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Bioprospecting potential of novel yeast *Clavispora lusitaniae* for production of carbonated low alcoholic beverage from lemon var. *Citrus latifolia*

Richa Arora

Department of Microbiology, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara-144411, Punjab, India

ABSTRACT

The pure yeast isolate 84 from whey beverage with potential to ferment lemon juice was characterized as *Clavispora lusitaniae*. The lemon var. *Citrus latifolia* beverage was prepared with low alcohol content and high carbonation using using *Clavispora lusitaniae* @ 0.5% in lemon juice and fermenting under standardized Brix 16 ^{o}B, acidity 0.3-0.4 percent, and temperature 30±5 ^{o}C. The quality parameters of *Citrus latifolia* beverage were 10 percent juice, pH 2.6, TSS 14.4^{o}B, acidity 0.40 percent, ascorbic acid 1.80 mg/100ml, alcohol 0.767 (%v/v), and CO_2 (Bar) 1.20 and Total Plate Count $3.3x10^7$cfu/ml and ranked between "liked very much to moderate" and had a shelf life of three months. The volatile components detected by HPLC showed the presence of ethanol (0.85 %). The major elements (K, Mg, Na), essential elements (Ca, P, Fe) and trace

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elements (Zn, Cu, Mn) were retained in all the beverages during storage period of three months.

Keywords: Low-alcoholic naturally carbonated, *Clavispora lusitaniae*, fermentation, lemon, organoleptic evaluation, sensory attributes, clarification, shelf-life

INTRODUCTION

India holds second position in the production of fruits with annual production of 50 Mt of fruits per year where 25 % is perished owing to poor storage and handling practices [1]. The perishable fruits and vegetables become vanished owing to degradation due to microbes, physiological spoilage, loss of moisture, physical damage during harvesting, transporting and packaging [2]. Several fruits with astringent taste including pineapple, amla, lemon etc. are non-palatable owing to very high acidic content. However, such fruits possess huge potential for processing as they have exceptional therapeutic and nutritional values.

Lemon [(Citrus limon (L) Burman] belonging to family *Rutaceae* is a cultivated hybrid derived from wild species such as citron and mandarin. Gopalan et al [3] reported that one hundred gram edible portion of lemon fruit contains ascorbic acid (39 mg), minerals (P- 10 mg, Ca-70 mg and K-270 mg), carbohydrates (11.1g) and 57 Kcal of energy. Lemon juice is reported to have antiseptic, antiscorbutic, antioxidative, anti-inflammatory effects, dietic and medicinal values and reduces cholesterol. High levels of minerals, phenolics, flavonoids and ascorbic acid account

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for good nutritive value of lemon [4]. Ascorbic acid and tannic acids obtained from juice help to provide daily losses of antioxidants. Owing to this high therapeutic and nutritional attribute, there is magnanimous opportunity for lemon juice processing into a value added product while retaining nutritional, organoleptic and sensory profile with enhanced shelf life. Fermentation debitters the beverage, retains nutrients and additionally CO_2 produced adds sparkle and tangy taste to beverage. The present study was carried out with an objective of exploring new technology for profitable conversion of lemon to fermented beverage with low alcohol content and more carbonation.

MATERIALS AND METHODS

Procurement of yeast isolate

A yeast isolate 84 with the capacity to produce 1.5 bar CO_2 and 1 per cent ethanol was provided by Punjab Agricultural University, Ludhiana-141 004, Punjab, India.

Characterization of yeast isolate

The biochemical characterization was done by examining fermentation of sugars and assimilation of carbon compounds [5]. The yeast isolate was identified by sequencing the D1/D2 domain of 26S rRNA and Internal Transcribed Spacer (ITS) region.

Procurement of lemon var. *Citrus latifolia*

Lemon var. Tahiti lime (*Citrus latifolia*) was procured from Punjab Agricultural University, Ludhiana-141 004, Punjab, India.

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Extraction of juice

After manual selection and washing of healthy lemons with water, it was cut in 2 parts. Juice was extracted using lemon squeezer, followed by pasteurization at 82 ^{0}C for 15 s.

Sugar solution preparation

500 g sucrose was obtained from local shop in Ludhiana and boiled in 1 L water for 10 minutes. The solution was cooled to 30 ^{o}C and stored aseptically.

Inoculum development

Fresh yeast was inoculated in diluted lemon juice (10 %) with inoculum volume of 0.5 % v/v followed by incubation at 20 ^{o}C for 24 h under aerobic conditions till 10^6-10^7 cells/ml were obtained.

Physicochemical analysis of lemon juice

The fresh lemon juice was analysed for total soluble solids, acidity, pH, sugars (total and reducing), brix acid ratio, juice yield and ascorbic acid content.

Fermentation of lemon juice

10 % pasteurised lemon juice diluted with boiled water and sugar solution with 16 ^{o}B was inoculated with yeast 84 with 0.5 % v/v inoculum

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followed by incubated at 20 ºC for 36 h followed by refrigeration for 24 h. Finally, the beverage was siphoned off and stored in sterilized glass bottles at 4 ºC.

Evaluation of quality parameters of lemon beverage for determination of its shelf life

Samples were taken from fermented beverage every fortnightly to determine its shelf life. The samples (in thrice) were analysed w.r.t. physicochemical, organoleptic and microbiological profiles as follows:

Physicochemical analysis of fermented beverage

Total soluble solids were analysed by using a refractometer (UNICO). pH of raw juice and fermented beverage was analysed with a digital pH meter (ECI, Hyderabad). Citric acid content was estimated by [6]. TSS was divided with total acidity to obtain brix-acid ratio. The analysis of several parameters was done by following standard procedures, viz. reducing sugars [7], total sugars [8], ascorbic acid [9]. Zahm and Nagel piercing device was used to estimate the CO2 content. Various volatile compounds viz. methanol, acetaldehyde, butanol, isopropanol, propanol, ethanol, ethyl acetate were evaluated by using HPLC (TR Wax column, GC Headspace injection and FID detector).

Organoleptic analysis of fermented beverage

Several organoleptic characteristics of the beverage viz. flavor, color, astringency, bouquet, appearance, body, aroma, overall acceptability were evaluated by a committee. The committee provided the scores on the basis of hedonic scale [10].

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Microbiological analysis of fermented beverage

The beverage samples were enumerated on GYE (Glucose, yeast extract) agar for yeast count by spread plate technique.

Mineral analysis

Mineral analysis (Al, B, Ba, Be, Bi, Ca, Zn, S, P, Ni, Na, Mo, Mn, Mg, K, Fe, Cu and Co) of beverage, prior to and after fermentation of beverage was carried out in Department of Soils using ICAP-AES, PAU, Ludhiana.

Statistical Analysis

The triplicated data was analyzed for mean and standard deviation by using CPCS1 and GSTATO4 softwares.

RESULT AND DISCUSSION

Characterisation of the screened yeast isolate

The biochemical characterization of the yeast 84 for carbon assimilation and fermentation is shown in Table 1. Further, with the molecular level analysis, the isolate 84 was characterized as *Clavispora lusitaniae*. The phylogenetic tree (Fig. 1) showed that *Clavispora lusitaniae* has close homology with *Clavispora lusitaniae* strain XJURML-1. Further, the growth profile of the culture is shown in Fig. 2. Biomass did not increase significantly after 48 hours up to 72 hours (0.53-0.8g). The maximum specific growth rate constant and generation time (h) with respect to cell dry weight (g/100ml) was calculated as 0.075 and 9.24 respectively. These results are in accordance with Giovanelli et al [11] who reported a

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maximum specific growth rate of 0.13 for the yeast *Saccharomyces cerevisiae* strain no. 7013 during aerobic fermentation studies in terms of cell count.

Physico-chemical analysis of raw *Citrus latifolia* juice

Several physico-chemical parameters of raw juice of *Citrus latifolia* (Tahiti lime) were % titrable acidity 5.68, TSS 9.0, pH 2.4, Brix acid ratio 1.58, ascorbic acid 27.67 mg/100ml, juice yield 43.33 %, reducing sugars 3.38 % and total sugars 4.06 % (Table 2). The juice formulations in beverages for all the citrus varieties were selected to attain 0.3-0.4 % acidity which is the key parameter in evaluation of sensory quality of the beverage. Titrable acidity determines the final organoleptic attributes like bouquet, aroma, color, appearance, body, astringency, flavor and overall acceptability of fermented fruit juice.

Evaluation of quality parameters of lemon beverage for determination of its shelf life

Physicochemical analysis of fermented beverage

The total soluble solids of fermented *Citrus latifolia* beverage showed significant reduction in brix from 16.0 to 14.4 ^{0}B, whereas brix acid ratio reduced from 50.54 to 36.80 (Table 3, Fig. 3). The pH of the beverage reduced from 2.7 to 2.6 and acidity significantly shooted up from 0.32 to 0.40 at the end of 90 days. pH strongly influences fermentation properties such as color, oxidation, chemical and biological stability. Acidic pH improves the stability by inhibiting bacteria and causes sugar fermentation to progress more evenly. The increase in acidity and decrease in pH

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was significant and ascribed to the produce CO_2 that forms weak acid in water. Similar studies have been reported by Ogiehor et al [12] which states decrease in pH from 5.10 to 2.90 while upsurge in acidity from 0.021 to 0.060 of zobo beverage produced from *Hibiscus sabdarifa* for 21 days of storage.

14.16 % reduction in total sugars was observed as it decreased from 15.32 per cent to 14.86 per cent after 30 days and 13.15 per cent after 90 days. The percentage decrease in reducing sugars is 27.65 per cent reducing sugars as the sugars reduced from 8.39 per cent to 7.88 per cent after 30 days and 6.07 per cent after 90 days respectively. However, there is significant reduction in sugar level with the progress of the fermentation with increasing number of days ($p < 0.05$). The production of ethanol initiated at the end of 15 days (0.023 % v/v) and it shooted to 0.767 % v/v at the end of 90 days. With reduction in pH from 6 to 3, the yeast cells became sensitive to ethanol. With gradual extent of fermentation, CO_2, alcohol and glycerol produced the sugar content reduced which was found to be proportional to formation of alcohol, glycerol and CO_2. The yeast *Clavispora lusitanae* produced high amounts of glycerol instead of ethanol which benefitted in the production of beverage with less ethanol content. Similar results have been reported by Eglinton et al [13], where *Saccharomyces cerevisiae* produced glycerol in wine as the main fermentation product that contributed to the sensory profile.

Ascorbic acid content of lemon var. *Citrus latifolia* were 27.67 mg/100 ml respectively. In beverage it reduced from 13.00 to 6.65 mg/100ml after 30 days which decreased to 2.65 mg/100ml after 60 days and 1.70 mg/100ml after 90 days. This could be due to the

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photooxidation of ascorbic acid where hydrogen atom is removed and dehydroascorbic acid is hydrolysed to diketoglulonic acid [14]. Loss of ascorbic acid can be compensated by the use of colored bottles for storage of the beverages to prevent photooxidation and by creating anaerobic conditions under high carbon-dioxide pressure. Obire et al [15] reported the results of fermented waste fruit juice which reveals that after 22 days of fermentation there is reduction in ascorbic acid from 35.6 to 25.0, 163 to 25.0 and 16.7 to 5.2 mg /100ml in banana juice, pawpaw juice and pineapple juice, respectively.

The CO_2 pressure (0.53 bar) started building up after one month, where it rised to 0.93 bar after 2 months followed by 1.20 bar after 3 months. All volatile components including methanol, acetaldehyde, butanol, isopropanol, propanol, ethyl acetate were found to be absent except ethanol which was 0.85 %.

Organoleptic analysis of fermented beverage

The data on sensory scores (Table 4) of various organoleptic characteristics shows that appearance, aroma, body, flavour, astringency and overall acceptability of beverage did not differ significantly and ranked between scores liked very much to moderately since carbon dioxide augments the organoleptic attributes owing to high acidity, sparkling effect and fizziness. The body of the beverage is mainly determined by the primary fermentation products viz. ethanol and glycerol. Lower fermentation temperature results in fresher and fruitier character of the beverage and carbonation improves the color, body, aroma, taste and overall acceptability of beverage.

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Flavour is mainly determined by amount of volatile released and taste imparting compounds of non-volatiles [16]. The mean sensory scores of color decreased from 7.6 upto 6.8 till at the end of storage period of 90 days (Table 4). Roth et al [17] reported that change in color from green to yellow during ripening had an impact on sweetness ratings of beverages. The mean sensory scores for bouquet significantly shooted up from 7.2 to 8.2 after 30 days and astringency increased from 8.0 to 8.4 with increasing number of days. Apart from the individual effect, each organoleptic parameter also influences the perception of other parameters also. A somatosensory tactile stimulus interacts with taste and aroma to modulate the perception [18]. Both the primary and secondary products of yeast metabolism organic acids, higher alcohols, glycerol, aldehydes, ethanol, ketones, esters etc. contribute to the bouquet of beverage. Dickinson [19] reported that, aromatic alcohol 2- phenylethanol contributes to sweet aroma of beer.

Microbiological analysis of fermented beverage

The viable cell count rised from 2.7 x 10^6 to 3.3 x 10^7 cfu/ml. However, it did not increased significantly during storage. This may be due to dual effect of high inoculum concentration, high CO_2 pressure, anaerobic conditions, and low temperature during storage.

Effect of fermentation on minerals

In *Citrus latifolia* beverage, K level significantly decreased from 86.3850 mg/l to 80.9300 mg/l while Cu, P and S increased significantly from 0.0045 mg/l, 5.9350 mg/l and 34.8550 mg/l to 0.0985 mg/l, 9.0000 mg/l and 36.7050 mg/l respectively ($p<0.05$) as evident from

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Table 5. Cu in cooperation with Zn^{2+} ions acts in the structure of Cu, Zn-superoxide dismutase, which is responsible for the detoxification of yeast cells. Joshi and Sandhu [20] reported that with increase in ethanol content there is considerable decrease in macroelements, titrable acidity, tannins, and colour units while viscosity, total esters and micro-elements increased.

Conclusions

The yeast isolate 84 was characterized as *Clavispora lusitaniae*. The final *Citrus latifolia* beverage with 10 % juice has pH 2.6, TSS 14.4^0B, per cent acidity 0.40, ascorbic acid 1.80 mg/100ml, alcohol % (v/v) 0.767 and CO_2 (Bar) 1.20. The average sensory scores for aroma significantly augmented from 7.8 to 8.2 after 30 days and astringency increased from 8.0 to 8.4 with increasing number of days, K level significantly decreased from 86.3850 mg/l to 80.9300 mg/l while Cu, P and S increased significantly from 0.0045 mg/l, 5.9350 mg/l and 34.8550 mg/l to 0.0985 mg/l, 9.0000 mg/l and 36.7050 mg/l respectively ($p<0.05$). The beverage was found to be stable for a period of three months.

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Table 1: Biochemical characterization of yeast isolate 84

Carbon assimilation		**Fermentation**	
Carbon source	**Yeast isolate 84**	**Carbon source**	**Yeast isolate 84**
D-Galactose	+	D-Glucose	+
L-Sorbose	+	D-Galactose	_
D-Glucosamine	+	Maltose	_
D-Ribose	+	Me alpha-D-glucoside	_
D-Xylose	+	Sucrose	_
L-Arabinose	+	Alpha, alpha-Trehalose	_
D-Arabinose	_	Melibiose	_
L-Rhamnose	_	Lactose	_
Sucrose	+	D-Xylose	+
Maltose	+	Cellobiose	_
Alpha, alpha-Trehalose	+	Melezitose	_
Me alpha-D-glucoside	+	Raffinose	+

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Cellobiose	_	Inulin	_
Salicin	_	Starch	_
Arbutin	_		
Melibiose	_		
Lactose	_		
Raffinose	V		
Melezitose	+		
Inulin	_		
Starch	_		
Glycerol	+		
Erythritol	_		
Ribitol	+		
Xylitol	_		
L-Arabinitol	_		
D-Glucitol	+		
D-Mannitol	+		
Galactitol	_		
myo-Inositol	_		
D-Glucono-1,5-lactone	+		
2-Keto-D-gluconate	+		
5-Keto-D-gluconate	V		
D-Gluconate	+		

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D-Glucuronate	V
DL-Lactate	+
Succinate	+
Citrate	+
Methanol	_
Ethanol	+

*V – variable growth

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Table 2: Physicochemical characteristics of raw lemon juice

Parameters	Raw lemon juice
pH	2.4
TSS ^{o}B	9.0
Acidity %	5.68
Brix-acid ratio	1.58
Total sugars %	4.06
Reducing sugars %	3.38
Ascorbic acid (mg/100ml)	27.67
Juice yield %	43.33

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Table 3: Effect of storage on physicochemical properties of Lemon beverage var. *Citrus latifolia*

Parameters	Fresh	15d	30d	45d	60d	75d	90d	CD (5%)
pH	2.7	2.8	2.7	2.7	2.6	2.6	2.6	0.127
TSS ^{o}B	*16.0	16.0	15.6	15.5	15.3	14.9	14.4	0.132
Acidity %	0.32	0.32	0.33	0.34	0.37	0.39	0.40	0.018
Brix-acid ratio	50.54	49.02	47.16	46.01	41.01	38.54	36.80	2.373
Total sugars %	15.32	15.31	14.86	14.51	14.10	13.77	13.15	0.043
Reducing sugars %	8.39	8.24	7.88	7.62	7.14	6.54	6.07	0.067
Alcohol (%,v/v)	-	0.023	0.113	0.263	0.473	0.703	0.767	0.007
Ascorbic acid (mg/100ml)	13.00	10.10	6.65	5.57	2.65	2.00	1.70	0.323
CO_2 (Bar)	-	-	0.53	0.60	0.93	1.20	1.20	0.085
Total Plate count (yeast) (cfu/ml)	-	$2.7x10^{6}$	$3.8x10^{6}$	$4.3x10^{6}$	$1.9x10^{7}$	$2.4x10^{7}$	$3.3x10^{7}$	-

*% Juice in beverage - 10%

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Storage temp- 4 ± 2^0C
**Hydrolysis of sucrose
*****Multiple Regression Equation**

X1= - 2.19111 – 0.0378543 X2 + 8.80837 X3
[X1= per cent Alcohol, X2= ^{o} Brix, X3= per cent Acidity]

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Table 4: Effect of storage on sensory attributes of lemon beverage var. *Citrus latifolia*

Sensory attributes	Fresh	15d	30d	45d	60d	75d	90d	CD (5%)
Appearance	7.4	7.4	7.4	7.4	7.4	7.4	7.4	NS
Color	7.6	7.6	7.6	7.6	6.8	6.8	6.8	0.657
Aroma	7.8	8.2	8.2	7.8	7.8	7.8	7.8	NS
Bouquet	7.2	7.2	8.2	7.8	7.2	7.2	7.2	0.580
Body	7.0	7.07	7.28	7.07	7.3	7.0	7.0	NS
Flavor	8.2	8.2	8.2	7.8	7.6	7.4	7.4	NS
Astringency	8.0	8.0	7.8	7.8	8.2	8.4	8.4	NS
Overall acceptability	7.6	8.0	8.0	7.6	7.4	8.0	7.4	NS

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*Mean value of five replicates

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Table 5: Mineral analysis of Lemon beverage var. *Citrus latifolia*

Mineral	Lemon beverage (Before fermentation) (mg/l)	Lemon beverage (After fermentation) (mg/l)	CD (5%)
Al 3092	1.3855	0.9675	0.004
B 2497	0.3215	0.2780	0.009
Ba 4554	0.6775	0.6645	0.003
Ca 3968	91.6500	90.4500	0.329
Co 2286	0.0005	0.0004	NS
Cr 2835	0.5275	0.5855	0.003
Cu 3247	0.0045	0.0985	0.003
Fe 2599	0.9245	0.9365	0.003
K 7664	86.3850	80.9300	0.301
Mg 2852	34.0850	33.3350	0.067

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Mn 2576	0.0850	0.0775	0.005
Na 5895	35.7300	36.4650	NS
Ni 2316	0.0125	0.0185	0.003
P 1774	5.9350	9.0000	0.431
Pb 2203	0.0355	0.0315	0.003
S 1820	34.8550	36.7050	0.150
Se 1960	0.0215	0.0285	0.003
Zn 2138	0.4750	0.5450	0.030

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PHYLOGENETIC TREE (Using Neighbor Joining Method):

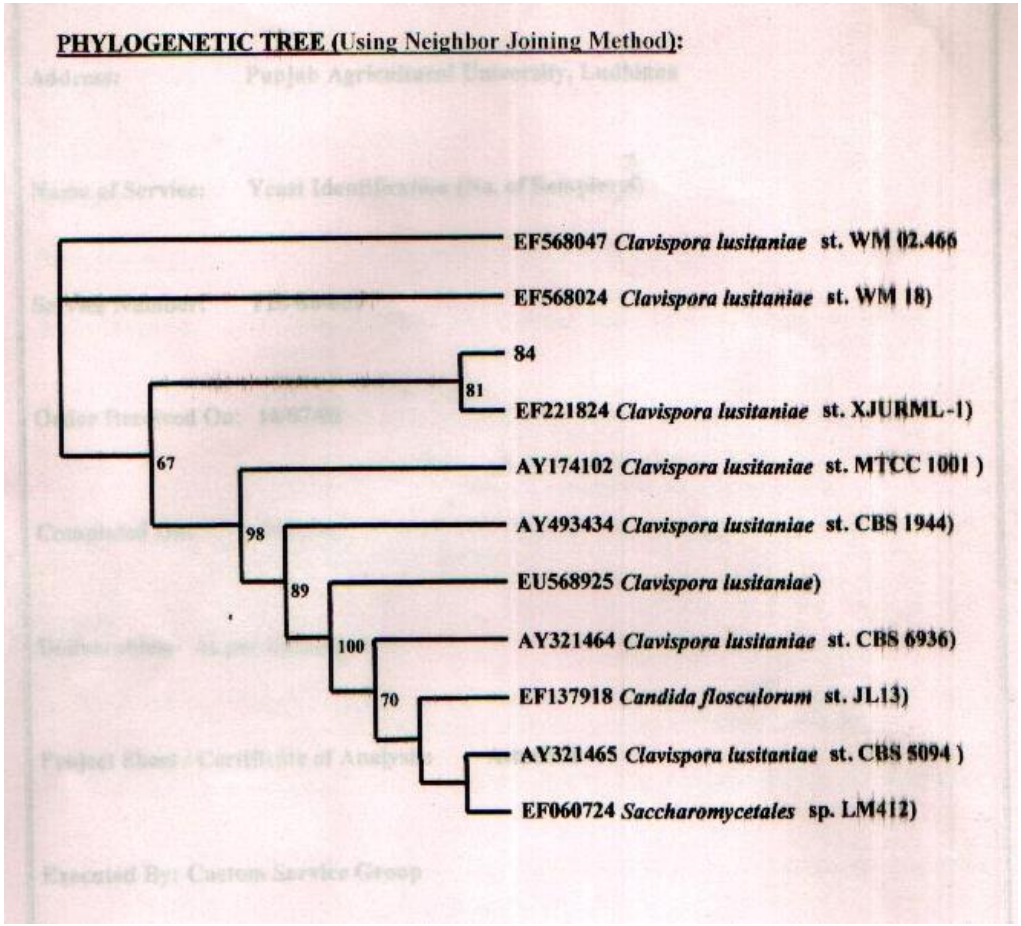

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Fig. 1 Phylogenetic tree for yeast isolate 84

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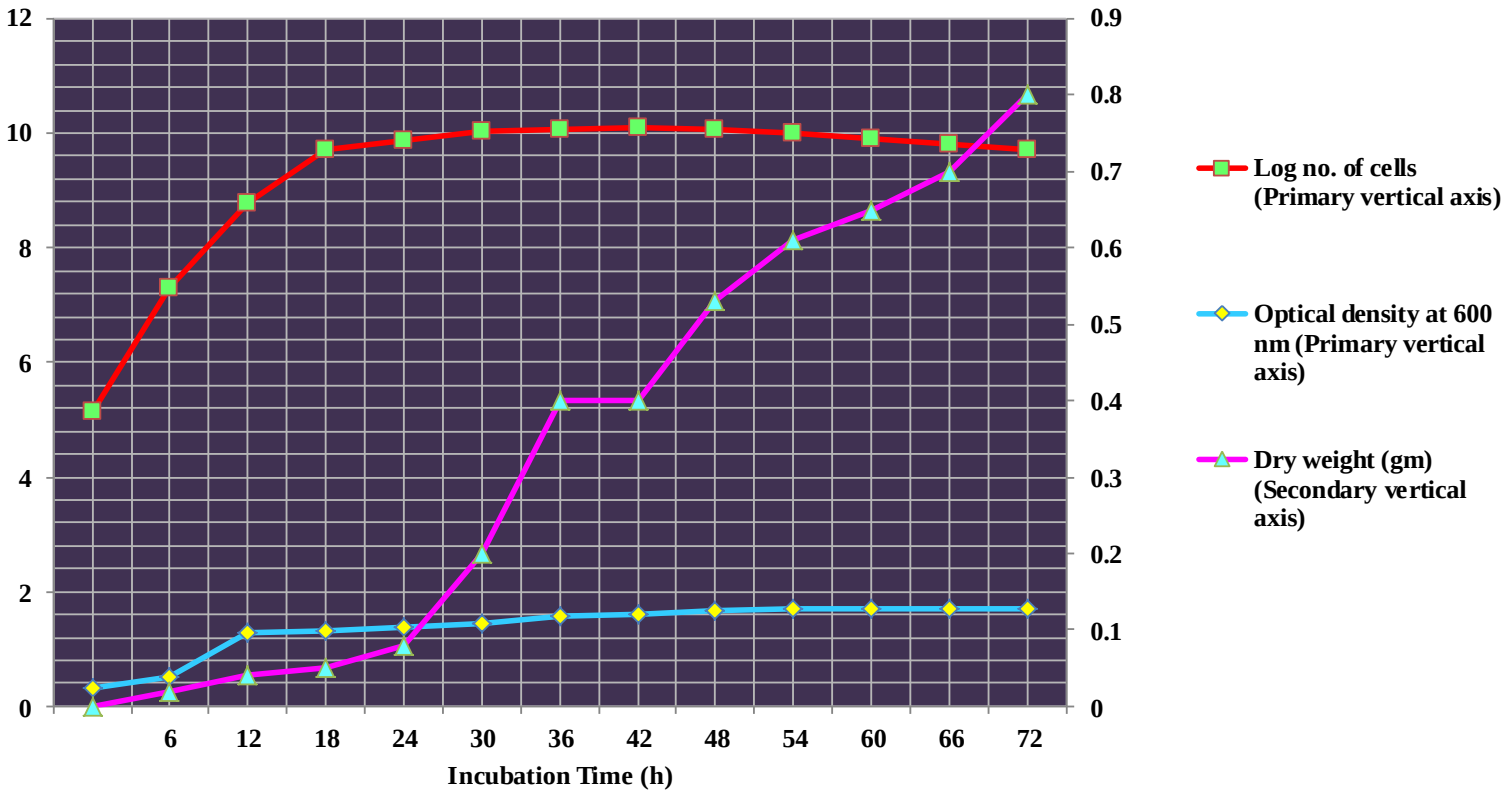

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Fig. 2 Growth profile of *Clavispora lusitaniae*

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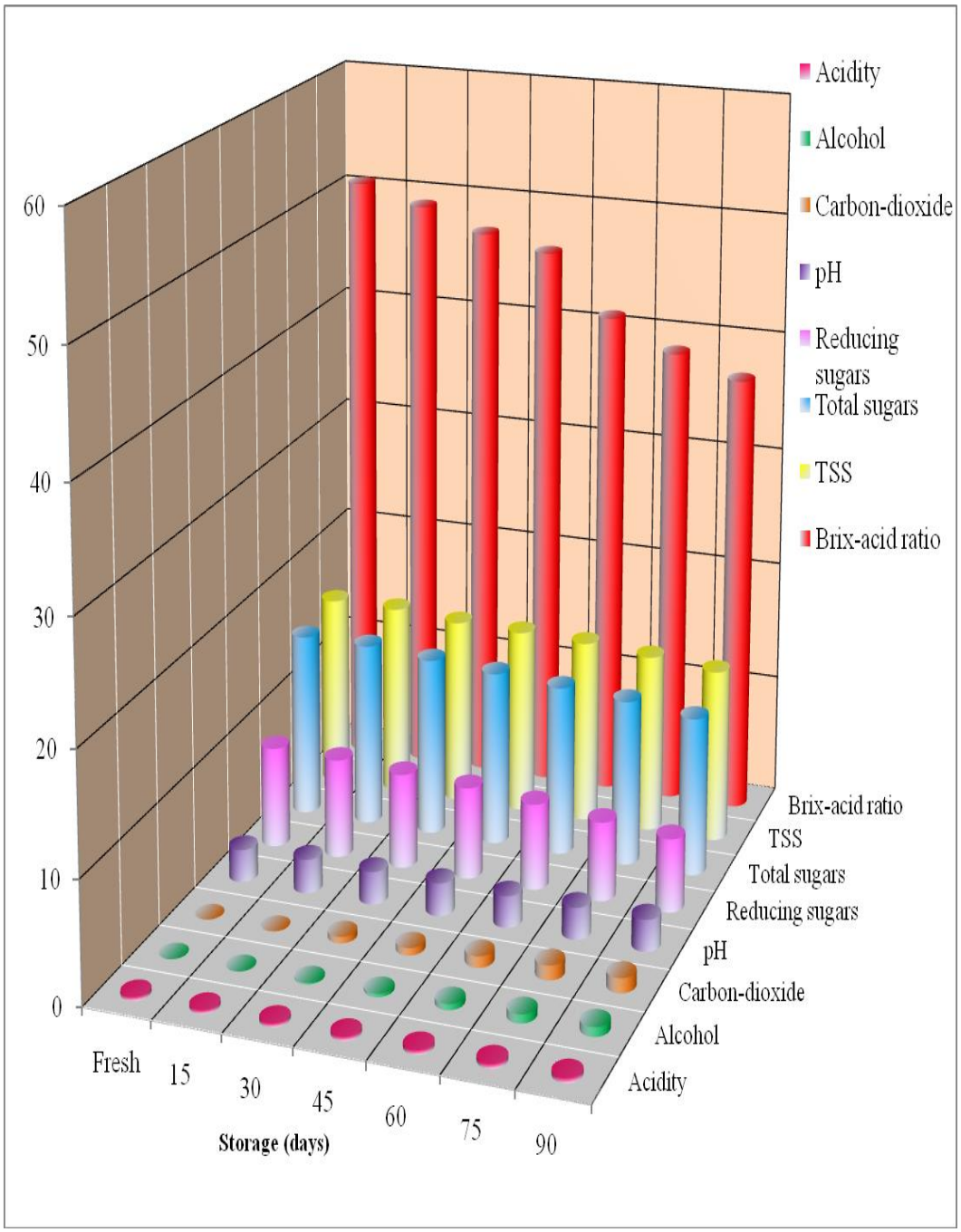

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Fig. 3 Effect of storage on physicochemical properties of Lemon beverage var. *Citrus latifolia*