MICROBIOLOGICAL PREPARATION OF NON-ALCOHOLIC NATURALLY CARBONATED BLENDED BEVERAGE FROM GUAVA AND LEMON

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in

MICROBIOLOGY
(Minor Subject: Biochemistry)

By

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CERTIFICATE I

This is to certify that the thesis/dissertation entitled, "**Microbiological preparation of non-alcoholic naturally carbonated fermented blended beverage from guava and lemon**" submitted for the degree of M.Sc, in the subject of **Microbiology** (Minor subject: **Biochemistry**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Davneet Kaur (L-2008-BS-180-M)** under my supervision and that no part of this thesis/dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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Dr. (Mrs.) Param Pal Sahota
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CERTIFICATE II

This is to certify that the thesis entitled, "**Microbiological preparation of non-alcoholic naturally carbonated blended beverage from guava and lemon**" submitted by **Davneet Kaur (L-2008-BS-180-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of M.Sc, in the subject of **Microbiology** (Minor subject: **Biochemistry**) has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination on the same.

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ABSTRACT

A reliable, controllable and reproducible technology for preparation of non-alcoholic naturally carbonated fermented beverage from guava var. *Allahabad Safeda*, *Lucknow-49*, *Punjab Pink* and its blends with lemon var. *Baramasi*, *Citrus latifolia* after fermentation with yeast *Clavispora lusitaniae* under optimized fermentation conditions has been developed. The specific growth rate (h^{-1}) and generation time (h) of yeast in guava and guava-lemon beverage (1:1) were 0.39, 1.77 and 0.35, 1.93 respectively. On the basis of physicochemical, microbiological and sensory evaluation the guava varieties *Allahabad Safeda* with 15 per cent juice, pH 3.9, TSS 11.7°B, acidity 0.53%, ascorbic acid 13.5 mg/100ml, alcohol (%v/v) 0.89, CO_2 1.53 bar and total plate count $33x10^9$ cfu/ml and *Punjab Pink* 15 per cent juice, pH 4.0, TSS 12.7°B, acidity 0.49%, ascorbic acid 9.7 mg/100ml, alcohol (%v/v) 0.81, CO_2 1.56 bar and total plate count $98x10^8$ cfu/ml, ranked highest for appearance (7.4), aroma (7.4), body (7.38), astringency (7.3) and overall acceptability (7.25) during storage period of 90 days under refrigerated conditions (4^0C). The best blend *Punjab Pink-Citrus latifolia* (1:1) 12.5 per cent juice, pH 2.7, TSS 12.14°B, acidity 0.51%, ascorbic acid 6.2 mg/100ml, alcohol (%v/v) 0.92, CO_2 1.50 bar and total plate count $27x10^9$ cfu/ml with shelf life of 90 days under refrigerated conditions (4^0C) was rated as liked very much. The optimized temperature and potassium metabisulphite concentration to inhibit the starter yeast culture was standardized as 55^oC and 700ppm, respectively, organoleptically heat treated beverage was superior than potassium metabisulphite treated beverage.

Keywords: Non-alcoholic naturally carbonated, *Clavispora lusitaniae*, fermentation, guava, lemon, organoleptic evaluation, blended, sensory attributes, shelf-life

Signature of Major Advisor **Signature of the Student**

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ਸਾਰੰਸ਼

ਸਰਵੋਤਮ ਖ਼ਮੀਰੀਕਰਨ ਹਾਲਾਤਾਂ ਅਧੀਨ ਅਮਰੂਦ ਰੂਪ-ਭੇਦ *ਅਲਾਹਾਬਾਦ ਸਫੈਦਾ*, *ਲਖਨਊ - 49*, *ਪੰਜਾਬ ਪਿੰਕ* ਅਤੇ ਇਸਦੇ ਨਿੰਬੂ ਰੂਪ-ਭੇਦਾਂ *ਬਾਰਾਮਾਸੀ*, *ਸਿਟਰਸ ਲੈਟੀਫੋਲੀਆ* ਨਾਲ ਖ਼ਮੀਰ *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੈਨੀ* ਨਾਲ ਖ਼ਮੀਰਣ ਬਾਅਦ ਇਕ ਵਿਸ਼ਵਾਸਯੋਗ, ਕੰਟਰੋਲ-ਯੋਗ ਅਤੇ ਪੁਨਰ-ਉਤਪਾਦਨ-ਯੋਗ ਤਕਨਾਲੌਜੀ ਵਿਕਸਤ ਕੀਤੀ ਗਈ। ਅਮਰੂਦ ਅਤੇ ਅਮਰੂਦ-ਨਿੰਬੂ ਪੇਅ/ਦ੍ਰਵ (1:1) ਵਿਚ ਵਿਸ਼ੇਸ਼ੀਕ੍ਰਿਤ ਵਿਕਾਸ ਦਰ (ਪ੍ਰਤੀ ਘੰਟਾ) ਅਤੇ ਜਣਨ ਸਮਾਂ (ਘੰਟਾ) ਕ੍ਰਮਵਾਰ 0.39, 1.77 ਅਤੇ 0.35, 1.93 ਸਨ। ਫਿਜ਼ੀਕੋਕੈਮੀਕਲੀ, ਸੂਖਮ-ਜੈਵ ਵਿਗਿਆਨਕ ਅਤੇ ਸੰਵੇਦਾਤਮਕ ਮੁਲਾਂਕਣ ਦੇ ਆਧਾਰ ਤੇ ਅਮਰੂਦ ਦੀਆਂ ਕਿਸਮਾਂ *ਅਲਾਹਾਬਾਦ ਸਫੈਦਾ*, ਜਿਸ ਦਾ 15 ਪ੍ਰਤੀਸ਼ਤ ਰਸ, ਪੀ ਐਚ 3.9, ਟੀ ਐਸ ਐਸ 11.7 ਡਿਗਰੀ ਬੀ, ਤੇਜ਼ਾਬੀਕਰਨ 0.53%, ਅਸਕੋਰਬਿਕ ਤੇਜ਼ਾਬ 13.5 ਮਿਲੀਗ੍ਰਾਮ/100 ਮਿਲੀਲੀਟਰ, ਅਲਕੋਹਲ (% ਵੀ/ਵੀ) 0.89, ਕਾਰਬਨ-ਡਾਇਕਸਾਈਡ 1.53 ਬਾਰ ਅਤੇ ਸਮੁੱਚੀ ਖਮੀਰ ਦੀ ਪਲੇਟ ਗਿਣਤੀ 33×10^9 ਸੀ ਐਫ ਯੂ/ਮਿਲੀਲੀਟਰ ਅਤੇ ਪੰਜਾਬ ਪਿੰਕ, ਜਿਸ ਦਾ 15 ਪ੍ਰਤੀਸ਼ਤ ਰਸ, ਪੀ ਐਚ 4.0, ਟੀ ਐਸ ਐਸ 12.7 ਡਿਗਰੀ ਬੀ, ਤੇਜ਼ਾਬੀਕਰਨ 0.49%, ਅਸਕੋਰਬਿਕ ਤੇਜ਼ਾਬ 9.7 ਮਿਲੀਗ੍ਰਾਮ/100 ਮਿਲੀਲੀਟਰ, ਅਲਕੋਹਲ (% ਵੀ/ਵੀ) 0.81, ਕਾਰਬਨ-ਡਾਇਕਸਾਈਡ 1.56 ਬਾਰ ਅਤੇ ਸਮੁੱਚੀ ਖਮੀਰ ਦੀ ਪਲੇਟ ਗਿਣਤੀ 98×10^8 ਸੀ ਐਫ ਯੂ/ਮਿਲੀਲੀਟਰ, ਜਿਸ ਨੂੰ ਦਿੱਖ ਲਈ ਸਭ ਤੋਂ ਉਤੇ ਰੈਂਕ (7.4) ਦਿੱਤਾ ਗਿਆ, ਖੁਸ਼ਬੋ (7.4), ਬੱਾਡੀ (7.38), ਤੀਬਰਤਾ (7.3) ਅਤੇ ਸਮੁੱਚੀ ਸਵੀਕ੍ਰਿਤੀ (7.25) ਹੈ, ਜੋ ਫਰਿੱਜ ਹਾਲਾਤਾਂ (4 ਡਿਗਰੀ ਸੈਂਟੀਗ੍ਰੇਡ) ਅਧੀਨ 90 ਦਿਨਾਂ ਦੇ ਸਟੋਰੇਜ਼ ਕਾਲ ਦੌਰਾਨ ਸੀ। ਸਭ ਤੋਂ ਵਧੀਆ ਮਿਸ਼ਰਣ *ਪੰਜਾਬ ਪਿੰਕ-ਸਿਟਰਸ ਲੈਟੀਫੋਲੀਆ* (1:1) 12.5 ਪ੍ਰਤੀਸ਼ਤ ਜੂਸ, ਪੀ ਐਚ 2.7, ਟੀ ਐਸ ਐਸ 12.14 ਡਿਗਰੀ ਬੀ, ਤੇਜ਼ਾਬੀਕਰਨ 0.51%, ਅਸਕੋਰਬਿਕ ਤੇਜ਼ਾਬ 6.2 ਮਿਲੀਗ੍ਰਾਮ/100 ਮਿਲੀਲੀਟਰ, ਐਲਕੋਹਲ (%ਵੀ/ਵੀ) 0.92, CO_2 1.50 ਬਾਰ ਅਤੇ ਸਮੁੱਚੀ ਖਮੀਰ ਦੀ ਪਲੇਟ ਗਿਣਤੀ $27x10^9$ ਸੀ ਐਫ ਯੂ ਪ੍ਰਤੀ ਮਿਲੀਲੀਟਰ, ਜਿਸਦੀ ਸੈਲਫ਼ ਜੀਵਨ-ਕਾਲ ਫਰਿੱਜ ਹਾਲਾਤਾਂ (4 ਡਿਗਰੀ ਸੈਂਟੀਗ੍ਰੇਡ) ਤੇ 90 ਦਿਨ ਸੀ, ਨੂੰ ਸਭ ਤੋਂ ਵੱਧ ਪਸੰਦੀਦਾ ਅੰਕਣ ਕੀਤਾ ਗਿਆ। ਆਰੰਭਿਕ ਖ਼ਮੀਰ ਕਲਚਰ ਨੂੰ ਰੋਕਣ ਲਈ ਸਰਵੋਤਮ ਤਾਪਮਾਨ ਅਤੇ ਪੋਟਾਸ਼ੀਅਮ ਮੈਟਾਬਾਈਸਲਫੇਟ ਸੰਘਣਤਾ ਨੂੰ 55 ਡਿਗਰੀ ਸੈਂਟੀਗ੍ਰੇਡ ਅਤੇ 700 ਪੀ ਪੀ ਐਮ ਤੇ ਕ੍ਰਮਵਾਰ ਮਿਆਰੀਕ੍ਰਿਤ ਕੀਤਾ ਗਿਆ। ਔਰਗੈਨੋਲੈਪਟਿਕ ਢੰਗ ਨਾਲ ਤਾਪ-ਪ੍ਰਤੀਪਾਦਿਤ ਪੇਅ/ਦ੍ਰਵ, ਪੋਟਾਸ਼ੀਅਮ ਮੈਟਾਬਾਈਸਲਫ਼ਾਈਟ ਪ੍ਰਤੀਪਾਦਿਤ ਪੇਅ ਤੋਂ ਉੱਤਮ ਦਰਜੇ ਦੀ ਸੀ।

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ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖ਼ਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖ਼ਰ**

CONTENTS

Chapter I

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 (John 2008). The annual horticultural production is estimated around 141 million tones, which is over 18 percent of Indian gross agricultural output. 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 (Kumar *et al* 2009). The post harvest loss of fresh fruits and vegetables are estimated to be 20- 30 %.

India is the largest producer of guava and sixth largest producer of citrus in the world. The area under cultivation for guava and citrus is 2.2 lac ha and 4.8 lac ha respectively with annual production of 17.8 lac MT (Anon 2005) and 43 lac MT. The fruit is available in plenty during the season of production which causes glut in market (Divya and Kumari 2009)

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 (Aniche 2003). The perishable nature of ripened fruits poses serious public health problem through contamination by moulds.

Consumers demand products which are minimally processed and free from contaminants, adulterants and harmful pathogenic microorganisms. The growing interest in new functional foods with special nutritional characteristics, minimally processing free from additives has led to the development of new beverages based on fruit juices. The proliferation of ready- to- drink beverages has caused the market to focus its interest on these products. 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 (Kaur *et al* 2009). 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.

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 non alcoholic naturally carbonated beverage with retention of all the nutrients of the fruit.

Compared to fruit juices the formulations of 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. In Punjab guava ranks second in cultivation after citrus and occupies an area of 8022 ha with an annual production of 160463 M. tones (Anon 2009) 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) (Kaur *et al* 2009) . 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) (Hui 2006). 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 (Jawaheer *et al* 2003). Vitamin C is needed for the formation and maintenance of intercellular substances or tissues, building resistance to infections and in the absorption of calcium and iron (Egbere *et al* 2009) while dietary fibre is mainly credited for regulating bowel movement and involved in lowering blood cholesterol and glucose adsorption(Thongsombat *et al* 2007).

Lemon (*Citrus limon* L.) belongs to family Rutaceae is cultivated hybrid deriving from wild species such as citron and mandarin. The area under lime and lemon cultivation is 634 ha with an annual production of 4774 M. Tones in Punjab (Anon 2009). It is a rich source of ascorbic acid (39 mg/100g) , carbohydrates (11.1g/ 100g) and minerals (K- 270 mg, Ca- 70 mg, P- 10 mg/100g) (Gopalan *et al* 2002). The nutritive value of this fruit lies in its high contents of acidity, ascorbic acids minerals, flavonoids and phenolics (Sharma 1996). Its nutrients, vitamins and flavonoids help to prevent unwanted damage to cell membranes and other structures of the body by neutralizing free radicals (Zvaigzne *et al* 2009). Antioxidants in lemon fruit possess antitumor activity. Ascorbic acid and tannic acids obtained from the juice help to provide daily losses of antioxidants.

The production of non 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.

In the present work the ability of the yeast species are exploited to achieve the following objectives:

1. Preparation of non-alcoholic naturally carbonated fermented beverage from guava (var. *Allahabad Safeda, Lucknow- 49, Punjab Pink*) using optimized fermentation conditions.
2. Blending of guava juice with lemon in different proportions with scaling upto 20 liters.
3. To study the microbiological, physicochemical and organoleptic qualities of beverages.

Chapter II

REVIEW OF LITERATURE

The work related to present study on microbiological preparation of non- alcoholic naturally carbonated blended beverage from guava and lemon has been reviewed under the following subheadings

2.1 Yeast characterization

Fonesca *et al* (2000) isolated an undescribed anamorphic yeast species of ascomycetous affinity, *Candida tartarivorans*, from dried wine lees in Portugal using a selective medium with L (+)-tartaric acid as the sole source of carbon and energy. The single isolate (IGC 4854) showed the following characteristics: sympodial holoblastic conidiogenesis, absence of asci with ascospores, a negative colour reaction with Diazonium Blue B (DBB), production of elaborate pseudomycelium, and ability to grow with inositol as sole source of carbon. Analysis of the physiological data pointed to a close relationship with other inositol-assimilating taxa, namely the genera *Arxula, Stephanoascus*, *Sympodiomyces, Zygoascus* and selected *Candida* species. The comparative analysis of the D1/D2 variable domain of the 26S rRNA gene of all available sequences for ascomycetous yeasts showed that strain IGC 4854 did not match with any other species in the database. The closest relative was *Candida auringiensis* Santa Maria but the two species differed in 24 nucleotide positions.

Lachance *et al* (2000) studied the ribosomal DNA of the cactophilic yeast species *Clavispora opuntiae* in order to clarify the global distribution of the yeast. Over 500 strains, including isolates from several new localities worldwide, were characterized by rDNA

restriction mapping. An unusual restriction pattern previously encountered only in one strain from Conception Island (Bahamas) was found in several Brazilian isolates. Sequences of the D1/D2 and D7/D8 divergent domains of the large subunit (LSU) and of the intergenic spacers (IGS) confirmed that these strains represent a genetically distinct variety of *Clavispora opuntiae.*

Escalante-Minakata *et al* (2008) identified eleven different micro-organisms by restriction and sequence analysis of the amplified region, between 18S and 28S rDNA and 16S rDNA genes in the mezcal fermentation from Agave salmiana. Three of them were the following yeast: *Clavispora lusitaniae, Pichia fermentans* and *Kluyveromyces marxianus*. The bacteria found were *Zymomonas mobilis* subsp. *mobilis* and *Zymomonas mobilis* subsp. *pomaceae*, *Weissella cibaria*, *Weissella paramesenteroides*, *Lactobacillus pontis, Lactobacillus kefiri, Lactobacillus plantarum* and *Lactobacillus farraginis.* The phylogenetic analysis of 16S rDNA and ITS sequences showed that microbial diversity present in mezcal is dominated by bacteria, mainly lactic acid bacteria species and *Zymomonas mobilis*. *Pichia fermentans* and *K. marxianus* could be micro-organisms with high potential for the production of some volatile compounds in mezcal.

Ten different versions of the D1/D2 divergent domain of the large-subunit ribosomal DNA were identified among interbreeding members of the yeast species *Clavispora lusitaniae* (Lachance *et al* 2003). One major polymorphism, located in a 90-bp structural motif of the D2 domain, exists in two versions that differ by 32 base substitutions. Three other polymorphisms consist of a two-base substitution, a two-base deletion, and a single-base deletion, respectively. The polymorphisms are independent of one another and of the two mating types, indicating that the strains studied belong to a single, sexually active Mendelian population.

A total of 194 bacterial isolates and 187 yeast isolates from the surfaces of four Irish farmhouse smear-ripened cheeses were identified at the midpoint of ripening using pulsed-field gel electrophoresis (PFGE), repetitive sequence-based PCR, and 16S rRNA gene sequencing for identifying and typing the bacteria and Fourier transform infrared spectroscopy and mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) analysis for identifying and typing the yeast. The yeast microflora was very uniform, and *Debaryomyces hansenii* was the dominant species in the four cheeses. *Yarrowia lipolytica* was also isolated in low numbers from one cheese (Jérôme Mounier *et al* 2005).

The yeasts with similar physiological properties has been examined using recently published phylogenetic analyses of 26S domain D1/D2 rDNA nucleotide sequences from all currently recognized ascomycetous yeasts. Unique metabolic pathways were examined, a relationship between physiology and rDNA phylogeny was evident. Yeasts subject to the petite mutation, resulting in respiratory deficiency, belong to three different clades, viz. a

Saccharomyces clade delimited by *S. cerevisiae* and *S. rosinii*, the *Dekkera/Brettanomyces* clade, and some *Schizosaccharomyces* species (*'Archiascomycete'* clade). However, petite mutants were also found in *Zygosaccharomyces fermentati* and some other more distantly related species. Yeasts able to assimilate n-hexadecane, uric acid or amines as sole carbon source are broadly distributed over the ascomycetous phylogenetic tree. However, species that assimilate adenine as sole carbon source are closely related. Most of these species also assimilated glycine, uric acid, n-hexadecane, putrescine and branched-chain aliphatic compounds such as isobutanol, leucine and isoleucine. Among the *Saccharomycetales*, species utilizing all or the great majority of these eight compounds are in the *Stephanoascus/Arxula/Blastobotrys* clade. *Candida blankii*, which is distantly related to this clade, proved to be an exception and assimilated six of eight of these compounds (Middelhoven and Kurtzman 2003).

2.2 Factors affecting Fermentation

Fermentation is defined as any energy generating process in which organic compounds act as both electron donor and electron acceptor, that is, an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptor. Fermentation technology is any microbial process or technology for the production of product having commercial value by mass culture of microorganisms.

The fermentation of fruit juices in a complex biochemical process, during which yeasts utilize sugars and other constituents of the fruit juices as substrates for their growth converting them to ethanol, carbon dioxide and other metabolic end products that contribute to the chemical composition and sensory quality of the fermented product. There are number of factors which will affect yeast fermentation performance like yeast strain employed, fermentation temperature media composition, mode of substrate feeding, osmotic pressure ethanol concentration, membrane composition etc (D'Amore 1992).

2.2.1 Effect of temperature

Temperature affects all types of growth metabolism, survival of fermenting organism and the fermentation process. At low temperature yeasts tend to be less sensitive to the toxic effects of high alcohol concentration. The growth rate of yeast cells is strongly influenced by fermentation temperature. This is particularly evident during exponential phase. At warmer temperature (> 20^{o}C) yeast cells experience a rapid decline in viability at the end of the fermentation. At cooler temperatures cell growth is retarded but viability is enhanced cool temperatures prolong the lag phase of fermentation and slows the rate of fermentation. Excessively high temperature may disrupt enzyme and membrane functions, resulting in struck fermentation (Sener *et al* 2007). Fermentation is reported to cease at 30^{o}C with 342g/ l sugar in the medium before whole of the sugar fermented while 25- 30^{o}C caused a negative effect on survival of *Saccharomyces cerevisiae*.

White wines are preferred to be fermented at lower temperatures i.e. 10- 20^{o}C to enhance the production and retention of flavor volatiles. Such trends have required the selection and use of *S. cerevisiae* strains which exhibit growth rates at lower temperatures (Fleet and Heard 1993; Jackson 2000). The fermentation conducted at temperature less than 15- 20^{o}C may show a greater contribution from *Hanseniaspora* and *Candida* species, which would have greater impact on wine flavor (Heard and Fleet 1988, Erten 2002). Temperature can affect the sensitivity of yeasts to alcohol concentration, growth rate, rate of fermentation, viability, length of lag phase, enzyme and membrane function etc (Sener *et al* 2007).

Jairath (2009) found that fermentation metabolism of *Clavispora lusitaniae* can be carried out at wide temperatures form 10- 40^{o}C. Sanchez *et al* (2004) studied the influence of temperature between 10- 40^{o}C on the fermentation of D- xylose with *Pachysolen tannophilus* ATTC 32691 to produce ethanol and xylitol. Maximum overall xylitol yield occurred at 15^{o}C.

Oliverio *et al* (2010) stated that the fermentation conducted at a controlled temperature below 32^{o}C will result in an increased yield and will also provide the very high gravity fermentation. The low temperature of the mash leads to a low level of contaminants, assuring good quality and greater fermentation yield.

Yeast can respond to the physical effect of high temperatures (increased membrane fluidity) by changing their fatty acid composition. With increasing temperature, the proportion of saturated fatty acids esterified into membrane lipids increased at the expense of unsaturated acyl chains. In general, the higher the fermentation temperature the greater the inhibitory effect of ethanol and the lower the ethanol production rate (D'Amore 1992).

2.2.2 Effect of pH

The pH of the medium may change in response to metabolic activities of microorganisms. Also during the course of fermentation nitrogen source can significantly affect the pH (Haapala *et al* 1994) and therefore initial pH of the medium must be adjusted carefully. During fermentation of grape juice pH of the medium varied between 2.8 and 4, however the growth and fermentation rates of *S. cerevisiae* decreased when the pH was decreased from 3.5 to 3 (Heard and Fleet 1988).

According to Charoenchai *et al* (1998) variation in the medium pH between 3-4 did not significantly affect the growth rate of yeast. Vyas and Joshi (1982) maintained an initial pH in the range of 3.3- 4.9 for making plum wine of acceptable qualities. Grewal (1986) reported a pH range of 4.0- 5.0 as optimum for fermentation of plums.

Jairath (2009) reported that optimum pH range for growth of yeast *Clavispora lusitaniae* is 3.0- 5.0. Yeast can maintain internal pH values quite well in solutions of different pH as cells either keep H^{+} from entering or expel H^{+} ions as rapidly as they enter the cell and it can be grown quite well at pH levels between 3.6- 6.0. The yeast *Clavispora lusitaniae* can tolerate pH values of as low as 2.7, but a change in pH can affect the formation

of fermentation products. Growth of *Clavispora lusitaniae* in different fruit juices resists the change in the pH of the juices due to buffering capacity of fruit juices. At higher pH values the lag phase is reduced and fermentation activity increases.

2.2.3 Effect of fermentable sugars

Yeast owes its inverting and fermentative property to the various enzymes present in it like sucrase, zymase, maltase, lactase, reductase, carboxylase etc. But yeast of different species do not all contain the same enzymes and hence different yeast behave differently towards the various sugars. A particular yeast may ferment one sugar but not another. Sanchez *et al* (2010) reported that a recombinant industrial *Saccharomyces cerevisiae* strain, TMB3130 was used to improve the simultaneous conversion of xylose and arabinose to ethanol. The strain TMB 3130 displayed an increased consumption rate of xylose and arabinose under aerobic and anaerobic conditions. Improved anaerobic ethanol production was achieved at the expense of xylitol and glycerol but arabinose was stochiometrically converted to arabitol.

Natural habitats of yeasts were examined for the presence of strains able to produce ethanol from d-xylose (Nigam *et al* 1985b). Black knots, insect frass, and tree exudates were screened by enrichment in liquid d-xylose-yeast extract medium. Among the 412 isolates examined, 36 produced more than 1 g of ethanol per liter from 20 g of d-xylose per liter, all under aerated conditions. Some strains produced more biomass than ethanol, and among these, ethanol may or may not be assimilated rapidly after depletion of d-xylose. Ethanol production appeared best at low pH values and under mild aeration. Possible correlations between the nutritional profiles of the yeasts and their ability to produce ethanol from d-xylose were explored by multivariate analysis. d-Xylose appeared slightly better utilized by yeasts which rate poorly in terms of fermentation. The fermentation of d-glucose had no bearing on d-xylose fermentation. No specific nutritional trait could discriminate well between better d-xylose fermentors and other yeasts

The growth parameters of *Debaryomyces hansenii* with respect to the utilization of pentoses and hexoses in mixtures and as single carbon sources were studied by Nobre *et al* (1999). Growth on pentoses was slower than on hexoses, but the values obtained for biomass yields were very similar in both types of sugars. Glucose and xylose were transported by cells grown on glucose, via a specific low- affinity facilitated diffusion system. Cells derepressed by growth on xylose exhibited two distinct high-affinity transport systems for glucose and xylose.

Smith *et al* (1997) reported that incremental improvement in ethanol yield appeared to vary with the degree of fortification ranging from 5.8%for unfortified spent sulfphite liquor (SSL) to 27% for the highest level of fortification tested. Decreasing rates of fermentation were observed for SSL fortified with glucose, mannose and galactose respectively. Sugar

uptake rates in SSL fortified with glucose, galactose and mannose were 6.8, 2.8, and 2.0g/l/h respectively. *S.cerevisiae* cells take up xylose with the same sugar permeases it uses for the uptake of d-glucose (Kotter and Ciriacy 1993; Hamachar *et al*, 2002) .However xylose uptake is inefficient compared to that of glucose and especially at low concentrations it limits the utilization of xylose (Gardonyi *et al* 2003)

2.2.4 Effect of substrate concentration

Yeast cells sense the amount and quality of external nutrients through multiple interconnected signaling networks, which allow them to adjust their metabolism transcriptional profile and developmental program to adapt readily and appropriately to changing nutritional states (Zaman *et al* 2008).The highly interconnected signaling networks provide the cell with a highly nuanced view of the environment and that the cell can interpret the information through a sophisticated calculus to achieve optimum responses to any nutritional condition.

In a fermentative process, the variable used to establish the process kinetic has been usually the variation of biomass (synthesis) in the system, variation of substrate content, variation of a product (metabolite), determination of O_2 consumed or CO_2 evolved, heat evolved etc. Among these variables, biomass synthesis during the process and substrate consumption determination usually represents kinetically any fermentation process (Pandey *et al* 2001).

Considering biomass variation during running time in the process it can be defined as a general manner that this variation depends on several factors, so:

$$dx/dt = f(x,s,T) \qquad (1)$$

where

x : biomass concentration (g biomass/l)

t : time (h)

s : substrate (g substrate/l)

T : temperature (^oC)

First term in expression (1) is a velocity or kinetic term. It represents a unit change (biomass concentration) by time unit which can be used to determine parameters such as maximum cell concentration obtained in the fermentation, time delay in the rise of biomass content in the reactor, substrate consumed in the process at any time, yield obtained in the process.

A mathematical model that related biomass synthesis with substrate consumed. The model is:

$$\mu = \mu_{max.} S/ (K_s + S) \qquad (2)$$

where

μ : specific growth rate (h^{-1})

μ_{max} : maximum specific growth rate (h^{-1})

S : substrate concentration (g/l)

K_s : affinity constant biomass/substrate (g/l)

Specific growth rate (μ) is related with velocity or rate (dx/dt) as well as the intensity relating the velocity or rate term with the quantity of biomass present at a particular time in the fermenter. The parameters μ, μ_{max} and K_s determine values for a particular process. At the same time it must be pointed out that specific growth rate is a definition and so is independent of the process. This definition is expressed as:

$$\mu = (1/X)\,(dX/dt) \qquad (3)$$

where

X : biomass concentration at a particular time t (g/l)

t : time (h)

μ : specific growth rate (h^{-1})

Specific growth rate (μ) defined through expression (3) had permitted the representation of kinetic pattern of microbial growth through different phases known as growth phases, expressed as lag phase ($\mu \sim 0$), accelerated growth phase ($\mu > 0$), exponential growth phase ($\mu = \mu_{max}$), deaccelerated growth phase ($\mu_{max} > \mu > 0$), stationary growth phase ($\mu = 0$) and negative growth rate phase ($\mu < 0$).

Applying to equation (3), the statement that in the exponential phase $\mu = k$, where k is a constant, it can be stated that:

$$\ln X/X_0 = \mu t \qquad (4)$$

where

X : biomass concentration at time t of the exponential growth (g/l)

X_0 : biomass concentration at time $t = t_0$ (g/l)

μ : specific growth rate at the exponential phase (h^{-1})

t : time that corresponds to the biomass concentration X (h)

From equation (4), doubling time value can be obtained i.e. the time that takes the biomass concentration to achieve its double value:

$$t_d = \ln 2/\mu_{max} = 0.693/\mu_{max} \qquad (5)$$

where t_d is the doubling time (h).

Yield based on substrate consumption ($Y_{x/s}$) and is defined as:

$$Y_{x/s} = S_0\text{-}S_t / X_t\text{-}X_0$$

where

$Y_{x/s}$: yield based on substrate consumption (dimensionless)

S_0 : initial substrate concentration (g/l)

S_t : final substrate concentration (g/l)

X_0 : initial biomass concentration (g/l)

X_t : final biomass concentration (g/l)

Kocher *et al* (2006) found 20% sugar to be optimum for ethanol production from sugarcane juice. Charoenchai *et al* (1998) recorded increasing the sugar concentration of juice from 200g/l to 300g/l decreased the growth rate and final cell biomass with no evidence of non-Saccharomyces species growing faster than Saccharomyces .Juices that have been processed or clarified may not have sufficient nitrogen nutrients to allow yeast growth and complete fermentation(Henschhe and Jiranek 1993). The nitrogen demand of yeast increases significantly with the increasing sugar concentration of the must and also varies with the strains of yeast. Non Saccharomyces are more demanding of vitamins than *S.cerevisiae* which could be factor limiting their contribution especially in spontaneous fermentation (Fleet 2001).

Devine and Slaughter (1980) studied the effect of medium composition on ethanol production. Panchal and Stewart (1980) reported that when the substrate concentration is increased beyond 25 per cent, the effect of osmotic pressure becomes pronounced which seriously affects fermentation efficiency and leads to decreased ethanol production. Increasing wort sugar concentration was reported to have a detrimental effect on the fermentation performance adversely affecting the yeast physiology and altering the physical and flavor properties of beer product (Brothwick *et al* 1997; D'Amore 1992; Younis and Steward 1997)

Okunowo *et al* (2005) examined different yeast strains on orange juice where the fermentation efficiency varied between 48.05%-99.46% with *S.cerevisiae* and *S.carlsbergenesis* while the sugar utilization was least (2.76g/day) with *S.carlsbergenesis* and highest with (3.07/day) with *S.cerevisiae*.

To investigate about sugar concentration efficiency on fermentation process, different batches of fermentation medium with various initial concentration of sugar (50, 100, 150, 200, 250g/l) were prepared with ph and temperature adjusted to 4.5 and 32^0C.maximum and minimum ethanol yield were observed at 100 and 150g/l sugar concentration respectively (Asli 2010) .High substrate concentrations have been shown to inhibit yeast growth and fermentation performances a result of high osmotic pressure and low water activity.

Anshula (1987) reported 15^oB to be optimum for fermentation of Jamun. Cheema (1989) has reported 10 ^{o}B as optimum TSS for fermentation of guava. Neelam (1987) has reported 20 ^{o}B to be optimum for efficient alcoholic fermentation of pear and mango juice, respectively. Singh (1993) and Sree (1995) reported 15^oB to be optimum for ginger juice and mint mash fermentation, respectively. The fermentation rate in cashew apple juice with three different TSS (20, 22 and 24°B) was higher for a week which was found to decrease

thereafter. Further, with the increase in the initial sugar concentration the fermentation rate was decreased which indicated that the higher sugar concentration had an adverse effect on the fermentation efficiency of the yeast. The fermentation efficiency of the yeast was found between 57.94 – 61.01% which was comparatively low as the tannin contents in cashew apple juice and initial sugar concentration may had an adverse effect on the efficiency of the yeast. The decline in the rate of fermentation towards the completion of fermentation may be due to more alcohol production which inhibited the fermentation efficiency of the yeast.

Bertolini *et al* (1991) reported new yeast strains for alcoholic fermentation at higher sugar concentration. Joshi and Sharma (1994) reported that apricot musts having initial TSS 30^0B showed slightly lower rate of fermentation, more residual TSS and reducing sugar, better appearance and overall quality as compared to must having initial TSS 24 ^{o}B. Optimum TSS 24 ^{o}B for the production of kinnow wine has been reported by Singh *et al* (1998).

2.3 Chemical characteristics

2.3.1 Juice/ Pulp per cent

The composition of guava varies depending on the cutivar, stage of maturity and season .Guava is a good source of pectin, calcium, many vitamins like ascorbic acid, niacin, thiamine, vitamin A and antioxidants. According to Palaniswamy and Shanmugavelu (1974) the pulp percentage ranged from 56.2% in Red Fleshed to 95.4% in L-49. Anakapalli and Chittidar recorded above 90% pulp. Jain and Nema (2007) reported the pulp yield in five different cultivars of guava lied between 54.0-54.8%.The maximum pulp yield was recorded in Allahabad Safeda (54.8%) followed by Lucknow-49 (54.6%), Apple color (54.3%) and Chittidar (54.2%). The minimum pulp yield was found in Red Fleshed (54.0%).

Mitra *et al* (1983) studied on physic-chemical characters of some guava varieties of West Bengal and reported that the varieties Red Fleshed and Apple Colour had low percentage compared to other varieties. At Lucknow, Tandon *et al* (1983) found the pulp percentage ranged from 96.2%in Chittidar to 98.3% in Kerala. Singh (2007) observed significant difference in pulp per cent among seventeen genotypes during winter season. The highest pulp (99.25%) was reported in Sardar Selection 4/10 which was found statistically at par with Sardar Selection 6/8(98.75%),Dharidar (98.51%) and Hybrid Bahadurgarh (98.49%). The minimum pulp yield was noticed in B.S.S 6/12.

Citrus juices are an excellent source of potassium, vitamin C, folic acid, inositol and bioflavonoids that not only give citrus juice its flavor and color but are potent antioxidants. Ziena (2000) reported that Fruits of Seedless lime (*Citrus latifolia Tan*) has juice yield of 55.6% when fruit is dark green and it increases to 59.4% when it is light greenish yellow.

Mature ‘Kagzi’ and Tahiti limes yielded 61 and 51.2 % juice respectively (Rao *et al* 1977). Sandhu *et al* (2000) reported that the juice content in three species of Kagzi lime, Baramasi lemon and Hill lemon was more than 40 percent.

In an evaluation trial of lemons in Hissar, juice content varied from 21.33 percent in cultivar Eureka to 42.33 percent in Seedless and Baramasi cultivars (Arora and Daulta 1981). Suddamath and Iyer (1982) reported that juice content of Nepali round and Tahiti lime was quite high (more than 46 %) whereas fruits of Nepali oblong had only 32 percent juice.

2.3.2 Total Soluble Solids

Soluble solids can be measured from the refractive index and refractometers are calibrated to give ^{o}Brix or per cent total soluble solids values directly. Refractometer reading changes with temperature as the refractive index of the sugars changes with the temperature. Kahlon *et al* (1993) studied the variability pattern for fruit quality in seedling population of guava and recorded the TSS content from 7.8 to 14.4 per cent and 8.6 to 14.8 per cent during rainy and winter season respectively. Jain and Nema (2007) observed total soluble solids in five cultivars of guava ranging from 11.8 brix to 12.8 brix. The maximum and minimum Brix was found in Allahabad Safeda and Red Fleshed respectively.

Singh (1988) studied the chemical composition of the fruit in different guava varieties and showed marked variations. The TSS content ranged from 8.0 per cent in Hafsi to 15.0 per cent in Apple Colour. Chatterjee *et al* (1992) recorded the cv. Allahabad Safeda posses maximum TSS of 9.65 per cent followed by Sardar having 8.85 per cent and 8.45 per cent in Red Fleshed.

Hegde and Chharia (2004) noted Maximum TSS content (13.83%) on 155 days after fruit set in winter season crop. During rainy season, maximum TSS (9.31%) was recorded on 120 days after fruit set and minimum was observed on 30 days after fruit set. At Faizabad, Pandey and Singh (1999) found that fruits of L-49 have the highest TSS (14.20%), followed by Allahabad Safeda (13.45%) and Apple Colour (12.60%).

Sharma *et al* (2010) reported the total soluble solids ranged from 9.4 brix to 13.5 brix in 22 genotypes of guava. The maximum total soluble solids was recorded in ‘Hybrid Red Supreme’ (13.5) followed by ‘Hisar Safeda’ (13.2) and ‘Patillo’ (13. 1), while minimum total soluble solids was recorded in ‘Chakaiya Rehmannagar’ (9.4) which was at par with ‘Chinese’ (9.6), ‘Super Max Ruby’ (10.1). Aulakh (2005) recorded maximum TSS in the fruit of Behat Coconut (11%) followed by Tehsildar (10.6%) and L-49 (10.5%) and minimum in Baraf Khana (8.2%).

TSS and TSS/acid ratio are the reliable indices for assessing the maturity in citrus. Total soluble solids content in Hill lemon was found to be lower as compared to Baramasi lemon and Kagzi lime selections (Sandhu *et al* 2000). Prasad *et al* (1999) studied eight lemon cultivars for variability and reported that TSS content of cultivars varied from 5.98-6.90 percent. However, the genotypic coefficient of variation and genetic advance was lower for this character, indicating the role of environmental factors in this variation.

2.3.3 Acidity

Guava is classified as sub acid fruit, since their soluble solids are composed mainly of organic acids and sugars, which are used as the main index of maturity and one of the major analytical measures of flavor quality**.** Mainly the citric, malic, glycolic, tartaric and lactic acids contribute towards the acidity of guava fruit (Hui 2006). Under Ludhiana conditions, at ripe stage the total acidity of Allahabad Safeda and Sardar guavas cvs. was 0.25 per cent and 0.19 per cent in rainy season crop and 0.31 and 0.33 per cent in winter season crop respectively.

Sharma *et al* (2010) reported that the acidity ranged between 0.37% to 0.96% for the different genotypes of guava. Maximum acidity was recorded in 'Chinese guava' (0.96%), which was at par with 'Spear Acid' (0.95%). The minimum acidity was noted in 'Hisar Safeda' (0.37%), followed by 'Lucknow-49' and 'Patillo' (0.42%).The Vietnamese variety had the highest acidity of 0.52% followed by Taiwan(0.51%) and GU5 (0.496%).

Jain and Nema (2007) observed maximum and minimum acidity in Allahabad Safeda(0.48%) and Red Fleshed (0.38%) respectively. Aulakh (2005) concluded that the amount of titrable acidity increased continuously from 30 days after fruit set to 135 days after fruit set in winter season (0.31%to 0.62%). Similarly during rainy season it increased upto 110 days after the fruit set (0.28% to 0.58%).

Acidity contributing to the tartness in most citrus fruits is largely due to citric acid accounting from 85 to 95 % of total acids in various cultivars. In addition, traces of malic acid tartaric, benzoic, oxalic and succinic acids have also been reported (Kale and Adsule 1995).

In a trial on selections of lime and lemon under Ludhiana, conditions in Punjab, Sandhu *et al* (2000) reported that maximum acidity was recorded in Kagzi lime followed by Baramasi lemon and Hill lemon. In addition, acid content in January harvested fruits of Baramasi lemon and Kagzi lime was lower than July harvested fruits.

Selection from existing natural variability led to identification of sour mutant of sweet lime which had much higher acid percentage in juice (5.40 %) than common sweet lime (Govind and Singh 2000). Prasad *et al* (1999) studied variability for chemical characters in lemon cultivars and reported that acidity varied from 0.47 g/100 ml to 0.55 g/100 ml. Singh and Govind (2000) reported that acidity percentage was minimum (4.2 %) in Hill lemon and was maximum (5.8 %) in Gol neembu.

2.3.4 Ascorbic acid

Ascorbic acid (vitamin C) is a white crystalline compound of a simple structure that is closely related to the monosaccharides and which is naturally synthesized in certain plants and by some microorganisms (Egbere *et al* 2009). Vitamin C is essential for the synthesis of collagen which is the major fibrous element of skin, bone, blood vessels and teeth (Zvaigzne *et al* 2009). It participates in numerous biochemical reactions suggesting that vitamin C is

important for every body process from bone formation to scar tissue repair (Rickman *et al* 2007). Humans need a daily intake of 30-45 mg per day while deficiency of this vitamin leads to scurvy which can be prevented with only 10 mg vitamin C/day.

Oranges are well known as fruits rich in vitamin C but guava is far superior with vitamin C content three to six times higher than that in orange. Although guava is a rich source of ascorbic acid, its level is subjected to wide variations because of geographical location, horticultural practices, season and cultivar (Jawaheer *et al* 2003).White fleshed guava is reported to be a better source of vitamin C (142.6 mg/100g) than pink fleshed guava and is also rich in other antioxidants such as phenolics and ß-carotenes (Luximon-Ramma *et al* 2003).

Pandey and Singh (1998) reported ascorbic acid content of guava cultivars ranged from 149.0 to 250 mg per 100g of pulp and L-49 showed highest value followed by Allahabad Safeda and Sangam. Bal and Dhaliwal (2004) recorded the highest ascorbic acid to the tune of 210 mg/100g in pulp of Sardar and Allahabad Safeda guava. Phattaraworrasuth and Chiewchan (2008) reported that the vitamin C content in Panseethong and Klom Salee, the well known cultivars of guava in Thailand, was 68.8 and 104.4 mg/100g of fresh guava respectively. Thaipong *et al* (2005) obtained the highest vitamin C in pink flesh clone 'Fan Retief' (397 mg/100g FW) over the fruits of cvs. Allahabad Safeda (379 mg/100g FW), Advanced Selection (259 mg/100g FW) and Ruby Supreme (174 mg/100g FW).

Sandhu *et al* (2000) evaluated certain strains of lime and lemon under Ludhiana conditions and reported that Baramasi lemon II and III had ascorbic acid content of 41.7 and 42.6 mg/100ml juice in July, and 49.5 and 48.4 mg/100ml juice in January harvested fruits, which was much higher than other lime/lemon selections.

Arora and Daulta (1981) evaluated four cultivars of lemon and reported that vitamin C was highest in Kagzi Kalan (31.97mg/100ml) followed by Baramasi and the lowest in Eureka (17.82 mg/100ml). Suddamath and Iyer (1982) reported that ascorbic acid content among nine cultivars varied from 17.50 to 42.66 mg/100ml of juice.

Mehta and Bajaj (1984) carried out investigations on three citrus species viz. Blood Red orange, Kinnow mandarin and Villafranca lemon and reported that Blood Red contained maximum (82.2 mg/100g) ascorbic acid followed by Villafranca (36.4 mg/100g) and Kinnow 924.5 mg/100g) varieties.

2.4 Carbonated beverages

Khurdiya *et al* (1996) prepared carbonated guava beverage by converting juice into sugar syrup base having 40% guava juice at 40°B and 1% acidity and filling it with chilled carbonated water at 80psi pressure of CO_2 gas. The carbonated guava beverage could be stored for three months at room (23-41°C) and low (3-5°C) temperatures and found acceptable with respect to color, flavor and overall quality. Darbyshire *et al* (1983) invented

process for the manufacture of naturally carbonated beverage. They observed that a beverage is fermented with a combination of at least one yeast and at least one lactobacillus for their symbiotic ability and capability to produce its synergistic organoleptic effect which eliminate all after taste yeast.

Tracey (1989) prepared carbonated dairy or non dairy milk product optionally in combination with other beverages e.g. fruit juice, by injecting carbon dioxide or a mixture of gases under pressure of 50-200 K Pa at 40^0 C into the milk. The final product prepared had a reasonable shelf life. Passe *et al* (1997) observed the effects of beverage carbonation on sensory responses and voluntary fluid intake following exercise. It was concluded that levels of carbonation equal to or in excess of 2.3 volume carbon dioxide had negative impact on drink acceptability.

Jairath (2009) prepared non-alcoholic naturally carbonated beverage from amla var. Francis with 14^oB, 0.73 (% v/v) alcohol and Co_2 pressure of 1.20 Bar. On the basis of organoleptic evaluation, all the sensory attributes varied non-significantly throughout storage period of three months under refrigerated conditions. Sahota *et al* (2009) prepared low alcoholic self carbonated blended beverages from Carrot var. selection-21 and Amla var. Chakaiya in the ratio of 3:1, 1:1, 1:3 after optimizing the inoculum concentration TSS adjusted to 16^oB,and carrot-amla (1:1) was rated the highest

Hotchkiss and Chen (1996) described the process to inhibit or reduce the growth of bacteria & other pathogens in a liquid by adding carbon dioxide to the liquid and thermally inactivating the bacterial and other pathogens. Kaushal (2004) reported that total soluble solids (TSS) and CO_2 gas pressure for carbonation are the key parameters that affect the organoleptic quality of carbonated beverages. Apple juice beverage as well as pear juice beverage with 14^oB TSS carbonated at 80 psi CO_2 gas pressure was adjudged to be best from overall sensory point of view.

Gupta (1997) standardized the technology for carbonated pome fruit juice beverages. 10 per cent (apple/pear/apple-pear (1:1:1) juice) with synthetic cola concentrate at 100 psi and 120 psi of carbonation were adjudged the best as these had highest physio-chemical and sensory qualities. Clark (2005) prepared the carbonated fortified milk based beverage and method for suppressing bacterial growth in the beverage. Carbonation of the beverage reduced the bacterial count and also reduced the degradation of essential nutrients in the beverage.

Ranganna (1994) reported that commonly used concentration of CO_2 in carbonated fruit juice beverages (0.1 to 0.8 %) is lower than that required for complete inhibition of microbial activity (14.6 g/L), yet the level is significant in supplementing the lethal effect of acidity on pathogenic bacteria. Shaikh and Rathi (2007) utilized the buttermilk for the preparation of carbonated fruit flavored beverages. The beverage containing 12% sugar, 24%

pineapple juice and processed by ultrafilteration was best as compared to other combinations

Kaushal (2002) prepared carbonated beverage from apple juice, pear juice and reconstituted apple juice, TSS of 14°B and CO_2 gas pressure of 80 psi were found to be optimum. Carbonated beverage showed good storage stability for a period of 9 months from nutritional, microbiologically and sensory quality point of view.

Sherwood and Jenkins (2007) prepared an improved carbonated protein beverage drink composition which provides relatively high protein content ranging from 2% by weight to about 15% by weight while simultaneous employing a carbonation concentration between about 0.1 volume of carbonation (per volume of liquid drink solution or liquid drink suspension) to about 4 volumes of carbonation.

2.5 Ready to serve beverage

Sahu *et al* (2005) prepared ready to serve whey based mango-lemongrass beverage in which the volumes of mango pulp (12%), sugar (80%), water (48%) and paneer whey (32%) were kept constant while the volumes of lemongrass distillate was varied from 0-2.5% (v/v), having the properties of 15.2°B TSS, 4.35 pH, 23.29% total sugars and 0.19% acidity. The organoleptic scores of the beverages improved as the concentrations of lemongrass distillate increased from 0 to 1.5%.

Pandey and Singh (1999) carried the studies on preparation and preservation of guava ready to serve beverage. Recipies for commercial preparation of guava ready to serve beverage were evaluated. The varietal and suitability and storage stability were also examined. The recipe containing 10% pulp and 11% TSS with 0.25% acidity was found most ideal. The ready to serve beverage prepared from Sardar (L-49) was found better than that of Allahabad safeda, Apple colour and Sangam. Storage stability of the product found four months at ambient temperatures.

Barwal *et al* (2005) carried out the studies on processing and development of ready to serve beverage from bittergourd fruit. Three levels of bitter gourd (cv Solan Hara) juices (10, 15 and 20%) were tested at three levels (10.0, 12.5 and 15ºB) of TSS. The beverages were analysed for various physicochemical and sensory properties during a storage period of upto 180 days. A beverage 10% juice at 12.5°B was judged the best. The product had appealing flavor, body and sugar acid blend.

Jain and Khurdiya (2004) blended Indian goose berry for the preparation of ready to serve beverages for boosting their nutritional quality in terms of vitamin C content. On the basis of overall sensory quality and vitamin C content, ready to serve beverage prepared by blending gooseberry and Pusa Navrang grape juice in 20:80 ratio was found to be the best. Kumar and Manimegalai (2001) prepared blended RTS beverages from pineapple-pear and pineapple-pomegranate (1:1), pomegranate-pear-pomegranate (1:1:1) and pineapple juice

alone and concluded on the basis of sensory properties that blend of pineapple-pear-pomegranate (1:1:1) was most acceptable as compared to other.

Singh *et al* (1999) attempted to develop a soft beverage from paneer whey and guava. Three types of guavas were tried with different sugar levels. The guava extract and whey ratio were tried at 1:5, 1:4 and 1:3. The guava whey beverage formulated by using Banarsi Surkha variety of guava extract at 1:3 ratio with 8% sugar level and lemon colour scored highest. The carbonation enhanced the acceptability of guava-whey beverage.

Saxena *et al.* (1996) prepared RTS drinks from mixed juices from grapes-mango and grape-pineapple in different ratio and reported that all the ratio were acceptable but superior one was 1:1 due to balanced taste and flavor. Vaidya *et al.* (1998) prepared RTS from blended juice taking guava, pomegranate and ber in various proportion like 0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 100:0 and found the combinations of guava-pomegranate (30:70) and guave-ber (40:60) to be superior over the other.

Saravanakumar and Manimegalai (2005) prepared beverage by blending papaya juice at 10% with whey maintaining TSS and acidity at 15ºB and 0.3% respectively. Storage stability, microbiological and sensory changes were evaluated periodically during three months under refrigerated conditions. There was an increase in acidity and reducing sugars and decrease in pH, TS and ascorbic acid but TSS did not change. Sensory properties were highly acceptable even after storing the beverage for 3 months under refrigerated conditions.

Krishnaveni *et al* (2001) prepared ready to serve beverage from two varieties of jack fruits with 10% pulp, 18°B, TSS and 0.25% acidity and packed in coloured (green) and colourless bottles are stored at room temperaturesto study the storage stability. Retention of ascorbic acid and β carotene contents were much better in the the samples stored in green coloured bottles. The sensory quality attributes were found to be highly acceptable even after storing for 6 months at room temperatures. Shrera (2005) utilized Hill lemon (*Citrus pseudolimon* Tan) and Tulsi (*Ocimum sanctum* L) for the development of RTS and appetizer. RTS beverages were prepared from 5% Hill lemon juice, 10% tulsi extract, 14°B TSS were found better than RTS beverages prepared from other combination and showed good storage stability for a period of six months on the basis of nutritional, microbiological and sensory attributes.

2.6 Blended beverages

Fruits which are rich in nutrients but are not accepted due to poor taste and flavor can be blended with other fruits to improve their acceptability and make use of available nutrients (Khan *et al.* 1988). Akubor (2003) studied the influence of storage on the physicochemical, microbiological and sensory properties of heat and chemically treated melon-banana beverage. Vitamin C, soluble solids, titrable acidity and pH of beverages treated with sodium benzoate and sodium benzoate with pasteurization showed no significant changes with

storage. A combination of sodium benzoate and heat treatment maintained acceptability of beverage for 35 days. The 121°C – 15 minutes treated beverage remained acceptable upto 12 days.

Sampedro *et al* (2009) reported that during the shelf life of orange juice-milk based beverage at 8-10° C, the untreated sample spoiled after 1 week whereas the pulse electric field and thermally processed samples remained stable during the entire four week storage. Furthermore, slight decrease in pH, °Brix occured in the untreated samples, during storage while a slight increase in PME and no change in pH and ºBrix values were observed in PEF and thermally processed samples.

Naik *et al* (2009) prepared whey based watermelon beverage by blending watermelon juice (25%), sugar (7%) and different concentrations of betel leaves distillate (0, 1, 2,3%) to channa whey (75-78%) the prepared beverage has red colour, highly acceptable taste and acceptability. The storage study showed that there is an increasing trend TSS, acidity and reducing sugars and a decreasing trend in the pH and ascorbic acid but total sugar has non significant effect during storage. The sensory quality of fresh beverage containing 2% betel leaves distillate on the preparation as well 30 days of storage were found to be highly acceptable.

Marques De Carvalho *et al* (2007) evaluated the physicochemical, microbiological and sensory stability of a blended non-alcoholic beverage composed of coconut water and cashew apple juice (80:20 v/v) during six months. The results showed that the beverage presented good stability in all analysed parameters except for vitamin C. The beverage colour also presented changes during the storage time. Despite these alterations, the product acceptance during the storage period did not show any rejection.

Da S Lima *et al* (2009) prepared beverage formulated with acerola fruit juice (25%) and green coconut water (75%) with added caffeine. Physicochemical and sensory analysis was performed during six months storage at room temperatures (27°C). The vitamin C content decreased significantly during storage. The product was acceptable during six months of storage.

Selected characteristics of whey fortified banana beverages stored at 4, 20, 30 and 40°C were monitored at specific time intervals over a 60 day storage period, the sensory characteristics of the whey-banana beverage stored at 4°C were studied and the product was sour, sweet, smooth beverage with distinctive banana flavour and minimum off flavour (Ernest *et al* 2005).

Dhaliwal and Hira (2004) prepared four different combinations of carrot juice with two levels each of spinach (45 and 9%) and pineapple juice (30% and 40%) and stored for six months in glass bottles at room temperature (32-40°C). No significant changes were observed in pH, acidity, total solids, viscosity and mineral contents of the juices during storage. The

storage resulted in 80-88.75% losses in ascorbic acid and 52.02-61.41% losses in β-carotene contents.

2.6 Non alcoholic beverages

Egbere *et al* (2009) prepared Kunun-zaki; a fermented non alcoholic drink fortified with 90mg/L of vitamin C and studied the effects of pasteurization on survival patterns of microflora as well as vitamin C retention in it. The results show that there was gradual decline in vitamin C retention and steep decline in microbial load reduction during pasteurization.

Sahota and Sunil (2006) developed a reliable, controllable and reproducible technology for preparation of non-alcoholic naturally carbonated plum beverage by inoculating yeast and optimized the fermentation condition, such as TSS 14°B, alcohol content 0.7 % (w/v), CO_2 pressure 3psi.The nutrients and anti-microbial activity of sorrel drinks, non-alcoholic beverages from Roselle was studied and found that unfortified Roselle beverage had low anti-microbial properties (Obon and Eluziyan 2004). Fortified beverage with pineapple and lemon greatly enhanced the antimicrobial properties.

Osuntogun and Aboaba (2004) carried out the studies for microbial and physicochemical quality of four non alcoholic beverages- ginger beer, soya milk, soborado drink and kunun-zaki so as to determine the most effective mode of storge and consumer acceptability. A combination of pasteurization and refrigeration was found to be most suitable for prolonged shelf life and consumer acceptability. The organisms isolated included Lactobacillus, Streptococcus, Saccharomyces, Candida, Leuconostoc, etc. Chemical analysis showed that the major food components were retained.

Mugula *et al* (1994) prepared powder for instant non-alcoholic pawpaw beverage by traditional sundrying and controlled overdrying. The reconstituted beverage was organoleptically acceptable. Sundrying resulted into losses of vitamin A and C and total sugars by 97, 98 and 87% while over drying losses were 92, 98 and 97%, respectively.

Wireko-Manu *et al* (2010) produced non alcoholic beverage flavored with citrus lime and ginger from two varieties of sweet potato tubers. pH , total sugars, total solids, Brix, total titrable acidity, vitamin C and A were determined and sensory evaluation was conducted on the products to assess the acceptance preference. Generally, the beverage had good consumer preference with the ginger flavored being the most preferred.

Von-Mollendorff *et al* (2006) stated that Boza, a low pH and a low alcohol beverage produced from cereals, is a healthy beverage being rich source of bacteriocin producing lactic acid bacteria with antimicrobial activity against a number of spoilage and pathogenic bacteria. A low alcoholic fruit beverage prepared by separation of pulp from the peel, an unfermented and a partially fermented juice fraction with other ingredients (Miclo 1993).

Iresel *et al* (1995) produced a non-alcoholic beer by limited fermentation with immobilized cells of *Saccharomyces cerevisiae* in a packed bed reactor. Under combined

stress factors such as low temperature 2 – 4^0C and anaerobic conditions, only a small amount of glucose is metabolized resulting in low concentration of the ethanol (<0.08%). Eglintun *et al* (2002) found that glycerol is a major fermentation product of *Saccharomyces cerevisiae* that contributes to the sensory characteristics of wine and produce less ethanol than wild-type strains.

A technology developed for low alcohol < 5.5 % beverage based on sugar wine and small fruit berries (Taccard 2001). Munene *et al* (2002) found that the ratio of glycerol to bioethanol could be altered in favour of glycerol by adjusting fermentation parameters as osmotic pressure, pH, and temperature and yeast cell inoculum. Pandove (2007) prepared low alcoholic self carbonated beverages from carrot and amla after optimising inoculum concentration of. *Saccharomyces cerevisiae* var. *ellipsoideus* culture at the rate of 0.5% v/v. On the basis of organoleptic evaluation, low alcoholic self carbonated carrot-amla (50:50) beverage was rated the best, with shelf life of three months.

Gokavi *et al* (2005) developed an oat-based symbiotic non-alcoholic beverage to get the combined benefit of the probiotic property of cultures, isolated from the traditional Bulgarian cereal-based beverages and the prebiotic property of dietary fibre oats. Durojaiye *et al* (2003) studied refrigeration and pasteurization delayed and decreased the decline of pH, increased the shelf life of the kunu, a non-alcoholic beverage from millet and reduced the sedimentation of suspended particles. Kitabatake *et al* (2003) prepared non-alcoholic beverage 'Togwa', and observed decrease in pH.

Ade-Omowaye *et al* (2006) carried out the development and quality evaluation of non-alcoholic beverages from maize based product, titrable acidity was found to be 0.22, 0.18, 0.05 and 0.30 % for plain, fruit flavored, soy fortified and soured beverage respectively. Fruit flavored ranked highest in preference followed closely by the plain beverage, while soy-fortified samples was the least acceptable. Singh and Nath (2004) showed that bael fruit beverages may be fortified with the protein-acidic polysaccharide complexes, viz. CMC-WPC complex and pectin-WPC complex. The bael fruit beverage with 16^oB and 1.75% CMC-WPC was rated the best; fortification to a higher protein level reduced its acceptability.

Chapter III

MATERIALS AND METHODS

3.1 Yeast culture

The yeast culture, *Clavispora lusitaniae* (Annexure I), was procured from the Department of Microbiology, Punjab Agricultural University, Ludhiana.

3.1.1 Growth kinetics of yeast in beverage

A loopful of 24 hrs old actively growing yeast culture 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, guava juice and blended guava –lemon juice with 15º B. Growth curves were obtained in terms of viable cell count ($\log_{10}$ no. of cells per ml).

3.1.2 Enumeration

Total yeast count was enumerated on GYE agar by serial plate dilution method.

3.1.3 Maintenance of Yeast culture

Yeast cultures were maintained at 4°C in GYE broth containing 20% glycerol.

3.2 Fruits

a) Guava

Guava var. *Allahabd Safeda* (Plate 1), *Lucknow*-49 (Plate 2) and *Punjab Pink* (Plate 3) were procured from Department of Horticulture, PAU, Ludhiana and PAU Regional Fruit Research Station, Bahadurgarh.

b) Lemon

Lemon var. Baramasi (Plate 4) and *Citrus latifolia* (Plate 5) were procured from the Department of Horticulture, PAU, Ludhiana.

3.3 Selection of fruits

Healthy fruits were selected after manually sorting and discarding defective fruits. Fruits were washed in chlorinated water and then used for the extraction of juice. Juice was extracted aseptically under hygienic conditions.

3.4 Extraction of juice

3.4.1 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.

3.4.2 Lemon

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

3.5 Preparation of sugar solution

Granulated sucrose was procured from local market. 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.

3.6 Preparation of non-alcoholic naturally carbonated fermented beverage from Guava

3.6.1 Physico-chemical analysis of guava pulp

The physico-chemical analysis, TSS, pH, acidity, Brix acid ratio, total sugars, reducing sugars, ascorbic acid, total phenols and juice yield of guava pulp was done. 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%.

3.6.2 Inoculum preparation

The inoculum was prepared in pasteurized, diluted juice (15°Brix). A loopful culture of 24 h old yeast culture was inoculated in 100 ml diluted guava juice in 250 ml Erlenmeyer flask and incubated at 30^0C for 24 hrs to achieve concentration of 10^5-10^6 cells/ml.

3.6.3 Fermentation

The diluted juice was inoculated @ 0.5% v/v with freshly prepared inoculum and incubated at 30 ± 5^0C for 36 hours in batch scale glass digester.

3.6.4 Bottling and Storage

The beverage was refrigerated for 24 hours, siphoned, bottled and then stored in refrigerated conditions.

3.6.5 Shelf life studies of Guava beverage

Shelf life of non-alcoholic naturally carbonated fermented guava beverage of all the three varieties *Allahabad Safeda, Punjab Pink* and *Lucknow-49* stored at refrigerated temperature (4^0C) was studied and evaluated fortnightly for physicochemical, microbiological and sensory qualities.

3.7 Chemical Analysis of Juice

3.7.1 pH

pH of the juice was determined using a digital pH meter (Electronic Corporarion of India Ltd., Hyderabad, type 101).

3.7.2 Total Soluble Solids

Percent total soluble solids (%TSS) in juice and beverage were determined by using Erma hand refractometer of 0-32ºB (UNICO make). A drop of distilled water at 20^0C was placed on clean and dry prism and calibration was done at zero line on the scale. Then the samples of juice and beverage were analyzed for their TSS value by reading the line of demarcation on the scale.

Plate 1: Guava var. *Allahabad Safeda*

Plate 2: Guava var. *Lucknow-49*

Plate 3: Guava var. *Punjab Pink*

Plate 4: Lemon var. *Baramasi*

Plate 5: Lemon var. *Citrus latifolia*

3.7.3 Titrable Acidity

Total acidity expressed as Citric acid was estimated following the procedure of AOAC (1999). Titrable acidity was determined by titrating known quantity of water extract of fresh fruit (10ml) against standardized 0.1N NaOH using a few drops of 1% phenolphthalein solution as indicator to pink end point which should persist 15 seconds. Results were expressed as % anhydrous citric acid for fresh fruit.

$$\text{Acidity (\% citric acid)} = \frac{\text{Titre x Normality of alkali used x Volume made x Equivalent weight}}{\text{Wt. of sample x Aliquot used x 1000}} \times 100$$

3.7.4 Brix - Acid Ratio

Brix - acid ratio was calculated by dividing TSS value with total acidity of the juice and carbonated beverage.

3.7.5 Total sugars

Total sugars were estimated by phenol-sulphuric acid method of Dubois *et al* (1956) using glucose as standard. For estimation purposes, aliquots of 0.1-0.5 ml sugar solution/beverage were taken in test tubes and distilled water was added to make the volume 1 ml. It was followed by addition of 1 ml 5% phenol and 5 ml conc. sulphuric acid. The acid was poured directly in the centre of the test tube to ensure that temperature rises to 70^0C for optimal color development. The test tubes were kept for 10 min at room temperature and then cooled under tap water. A stable yellow orange color developed after about 20 min. Absorbance was recorded at 490 nm using spectrophotometer (Spectronic-20) against a reagent blank.

The concentration of total sugars was calculated from the standard curve by using glucose (20-100µg/100ml).

3.7.6 Reducing sugars

Reducing sugars were estimated by the method of Miller (1959). Test tubes containing 3 ml sample and 3 ml DNS reagent (10 g of 3, 5 dinitrosalicyclic acid, 2.0 g phenol and 0.5 g sodium sulphite solution dissolved in 500 ml 1% sodium hydroxide solution and the volume was made 1000 ml by adding additional alkaline solution, filtered and stored in a dark colored bottle) were heated for 15 min in a boiling water bath. 1 ml Rochelle salt solution (40 g sodium potassium tartarate was dissolved in distilled water and the volume was made to 100 ml) was added to each tube and the tubes were allowed to cool to room temperature. O.D. was measured at 575 nm using spectrophotometer.

The concentration of reducing sugars was calculated from the standard curve by using glucose (20-100µg/100ml).

3.7.7 Ascorbic acid

The titrametric method using 2, 6-dichlorophenol indophenol dye was used to estimate ascorbic acid (AOVC 1996). Dye factor (i.e. mg of ascorbic acid per ml of dye) was

determined by 5ml standard ascorbic acid solution and 5ml 0.4% oxalic acid against dye solution to a pink colour. Known weight of crushed sample (10g) or known volume of beverage (10ml) was taken and 100 ml of volume was made up with 0.4% oxalic acid solution. The mixture was filtered through Whatman filter paper no. 4. To a measured volume of aliquot (10ml), 15 ml of oxalic acid (0.4%) was added followed by titration against standardized dye (0.04%) to a pink end point which should persist for at least 15 sec. Fresh dye solution and standardized ascorbic acid was prepared before each analysis.

$$\text{mg of ascorbic acid / 100g} = \frac{\text{Titre x Dye factor x Volume made}}{\text{Aliquot taken x Weight of sample}} \times 100$$

3.7.8 Total phenols

3.7.8.1 Extraction of total phenol

A known volume of beverage (1ml) was taken in 100 ml conical flask. To this 10ml HCl (0.3N) in methanol was added and kept on environmental shaker at 150 rpm for 1hr. after shaking, crude extract was filtered through Whatman No.1 filter paper. The filtrate so obtained was evaporated to dryness in a boiling water bath. To this residue, hot water was added and final volume was adjusted to 25ml with distilled water.

3.7.8.2 Estimation of total phenols

Total phenols were estimated by method of Malik and Singh (1971). To 0.05ml of the above extract, 1ml each of Folin-Ciocalteu reagent (diluted 1:2 with distilled water) and sodium carbonate reagent (35 grams of sodium carbonate dissolved in 60ml distilled water and final volume made to 100ml) was added and then mixed. After 10 min, 2ml of distilled water was added and intensity of color was recorded at 620 nm against reagent blank.

The concentration of total phenols was calculated from the standard curve prepared by using gallic acid (10-40µg/ml).

3.7.9 Alcohol estimation

Percent Alcohol (v/v) was calculated by the Spectrophotometric determination method of ethanol (Caputi *et al* 1968).

One milliliter of fermented wash was taken in 500 ml pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of Potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid & volume raised to 1 litre). About 20 ml of distillate was collected in each sample and flasks were kept in water bath maintained at 62.5 ^{O}C for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50 ml. Five ml of this was diluted with 5 ml of distilled water for measuring the optical density at 600 nm using a Baush & Laumb Spectronic-20.

A standard curve was prepared under similar conditions by using standard solution of ethanol containing 1-5% (v/v) ethanol in distilled water.

3.7.10 Carbon Dioxide volumes determination

Carbon Dioxide volumes in beverage bottles were determined by Zahm and Nagel piercing device. The rationale for this method of carbon dioxide determination was the measurement of the equalized head space pressure at a given temperature. The two factors were plotted on a table in order to find the CO_2 volumes.

3.8 Microbiological analysis

Total yeast count was enumerated on GYE agar by serial plate dilution method.

3.9 Blending of guava juice with lemon in varying proportion for preparation of non-alcoholic naturally carbonated fermented beverage

3.9.1 Selecting and standardizing the best blend of guava: lemon on the basis of sensory evaluation

Guava juice was blended with diluted lemon juice in the ratio of 1:2, 1:1, and 2:1. Blended juice was pasteurized at 82^0C for 15 seconds, cooled and brix adjusted to 15ºB by adding sugar solution. The best ratio of the blended beverage was selected on the basis of sensory evaluation.

3.9.2 Inoculum preparation

The inoculum was prepared in blended juice. It was boiled for 5 minutes and adjusted the brix to 15^0B by adding sugar solution. A loopful culture of 24 h old yeast culture was inoculated in 100 ml blended juice in 250 ml Erlenmeyer flask and incubated at 30^0C for 24 hrs to achieve concentration of 10^5-10^6 cells/ml.

3.9.3 Fermentation

The diluted juice was inoculated @ 0.5% v/v and incubated at 30 ± 5^0C for 36 hours aerobically.

3.9.4 Bottling and Storage

The beverage was refrigerated for 24 hours, siphoned, bottled and stored in refrigerated conditions.

3.9.5 Shelf life studies of the standardized guava: lemon blended beverage

Shelf life of the standardized non-alcoholic naturally carbonated fermented blended guava and lemon beverage stored at refrigerated temperature (4^0C) was studied by evaluating fortnightly for physicochemical, microbiological and sensory qualities fortnightly till a period of 90 days.

3.9.6 Chemical and microbiological analysis of juice

As in 3.8 and 3.9, respectively.

3.10 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)

3.10.1 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 - 5 minutes.

3.10.2 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.

3.11 Sensory evaluation

The organoleptic evaluation of guava and blended guava-lemon beverages (untreated, heat treated and potassium metabisulphite treated) was done on the basis of appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability by a panel of judges. Consumer acceptance for the products was evaluated on a nine point "Hedonic scale" (Amerine *et al* 1965) with following scale:

Scale	Sensory Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like/Dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

3.12 Statistical analysis

Statistical analysis of the data was carried out using GSTAT04 and CPCS1 software developed by Department of Mathematics, Statistics and Physics PAU, Ludhiana.

Chapter IV

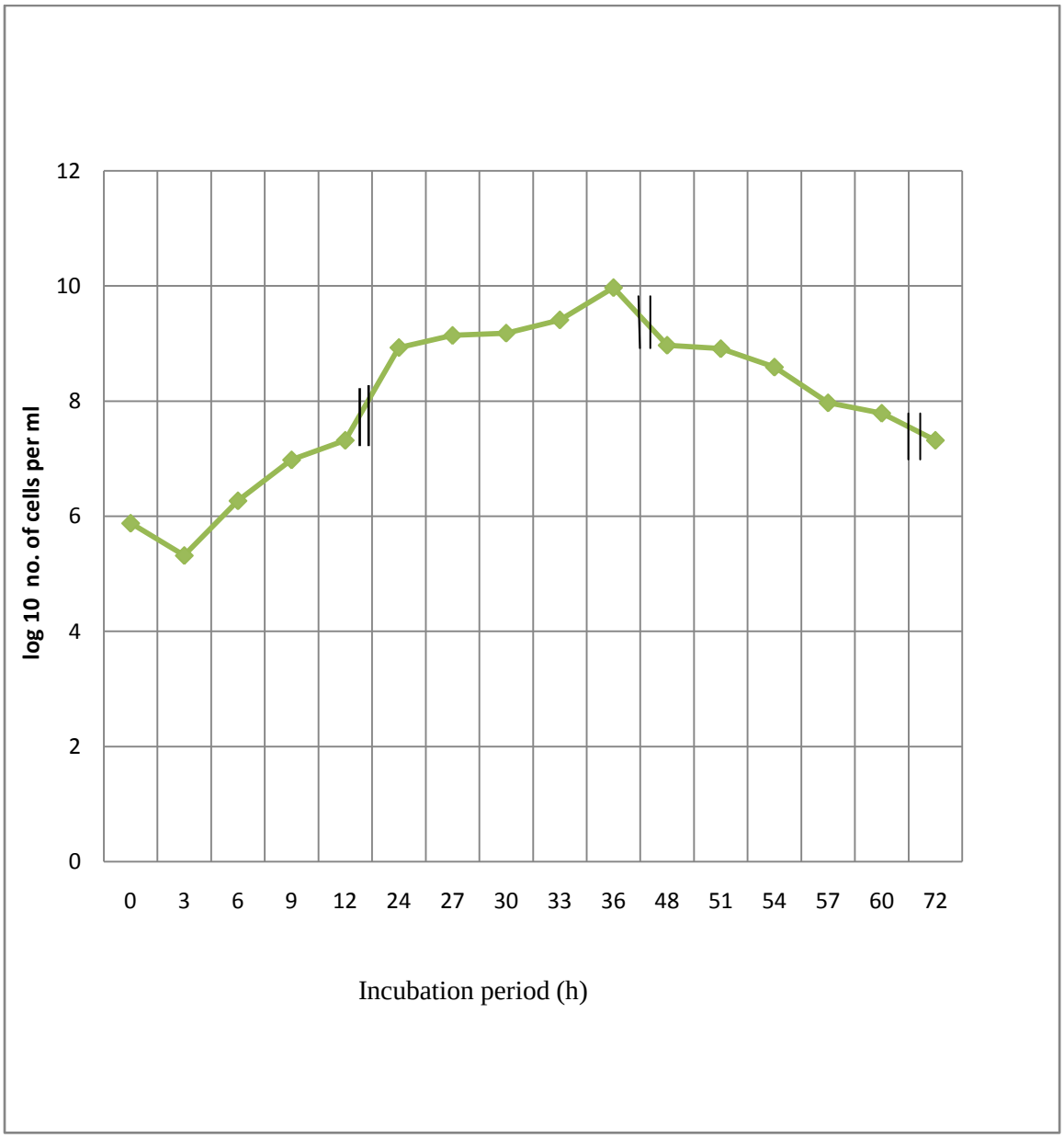
RESULTS AND DISCUSSION

4.1 Studies on growth kinetics of yeast *Clavispora lusitaniae* in guava juice during fermentation

Growth kinetics of yeast *Clavispora lusitaniae* was performed in guava juice, with the physicochemical properties as pulp 15 per cent, pH 4.1, TSS 15°B at temperature 30±2°C during the fermentative phase. Characteristic growth phases could be recognized by monitoring viable cell count (log_{10} no. of cells per ml) during batch fermentation. The growth curve (Figure 1) under aerobic conditions with respect to viable cell count (log_{10} no. of cells per ml) was found to show normal sigmoid curve with first a short lag period of 3 h followed by a phase of exponential growth upto 24 h as indicated by sharp increase in the viable cell count (log_{10} no. of cells per ml) from 5.32 to 8.93. This may be because yeast cells acclimatize fastly to any nutrient composition by changing the lipid composition of their membranes during lag phase followed by exponential phase, while during stationary phase in between 24 h to 36 h there was almost a static viable cell count. This may be due to the fact that after an initial lag phase, exponential growth proceeds primarily by fermentation, whereas respiration is substantially repressed. After the exhaustion of the initial fermentable carbon source, cells metabolically adapt to respiration using ethanol during (diauxic) lag phase that is followed by a second exponential growth phase, although at a slower rate. It has been observed that when cells start growing on non fermentable substrates (glycerol) the second lag phase and exponential phase do not appear, and the initial exponential growth is immediately followed by the stationary phase. Cells may be entering the stationary phase as a result of carbon starvation, but exhausting other nutrients, including nitrogen, phosphorus and sulphur also force cells to enter the stationary phase (Harder and Dijkhnizen 1983).

Stationary phase cells are characterized by altered physiological properties which may exhibit increased resistance to various stresses. The viable cell count decrease from 36 h onwards upto 72 hrs showing a death phase due to nutrient limitation and competition. Death of cells occurs under natural conditions and can be boosted by adjusting the environmental factors beyond the degree of tolerance of microbial cells. The maximum specific growth rate (h^{-1}) and generation time (h) with respect to viable cell count (log_{10} no. of cells per ml) were calculated as 0.39 and 1.77 respectively. These results are in accordance with Dijken *et al* (2000) who reported the specific growth rates of four different *Saccharomyces cerevisiae* on glucose between 0.34 – 0.44 h^{-1}. The specific growth rate (generation time) depends on the number of environmental factors such as presence of nutrients, chemical composition of growth medium, temperature, pH, a_w, oxygen tension and concentration of excreted metabolites.

Figure 1: Growth kinetics of yeast *Clavispora lustitaniae* in guava juice during fermentation



4.2 Physicochemical characteristics of three different Guava varieties

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 flavour and aroma to the beverages during pasteurization and storage (Jairath 2009).

The physicochemical composition of guava pulp evaluated on the basis of chemical analysis (Table 1) 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 respectively.

Jain and Nema (2007) 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 its rich antioxidant properties and flavor guava is ideal for preparation of ready-to-serve beverages.

Table 1: Physicochemical characteristics of three different Guava varieties

Parameters	*Allahabad Safeda*	*Lucknow-49*	*Punjab Pink*
TSS (°B)	10.47	10.56	10.73
Acidity (%)	0.38	0.43	0.41
pH	4.1	4.0	4.1
Brix-acid ratio	27.55	24.56	26.17
Total sugars (%)	6.93	5.57	6.40
Reducing sugars (%)	3.84	3.36	3.57
Ascorbic acid (mg/100mg)	193.7	215.0	184.08
Total phenols (mg/100mg)	314	278	253
Pulp yield (%)	56.75	71.42	66.66

4.3 Shelf life studies of Guava beverage

Shelf life of non-alcoholic naturally carbonated fermented guava beverage of three varieties *Allahabad Safeda, Lucknow-49* and *Punjab Pink* stored at refrigerated temperature (4^0C) was studied for a period of 90 days evaluated fortnightly for physicochemical, microbiological and organoleptic qualities.

4.3.1 Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*

The results (Table 2, Figure 2) show a significant decrease in brix from 15.0°B to 11.2°B and brix acid ratio from 68.18 to 19.65. The pH of the beverage also decreased from 4.2 to 3.8 with subsequent increase in the acidity from 0.22 to 0.57 per cent after fermentation during storage period of 90 days. 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 23.73 per cent as it decreases from 13.27 per cent to 11.76 per cent and gradually decreased to 10.12 per cent at the end of 90 days while the percentage decrease for the reducing sugars was 43.28 per cent as it decreased gradually from 7.3 per cent to 4.14 per cent after 90 days.

The percentage decrease in the ascorbic acid and total phenols was found to be 68.19 percent and 17.73 percent respectively, after fermentation during storage period of 90 days. Storage at low temperature (4°C) and citric acid have a protective role on L-ascorbic acid and tends to slow its oxidation due to its metal sequestrating properties (Jairath 2009). The alcohol production starts after 15 days (0.34% v/v) and reach 0.74 percent v/v after 60 days and up to 1.05 percent v/v after 90 days. The shelf life is standardized on the basis of alcohol production < 1.0%. The CO_2 pressure starts building after 15 days and reaches up to 1.60 bars after 90 days. Viable cell count increased from 34 x 10^6 to 33 x 10^9cfu/ml at the end of 90 days. Its shelf life was found to be 75 days.

Krishnaveni *et al* (2001) observed a decreasing trend in pH, total sugars and ascorbic acid and an increasing trend in the acidity during the six month storage of the jack fruit ready to serve beverage.

Table 2: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS (ºB)	15	14.6	14.0	13.4	12.9	11.7	11.2	0.048
Acidity (%)	0.22	0.28	0.37	0.47	0.49	0.53	0.57	0.0058
pH	4.2	4.2	4.1	4.0	4.0	3.9	3.8	0.045
Brix-acid ratio	68.18	52.14	37.83	28.51	26.32	22.07	19.65	0.67
Total sugars (%)	13.27	12.83	11.76	11.05	10.84	10.48	10.12	0.037
Reducing sugars (%)	7.3	6.9	5.96	5.23	4.92	4.58	4.14	0.051
Ascorbic acid (mg/100ml)	31	27.2	22.3	18.4	16.3	13.5	9.86	0.75
Total phenols (mg/100ml)	43.3	41.4	39.8	38.5	37.7	36.4	35.62	0.029
Alcohol (%,v/v)	-	0.34	0.42	0.67	0.74	0.89	1.05	0.031
CO_2 (bar)	-	0.85	0.92	1.18	1.47	1.53	1.60	0.028
Total yeast count (cfu/ml)	34×10^6	52×10^7	96×10^7	41×10^8	39×10^8	96×10^8	33×10^9	-

% Juice in beverage – 15%

Storage temp - 4±2ºC

* Multiple Regression Equation

$Y = 0.74 – 0.072X_1 + 1.926X_2$

where

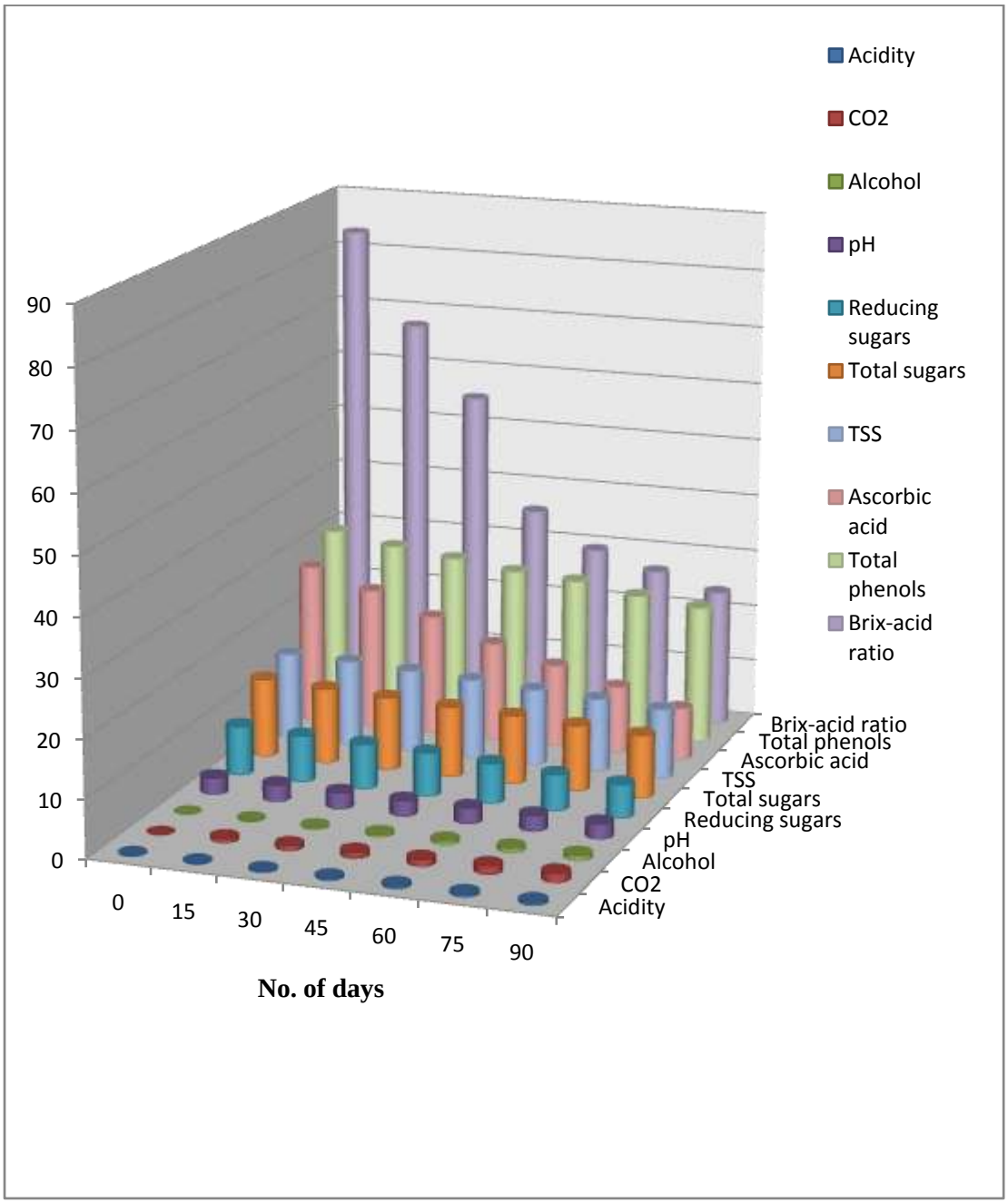
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

** $R^2 = 0.97$

Figure 2: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*



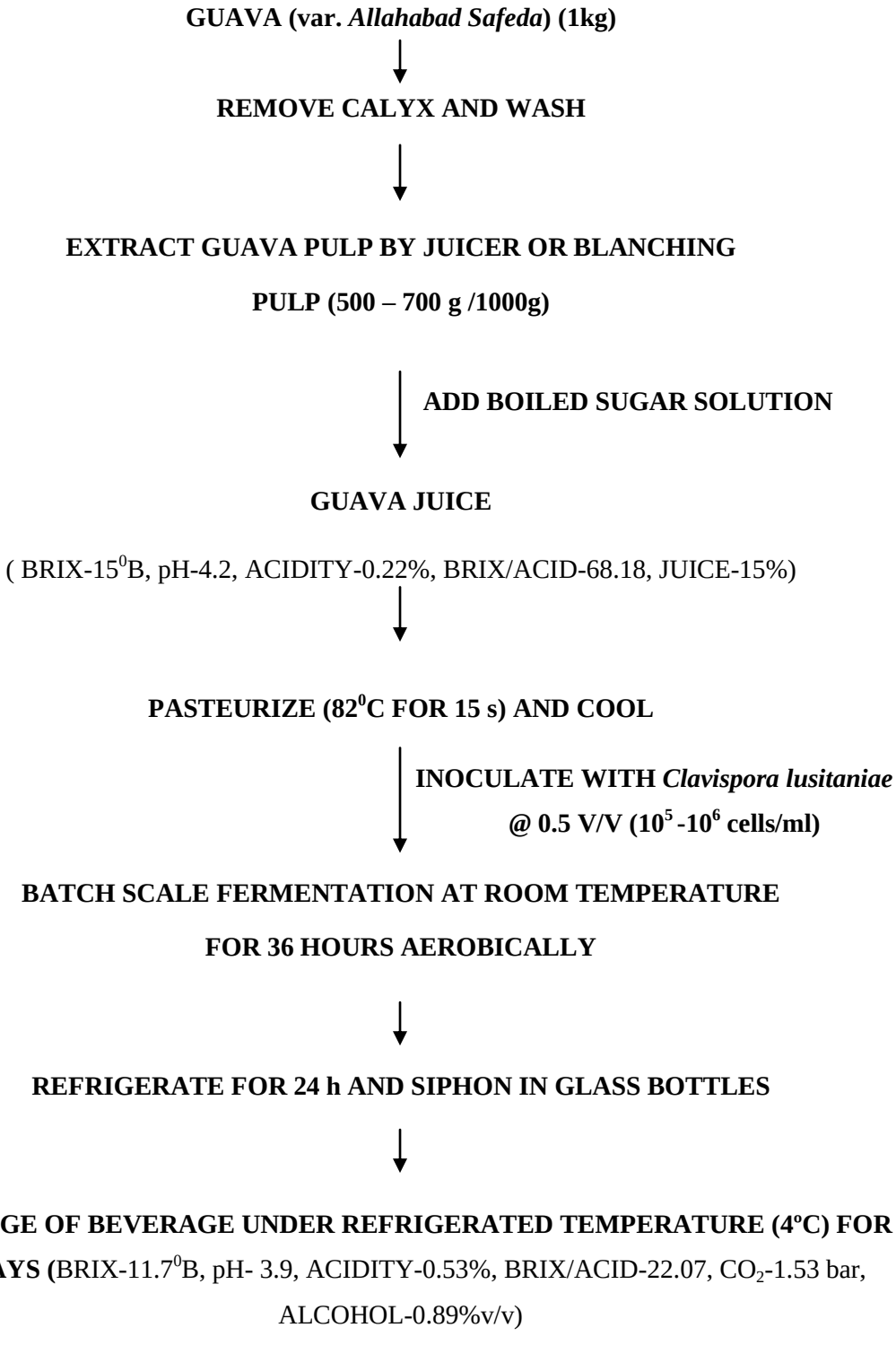


Figure 3: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*

4.3.2 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*

The data on sensory scores of various organoleptic characteristics (Table 3) show that appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability of guava var. *Allahabad Safeda* beverage differ significantly and scores ranged between liked moderately to liked slightly during 90 days of storage. Besides ethanol, a variety of by-products are also produced that influence the sensory characteristics of fermented non-alcoholic naturally carbonated beverages (Jairath 2009).

Aroma and body scores increased from 7.2 to 7.5 and 7.4 to 7.5, respectively upto 45 days whereas flavor and astringency scores increased from 6.5 to 7.0 and 7.0 to 7.5 up to 60 days. The overall acceptability scores were highest up to 15 days of storage. Khurdiya *et al* (1996) reported that the carbonated guava beverage was found acceptable with respect to color, flavor and overall quality during three months of storage. Heat processing improved the flavor of the carbonated guava beverage.

Murtaza *et al* (2004) reported the strawberry beverages stored at refrigeration temperatures (4-6°C) was ranked the best for color, flavor and taste as compared to others stored at room temperatures (25°C). The loss of flavor and taste after 60 days may be due to degradation of ascorbic acid and furfural production.

Table 3: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Allahabad Safeda*

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.0	7.0	7.1	7.0	7.0	7.0	7.0	0.033
Taste	6.9	7.0	7.0	7.1	7.0	7.0	6.9	0.018
Color	7.0	7.0	7.0	6.8	7.0	7.0	7.0	0.016
Aroma	7.2	7.2	7.4	7.5	7.3	7.3	7.3	0.014
Bouquet	7.0	7.15	7.1	7.0	7.2	7.2	7.1	0.016
Body	7.4	7.3	7.38	7.5	7.2	7.3	7.2	0.017
Flavor	6.5	6.8	7.0	7.0	7.0	6.8	6.8	0.02
Astringency	7.0	7.0	7.3	7.5	7.5	7.4	7.4	0.02
Overall acceptability	7.0	7.5	7.25	7.25	7.2	7.0	6.8	0.018

*mean value of three replicates

% Juice in beverage – 15%

Storage temp - 4±2ºC

4.3.3 Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow - 49*

The fermentation of guava beverage var. *Lucknow-49* (Table 4, Figure 4) with yeast results in significant decrease in brix from 15.0°B to 11.9°B and brix acid ratio from 68.18 to 21.25. The pH of the beverage decreased from 4.1 to 3.8 and acidity increased from 0.22 percent to 0.56 percent during fermentation. The decrease in pH and increase in acidity was significant. The percentage decrease in total sugars with storage period of 90 days was 26.85 percent as it decreased from 13.33 percent to 10.92 percent after 60 days and gradually to 9.75% at the end of 90 days while the percentage decrease in the reducing sugars was 38.38 percent 6.67 percent to 4.11 percent after 90 days. As ripened fruit is nutrionally rich in fermentable sugars, hydrolysis of sucrose by fermentable yeast takes place very early stage during fermentation and the glucose level is lower than the fructose level, thus confirms the faster use of glucose as the hydrolysis of sucrose leads to equal quantities of glucose and fructose. Thus the total and reducing sugars decrease with increase in fermentation time (Arora 2009)

During storage period of 90 days under refrigerated conditions, the ascorbic acid and the total phenols decreased from 32.6 mg/100ml to 6.3mg/100ml and 38.4 mg/100ml to 30.72 mg/100ml, respectively. Jairath (2009) reported a loss of 80 per cent ascorbic acid in non-alcoholic naturally carbonated fermented lemon beverage during three months of storage. The product formation i.e. alcohol starts after 15 days (0.32 % v/v) and reached 0.81 percent v/v after 60 days and up to 1.35 percent v/v after 90 days. The CO_2 pressure starts building after 15 days and reached up to 1.97 bars after 90 days. Markides (1986) reported that yeasts ferment the sugar to alcohol producing CO_2 as the by-product having the bottle pressure of about 500-600 KPa (5-6 atmospheres) at 10^0C; after the completion of secondary fermentation and for each 100 KPa of pressure rise, approximately 4g/l of sugar was required. Viable cell count increased to 21 X 10^9 after 90 days of storage period.

These results are in accordance with the observations made by Akubor (2003) during the storage studies of heat and chemically treated melon-banana beverage who reported that the soluble solids of the beverage decreased from 7 to 1.5°B, the titrable acidity increased from 0.1 to 0.6% while the pH dropped drastically from 6.18 to 2.82 by the 21st day of storage. The subsequent decrease in vitamin C level could be attributed to the storage effects i.e. the microorganisms in the beverage may have produced metabolites that probably reduced indophenols.

Table 4: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow-49*

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSSºB	15	14.7	14.1	13.7	13.0	12.6	11.9	0.40
Acidity %	0.22	0.27	0.36	0.41	0.47	0.52	0.56	0.004
Ph	4.1	4.1	4.0	3.9	3.9	3.8	3.8	0.057
Brix-acid ratio	68.18	54.44	39.16	33.41	27.66	24.23	21.25	1.344
Total sugars (%)	13.33	12.91	12.20	11.82	10.92	10.36	9.75	0.0407
Reducing sugars (%)	6.67	6.13	5.86	5.42	5.04	4.53	4.11	0.036
Ascorbic acid (mg/100ml)	32.6	27.5	21.9	17.4	13.2	9.7	6.3	0.17
Total phenols (mg/100ml)	38.4	36.8	35.5	34.1	33.5	31.9	30.72	0.04
Alcohol (%,v/v)	-	0.32	0.44	0.68	0.81	1.14	1.35	0.011
CO_2 (bar)	-	0.79	0.94	1.24	1.52	1.63	1.97	0.0064
Total yeast count (cfu/ml)	21×10^6	39×10^7	94×10^7	26×10^8	80×10^8	74×10^8	21×10^9	-

% Juice in beverage – 15%

Storage temp - 4±2ºC

*Multiple Regression Equation

$Y = 3.818 – 0.269X_1 + 1.27X_2$

Where

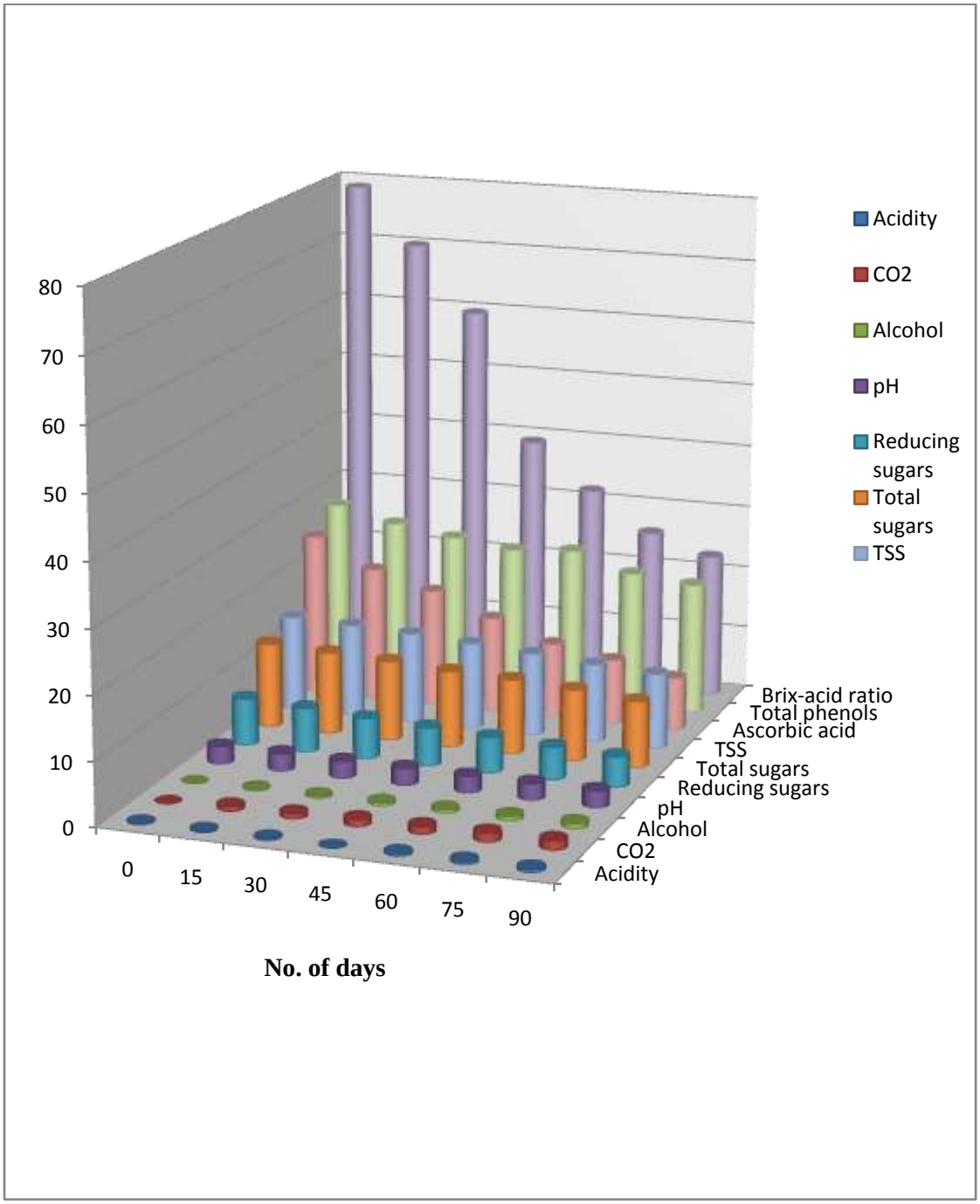
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.97$

Figure 4: Effect of storage time on physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow - 49*



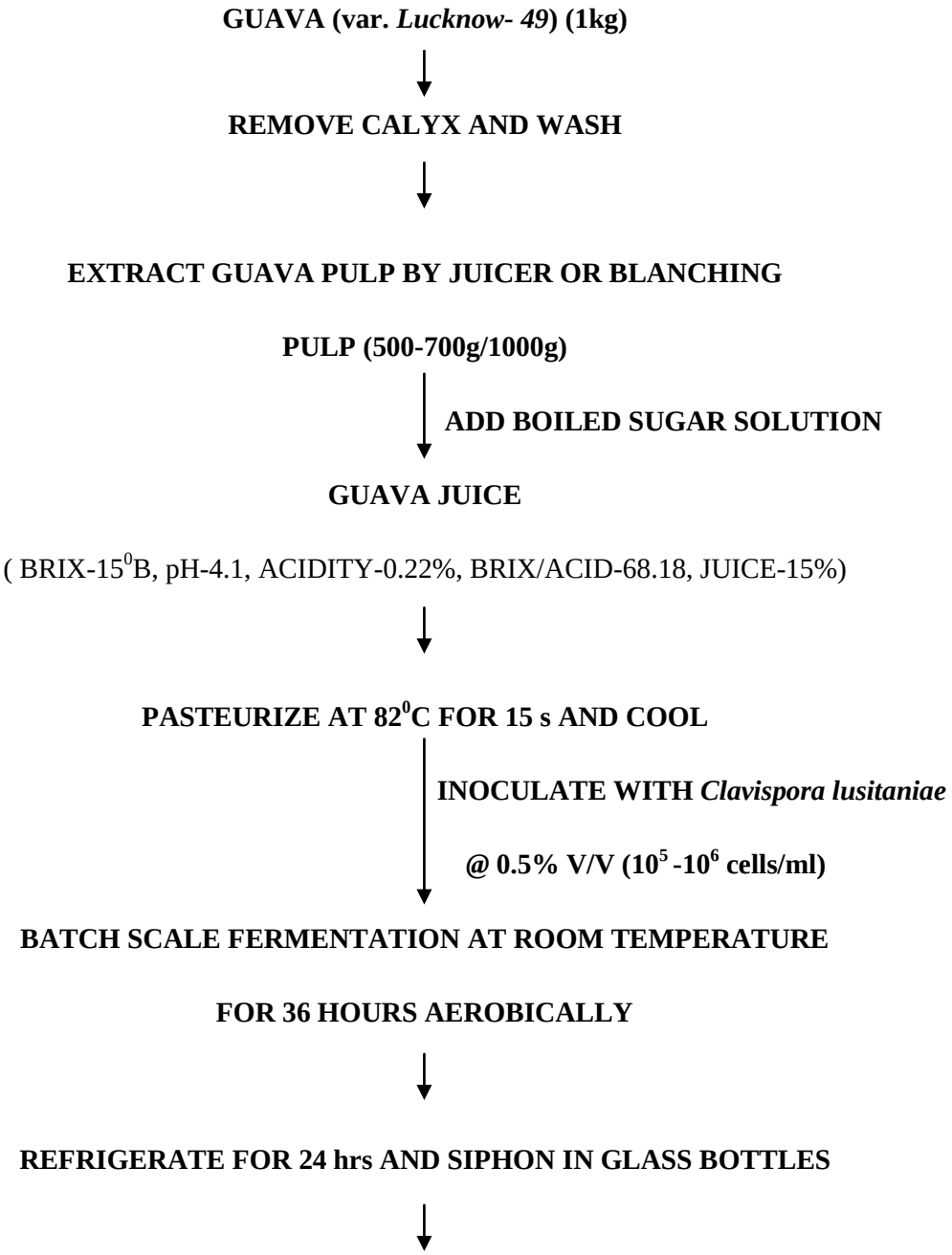


Figure 5: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow-49*

4.3.4 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow - 49*

Effect of storage on mean sensory scores of Lucknow 49 beverage varied significantly for various sensory attributes and ranked between scores liked moderately to liked slightly (Table 5). Appearance and taste scores were maximum up to 30 days. The scores for appearance and taste increased from 7.0 to 7.4 and 7.0 to 7.2, respectively. Bouquet, flavor and astringency scores were maximum up to 45 days. The scores for bouquet, flavor and astringency increased from 7.0 to 7.35, 6.5 to 7.0 and 7.0 to 7.55 up to 45 days, respectively. Akubor (2003) reported the thermally treated melon banana beverages had lower sensory rating for color and flavor during storage which could be due to interaction between reducing sugars and amino acids in the beverage at high temperatures. Volatiles from such interactions impart food characteristic, flavors that may be desirable or objectionable. The overall acceptability scores increased from 7.1 to 7.35 up to 30 days and then gradually decreased to 7.0 at the end of 90 days of storage, respectively due to increased acidity and alcohol content.

Chopda and Barrett (2001) reported that sensory panelists ranked the cloudy guava beverage prepared from aseptic guava puree highest over the juices from pasteurized, clear nectar, freeze dried puree powder or juice powder.

Table 5: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Lucknow – 49*

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.0	7.1	7.4	7.3	7.3	7.3	7.1	0.02
Taste	7.0	7.1	7.2	7.1	7.0	7.0	7.0	0.013
Color	7.2	7.2	7.2	7.2	7.1	7.0	7.0	0.017
Aroma	6.8	6.8	7.0	7.0	7.2	7.0	7.2	0.02
Bouquet	7.0	7.3	7.35	7.35	7.2	7.2	7.2	0.02
Body	6.8	7.0	7.0	7.0	7.0	7.1	7.0	0.024
Flavor	6.5	6.8	7.0	7.0	6.9	6.8	6.87	0.016
Astringency	7.0	7.35	7.5	7.55	7.4	7.4	7.4	0.015
Overall acceptability	7.1	7.3	7.35	7.3	7.2	7.0	7.0	0.017

*mean value of three replicates

% Juice in beverage – 15%

Storage temp - 4±2ºC

4.3.5 Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*

The results of guava beverage var. *Punjab Pink* (Table 6, Figure 6) during fermentation show significant change in physicochemical properties viz. brix from 15.0°B to 12.2°B and Brix acid ratio decreased from 75.0 to 22.14 at the end of 90 days. The pH decreased from 4.3 to 3.9 and acidity gradually increased from 0.20 percent to 0.56 percent during fermentation. This increase in acidity may be due to formation of acid by degradation of polysaccharides and oxidation of reducing sugars or by breakdown of pectic substances and uronic acid (Iqbal *et al* 2001; Hussain *et al* 2008).

The percentage decrease in total sugars was 22.4 percent with fermentation as its initial level 12.9 percent decreased to 11.83 percent after 30 days and 10.0 percent after 90 days. The percentage decrease in the reducing sugars was 48.10 percent as they decreased from 6.34 percent to 5.27 after 30 days and finally reached up to 3.29 percent after 90 days.

There was a significant reduction in ascorbic acid content and total phenols in the beverage. The percentage reduction of ascorbic acid was found to be 74.14 percent as it decreased from 29.4 mg/100ml to 12.2 mg/100ml after 60 days and gradually to 7.6mg/100ml after 90 days. The percentage decrease in total phenols was 21.3 percent as it decreased from 35.7mg/100ml to 28.08 mg/100ml after 90 days of storage. Zvaigzne *et al* (2009) also reported significant decrease in the content of phenolic substances in freshly squeezed grape fruit juice during storage at 4°C.The decrease in the polyphenolic substances is inversely proportional to the non-enzymatic browning reactions which increase slowly throughout the cold storage time (Roberds *et al* 1999).

The alcohol content after 15 days was 0.37 percent v/v and gradually increased to 0.63 percent v/v after 60 days and reached up to 1.13 percent v/v after 90 days. The CO_2 pressure of 0.83 bars at 30 days, it increased to 1.49 bars after 60 days and maximum was 1.78 bars at the end of 90 days. Viable cell count increased from $28x10^6$ to $98x10^8$ after 90 days of storage.

Table 6: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS(ºB)	15	14.8	14.3	13.9	13.3	12.7	12.2	0.05
Acidity (%)	0.20	0.22	0.29	0.36	0.44	0.49	0.56	0.008
pH	4.3	4.2	4.2	4.1	4.0	4.0	3.9	0.04
Brix-acid ratio	75.0	67.27	49.31	38.6	30.2	25.9	22.14	2.49
Total sugars (%)	12.9	12.36	11.83	11.57	11.05	10.6	10.0	0.04
Reducing sugars (%)	6.34	5.96	5.27	4.83	4.19	3.72	3.29	0.02
Ascorbic acid (mg/100ml)	29.4	24.3	20.1	16.5	12.2	9.7	7.6	0.049
Total phenols (mg/100ml)	35.7	33.9	32.7	31.6	30.4	29.1	28.08	0.029
Alcohol (%,v/v)	-	0.37	0.48	0.56	0.63	0.81	1.13	0.03
CO_2 (bar)	-	0.83	0.91	1.14	1.49	1.56	1.78	0.028
Total yeast count (cfu/ml)	28×10^6	46×10^7	89×10^7	32×10^8	76×10^8	67×10^8	98×10^8	-

% Juice in beverage – 15%

Storage temp - 4±2ºC

*Multiple Regression Equation

$Y = 8.32 – 0.52X_1 – 1.58X_2$

Where

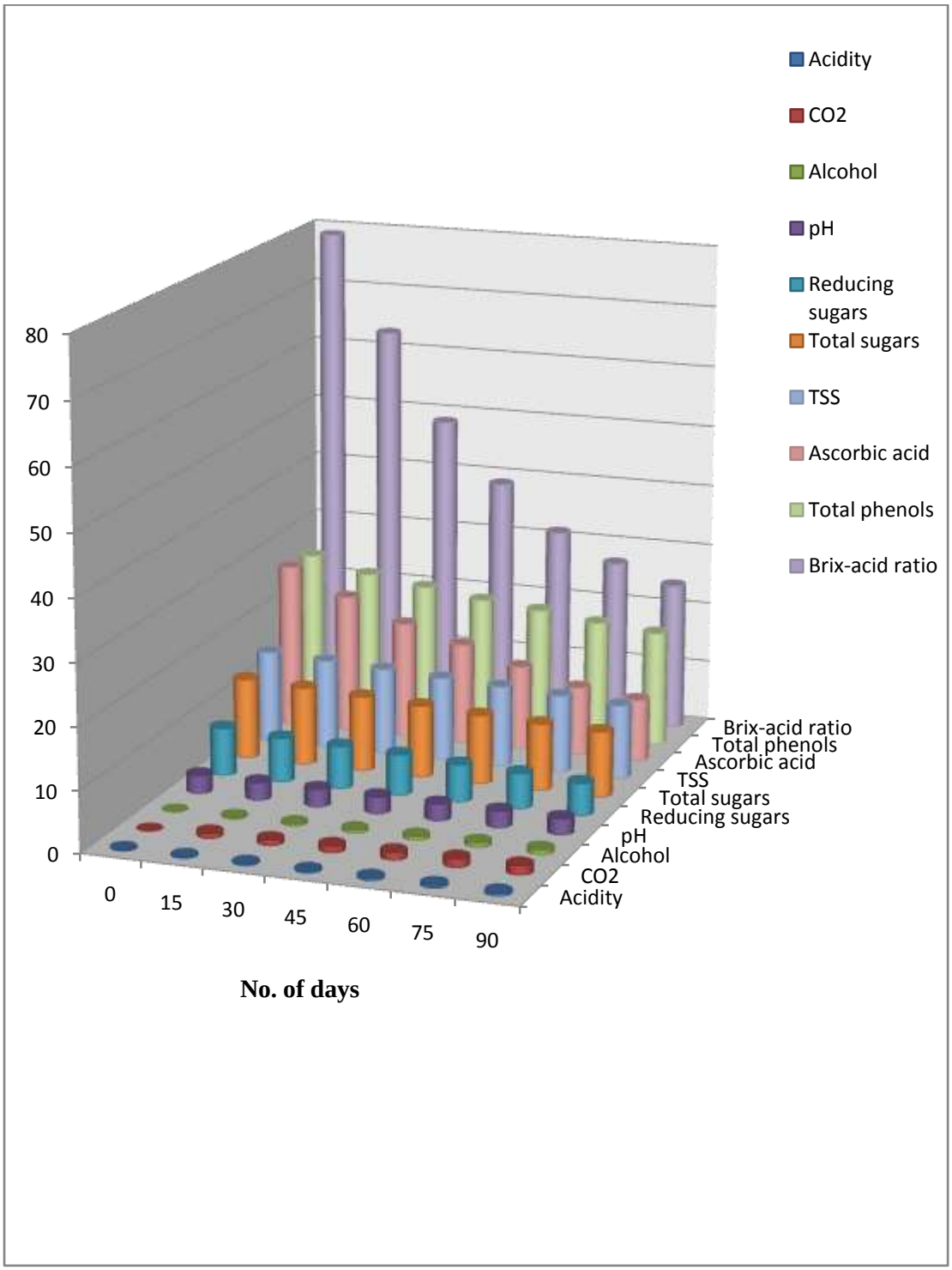
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.90$

Figure 6: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*



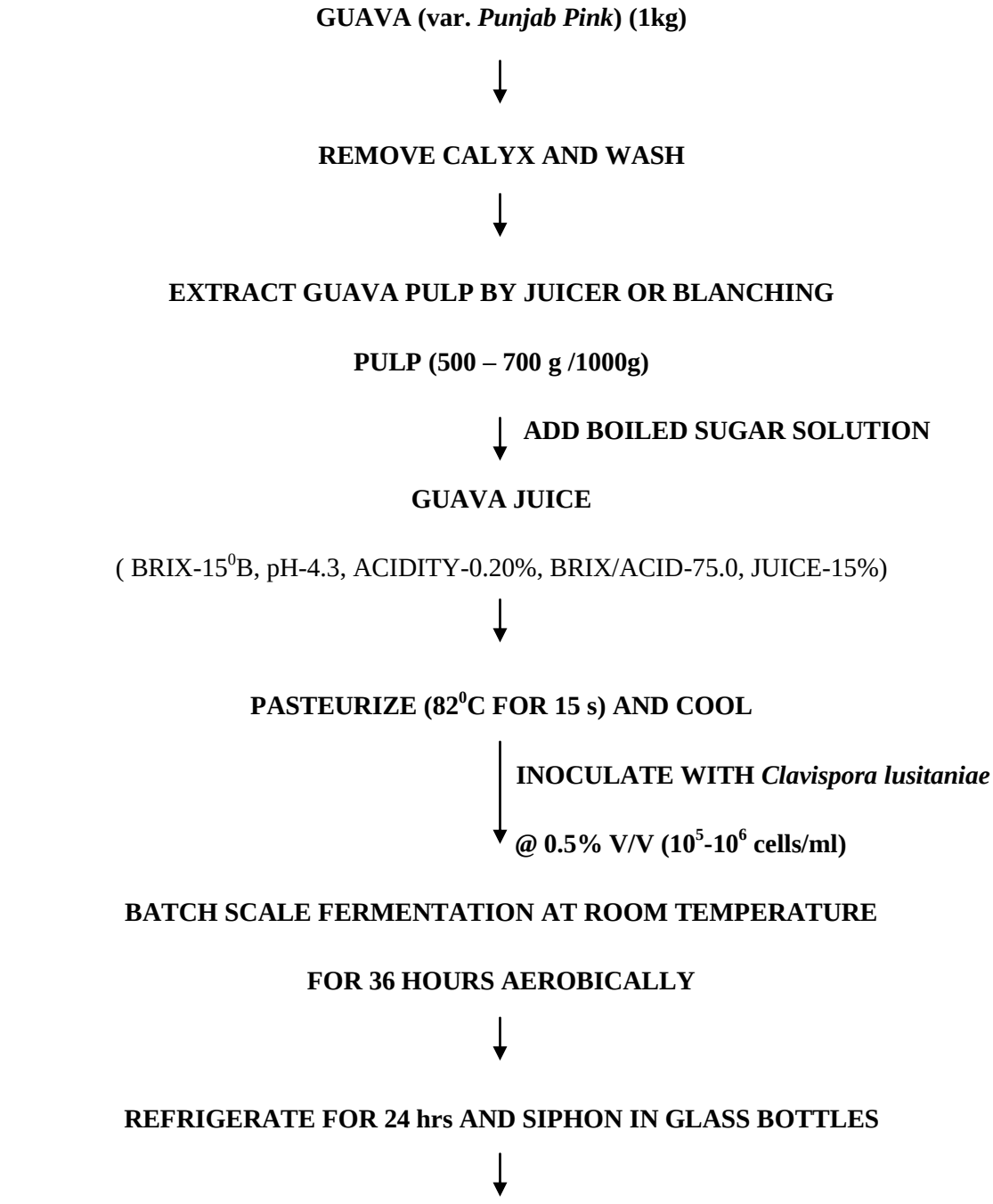


Figure 7: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*

4.3.6 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*

The flavor of guava is due to sugars, acids, phenolics, volatile and aroma active compounds present in it. Storage at 4°C was found to have significant effect on the mean sensory scores for appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability of *Punjab Pink* beverage (Table 7) ranked between scores liked moderately to slightly. The aroma and overall acceptability scores increased from 7.2 to 7.5 and 7.0 to 7.25 up to 5 days. Ogunjobi and Ogunwolu (2010) reported that esters and aldehydes together with tannins and acids already present enhance the taste and aroma of wine during storage. There was significant increase in flavor and astringency scores from 6.5 to 7.0 and 7.0 to 7.5, respectively up to 60 days.

Table 7: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented Guava beverage var. *Punjab Pink*

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.2	7.5	7.4	7.4	7.2	7.3	7.3	0.011
Taste	7.0	7.2	7.2	7.1	7.1	7.0	7.0	0.001
Color	7.0	7.0	7.2	7.0	7.1	7.0	7.0	0.01
Aroma	7.2	7.2	7.4	7.5	7.3	7.3	7.3	0.01
Bouquet	7.0	7.15	7.1	7.0	7.2	7.2	7.1	0.011
Body	7.4	7.3	7.38	7.5	7.2	7.3	7.2	0.01
Flavor	6.5	6.8	7.0	7.0	7.0	6.8	6.8	0.0096
Astringency	7.0	7.0	7.3	7.5	7.5	7.4	7.4	0.01
Overall acceptability	7.0	7.5	7.25	7.25	7.2	7.0	6.8	0.02

*mean value of three replicates

% Juice in beverage – 15%

Storage temp - 4±2ºC

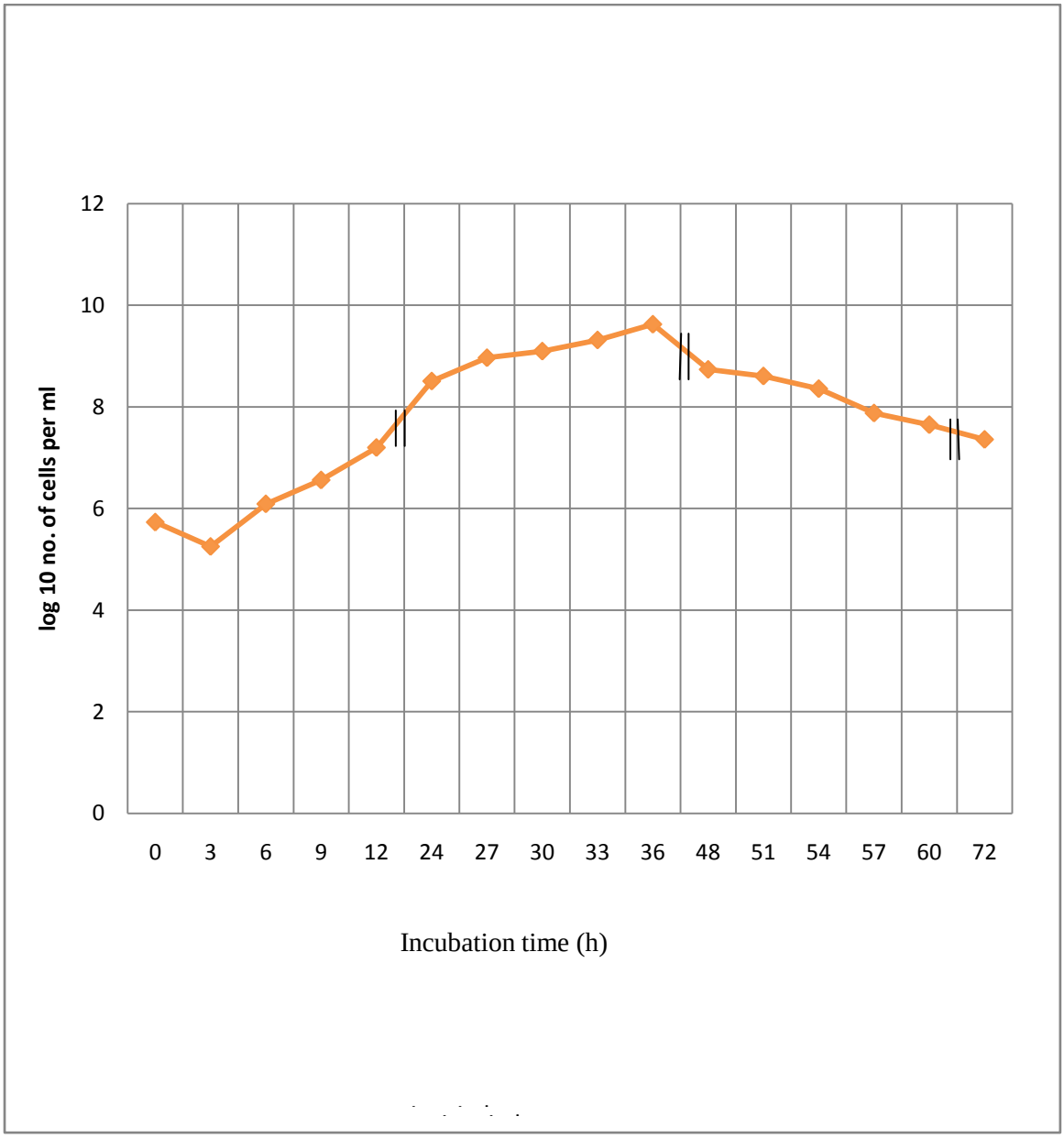
4.4 Studies on growth kinetics of yeast *Clavispora lusitaniae* in blended guava: lemon (1:1) juice during fermentation

The growth curve of yeast *Clavispora lusitaniae* (Figure 8) in blended guava-lemon (1:1) juice with juice per cent 12.5%, pH 3.0, 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). 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* (2007) 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 (Pandove 2007).

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 (2008) 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.

Figure 8: Growth Kinetics of *Clavispora lusitaniae* in blended guava: lemon (1:1) juice during fermentation



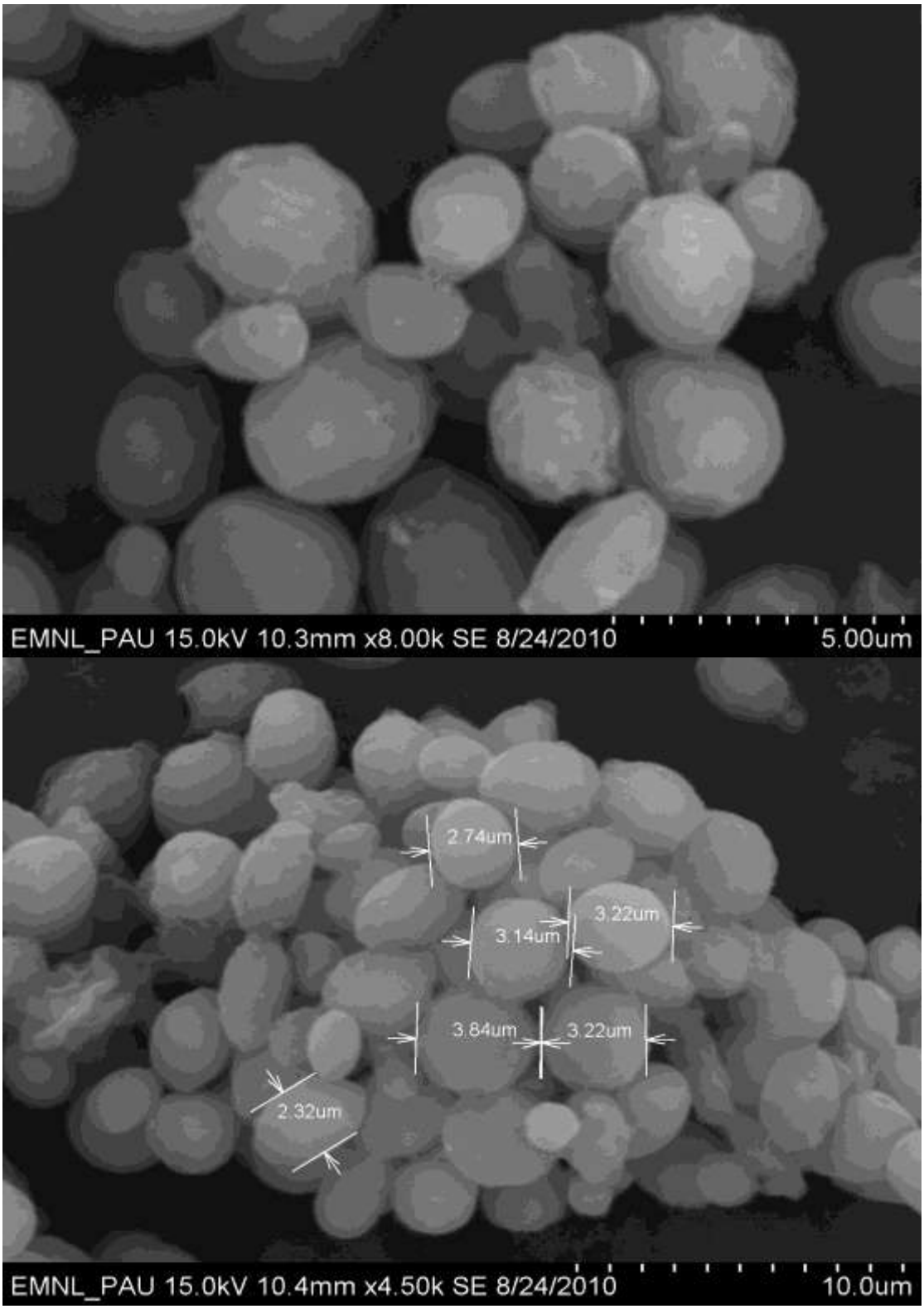


Plate 6: Scanning Electron Micrographs of yeast *Clavispora lusitaniae*

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

The trials for development of non-alcoholic naturally carbonated fermented beverage from different blends of guava and lemon with improved sensory scores and nutritional quality were carried out (Table 8). 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), 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 non-alcoholic naturally carbonated blended beverage from guava and lemon.

Pandove (2007) 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. Tiwari (2000) prepared RTS beverage from guava-papaya blends with 15 per cent pulp, 14°B and 0.3 per cent acidity and found that RTS prepared from guava-papaya (70:30) blend had organoleptically better consistency and flavor.

Table 8: 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

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

The results of blended *Allahabad Safeda* and *Baramasi* beverage (Table 9, Figure 9) 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 0.26 percent after 30 days and then increased to 0.45 percent after 60 days and finally reached up 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 was 20.08 percent as it reduced from initial level 13.64 percent to 12.71 percent after 30 days and then decreased to 12.03 percent at the end of 90 days. The percentage decrease in the reducing sugars was 32.12 percent as initial sugar level decreased from 8.56 percent to 7.82 percent after 30 days and then decreased to 5.81 percent at the end of 90 days. Burdurlu *et al* (2006) reported 17 – 85 per cent degradation of vitamin C in citrus juice concentrates during storage at different temperatures. Ocloo and Ayernor (2008) 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 17.2 mg/100ml after 45 days and reached up 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 increased to 0.65 percent v/v after 60 days and 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 35×10^8 to 15×10^9 cfu/ml during storage.

Table 9: 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	
TSSºB	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±2ºC

*Multiple Regression Equation

$Y = 2.20 – 0.15X_1 + 0.99X_2$

Where

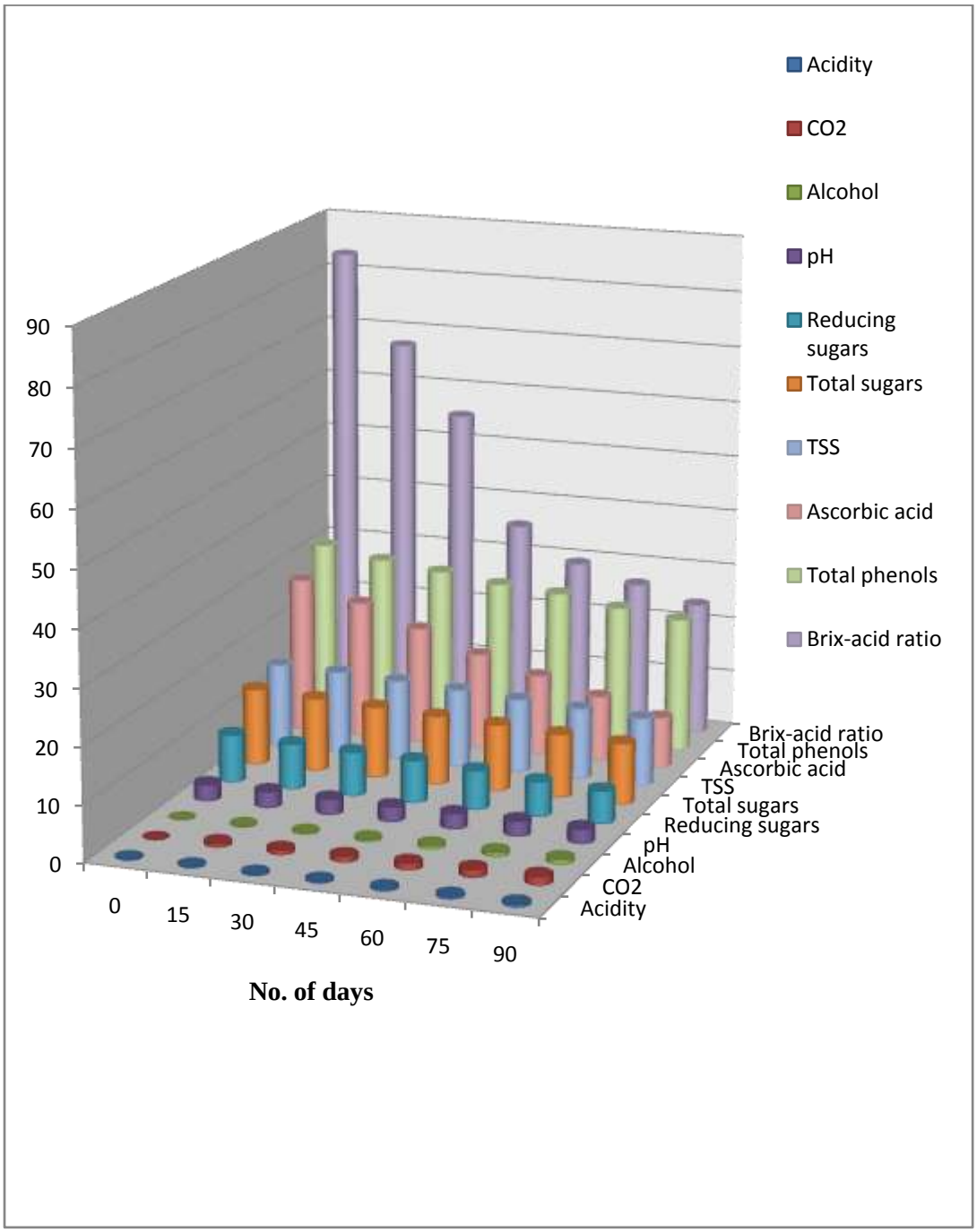
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.95$

Figure 9: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda* - *Baramasi* beverage (1:1)



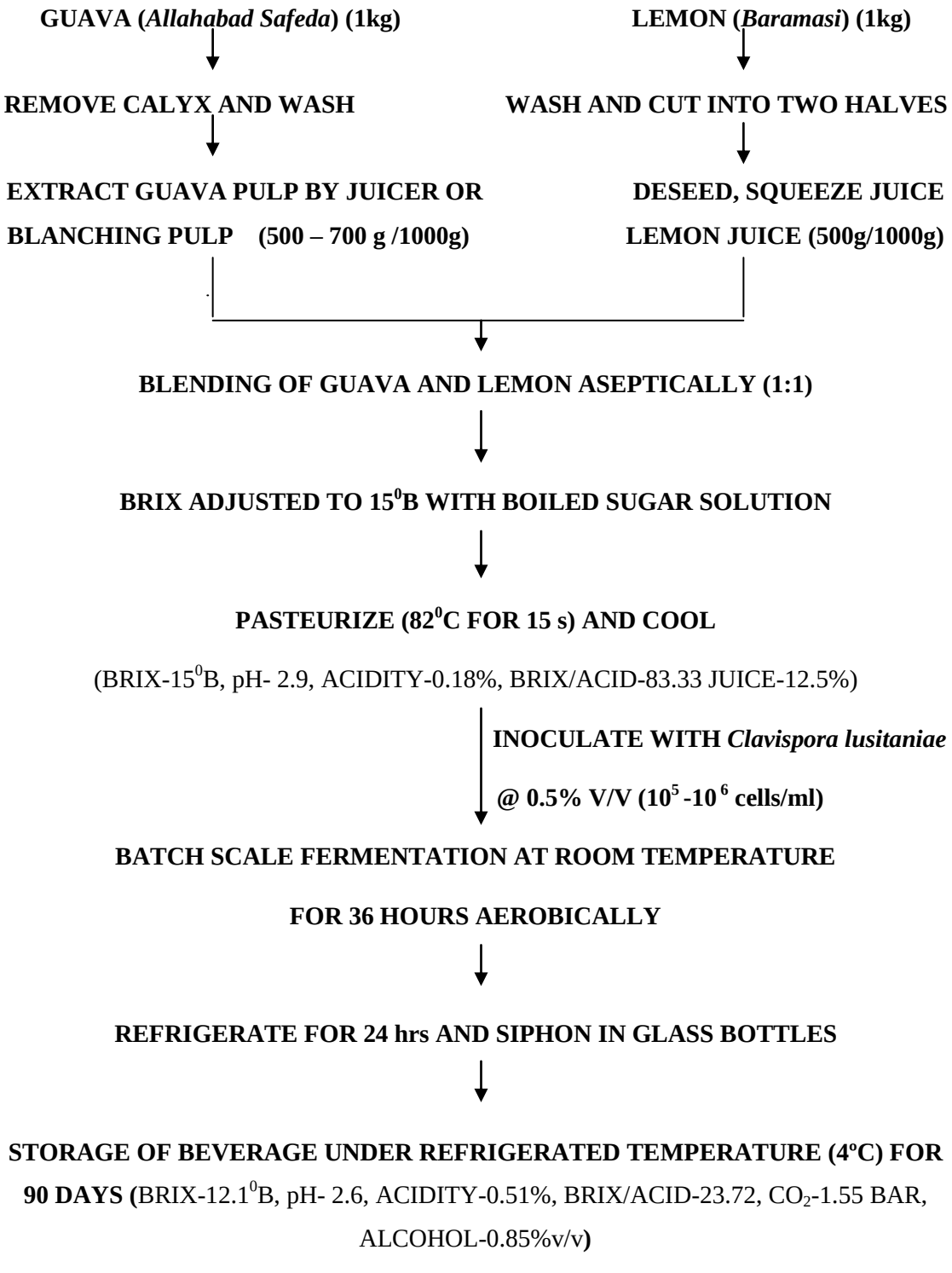


Figure 10: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda- Baramasi* beverage (1:1)

4.7 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda- Baramasi* beverage (1:1)

The mean sensory scores for appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability were ranked between liked very much to moderately after 90 days of storage (Table 10). The beverage had an appealing effervescent sparkling straw colored appearance. There was a significant variation among the different sensory attributes like taste, color, aroma, bouquet and body. Ethanol and glycerol are among the primary products of yeast fermentation, and they determine the body of the beverage. The formation of glycerol depends on various factors, such as temperature, pH, sugar concentration and yeast strain. The flavor and overall acceptability scores also increased significantly from 7.5 to 8.0 up to 30 days. The mean scores for astringency increased from 7.5 to 7.75 up to 30 days and then decreased to 7.1 at the end of 90 days of storage.

The deterioration in quality of guava can be due to non enzymatic browning reactions through the involvement of ascorbic acids and tannins. The organoleptic scores and overall acceptability of the whey based banana herbal beverage improved with increase in Mentha extract from 0-2% during storage (Yadav *et al* 2010).

Table 10: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda- Baramasi* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.3	7.3	7.3	7.2	7.2	7.3	7.2	0.012
Taste	7.4	7.6	7.6	7.5	7.5	7.5	7.4	0.027
Color	7.3	7.3	7.3	7.3	7.2	7.2	7.2	0.015
Aroma	7.0	7.25	7.4	7.35	7.4	7.4	7.34	0.012
Bouquet	7.2	7.34	7.3	7.25	7.25	7.2	7.0	0.011
Body	7.0	7.1	7.4	7.4	7.4	7.25	7.25	0.011
Flavor	7.5	8.0	8.0	7.8	7.8	7.8	7.7	0.3
Astringency	7.5	7.75	7.75	7.6	7.4	7.2	7.1	NS
Overall acceptability	7.5	8.0	8.0	7.5	7.4	7.4	7.4	0.011

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

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

The results of blended beverage in (Table 11, Figure 11) 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 0.24 percent after 30 days and finally reached up to 0.52 percent at the end of 90 days. Sirohi *et al* (2005) and Naik *et al* (2009) 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 decreased from 13.45 percent to 12.69 percent after 30 days and then decreased to 10.50 percent at the end of 90 days. The reducing sugars decreased from 7.45 percent to 5.63 percent after 60 days and then decreased to 4.82 percent at the end of 90 days. The percentage decrease in the total sugars was 21.93 percent while of reducing sugars was 35.30 percent.

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* (2003) 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 (Hand *et al* 2006). It is also very much dependent on pH i.e. the juice with higher pH are much less susceptible to browning (Phattaraworrasuth and Chiewchan 2008).

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 increased to 1.23 after 60 days and reached to 1.50 bars after 90 days. The viable cell count increased from $41 \times 10^6 – 25 \times 10^9$ cfu/ml.

Table 11: 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	
TSSºB	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±2ºC

*Multiple Regression Equation

$Y = 4.94 – 0.31X_1 – 0.63X_2$

Where

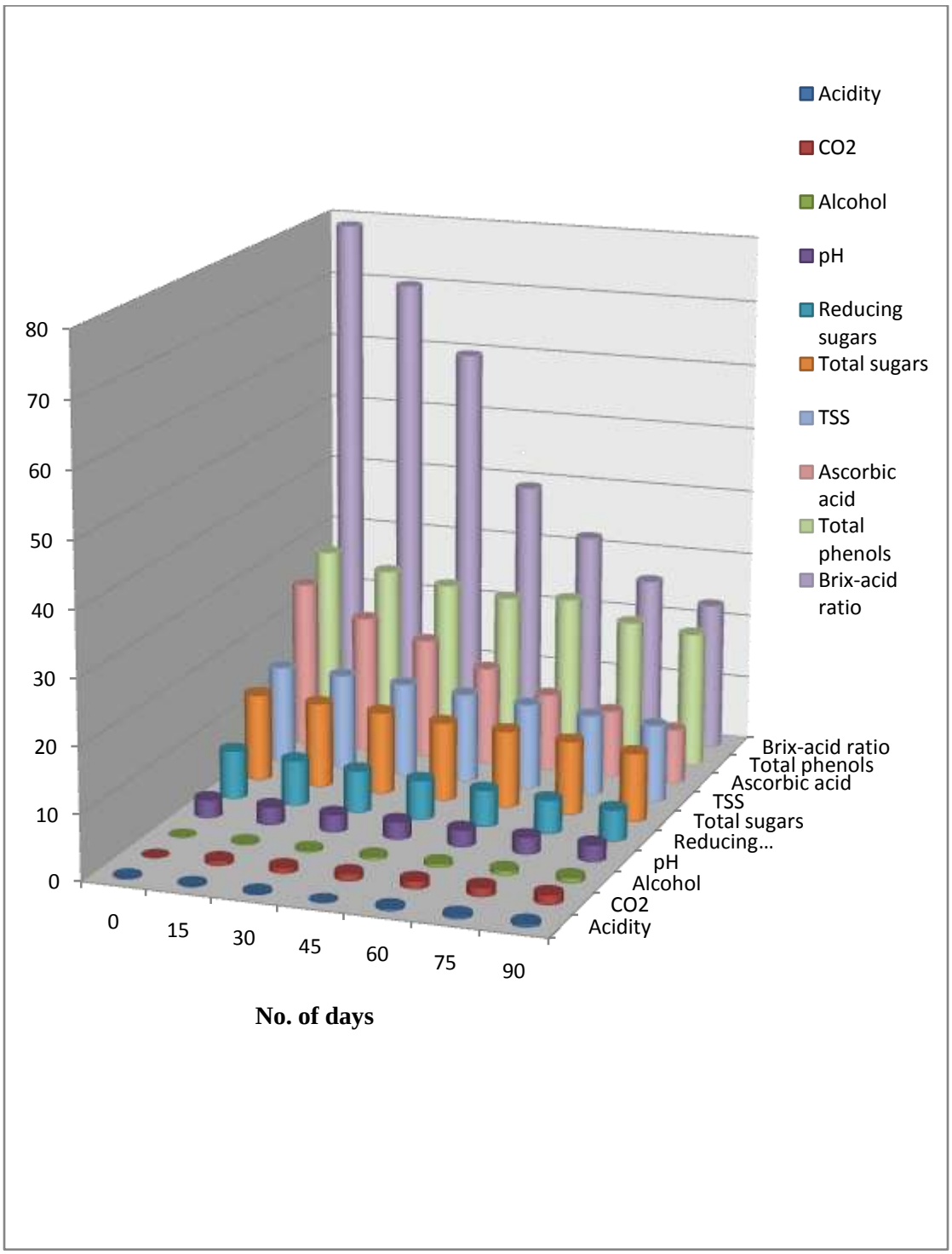
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.94$

Figure 11: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented *Lucknow-49* – *Baramasi* beverage (1:1)



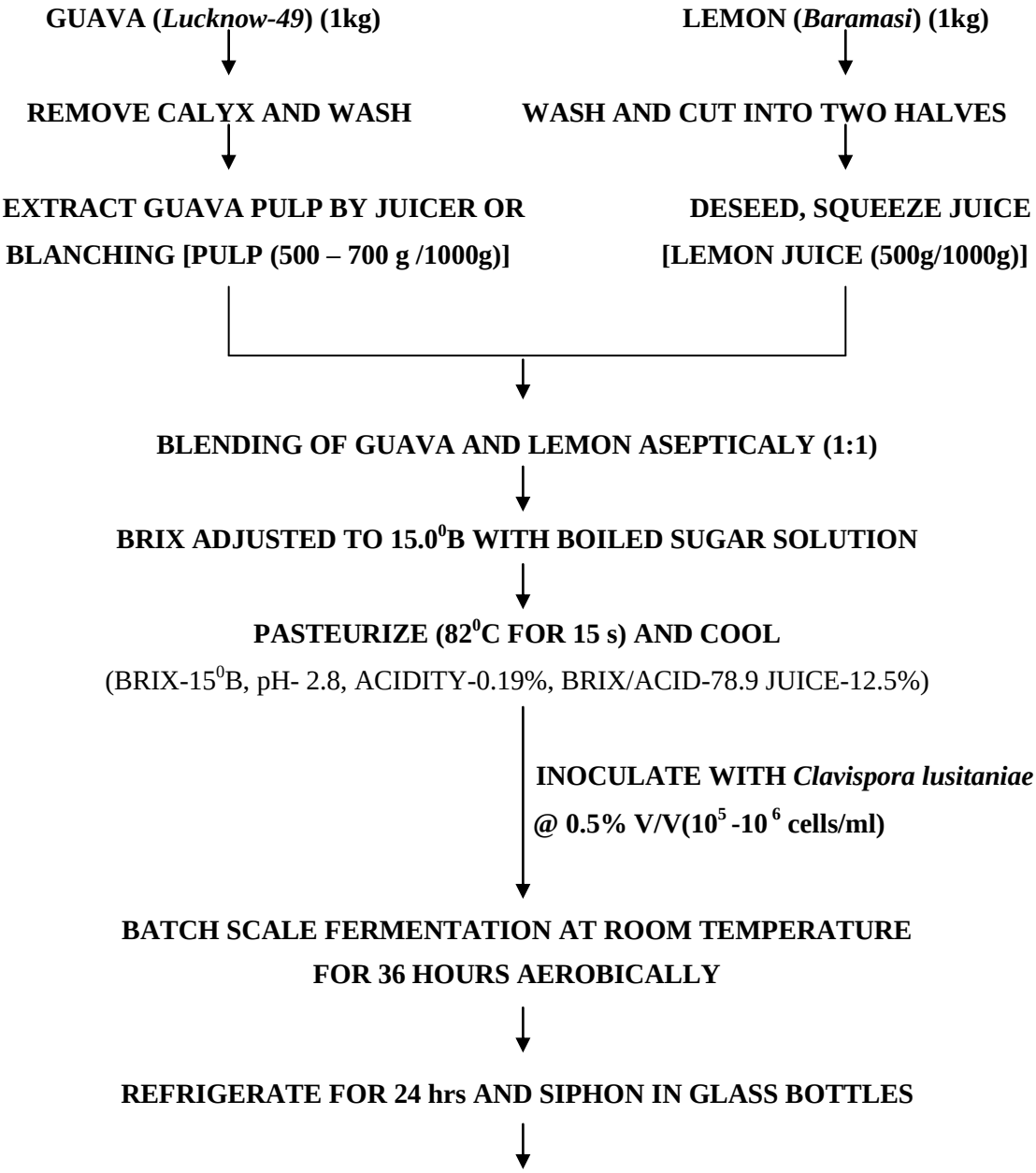


Figure 12: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Lucknow-49- Baramasi* beverage (1:1)

4.8 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Lucknow-49 – Baramasi* beverage (1:1)

The data on sensory scores of various sensory characteristics (Table 12) shows that appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability of beverage differ significantly and ranked moderately. The taste scores increased from 7.6 to 7.8 up to 45 days with a significant variation in other attributes like aroma, bouquet and body during storage period of 90 days. Kumar (1997) found that carbonated pure mandarin juice beverage at 100 psi pressure of carbonation was adjusted the best having highest sensory quality characteristics as well as acceptable storage life for the period of 6 months.

There was a significant decrease in scores for flavor and astringency after 30 days and 15 days respectively. The amount of aliphatic esters and terpenic compounds were mainly responsible for the unique flavor of guava. Singh *et al* (1999) reported the decrease in the organoleptic acceptability of various blended guava and pineapple ready to serve beverages was due to the changes in the composition of total soluble solids, total sugars, reducing sugars and ascorbic acid content over a storage time of 120 days.

Table 12: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Lucknow-49 – Baramasi* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.0	7.2	7.1	7.0	7.0	7.0	7.0	0.011
Taste	7.6	7.7	7.8	7.8	7.7	7.6	7.6	0.016
Color	7.5	7.5	7.4	7.45	7.45	7.4	7.4	0.010
Aroma	7.4	7.5	7.6	7.5	7.5	7.6	7.5	0.011
Bouquet	7.6	7.76	7.72	7.55	7.5	7.5	7.5	0.011
Body	7.0	7.3	7.35	7.3	7.2	7.2	7.2	0.011
Flavor	7.5	7.65	7.77	7.5	7.4	7.0	6.8	0.010
Astringency	7.7	7.63	7.57	7.5	7.32	7.25	7.25	0.002
Overall acceptability	7.6	7.5	7.5	7.5	7.4	7.3	7.3	0.010

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

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

The results of blended beverage in (Table 13, Figure 13) 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 0.29 per cent after 30 days and reached upto 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 (Divya and Kumari 2009). The percentage decrease in the total sugars was 18.96 per cent as it decreased from 13.18 percent to 12.32 percent after 30 days and 10.68 percent after 90 days. The percentage decrease in reducing sugars was 32.56 percent as it decreased from 7.83 percent to 6.96 percent after 30 days and gradually decreased to 5.28 percent after 90 days.

The ascorbic acid decreased from 26.9 mg/100ml to 10.2 mg/100ml and total phenols decreased from 26.4 mg/100ml to 18.72 mg/100ml at the end of 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 (Egbere *et al* 2009). Kabasakalis *et al* (2000) 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.

Table 13: 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	
TSSºB	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

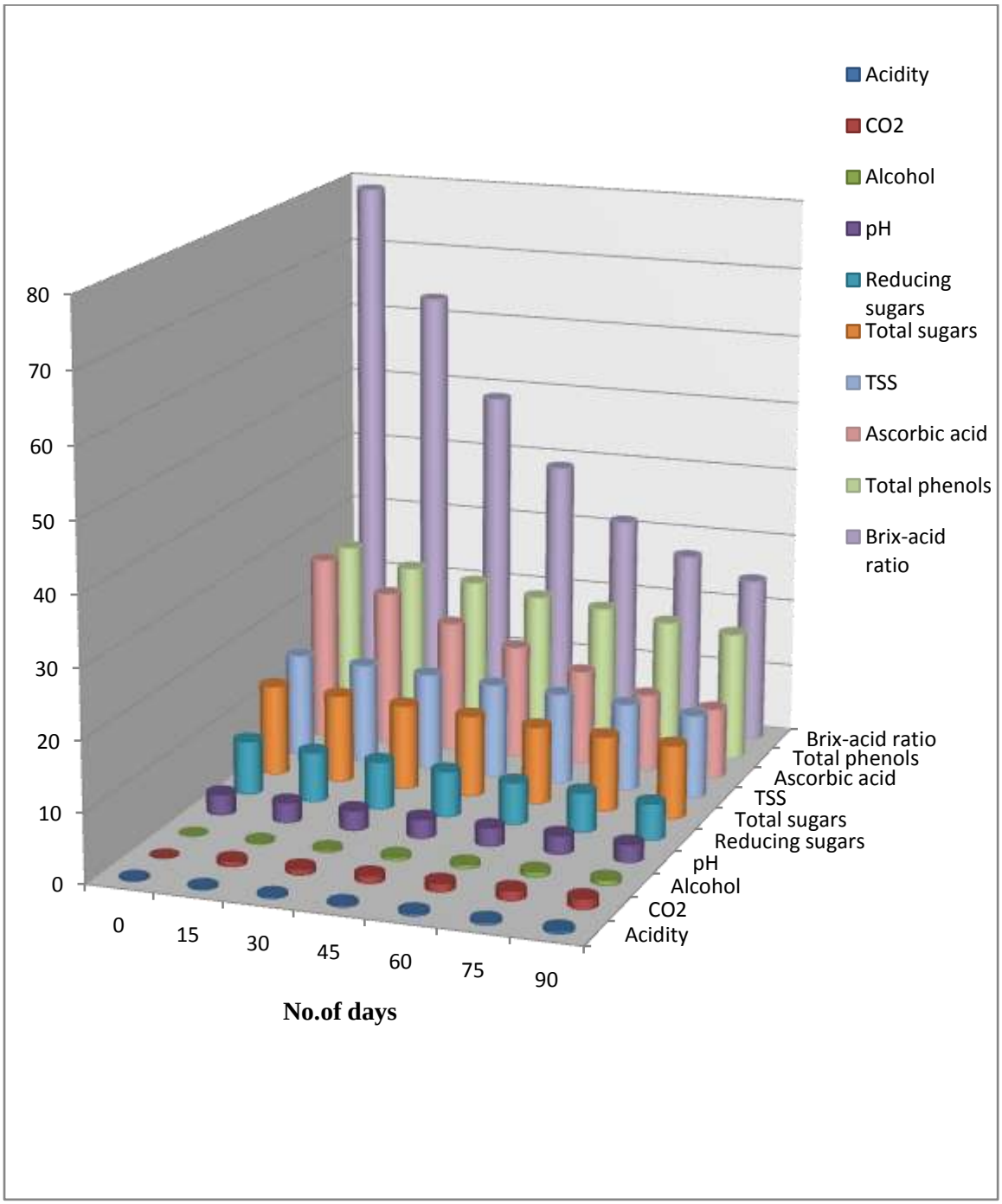
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.95$

Figure 13: Effect of storage time on microbiological and physicochemical properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink* – *Baramasi* beverage (1:1)



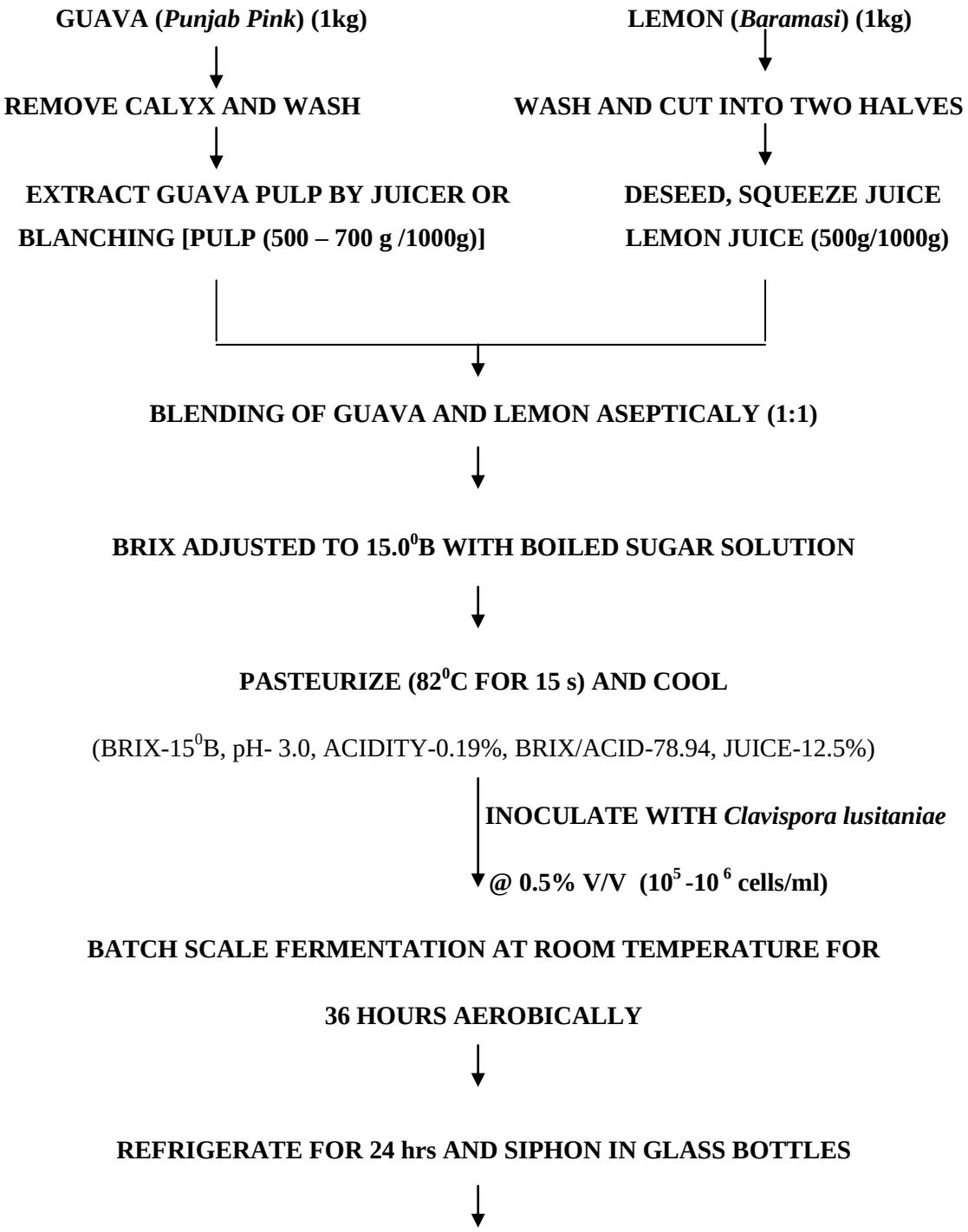


Figure 14: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Punjab Pink- Baramasi* beverage (1:1)

4.10 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Baramasi* beverage (1:1)

The blended beverage shows significant variation in the sensory attributes and the scores ranged between liked very much to moderately (Table 14). Various fermentation parameters affects lipid metabolism of yeast which is related to cell development, membrane integrity and the production of several by-products, especially those directly related to the various organoleptic properties of beverage. Both the primary and secondary products of yeast metabolism contribute to the bouquet of beverage including ethanol, glycerol, higher alcohols, organic acids, esters, aldehydes and ketones. The mean sensory scores for taste, flavor and astringency were maximum (7.6, 8.2 and 8.0 respectively) up to 45 days and the scores decreased till 90 days of storage. But due to the tangy taste and eye appeal of effervescence it was acceptable till 3 months. Akhtar *et al* (2010) reported that the panelists clearly identified the changes in flavor profile of the mango pulp samples rating the stored sample inferior as compared to the freshly prepared ready to serve mango beverages. He also reported that storage time perpetually decrease flavor score until 90 days storage, nevertheless the mango drink samples were still liked by the judges for color and flavor.

The mean sensory scores for overall acceptability increased from 7.5 to 8.0 up to 45 days and then decreased to 7.3 at the end of 90 days due to increase in carbonation and increase in acidic content after storage. Sener *et al* (2007) reported that the fruit esters are both synthesized and retained to a greater degree at cool temperatures during fermentation. Under the influence of oxygen from the air, the color and taste will change as a result of oxidation reaction.

Table 14: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink* – *Baramasi* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.2	7.4	7.4	7.3	7.3	7.3	7.3	0.02
Taste	7.3	7.5	7.6	7.6	7.5	7.5	7.4	0.018
Color	7.1	7.1	7.0	7.0	7.1	7.1	7.0	0.017
Aroma	7.0	7.5	8.0	7.75	7.5	7.5	7.5	0.017
Bouquet	7.2	7.35	7.4	7.36	7.36	7.3	7.34	0.013
Body	7.5	7.5	7.45	7.4	7.45	7.5	7.45	NS
Flavor	7.5	7.8	8.0	8.2	7.5	7.5	7.0	0.014
Astringency	7.5	8.0	7.8	8.0	7.8	7.9	7.9	0.012
Overall acceptability	7.5	7.75	8.0	8.0	7.8	7.5	7.3	0.011

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

Plate 7: Batch Scale Fermentation in Fermentors

Plate 8: Carbonation in glass bottles

4.11 Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

The results of blended beverage (1:1) (Table15, Figure 15) show significant decrease in Brix from 15.0°B to 12.73°B and brix acid ratio from 51.72 to 20.86. The pH of the beverage decreased from 3.0 to 2.8 and acidity significantly increased from 0.29 per cent to 0.61 per cent at the end of 90 days which is due to the production of various organic acids as the by-products of fermentation. Sampedro *et al* (2009) also observed decrease in the °Brix values of PEF treated and thermally processed orange juice-milk based beverage. The percentage decrease in the total sugars was 23.72 per cent as it decreased from 10.58 per cent to 9.46 percent after 30 days and 8.07 per cent after 90 days. The percentage decrease in reducing sugars was 33.11 per cent as the sugars reduced from 7.64 per cent to 5.11 per cent at the end of 90 days. However, there was significant reduction in sugar level with the progress of the fermentation with increasing number of days because yeast cells produce a lot of enzymes such as glycosidase which swiftly break down sugars and convert them into the secondary metabolites.

There was a significant decrease in ascorbic acid and total phenols during fermentation. The ascorbic acid decreased from 28.7 mg/100ml to 5.3 mg/100ml while total phenols decreased from 34.3 mg/100ml to 26.62 mg/100ml after 90 days. The fermenter being impermeable to oxygen; therefore, vitamin C destruction is probably caused by oxidation by residual oxygen in the head space followed by anaerobic decomposition and by the destructive effect of light especially since the beverage is packaged in transparent bottles. Akubor (2003) reported 93% loss of vitamin C during 50 days storage period of melon-banana beverage.

The alcohol formation began after 15 days (0.18% v/v) and increased to 0.51% v/v after 60 days and to 0.98% v/v after 90 days. The CO_2 pressure of 0.84 started after 30 days and increased to 0.90 bars after 60 days and after 90 days reached upto 1.50 bars. The viable cell count increased from $25x10^6$ to $28x10^9$cfu/ml after 90 days. Arora (2009) reported increase in alcohol per cent, CO_2 pressure and viable cell count to 0.86 per cent, 1.5 bar and $3.3x10^8$ during 90 days of storage of non-alcoholic naturally carbonated lemon beverage The evaluation of parameters like propanol, butanol, acetaldehyde, methanol, ethanol, ethyl acetate and isopropanol was done by GC Headspace Injection and TR Wax Column, detected by FID of which only ethanol was present (0.98%) and all other parameters were absent (Table 16).

Table 15: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS°B	15.0	14.80	14.50	14.0	13.65	13.11	12.73	0.04
Acidity %	0.29	0.34	0.40	0.46	0.48	0.54	0.61	0.004
pH	3.0	2.9	2.9	3.0	2.8	2.8	2.8	0.03
Brix-acid ratio	51.72	43.53	36.25	30.43	28.43	24.27	20.86	0.27
Total sugars (%)	10.58	10.02	9.46	9.23	8.72	8.39	8.07	0.044
Reducing sugars (%)	7.64	7.38	6.97	6.53	6.18	5.74	5.11	0.04
Ascorbic acid (mg/100ml)	28.7	24.9	20.1	17.4	13.9	9.6	5.3	3.04
Total phenols (mg/100ml)	34.3	32.2	31.1	30.2	29.0	27.8	26.62	0.249
Alcohol (%,v/v)	-	0.18	0.23	0.45	0.57	0.69	0.98	0.004
CO_2 (bar)	-	-	0.54	0.70	0.90	1.2	1.5	0.003
Total yeast count (cfu/ml)	25×10^6	41×10^7	77×10^7	25×10^8	70×10^8	96×10^8	28×10^9	-

% Juice in beverage – 12.5%

Storage temp - 4±2°C

*Multiple Regression Equation

$Y = 2.03 – 0.16X_1 + 1.69X_2$

Where

Y = per cent alcohol

X_1= °Brix

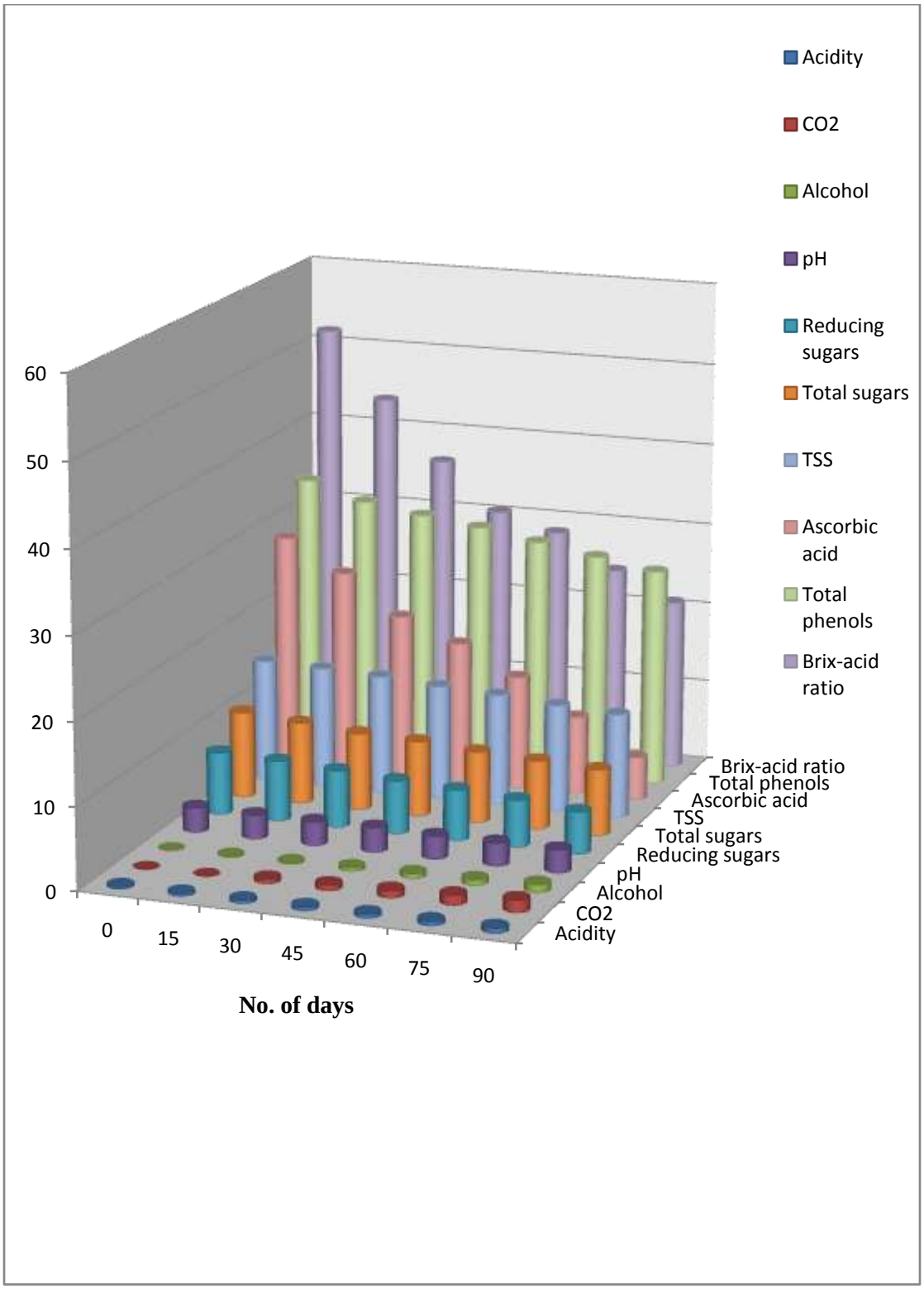
X_2= per cent acidity

**$R^2 = 0.98$

Table 16: Evaluation of volatile components in non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

S.No.	Parameter	Results	Standard/ Specification/ Method Followed
1.	Propanol	Absent	GC Headspace Injection, TR Wax Column, Detection by FID
2.	Butanol	Absent	
3.	Acetaldehyde	Absent	
4.	Methanol	Absent	
5.	Ethanol	0.98%	
6.	Ethyl acetate	Absent	
7.	Isopropanol	Absent	

Figure 15: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda* – *Citrus latifolia* beverage (1:1)



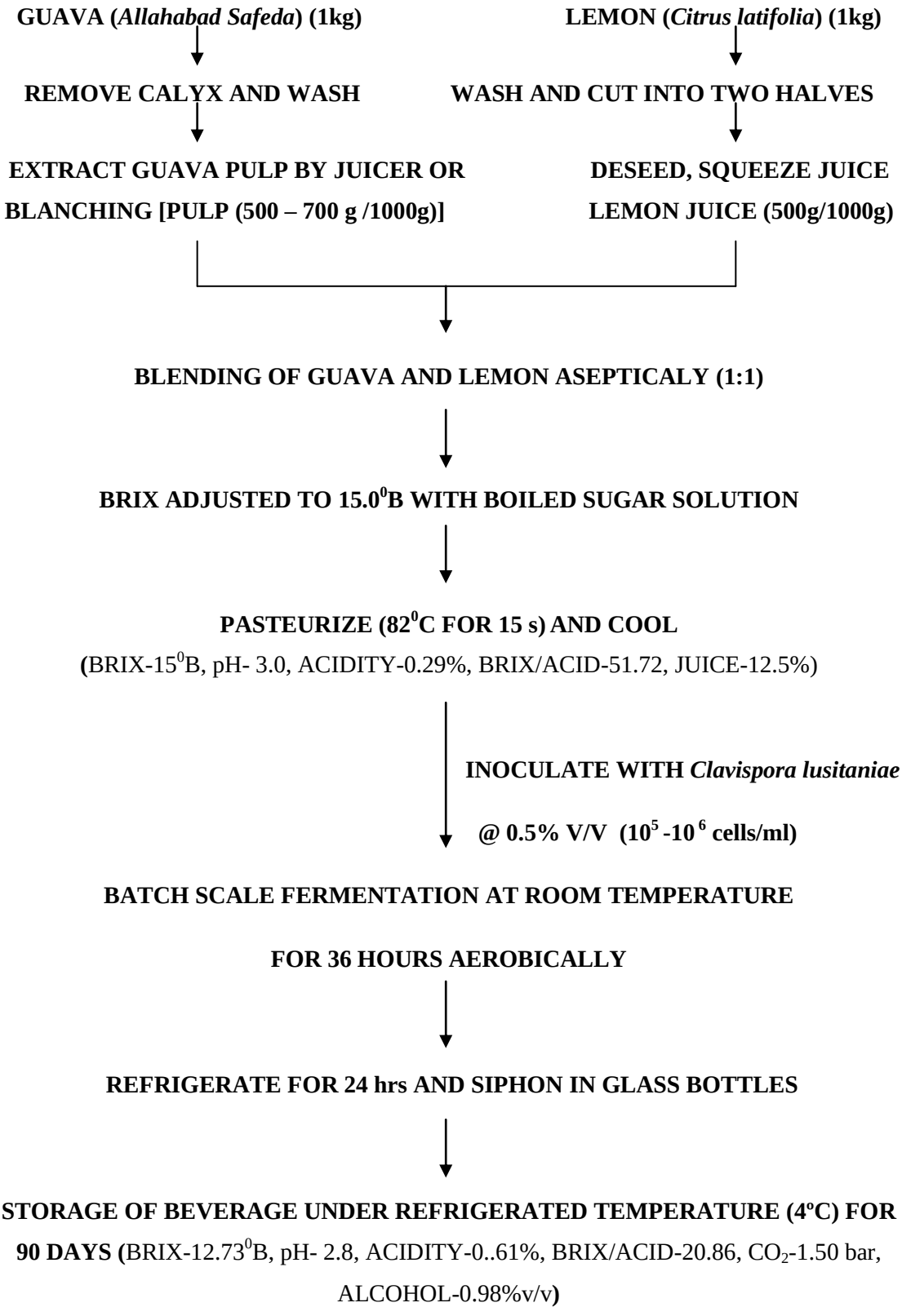


Figure 16: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

4.12 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

Effect of refrigerated storage on the mean sensory scores of *Allahabad Safeda – Citrus latifolia* beverage varied for the appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability (Table 17). Low temperature fermentation favors the production of beverages with more pronounced aromatic profile. The beverage is ranked between liked very much to moderately. Guava is classified as sub acid fruit, since their soluble solids are composed mainly of organic acids and sugars, which are used as the main index of maturity and one of the major analytical measures of flavor quality**.** The beverage was rated highest for taste, aroma and flavor over the other sensory attributes like appearance, color, bouquet, body and astringency up to 30 days due to masking effect of carbonation on bitterness. The tactile sensation of astringency is elicited primarily by the flavonoid phenols which are found abundant in fermented beverage. The hesperidin and naringin develop gradually after extraction as delayed bitterness compound of lemon. The mean scores for overall acceptability increased from 7.5 to 8.0 up to 30 days and then decreased to 7.3 after 90 days but still carbonation enhances the acceptability of the guava lemon beverage.

Table 17: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Allahabad Safeda – Citrus latifolia* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.4	7.4	7.4	7.4	7.4	7.3	7.3	0.01
Taste	7.7	7.9	8.1	8.0	7.8	7.8	7.8	0.015
Color	7.5	7.5	7.5	7.45	7.45	7.44	7.4	0.008
Aroma	7.5	7.8	8.0	7.8	7.8	7.8	7.5	0.11
Bouquet	7.3	7.3	7.2	7.2	7.2	7.15	7.15	0.011
Body	7.2	7.2	7.3	7.3	7.3	7.3	7.1	0.007
Flavor	7.7	7.9	8.1	7.8	7.5	7.0	6.9	0.008
Astringency	7.4	7.5	7.25	7.17	7.0	6.8	6.7	0.008
Overall acceptability	7.5	7.9	8.0	7.8	7.5	7.5	7.3	0.013

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

4.13 Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Lucknow-49- Citrus latifolia* beverage (1:1)

The results of the blended *Lucknow-49-Citrus latifolia* beverage (1:1) (Table 18, Figure 17) show significant decrease in brix from 15.0°B to 12.1°B, brix acid ratio decreased from 55.53 to 20.50 whereas the pH of the beverage increased to 3.0 after 45 days and then decreased to 2.7 after 90 days with increase in acidity from 0.27 per cent to 0.59 per cent during fermentation. The increase in acidity may be due to conversion of lactose to lactic acid and formation of organic acid. Yadav *et al* (2010) observed a significant increase in acidity and decrease in pH during the storage period of whey based banana herbal beverage.

The total sugars decreased from 12.49 per cent to 11.77 per cent after 30 days and further to 9.51 per cent after 90 days. The reducing sugars decreased from 7.98 per cent to 7.13 per cent after 30 days and reached upto 5.86 per cent at the end of 90 days. The percentage decrease in total sugars is 23.85 per cent while the decrease in reducing sugars is 26.56 per cent. Barwal *et al* (2005) also observed a decrease in the total sugars content during storage of ready to serve bittergourd beverage.

The percentage decrease in ascorbic acid is 71.10 per cent while of total phenols is 25.25 per cent. The ascorbic acid and total phenols decreased from 30.8 mg/100ml to 8.9 mg/100ml and 29.7 mg/100ml to 22.02 mg/100ml, respectively. Okunowo *et al* (2005) reported that the reduction in vitamin C during fermentation was needed for the metabolism and growth of yeast strain. Yeast contains little or no ascorbic acid but may absorb it from the medium to be used as possible source of carbon, also ascorbic acid helps to reduce molecular oxygen from the medium thereby promoting yeast growth. Da S Luna *et al* (2009) also reported 52.6 per cent reduction in the ascorbic acid content during storage of blended coconut water – acerola fruit juice beverage.

The concentration of alcohol was 0.30 per cent (v/v), 0.80 per cent (v/v), and 0.97 per cent (v/v) after 15, 60 and 90 days of storage of beverage respectively. The CO_2 pressure 0.65 bar started after 15 days and increased to 1.20 bar after 60 days and reached upto 1.50 bar at the end of 90 days of storage. The viable cell count increased from $23x10^6$ to $94x10^8$cfu/ml, a non significant increase during the storage due to combined effect of high initial concentration $23x10^6$ cfu/ml, anaerobic conditions, high CO_2 pressure and low temperature during storage. Pandove (2007) also reported the increase in alcohol content, CO_2 pressure and viable cell count from 0.4-0.8%, 0.9-1.5 bar and $1.0x10^7$-$9.5x10^8$cfu/ml respectively.

Table 18: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Lucknow-49- Citrus latifolia* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSSºB	15.0	14.6	14.1	13.4	13.0	12.5	12.1	0.04
Acidity %	0.27	0.35	0.42	0.45	0.51	0.56	0.59	0.036
Ph	2.8	2.8	2.9	3.0	2.8	2.8	2.7	NS
Brix-acid ratio	55.55	41.71	33.57	29.77	25.49	22.32	20.50	0.22
Total sugars (%)	12.49	12.16	11.77	11.08	10.46	9.83	9.51	0.035
Reducing sugars (%)	7.98	7.62	7.13	6.74	6.38	6.03	5.86	0.072
Ascorbic acid (mg/100ml)	30.8	26.7	21.3	18.6	14.2	11.7	8.9	0.043
Total phenols (mg/100ml)	29.7	27.5	26.4	25.3	24.2	23.1	22.02	0.149
Alcohol (%,v/v)	-	0.30	0.42	0.65	0.80	0.91	0.97	0.0038
CO_2 (bar)	-	0.65	0.86	1.20	1.20	1.50	1.50	0.02
Total yeast count (cfu/ml)	23×10^6	51×10^7	85×10^7	28×10^8	82×10^8	68×10^8	94×10^8	-

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

*Multiple Regression Equation

$Y = 1.18 – 0.11X_1 + 2.10X_2$

Where

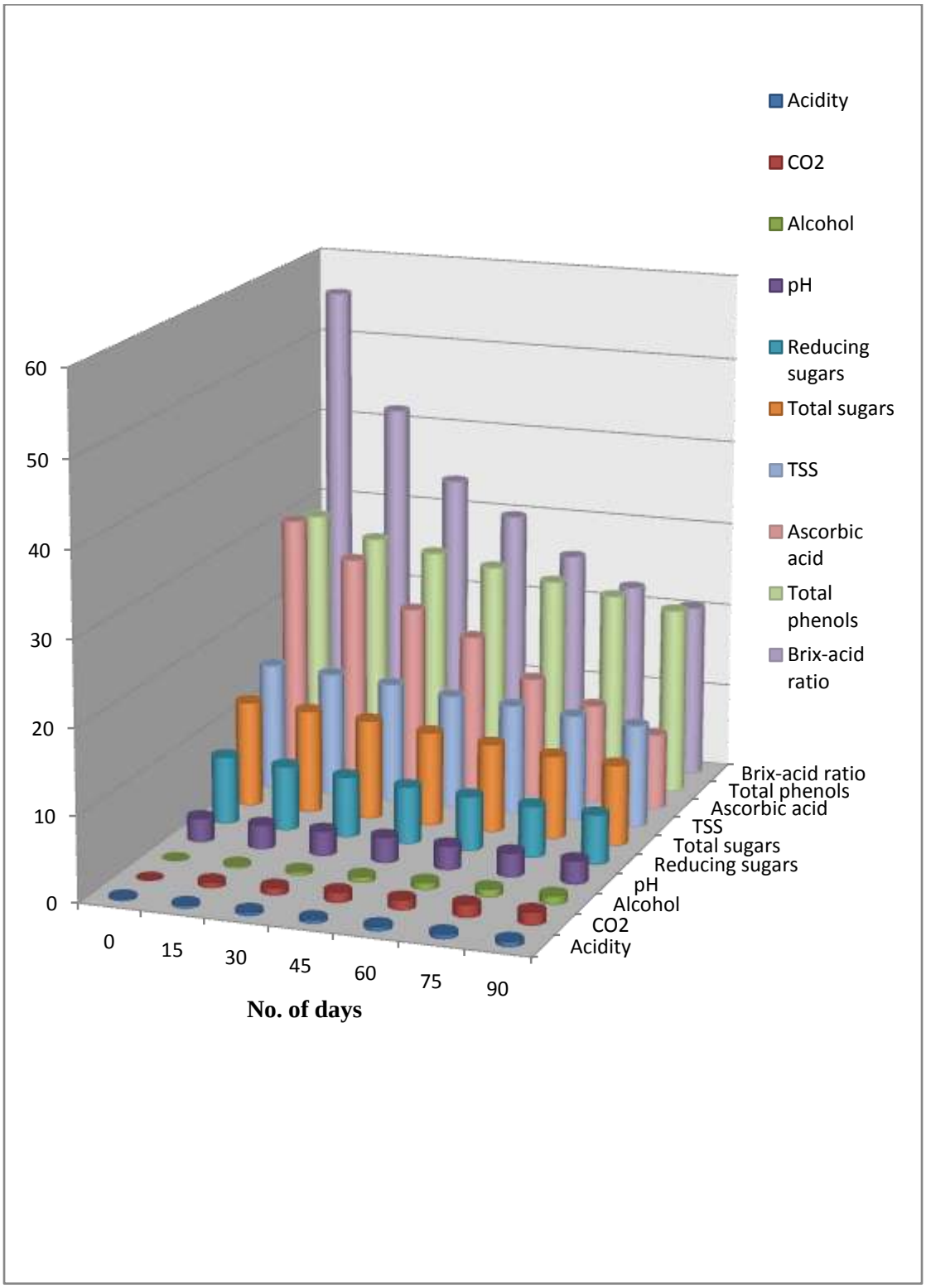
Y = per cent alcohol

X_1= ºBrix

X_2= per cent acidity

**$R^2 = 0.98$

Figure 17: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Lucknow-49- Citrus latifolia* beverage (1:1)



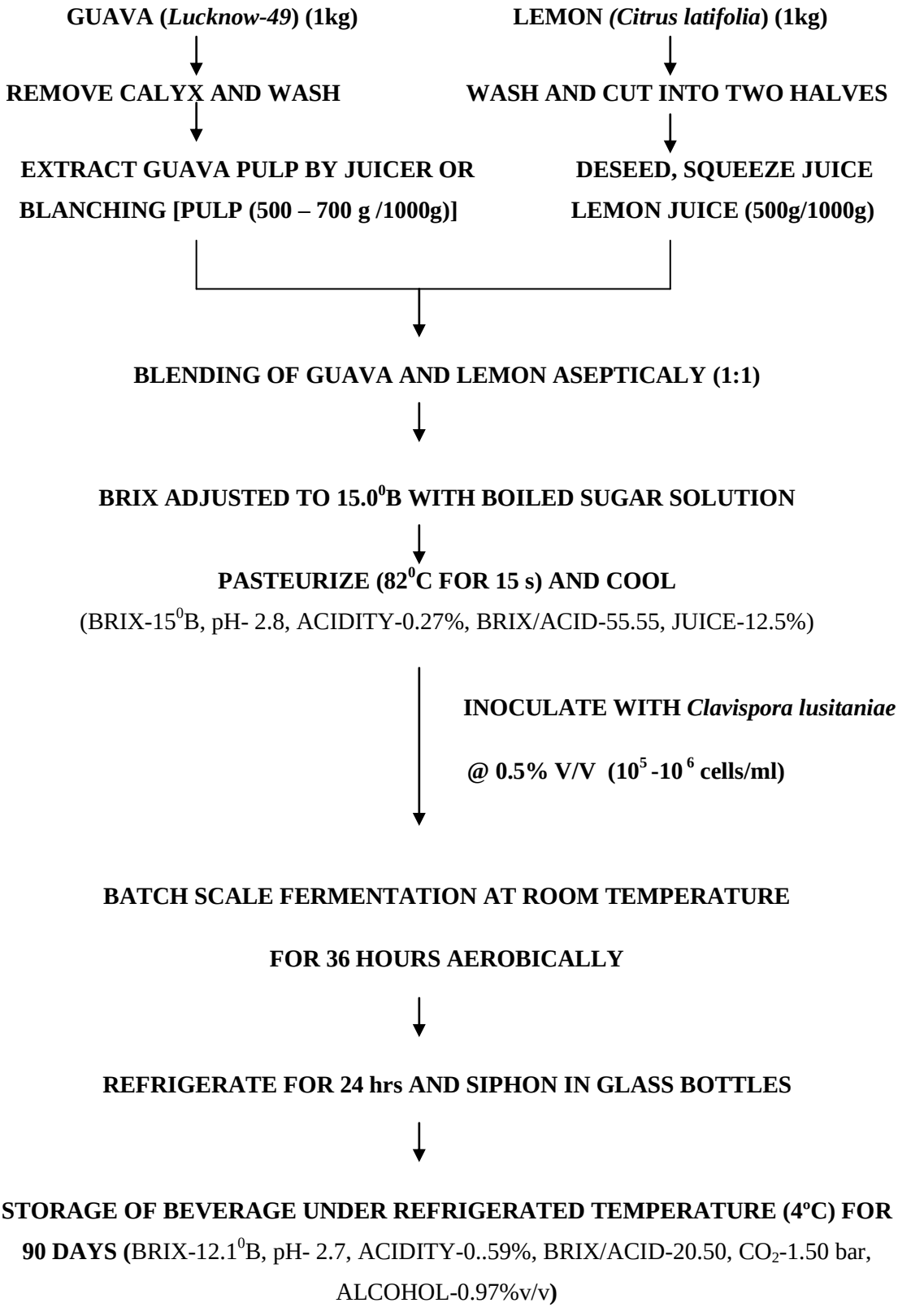


Figure 18: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Luckno- 49 – Citrus latifolia* beverage (1:1)

4.14 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Lucknow-49 – Citrus latifolia* beverage (1:1)

Storage was found to have significant effect on the mean sensory scores for various attributes like flavor, astringency and overall acceptability of the beverage and ranked between liked very much to moderately (Table 19). There was a least variation in the mean sensory scores of appearance, color and body. Di Maro *et al* (2007) reported that various non *Saccharomyces* species produce and secrete several enzymes that interact with grape- derived precursor compounds thus contributing to reveal the varietal aroma during spontaneous fermentation.

The mean sensory scores of flavor and astringency decreased from 8.0 and 7.5 to 7.0 and 6.7, respectively during period of 90 days. The overall characteristic scores are influenced by processing and storage of fruit juices. The decrease in the flavor score during storage could be possibly due to loss of volatile aromatic substances .The taste and aroma of beverage was most acceptable up to 30 days of storage. Periodical analysis of blended guava-whey beverage for various sensory attributes like color, taste, flavor and overall acceptability manifested a decline during the storage period (Divya and Kumari 2009). The beverage was maximum acceptable upto 15 days. Yau *et al* (1988) reported that carbonation enhances the sensory quality of beverage partly due to increased acidity, sparkle and unique taste.

Table 19: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Lucknow-49 – Citrus latifolia* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.5	7.5	7.4	7.4	7.4	7.4	7.4	0.01
Taste	7.7	7.9	8.0	7.9	7.9	7.8	7.8	0.02
Color	7.5	7.6	7.5	7.5	7.5	7.5	7.5	0.01
Aroma	8.0	8.0	8.0	7.8	7.78	7.75	7.75	0.01
Bouquet	7.5	7.4	7.4	7.4	7.4	7.3	7.35	0.01
Body	7.5	7.5	7.4	7.4	7.4	7.4	7.36	0.01
Flavor	8.0	8.1	8.0	7.7	7.5	7.1	7.0	0.008
Astringency	7.5	7.25	7.0	7.0	6.75	6.7	6.7	0.01
Overall acceptability	8.0	8.1	8.0	7.6	7.5	7.5	7.5	0.01

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

4.15 Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)

The results of blended beverage in (Table 20, Figure 19) shows a significant decrease in the brix from 15.0°B to 12.14°B, brix acid ratio from 65.21 to 23.80, pH from 2.9 to 2.7. The acidity gradually increased from 0.23 per cent to 0.32 percent after 30 days and finally reached 0.51 percent at the end of 90 days of storage. The total sugars decreased from 11.65 percent to 10.53 percent after 30 days and then decreased to 8.87 percent at the end of 90 days. The reducing sugars decreased from 8.96 per cent to 6.37 per cent after 30 days and then decreased to 4.75 per cent after 90 days of storage. The percentage decrease in total sugars was 23.86 per cent while of reducing sugars was 46.98 per cent. Yeast cells sense the amount and quality of external nutrients through multiple interconnected signaling networks, which allow them to adjust their metabolism transcriptional profile and developmental program to adapt readily and appropriately to changing nutritional states. Abbo *et al* (2006) also reported gradual decline in pH and °Brix and increase in acidity for soursop juice at 4°C during 8 weeks of storage.

The percentage decrease in ascorbic acid and total phenols was 80.19 per cent and 27.04 per cent, respectively. The ascorbic acid decreased from 31.3 mg/100ml to 16.8 mg/100ml after 45 days and then deceased to 6.2 mg/100ml at the end of 90 days of storage. Ascorbic acid the most important nutrients in the fruit juices, the significant decrease of ascorbic acid during cold storage can be due to its oxidation (El Sheikha *et al* 2009). Maraques De Carvalho *et al* (2009) reported 80% loss of vitamin C during storage of blended non alcoholic beverage composed of coconut water and cashew apple juice containing caffeine. The total phenols decreased from 28.42 mg/100ml to 20.72 mg/100ml after 90 days. Walkowiak- Tomczak (2007) stated that due to thermal processing, filtration and storage there is a considerable decrease in antioxidant activity of the product in relation to that of raw material.

The alcohol formation began at the end of 15 days (0.19% v/v) and reached up to 0.92% (v/v) after 90 days of storage. The CO_2 pressure of 0.56 bar started building after 15 days and increased to 1.20 bars after 60 days and then to 1.50 bars after 90 days. The viable cell count increased from 31 x 10^6 to 27 x 10^9cfu/ml during 90 days of storage. Saravana Kumar and Manimegalai (2005) reported an increase in acidity and decrease in pH, total sugars and ascorbic acid during 3 months storage of whey based papaya blended ready to serve beverage.

Table 20: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)

Parameters	Days							CD (5%)
	0	15	30	45	60	75	90	
TSS^{o}B	15.0	14.73	14.50	13.94	13.43	12.70	12.14	0.04
Acidity %	0.23	0.28	0.32	0.38	0.42	0.47	0.51	0.004
Ph	2.9	2.9	2.8	2.8	2.7	2.7	2.7	0.027
Brix-acid ratio	65.21	52.60	45.31	36.68	31.97	27.02	23.80	0.5
Total sugars (%)	11.65	11.09	10.53	10.31	9.75	9.23	8.87	0.05
Reducing sugars (%)	8.96	7.10	6.37	6.12	5.66	5.08	4.75	0.051
Ascorbic acid (mg/100ml)	31.3	26.4	20.2	16.8	12.1	8.4	6.2	0.04
Total phenols (mg/100ml)	28.4	26.6	25.5	24.1	22.8	21.6	20.72	0.05
Alcohol (%,v/v)	-	0.19	0.36	0.44	0.63	0.76	0.92	0.004
CO_2 (bar)	-	0.56	0.74	0.90	1.20	1.20	1.50	0.14
Total yeast count (cfu/ml)	31×10^6	48×10^7	90×10^7	35×10^8	24×10^8	84×10^8	27×10^9	-

% Juice in beverage – 12.5%

Storage temp - 4±2^{o}C

*Multiple Regression Equation

$Y = -0.80 + 0.0059X_1 + 3.20X_2$

Where

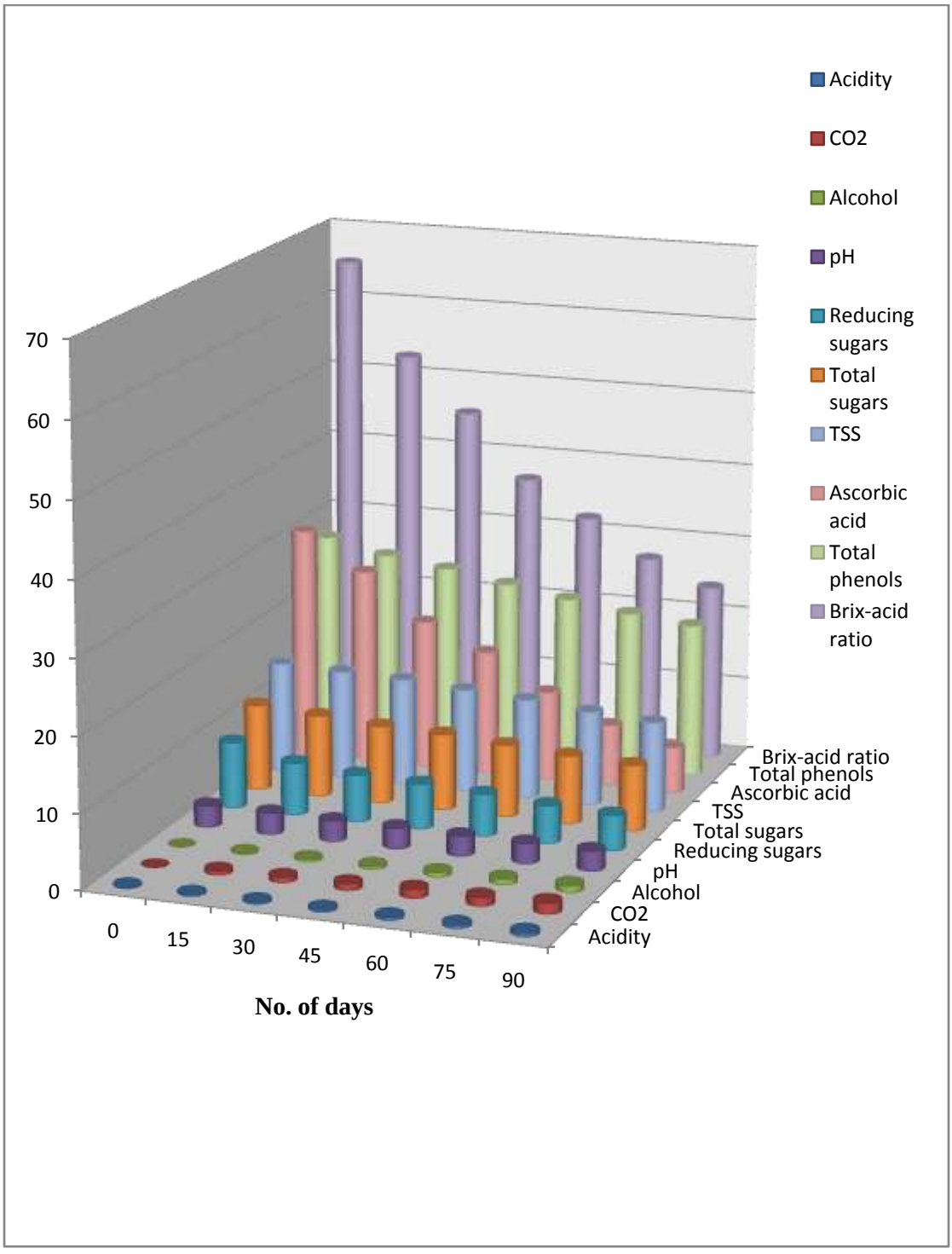
Y = per cent alcohol

X_1= ^{o}Brix

X_2= per cent acidity

**$R^2 = 0.98$

Figure 19: Effect of storage time on microbiological and physicochemical parameters of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)



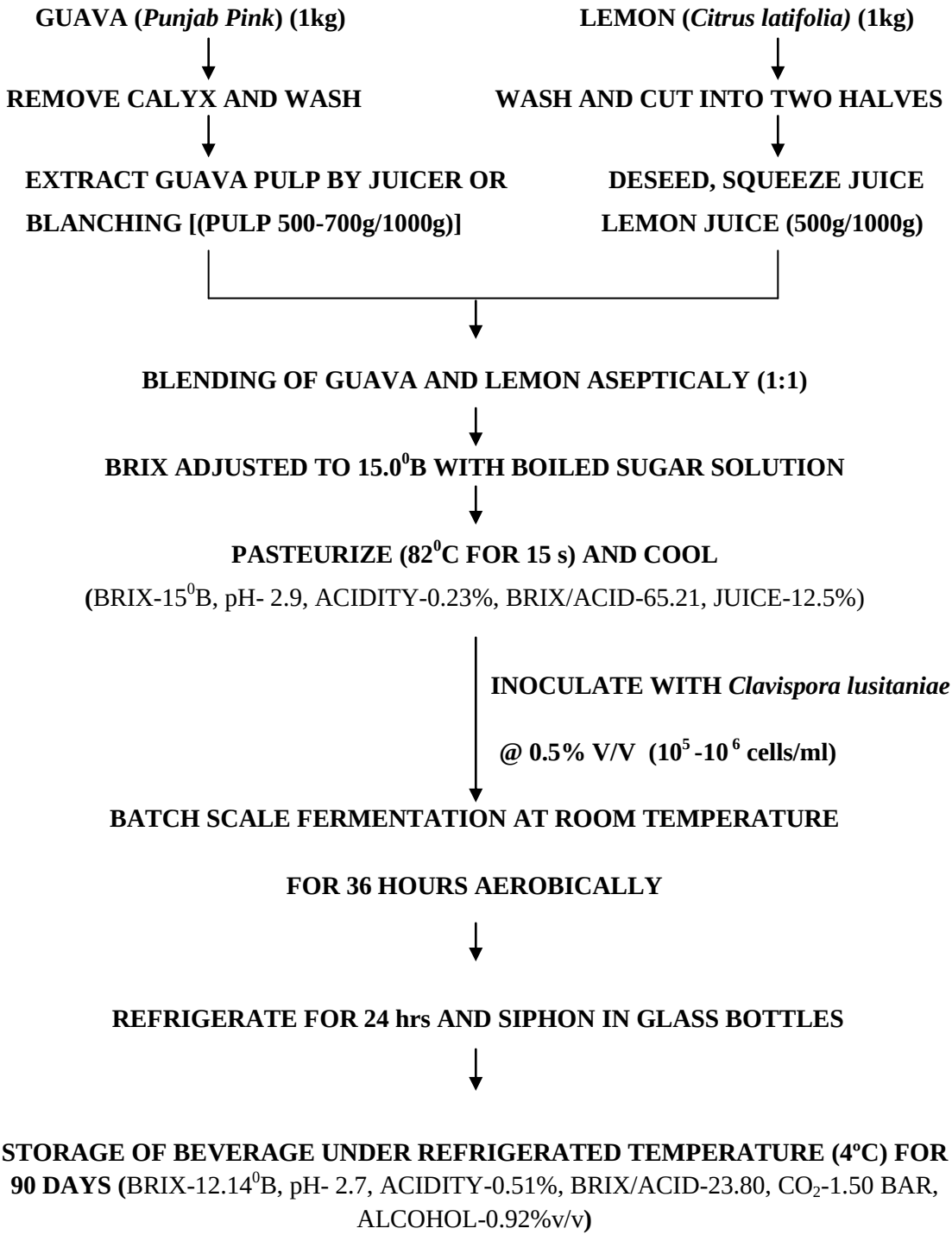


Figure 20: Flow diagram for the preparation of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)

4.16 Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)

The mean sensory scores (Table 21) for various sensory attributes like taste, color, aroma, bouquet, flavor and astringency of blended beverage varied significantly as the number of storage days increased. Cook *et al* (2003) found that somatosensory tactile stimuli interact with taste and aroma and hence modulating their perception. The formation of aroma compounds by yeast, i.e. short and medium-chain fatty acids and their corresponding ethyl esters, higher alcohols, and their corresponding acetate esters, is intrinsically linked to the metabolism of yeast cells. The scores were ranked between liked very much to moderately. The acidity is considered as one of the physicochemical properties which affect both organoleptic and keeping qualities of a product. The beverage had pleasing effervescent sparkling pink colored appearance. The beverage was rated highest for taste, aroma and flavor up to 30 days of storage. Phenolics also influence the various sensory attributes like color, astringency, bitterness and flavor of fruit beverages. The mean sensory scores for astringency decreased significantly from 7.5 to 6.72 during 90 days of storage. The beverage was most acceptable up to 30 days of storage. 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. The presence of yeast in beverage gave a desirable freshness to the fermented beverage due to production of carbon dioxide and ethanol.

Table 21: Effect of storage time on organoleptic properties of non-alcoholic naturally carbonated fermented blended *Punjab Pink – Citrus latifolia* beverage (1:1)

Sensory attributes	Days							CD (5%)
	0	15	30	45	60	75	90	
Appearance	7.5	7.5	7.6	7.6	7.5	7.5	7.5	0.013
Taste	7.8	8.0	8.1	8.0	7.9	7.9	7.9	0.014
Color	7.9	8.0	8.0	7.9	7.9	7.8	7.8	0.008
Aroma	8.5	8.75	8.73	8.7	8.7	8.5	8.5	0.013
Bouquet	7.0	7.25	7.38	7.37	7.38	7.3	7.25	0.011
Body	7.2	7.3	7.36	7.35	7.35	7.33	7.3	0.011
Flavor	8.1	8.4	8.5	8.3	8.0	7.7	7.5	0.011
Astringency	7.5	7.35	7.23	7.2	7.0	6.75	6.72	0.01
Overall acceptability	8.0	8.2	8.2	8.1	7.9	7.8	7.8	0.007

*mean value of three replicates

% Juice in beverage – 12.5%

Storage temp - 4±2ºC

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

The heat and potassium metabisulphite treated non-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, 6o^{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 22, Figure 21) without imparting bitter taste to the beverage. These results are in accordance with Arora (2009). The mean scores of the various sensory attributes varied non-significantly in both the treated beverages (Table 23). The heat treated beverage was best rated for appearance, color and bouquet. Organoleptically heat treated beverage was superior than potassium metabisulphite treated beverage.

A **B**

Plate 8

A: Guava var. *Allahabad Safeda* Beverage
B: *Punjab Pink – Citrus latifolia* (1:1) Beverage

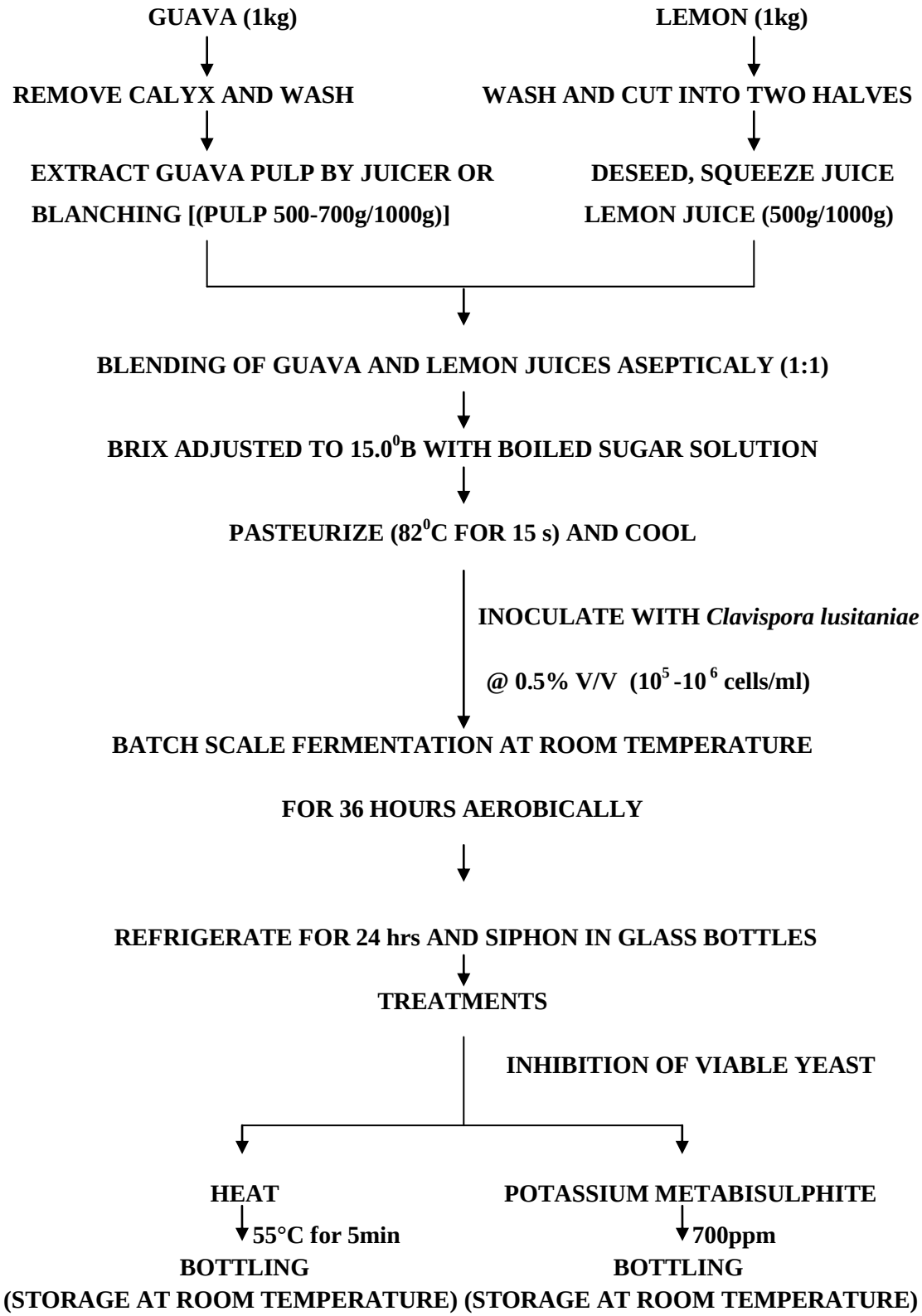


Figure 20: Inhibitory treatments for viable yeast cells in non-alcoholic naturally carbonated fermented guava: lemon (1:1) blended beverage

Table 22: 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			

Figure 21: Effect of temperature on the viability of yeast cells

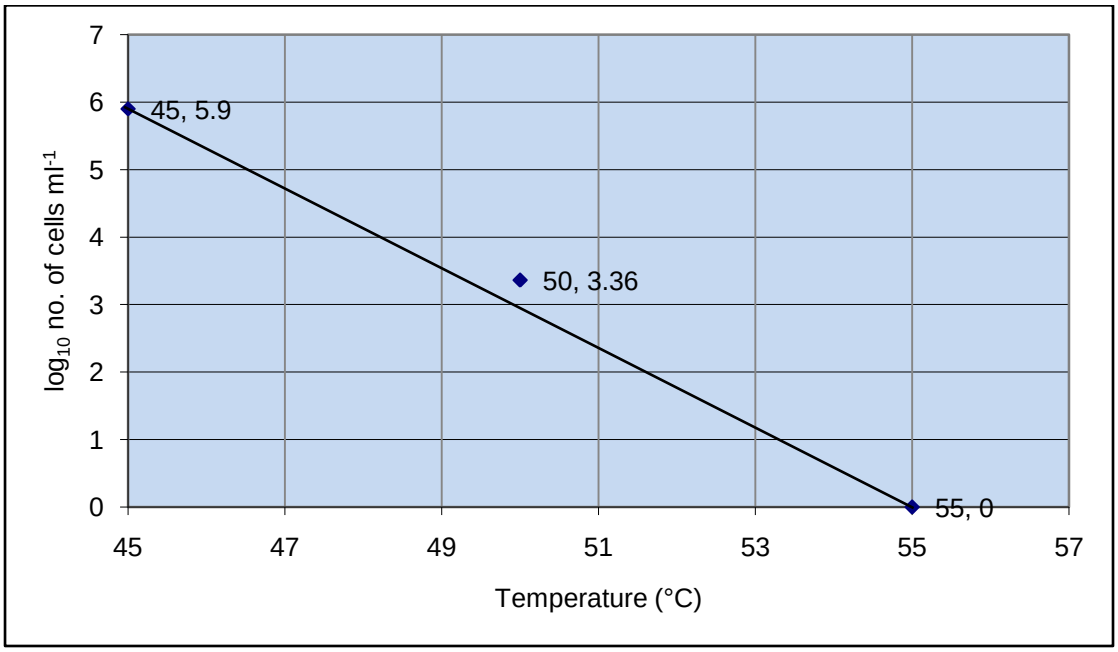


Table 23: Effect of clarification treatments on sensory attributes of non-alcoholic naturally carbonated fermented guava: lemon (1:1) blended beverage

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

Chapter V

SUMMARY

The studies on "Microbiological preparation of non-alcoholic naturally carbonated blended beverage from guava and lemon" were carried out to develop a reliable, controllable, reproducible fermentable technology for the preparation of non alcoholic naturally carbonated fermented beverage from guava and blended beverage from guava and lemon. Guava varieties *Allahabad Safeda*, *Lucknow 49* and *Punjab Pink* and Lemon varieties *Baramasi* and *Citrus latifolia* were procured from Department of Horticulture, Punjab Agricultural University, Ludhiana and PAU Regional Fruit Research Station, Bahadurgarh.

The growth curve of yeast *Clavispora lusitaniae* in guava beverage and blended guava-lemon beverage (1:1) under batch scale fermentation conditions with respect to viable cell count ($\log_{10}$ no. of cells per ml) were found to show normal patterns with first a short lag period of 3 hrs followed by phase of exponential growth upto 24 hrs and 32 hrs respectively as indicated by sharp increase in viable cell count ($\log_{10}$ no. of cell per ml) from 5.32 – 8.93 and 5.25 – 8.97 respectively followed by stationary and death phases. The specific growth rate (h^{-1}) and generation time (h) of yeast was 0.39 and 1.77 respectively in guava beverage and 0.35 and 1.93 respectively in blended guava-lemon beverage(1:1).

Three guava varieties used were *Allahabad Safeda*, *Lucknow- 49* and *Punjab Pink* and its blends in various proportions as (1:1, 2:1, 1:2) with Lemon var. *Baramasi* (*Citrus limon*) and *Tahiti lime* (*Citrus latifolia*) The best guava varieties standardized on the basis of physicochemical parameters of guava var. *Allahabad Safeda* beverage was 15% juice, pH 3.9, TSS 11.7°B, acidity 0.53%, ascorbic acid 13.5 mg/100ml, alcohol (%v/v) 0.89, CO_2 1.53 bar and total plate count $96x10^8$cfu/ml, *Lucknow-49* beverage with 15%per cent juice, pH 3.9, TSS 13.0°B, acidity 0.47%, ascorbic acid 13.2 mg/100ml, alcohol (%v/v) 0.81, CO_2 1.52 bar and total plate count $80x10^8$cfu/ml had a shelf life of 75 days and 60 days respectively. The shelf life is standardized on the basis of alcohol production < 1.0% v/v. The physicochemical parameters of *Punjab Pink* beverage 15% juice, pH 4.0, TSS 12.7°B, acidity 0.49%, ascorbic acid 9.7 mg/100ml, alcohol (%v/v) 0.81, CO_2 1.56 bar and total plate count $67x10^8$cfu/ml had shelf life of 75 days. The mean sensory score of the three varieties of guava beverage for various sensory attributes varied significantly during low temperature storage of 90 days and were ranked between liked moderately to slightly. Thus, on the basis of sensory evaluation guava var. *Allahabad Safeda* and *Punjab Pink* scored highest (7.5) after 15 days for overall acceptability and had sparkling appearance due to effervescence with shelf life of 75 days under refrigerated conditions (4°C).

The blending of guava-lemon juice for beverage preparation offered more combinations, guava due to its lower pH and higher titrable acidity and lemon for tartness

which provides appealing aroma, laxative texture and good mouthfeel to the beverage. Guava-lemon blend 1:1 scored highest for aroma (7.6), bouquet (7.55), body (7.4), flavor (8.0) and overall acceptability (7.57), and hence the blending ratio 1:1 was standardized for the microbiological preparation of non-alcoholic naturally carbonated blended beverage from guava and lemon.

The physicochemical characteristics of the *Allahabad Safe*da *-Baramasi* beverage, 12.5% juice, pH 2.6, TSS 12.1°B, 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 had shelf life of three months under refrigerated conditions (4°C). The mean scores for the various sensory attributes were ranked between liked very much to moderately during 90 days of storage. The mean scores of flavor, astringency and overall acceptability increased significantly from 7.5 to 8.0, 7.5 to 7.75 and 7.5 to 8.0 respectively within 30 days of storage.

The *Lucknow-49- Baramasi* beverage, 12.5% juice, pH 2.6, TSS 11.9°B, 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 showed shelf life of three months under refrigerated conditions (4°C). The mean scores for the various sensory attributes were ranked moderately. The taste scores increased from 7.6 to 7.8 upto 45 days with a significant variation in other attributes like aroma, bouquet and body during storage.

The composition of *Punjab Pink- Baramasi* beverage, 12.5% juice, pH 2.6, TSS 12.1°B, acidity 0.50%, ascorbic acid 10.2 mg/100ml, alcohol (%v/v) 0.81, CO_2 1.49 bar and total plate count $85x10^8$cfu/ml. The mean sensory scores ranged between liked very much and moderately under low temperature storage (4°C). The mean sensory score (7.5) for overall acceptability increased to 8.0 till 45 days. The beverage was acceptable for three months physicochemically and organoleptically.

The physicochemical parameters of stored *Allahabad Safeda – Citrus latifolia* beverage 12.5% juice, pH 2.8, TSS 12.73°B, acidity 0.61%, ascorbic acid 5.3 mg/100ml, alcohol (%v/v) 0.98, CO_2 1.50 bar and total plate count $28x10^9$cfu/ml had shelf life of three months. The beverage was rated highest for aroma (8.0), taste (8.1) and flavor (8.1) over the other sensory attributes like appearance, bouquet, color, body and astringency.

The shelf life of the *Lucknow-49 – Citrus latifolia* was found to be three months under refrigerated conditions (4°C). The final beverage, 12.5% juice, pH 2.7, TSS 12.1°B, acidity 0.59%, ascorbic acid 8.9 mg/100ml, alcohol (%v/v) 0.97, CO_2 1.50 bar and total plate count $94x10^8$cfu/ml. The physicochemical parameters of stored *Punjab Pink – Citrus latifolia* beverage 12.5% juice, pH 2.7, TSS 12.14°B, acidity 0.51%, ascorbic acid 6.2 mg/100ml, alcohol (%v/v) 0.92, CO_2 1.50 bar and total plate count $27x10^9$cfu/ml with shelf life of three months. The beverage was scored highest for taste (8.1), aroma (8.73) and flavor

(8.5) upto 30 days of storage. The beverage was ranked between liked very much to moderately. It was acceptable organoleptically for three months.

Thus, on the basis of sensory evaluation *Punjab Pink-Citrus latifolia* (1:1) scored highest after 15 days for overall acceptability with a shelf life of three months under refrigerated conditions (4°C).

The heat treatment and potassium metabisulphite treatment to inhibit the viable yeast cells in non-alcoholic naturally carbonated fermented blended beverage 59was standardized as 55^{o}C for 5 min and 700ppm, respectively. On the basis of organoleptic evaluation heat treated beverage was found superior to the one treated with potassium metabisulphite.

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ANNEXURE I

ALIGNED SEQUENCE DATA: (337 bp)

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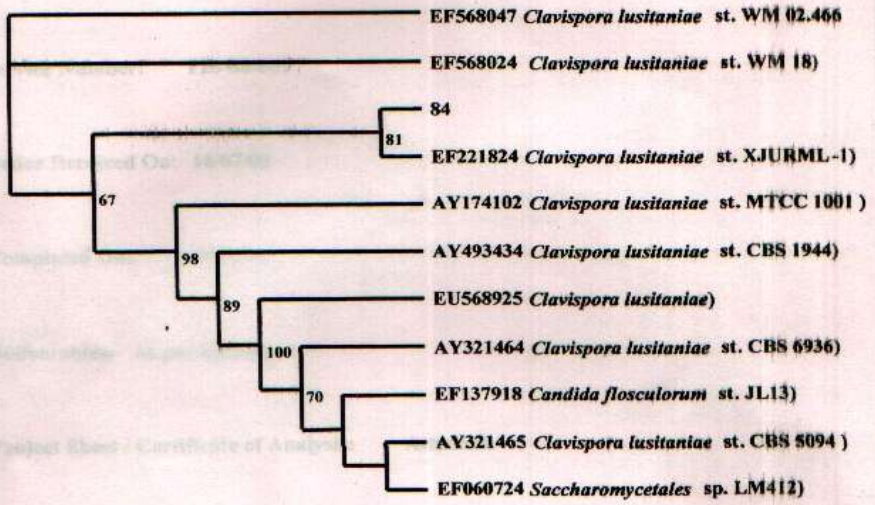
CCCCCGGTTAGGCGTTGCTCCGAAATATCAACCGCGCTGTCAAACACGTTTACAGCACGACATTTCGCCCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAG

PERCENTAGE HOMOLOGY 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	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											*

PHYLOGENETIC TREE (Using Neighbor Joining Method):



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OCPA	**:**	7.63/10
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