region was used to produce low alcoholic naturally carbonated fermented debittered beverage from Grapefruit. *C. lusitanae* produces enzyme naringinase. This enzyme is a mixture of α -L-rhamnosidase and β -D-glucosidase. The bitter component in citrus fruit, naringin can be hydrolyzed by α -L-rhamnosidase to rhamnose and prunin then by β -glucosidase to glucose and naringenin. The freshly prepared fermented Grapefruit beverage had TSS 14 °B, pH 4.7, acidity 0.26%, brix acid ratio 53.85, total sugars 11.6%, reducing sugars 3.34%, ascorbic acid 21.9 mg/100 ml, naringin 643.2 ppm, alcohol 0.00% (v/v), CO₂ 0.00 bar and total yeast count 5.83 (Log no.of cells/ml). Physico-chemical changes recorded after three months of storage at refrigerated temperature revealed TSS 12.0 °B, pH 4.2, acidity 0.54%, brix acid ratio 22.22, total sugars 8.97%, reducing sugars 1.94%, ascorbic acid 18.45 mg/100 ml, naringin 365.2 ppm, alcohol 0.76 % (v/v), CO₂ 1.35 bar and total yeast count 8.54 (Log no.of cells/ml). Naturally produced CO₂ by *C. lusitanae* during fermentation adds effervescence, sparkle, tangy taste to the beverage in addition to its antimicrobial properties. Thus bio-enzymatic debittering by *C. lusitanae* may become the new direction of citrus juice processing in the future, due to its economical viability with strong ability to remove the bitter taste from citrus juice beverage.

Keywords: *Clavispora lusitanae*, Economical viability, Internal transcribed spacer, Sequencing

INTRODUCTION

India is the world's second largest producer of fruits (57.73 million tonnes) with its projected value touching 98 Mt by the year 2020-2021 (Banerjee, 2009). The citrus occupies an area of about 0.81 Mha with production of 7.50 Mt and yield of 9.26 t/ha and ranks fifth in its production in the world (Anonymous, 2010). In the past three years, Punjab has recorded over 20 tonnes of citrus fruit per hectare while the national average productivity during the same period was just 8.82 tonnes per hectare, less than half of Punjab's production (Anonymous, 2013).

The health benefits of citrus fruit juices have been attributed to the presence of bioactive and antioxidant compounds such as ascorbic acid (53mg/100g), flavonoids, limonoids, coumarins and essential vitamins; folates (30µg), Niacin (0.282mg), Panthothenic acid (0.25mg), Pyridoxine (0.060mg), Riboflavin (0.040mg), Thiamine (0.1mg), Vitamin A (225 IU), Vitamin E (0.18mg) per 100g. Citrus fruits also contains electrolytes in the form of sodium (3-4mg/ml), potassium (300 mg in 178ml juice) and minerals like calcium (40mg), copper (39mg), iron (0.1mg), magnesium (10mg), manganese (0.024mg),

zinc (0.8mg) and β -carotenoides (71µg) per 100g (USDA National Nutrient Database).

The grapefruit (*Citrus paradisi*) is the fourth most economically important citrus fruit in the world. It is an excellent source of many nutrients, phytochemicals, vitamin C, and fiberpectin with pink and red hues that contain the beneficial antioxidant lycopene (Silver *et al.*, 2011). The nutritional and therapeutic value of citrus fruits provides ample scope for processing into a value added fermented product with retention of organoleptic properties, nutritional attributes, characteristics sensory properties, flavour, aroma, texture and long shelf life. But its tissues containing flavonoids like hesperidin, limonin, naringin may cause interference during the citrus fruit juice processing and cause for the bitter taste (Konno *et al.*, 1982). Naringin is the major component in grapefruit with very bitter taste and a threshold of 20 mg Kg⁻¹ in water and detectable limit less than 1.5 mg Kg⁻¹ (Chandler and Nicol, 1975). The enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency, and a convenient operation for removing the bitterness in large-scale commercial production (Yadav *et al.*, 2010). Naringin

can be hydrolyzed by the α -L-rhamnosidase activity into rhamnose and prunin (4,5,7-trihydroxyflavone-7-glucopyranoside), in which prunin can be further hydrolyzed by the β -D-glucosidase activity into glucose and naringenin (4,5,7-trihydroxyflavone) (Chien *et al.*, 2001).

The novel yeast isolate *Clavispora lusitaniae* mutant has been isolated and characterized molecularly and biochemically in the Department of Microbiology, Punjab Agricultural University, which produces naringinase (Jairath *et al.*, 2012, Sahota *et al.*, 2013) The enzyme naringinase is composed of α -L-rhamnosidase (EC 3.2.1.40) and β -D-glucosidase (EC 3.2.1.21) (Singh *et al.*, 2015). This yeast culture has potential to produce less than 1% alcohol and 1.5 bar pressure of carbon dioxide (Sahota and Pandove, 2010).

Hence, to safeguard the interest of progressive horticulturists, the utilization of Grapefruits for the production of beverages in the form of low alcoholic naturally carbonated fermented debittered beverages appear to be economically viable and value added technologies. The present work aims to redress the problem of tapping the bitterness by exploiting the potential of *C. lusitaniae* and thus making the fruit available throughout the year for the consumers to harness their health promotional properties.

MATERIALS AND METHODS

Fruits : Grapefruit *var.* Marsh Seedless was procured from Punjab Agricultural University, Regional Research Station, Bathinda-151001, Punjab, India.

Extraction of juice: Healthy fruits were washed with chlorinated water and cut into two halves, deseeded and juice was extracted aseptically using Electronic juicer.

Yeast culture: Yeast culture *C. lusitaniae* having the potential to produce less than 1% alcohol and 1.5 bar pressure of carbon dioxide (Sahota and Pandove, 2010) was obtained from the Department of Microbiology, PAU, Ludhiana. It has been isolated from whey beverage, phenotypically characterized and D1/D2 domain of 26S rRNA and Internal Transcribed Spacer (ITS) region sequenced (Jaitath *et al.*, 2012)

Inoculum preparation: Grapefruit juice was diluted with water (boiled and cooled), and brix adjusted to 14 ° B by adding sugar solution. A loopful of 24 hrs old yeast culture was transferred to juice in 250 ml Erlenmeyer flask and incubated at 20±5 ° C for 24 hrs to achieve concentration 10⁷–10⁸ cfu/ml, and used as starter culture.

Preparation of sugar solution: Granulated sucrose was procured from local market of Bathinda, Punjab, India. 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 grapefruit: Five fruits of uniform size were taken for physico-chemical analysis of grapefruit. The physico-chemical analysis, TSS, pH, acidity, brix acid ratio, naringin, total sugars, reducing sugars,

ascorbic acid and percentage juice yield of grapefruit was done.

Preparation of low alcoholic naturally carbonated fermented debittered grapefruit beverage: The raw grapefruit juice was diluted with water (boiled and cooled, 40% juice) and sugar solution was added to achieve the required TSS 14.0°B, followed by the culture @ 0.5% (v/v). It was incubated at 20± 5°C for 36 hrs. The beverage was refrigerated for 24 hrs, siphoned, bottled and stored at refrigerated temperature.

Quality evaluation and shelf life determination of grapefruit beverage: Shelf life of low alcoholic naturally carbonated fermented debittered grapefruit beverage, stored at refrigerated temperature (4°C) was studied and evaluated fortnightly for physico-chemical, microbiological and sensory qualities. The data was replicated thrice.

Physicochemical, microbiological and organoleptic 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). Total acidity expressed as citric acid was estimated following the procedure of AOAC (1999). Brix acid ratio was calculated by dividing TSS value by total acidity of the juice and carbonated beverage. Total sugars were estimated by phenol-sulphuric acid method of Dubois using glucose as standard (1956). 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 AOV (1996). Naringin was estimated by the method of Davis (1947). Carbon dioxide volume in beverage bottles was determined by piercing device (CO₂ tester). Total yeast count was enumerated on GYE agar by serial plate dilution method. The organoleptic evaluation of grapefruit beverages was done on the basis of appearance, color, aroma, bouquet, body, flavour, astringency and overall acceptability by a panel of five judges. Consumer acceptance for the products was evaluated on a nine point “Hedonic scale 2.”

Statistical analysis: The test of mean comparison according to critical difference (CD) was applied. Significance level was accepted at $p \leq 0.05$. The data of 3 replications was analyzed statistically by using GSTAT 04 and CPCS1 software developed by Maths, Statistics and Physics Department, PAU, Ludhiana.

RESULTS AND DISCUSSION

Physico-chemical characteristics grapefruit (*Citrus paradisi*): The acceptability and higher sensory score of beverages depends upon on physico-chemical properties including appearance, flavour, acidity and TSS. There may be changes in the physico-chemical characteristics and loss of some compounds that impart flavour and aroma to the beverages during pasteurization and storage. The physico-chemical composition of Grapefruit juice

Table 1: Physicochemical characteristics of Grapefruit juice.(*C. paradisi*).

Parameters	Value
TSS (°B)	7.7
Acidity (%)	0.35
pH	4.6
Brix-acid ratio	22
Naringin (ppm)	752
Total sugars (%)	14.5
Reducing sugars (%)	3.5
Ascorbic acid (mg/100ml)	33.5
juice yield (%)	39.33%

*Five fruits of uniform size were taken for physico- chemical analysis of grapefruit

evaluated on the basis of chemical analysis is: TSS 7.7°B, titrable acidity 0.35 per cent, pH 4.6, Brix acid ratio 22.0, total sugars 14.5 per cent, reducing sugars 3.5 per cent, ascorbic acid 33.5 mg/100g, naringin 752 ppm, and percentage juice yield from Grapefruit was 39.33% (Table 1).

Shelf life studies of grapefruit (*C. paradisi*) beverage: Shelf life of low alcoholic naturally carbonated fermented debittered Grapefruit beverage stored at refrigerated temperature (4°C) was studied for a period of 90 days and evaluated fortnightly for physico-chemical, microbiological and organoleptic qualities.

Effect of storage time on physico-chemical and microbiological properties of beverage: The results of Grapefruit beverage (Table 2) showed significant decrease in brix from 14.0°B to 12.0°B and brix acid ratio from 53.85 to 22.22. Similar results have been reported in lime juice (Sarolia and Mukherjee, 2002), kinnow sera, cane and kinnow cane juice (Khandelwal et al., 2006) and ready to serve mandarin (*Citrus reticulata*) diet drink (Ahmed et al., 2008).

The pH of the beverage also decreased from 4.7 to 4.2 with subsequent increase in the acidity from 0.26 to 0.54 per cent during storage period of 90 days. This decrease in pH and increase in acidity was attributed to formation of acidic compounds by degradation of reducing sugars (Akhtar et al., 2010). The increase in acidity may be ascribed to rise in the concentration of weakly ionized acid and their salts during storage (Akhtar et al., 2010).

The percentage decrease in total sugars with storage period of 90 days was 22.67 percent as it decreased from 11.60 to 8.97 percent at the end of 90 days while the percent decrease for the reducing sugars was 41.92 percent as it decreased gradually from 3.34 to 1.94 percent after 90 days. The sugars in citrus are mainly glucose, laevulose and sucrose. Similar results were also observed by Jairath (2012) for preparation of amla beverage.

The percentage decrease in the ascorbic acid was found to be 15.75 percent as it decreased from 21.90 to 18.45 percent during storage period of 90 days, this may be due to photo-oxidation of ascorbic acid as it is a strong reducing

Table 2: Effect of storage time on microbiological and physico-chemical attributes of low alcoholic naturally carbonated fermented grapefruit (*C. paradisi*) beverage.

Parameters	Days							CD (5%)
	Fresh	15	30	45	60	75	90	
TSS (°B)	14.00	14.00	13.10	13.50	12.20	12.50	12.00	NS
Acidity (%)	0.26	0.27	0.29	0.33	0.39	0.48	0.54	0.11
pH	4.70	4.70	4.60	4.60	4.60	4.20	4.20	NS
Brix-acid ratio	53.85	51.85	45.17	40.91	31.28	26.04	22.22	10.70
Total sugars (%)	11.60	11.20	10.30	9.56	9.18	9.00	8.97	NS
Reducing sugars (%)	3.34	3.11	3.19	2.19	2.43	2.16	1.94	0.714
Ascorbic acid (mg/100ml)	21.90	21.70	20.30	20.10	19.20	18.90	18.45	NS
Naringin (ppm)	643.20	615.20	439.90	412.80	384.00	370.60	365.20	125.80
Alcohol (% v/v)	0.00	0.14	0.38	0.45	0.54	0.74	0.76	0.14
CO ₂ (bar)	0.00	0.50	0.65	0.75	1.25	1.24	1.35	0.25
Total yeast count (Log no. of cells)	5.83	5.86	6.74	6.96	8.34	8.41	8.54	0.80

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

Table 3: Effect of storage on sensory attributes of low alcoholic naturally carbonated fermented debittered grapefruit juice (*C. paradisi*) beverage.

Sensory attributes	Fresh	15d	30d	45d	60d	75d	90d	CD (5%)
Appearance	7.50	7.50	7.50	7.50	7.30	7.30	7.30	NS
Color	7.50	7.50	7.50	7.40	6.95	6.86	6.86	NS
Aroma	8.17	8.11	7.93	7.93	7.87	7.87	7.64	NS
Bouquet	7.50	7.50	7.30	7.40	7.50	7.50	7.50	NS
Body	7.00	7.25	7.50	7.75	7.50	7.00	7.00	NS
Flavour	7.85	7.90	7.85	7.80	7.80	7.80	7.85	NS
Astringency	7.45	7.50	7.67	7.73	7.78	7.78	7.90	NS
Overall acceptability	7.20	7.50	7.45	7.20	7.00	7.45	8.00	NS

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

agent, readily losing hydrogen atom to become dehydroascorbic acid and finally hydrolysed losing its vitamin activity to ketogluconic acid. The decrease of ascorbic acid (vitamin C) in beverage during storage results from oxidation of ascorbic acid by ascorbic acid oxidase due to a combined effect of oxygen and light (Jairath *et al.*, 2012).

The decrease in naringin was 43.22 percent as it decreased gradually from 643.20 to 365.20 ppm after 90 days. This may be due to the production of enzyme naringinase by *C. lusitaniae*, (Sahota *et al.*, 2013) The enzyme naringinase is composed of α -L-rhamnosidase (EC 3.2.1.40) and β -D-glucosidase (EC 3.2.1.21) (Singh *et al.*, 2015).

The alcohol production started after 15 days (0.14% v/v) and reached up to 0.76 % v/v after 90 days. Sensitivity of yeast cells to ethanol marginally increased on decreasing the pH from 6.0-3.0. During fermentation process, CO₂, alcohol and glycerol produced is proportional to the amount of sugar fermented. The yeast strain produced large amount of glycerol at the expense of ethanol represent an advantageous alternative for the development of beverages with low ethanol content versus physical process which alter the organoleptic properties of the final product (Jairath *et al.*, 2012). The CO₂ pressure 0.5 bar started building after 15 days and increased to 1.25 after 60 days and after 90 days reached up to 1.35 bar. Viable cell count increased from 5.83 (Log no. of cells) to 8.54 (Log no. of cells) at the end of 90 days. The shelf life is standardized on the basis of alcohol production < 1.0%. Thus, shelf life of beverage has been found to be 90 days. Thus this technology will not only help in reducing the wastage of the citrus fruits, by removing the bitterness, but will also help in overcoming the problem of storage of beverages for a long period of time.

Effect of storage time on organoleptic properties of beverage: The beverage has been rated as liked very much during sensory evaluation due to its effervescence due to production of CO₂ by *C. lusitaniae*, improved tangy taste, color, appearance, texture, and aroma as well as enriched with the nutrients and typical flavour of the fruits. CO₂ is an antimicrobial agent itself acting as a critical solvent and weak organic acid (upon dissolution) penetrating plasma membrane and acidifying cell anterior of microorganism, thus responsible for enhancing shelf life of the beverage. The effectiveness of carbonation is the main factor in determining the quality and consumer acceptance of final beverage (Singh, 2015). 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. Grapefruit beverages were analyzed by Panellists for sensory scores till period of three months (Table 3). No significant difference in appearance, color, aroma, bouquet, body, flavour, astringency,

gency and overall acceptability of grapefruit was recorded.

Cook et al., 2003 found that somatosensory tactile stimuli interact with taste and aroma and hence modulating their perception. The beverage had pleasing effervescent sparkling appearance. The storage temperature can greatly affect the way beverage tastes and smells. Lower temperatures will emphasize acidity and tannins while muting the aromatics. Higher temperatures will minimize acidity and tannins while increasing the aromatics (skarletbeveragecompany.com). The presence of yeast in beverage gave a desirable freshness to the fermented beverage due to production of carbon dioxide (Pandove et al., 2016).

Conclusion

In the low alcoholic naturally carbonated fermented debittered Grapefruit beverage, the percentage decrease in naringin was 43.22 percent as it decreased gradually from 643.20 to 365.20 ppm after 90 days. This may be due to the production of enzyme naringinase by *C. lusitaniae*. The alcohol production started after 15 days (0.14% v/v) and reached up to 0.76 % v/v after 90 days. During fermentation process, CO₂ alcohol and glycerol produced is proportional to the amount of sugar fermented. Naturally produced CO₂ by *C. lusitaniae* during fermentation adds effervescence, sparkle, tangy taste to the beverage in addition to its antimicrobial properties. Thus, bio-enzymatic debittering by *C. lusitaniae* may become the new direction of citrus juice processing in the future, due to its economical viability with strong ability to remove the bitter taste from citrus juice beverage.

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