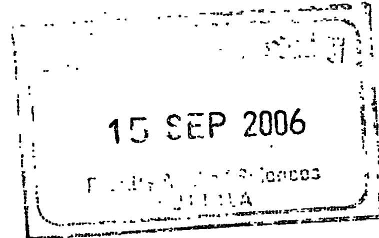
Identification of possible causes for presence of yeasts and moulds in end product of carbonated non alcoholic beverages and implementation of corrective actions and preventive measures.



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DECLARATION

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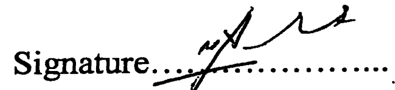
Signature 

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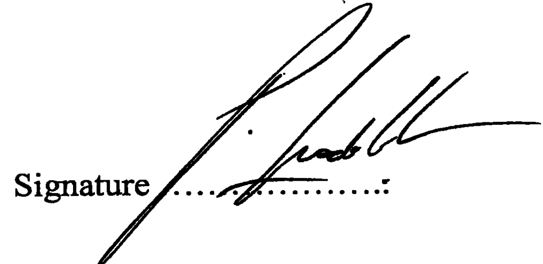
Signature 

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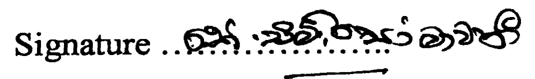
Signature 

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AFFECTIONALTY DEDICATED TO

MY PARENTS AND TEACHERS

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ABSTRACT

Soft drinks are simply defined as any non-alcoholic beverage containing syrups, essence or fruit concentrates that are mixed with water or carbonated water. Basic raw materials of carbonated soft drinks are purified water, sweeteners, carbon dioxide, permitted colours and flavours, acidulants, emulsions, heading agents, preservatives (SLS, 183:1997). These are not consumed for their food value, but rather for their thirst quenching properties or for their stimulating effect.

Soft drinks are more susceptible to microbial spoilage than other food products, due to both intrinsic and extrinsic factors. Microbial spoilage occurs due to three different kinds of microorganisms yeasts, moulds and bacteria (Varnnum, 1994). Yeasts are the dominant spoilage organisms in soft drinks. Spoilage pattern typically involve film formation, fermentation with gas production, turbidity and sediments and 'fruity' flavours. Mould spoilage involves visible growth together with bitter flavour and discoloration.

The study involves the identification of places that are highly vulnerable to contamination by yeasts and moulds. Pour plate method, 3M™ Petrifilm, swabs testing were used to count the yeasts and moulds. Mouthpiece of filler, Production environment, and stock syrup filter showed high count of yeasts and moulds.

Genus *Aspergillus* was isolated in the production environment by using streak method and confirms it by using slide culture technique. Concentration of Benzioc acid, a preservative for yeasts and moulds, measured by HPLC method was comparable with Sri Lanka standard (160 ppm).

Yeasts and moulds present in mouthpieces were studied for their tolerance by using a rinse with different concentration of chlorine solutions (175, 250, 400, and 500) ppm and the effectiveness was measured by using swab testing and finished product testing. Results showed that no yeast and moulds count was observed after washing with 400 ppm and higher chlorine solutions. According to the results analyzed by one way ANOVA, yeast and mould counts were nil in 1ml of finished product of Soda after the proposed pretreatment of mouth pieces ($p=0.011<0.05$).

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Abbreviation

AOAC	Association of Official Analytical Chemist
HPLC	High Performance Liquid Chromatography
Y & M	Yeasts and Moulds
CSD	Carbonated Soft Drink
EGB	Elephant House Ginger beer
EHQM	Elephant House Quality Manual
CIP	Clean In Place
SLS	Sri Lanka Standard
SMS	Sodium Meta bisulphite
ANOVA	Analysis of variance
UK	United Kingdom
Cm^2	Squire centimeter
$Inch^2$	Squire inches
ppm	part per million
Mpa	Mega Pascal
SO_2	Sulphur dioxide
UV	Ultra violet
$NaHCO_3$	Sodium bicarbonate
NaOCl	Sodium oxechloride
CO_2	Carbon dioxide
ATP	Adenosine tri phosphate

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CHAPTER 1

INTRODUCTION

1.1 Introduction

There are several kinds of beverages, namely the carbonated non-alcoholic beverages or soft drinks, the carbonated or non-carbonated mildly alcoholic beverages e.g. beer and wine, and the nonalcoholic, non-carbonated stimulating beverages coffee and tea. These are not consumed for their food value, but rather for their thirst quenching properties or for their stimulating effect.

Soft drinks are defined as any non-alcoholic beverage containing syrup, essences or fruit concentrates that are mixed with water or carbonated water (Green, 1978).

Carbonated Soft drink (CSD) is a ready to drink non-alcoholic beverage prepared from potable water, one or more of the ingredients (sweetening ingredients, fruit juices, artificial flavouring agent, foaming agents, permitted food colours, permitted preservatives clouding agents, emulsifying, stabilizing agents, acidulates, vitamins, edible common salt). Saturated with carbon dioxide and packed in hermetically sealed containers. These may be plain, flavoured or sweetened (SLS 183, 1997).

The sugar of carbonated soft drink is mostly sucrose, purchased as pure colorless syrup from the manufacture or made into syrup at the beverage plant from high purity crystalline sugar. The sugar syrup is later supplemented with the flavouring, colouring, and acid ingredients and may be stabilized with a preservative. The finished beverage will contain about 8% to 14 % of sugar (Potter, 1984).

Flavourings come in the form of synthetic flavour compounds, natural flavour extracts, and fruit juice concentrates. These flavours must be stable under the acidic conditions of the beverage and on exposure to light for a year or more, since the bottle drinks may be held this long or longer. The flavours do not have to be stable to heat much over $38^{0}C$ since the beverage is not commonly heat sterilized or pasteurized (Potter, 1984).

Some important colouring agents for soft drinks are the synthetic colors, particularly the certified coal tar colours. Caramel from burned sugar, a non-synthetic colour also is commonly used.

The amount of carbon dioxide used in the beverage will depend upon the particular flavour and brand of the beverage. The carbon dioxide enhances flavour, contributes acidic preservative action produce the tingling effect on the tongue, and gives the sparkling effervescent appearance to the beverage (Potter, 1984).

Acidulates are added additionally to the most carbonated drinks. The main resin for acidification is to enhance the beverage flavours. The principal acids used are phosphoric, citric, tartaric, and malic acid (Potter, 1984).

Water is the major ingredient in carbonated soft drinks and may be present to the extent of 92% (Potter, 1984).

Microbiological spoilage of soft drinks can occur and often manifests itself as an off-odor or off-flavour, sediment, turbidity, and/or excess CO_2 production in the beverage (International food safety hand book, 1999).

Most spoilage of soft drinks is associated with yeasts, which can survive and grow in carbonated beverages and sugar syrups. Yeast contamination causes off-odors, off-flavours, visible particles and gas production, which may increase pressure with in the package. *Sacchromyces, Zygosacccharomyces, Tourulopsis, Hunsenulla, Rhodotorula, Candida*, and *Brettanomyces* are commen yeast type found in carbonated soft drink (Jasper G.W, 1974).

Spoilage by other microbes such as Moulds is observed less frequently. Moulds require oxygen for growth, and most will not grow in carbonated beverages. However their spores and myceliums can survivel in carbonated beverages, and if a beverage losses CO_2, moulds may grow and cause off-odors, and off-taste in the product. Moulds can grow to large clusters, which are visible to the naked eye; *Aspergillus, penicillium* and *Rhizopus* are common mould type associated with contamination (Kees vander H., *etal,* 1999).

Both intrinsic and extrinsic factors are affecting to microbial growth in carbonated soft drink. Intrinsic factors are pH or acidity, water activity, osmotic condition, organic condition, mineral content. Extrinsic factors are nature of raw material, initial microbial load, handling, processing or treatment, package, storage condition (Green, 1978).

Hence the presence of yeast and mould could render the product unsafe for consumption, according to Sri Lanka Standard No. 183 of 1997. The manufacturer may undergo economic

losses due to the non-acceptance of such bottles in the market and will be liable for any health problems that may arise after the consumption.

Apart from rendering the product unsafe for consumption with their direct presence, yeast and mould growth can initiate unfavourable chemical reactions and may change the composition of the product by adding various toxic matters.

Therefore the preventive and elimination measures should best be addressed in terms of a Hazard Analysis Critical Control Point (HACCP) plan which can ensure that these measures are implemented in a standardized and sustainable manner.

1.2 Objectives

1.2.1 Major objective

To be gained finished carbonated nonalcoholic beverages free of yeasts and moulds.

1.2.2 Specific objectives

I. Identification of places of the production line where the contamination is mostly probable.
II. Identification of favourable condition for the growth of yeasts and moulds.
III. Implementation of preventive and corrective actions for possible causes.
IV. Identification of specific genera of yeasts and moulds grown in sweeten and non sweeten carbonate soft drink.

CHPTER 2

LITRETURE REVIEW

2.1 Classification of Drinks

2.1.1. Hard drinks / Alcoholic beverage

An alcolic beverage is a drink containing ethanol. Ethanol is a drug, and depressant, and even though alcohol is a legal drug in most parts of the world, many societies regulate or restrict the sale and use of it.

2.1.2. Soft drinks

The widest intrapritation is that the term encompasses all nonalcoholic drinks are soft drinks. But particularly beverages that are sweetened carbonated (aerated) and flavoured. Soft drinks are appropriate any time of the day, with or without solid food. Many of them tend to be habit forming without any claim of nutrition value (Wood Roof, J.G, 1974).

Soft drinks are defined as any non-alcoholic beverage containing syrups, essence or fruit concentrates that are mixed with water or carbonated water; it includes

a) Any fruit drink and any fruit juice, squash, crush or cordials.
b) Soda water, Indian or quinine tonic water, and any sweetened artificially carbonated water weather flavoured or unflavoured.
c) Ginger beer and any herbal or botanical beverage (Green, 1978).

Fruit juices

These are juices of various fruits, usually bottled for consumption without dilution, though some fruit juices are concentrated so that dilution is required before drinking. Such drinks are offered in bottle, jar or can, Ready-to- drink or in concentrated form, requiring dilution, and sometimes as powdered for reconstitution, and they are often fortified with extra Vitamin C (Green, 1978).

Squashes

The basic raw materials for squashes were prepared by separating the juice, etc. from the peel and sieving the result to remove gross solids. After pasteurization, these materials were preserved

with sulphur dioxide and stored in barrels until they were required to manufacture. These juices are practically free from oil and some from of addition has to be made during manufacture in order to give the products, when diluted and ready to drink sufficient flavour (Green, 1978).

Cordials

Cordial in the context of soft drinks, means 'a beverage containing sugar' which is of a concentration which requires dilution before drinking.

Vegetable juice

Accepting that tomato juices can be classified as a fruit juice, Vegetable juice have had little popularity in the U.K. on the European content, the juices of beetroot and carrot have been marketed (Green, 1978).

Carbonates

Carbonated soft drinks (CSD) is a ready to drink non alcoholic beverage prepared from potable water, one or more of the ingredients (Sweetening ingredients, fruit juices, artificial flavouring agent, foaming agents, permitted food colours, permitted preservatives clouding agents, emulsifying, stabilizing agents, acidulates, vitamins, edible common salt). Saturated with carbon dioxide and packed in hermetically sealed containers. These may be plain, flavoured or Sweetened (SLS, 183:1997).

Carbonates may be classified as;

a) Cola types with caramel flavouring and colouring.
b) Ginger ale type with ginger flavour.
c) Natural fruit juices colours and flavours.
d) Synthetic fruit colored and flavour types.
e) Sparkling water is soda and carbonated water (WoodRoof, J.H, 1974).

According to Green, 1978 Carbonates classified as,

a) Mineral water (E.g. Soda)
b) Sweetened carbonated flavoured drinks (E.g. Lemon crush)
c) Sweetened and carbonated fruit and vegetable drinks.
d) Carbonates prepared from extracts (E.g. Ginger beer)

e) Tonic water (E.g. quinine tonic water)
f) Colas (E.g. Coca cola)

2.1.3. Developments of carbonated soft drinks and It's origin

The origin of carbonated soft drinks goes back to late 1700 Greek and Roman times when naturally occurring mineral water was priced for medicinal and refreshing qualities (Potter et.al., 1995).The chemical character of natural gases was studied by Van Helmont and Hoffman, and the first artificial 'spring water' was produced by Dr. Gabriel Venel in 1950 by adding hydrochloric acid to sodium carbonate in a closed vessel. The British chemist Josep Prestly gave a higher contribution regarding the development of artificial spring water in 1772, published 'Direction for impregnating water with fixed air, studied about the carbonation of water, wine, beer, medicinal value and the delicate flavour of carbonated water which can be drowned by the stronger flavour of other ingredients. Antonine Lavoisiser identified fixation air described by Priestly as a combination of carbon and oxygen and called as 'gaz acide carbonique' In 1775 an English scientist, J. M. Nooth, developed a special apparatus for making effervescent water. It was improved by J. J. de Mazalihas in commercial scale, after that factories were opened in various cities in Europe in 1789-1821. Cavendish showed that carbon dioxide was more suitable in water at low temperatures and that assist by pressure, agitating and conducting the water during carbonation over a large area. Beginning of 19th century aerated or carbonated water was well accepted by England and America, scientist were encouraged to continue their studies regarding aerated water, men such as Benjamin Silliman and Joseph Hawkins bottled and sold artificial carbonate water. Quinine tonic water developed in 1985, becoming a means of protecting British forces abroad from malaria. Cola, extracted from the nuts and leaves of Bolivian Coca shrub was initially promoted as a brain tonic, shipped to America in Second World War. In 1950, the huge CSD in the world, 'Coca Cola' came to the market having a huge customer demand (Mitchell, 1998).

2.1.4 Consumption of soft drinks

Ranking according to Consumption of soft drinks, United State of America (USA) is getting the world's number one. Americans drink about 50 billion liters (10 billion gallons) of soft drinks per year. In the Philippines, per capita consumption each year is about 150 bottles of soft drink per year; in Thailand, it is 38 bottles. Even in comparable markets, such as Pakistan and Sri

Lanka, it is 30 and 23 bottles respectively and in India just 5 bottles per year (http://www. Vietnam news brief service.com, 2001).

2.1.5 Health and nutritional aspects of consumption

A policy statement issued by the American Academy of Pediatrics in 2004 outlined the following nutritional concerns associated with the increased intake of high-sugar drinks Contributes to overweight and obesity, Dental cavities due to the high sugar content and enamel erosion from the acidity of soft drinks.

2.2. Processing of carbonated Soft drinks

2.2.1. Raw materials

Basic raw materials of CSD are Purified water, Sweeteners, Carbon dioxide, Permitted colours and flavours, Acidulants, emulsions, heading agents, Preservatives (SLS, 183:1997).

2.2.1.1. Water

Water is always main ingredient and represents 86% of a carbonated drink. Water is used after the further treatment. This is because the water in a soft drink functions as the solvent for all the other ingredients. Therefor it's quality is very important. It should be natural in taste and colour, free of any harmful constituents and in accordance with each individual manufacturer's specification. In Sri Lanka it should confirm to SLS 614 stranded.

Clarification of water

Clarification is a process done for removing impurities in the water. This is series of coagulation, filtration and chlorination. During clarification process desired pH level must be adjusted.

Coagulation

Coagulation involves mixing a gelatinous precipitate, or floc (ferric sulphate or aluminum sulphate), in to the water the floc absorbed suspended particales and easily traprd by filters.

Filtration

The clarified water is poured through a sand filter (different sizes of sand layers) to remove fine partials of floc.

Sterilization

Add chlorine to sterilize the water from microorganism and organic compounds.

Declorination

Activated carbon filter dechlorinates the water and remove residual organic matter.

2.2.1.2. Sweeteners

The Sweetened product shall contain a minimum of 5% by mass of sugar (SLS, 183:1997). Sugar is natural product and normally added to assist taste. Other common sweeteners used in Soft drink are,

Dextrose
Liquid glucose
Fructose
Invert sugar
Sugar syrup (Anonymous, 1991)

2.2.1.3.Carbon dioxide

Carbon dioxide is the only gas suitable for providing the effervescence in Soft drink. It is non toxic, Inert, Virtually tasteless and allows for convenient bulk transportation and storage. When dissolved in water, CO_2 is sparingly soluble providing the characteristic mouth feel and taste to the product means some of it remains as the gaseous form, while a proportion dissolves in the water. CO_2 has a preserving property in some conditions, inhibiting the development of harmful aerobic microorganisms. CO_2 also serves to provide internal pressure in canning to prevent distortion in the container purity should not be less than 99% (SLS183, 1997).

2.2.1.4. Colours and flvours

Colours

The proper use of colour in soft drinks several important functions. It makes the product more aesthetically appealing, healps to correct for natural variations in colours and it helps preserve the identify or character by which drinks are recognized. Basically colours are two types. They are natural colours and synthetic colours. Where the natural colours are botanical extracts or entomological extracts and synthetic colours are artificially produced (Green, 1978).

Table 2.1 Colourings commenly used in Soft drinks in the UK

Name of colour	EEC designation
Tartrazine	E102
Sunset yellow FCF or Orange yellow S	E110
Carmoisine or Azorbuine	E122
Amaranth	E123
Indigo carmine or Indigotine	E132
Green S or Acid Brilliant Green BS Lisamine Green	E 142
Caramel	E150

Source: (Green, 1978, Soft drink technology-1)

Flavours

The flavored product shpuld have pleasant and characteristic flavour. The flavour of the product shall be in accordance with any claim maid or implied on the label. The product shall be free from off flavours and off odors (SLS183, 1997).

2.2.1.5. Preservatives

A preservative is a substance that enables products such as soft drink to have a longer shelf life by inhibiting or arresting the growth of microorganisms such as yeast, moulds and bacteria. A need for preservatives depends upon the type of the product and the processing used.

Table 2.2 Requirement for preservatives in carbonated beverages

Preservative	Requirement	EEC designation
Benzoic acid (mg/kg, max.)	160	210
Sulphur dioxide (mg/kg, max.)	70	200

Source: (SLS183,1997)

When a product contain more than one preservative, the quantity of each preservative expressed as a percentage of the maximum permitted limit of the preservative, shall be calculated. The sum of the percentages shall not exceed 100 (SLS183, 1997).

Benzoic acid

The sodium salt of benzoic acid is more soluble than the free acid and is most commonly used. The undissociated acid, formed from the salt in solution, is responsible for antimicrobial activity for Yeasts and moulds, which is optimum in the pH range 2.5-4.0 (Varnum, A.H, 1994).

Sodium Meta bisulphate

Antimicrobial activity is depending on the concentration of the undissosiated sulphrous acid molecule which is highest at low pH value (Varnum, A.H, 1994).

2.2.1.6. Accidulants

Acid is one of the basic properties of a soft drink. Acid is used in soft drink products to fulfill two main functions. Firstly acidity is a key function in the taste profile of a drink as it balances the sweetness. Secondary it inhibits the growth of microorganisms such as yeast and bacteria (Thoronger and Manning, 1983).

E.g. Citric acid
Ascorbic acid
Acetic acid
Ascorbic acid
Malic aacid
Othophospheric acid (SLS183, 1997).

2.2.1.7. Sodium bicarbonate

Soda water shall contain minimum of 560 ppm/kg Sodium bicarbonate (SLS 183:1997).

2.2.1.8. Heading Agents

A number of natural plant extract are used as heading liquids or formally agent in the beverage industries mainly in ginger beer (Green, 1978). The mechanism of heading agents is analogues to that of emulsifiers in that the interfacial tension between two phases, in the case of foam gas and liquid, is reduced. Heading agents also form an elastic protective barrier between entrapped air bubbles (Varnum, A.H, 1994)

2.2.1.9. Quinine Sulphate

The Tonic shall contain not more than 100 mg/kg quinine salt.

2.2.2. Packaging material

Three types of packaging materials (Glass, PET, Cans) are used (Elephant House Quality Manual, 2003).

Glass bottles

These are returnable packages are two types, Clear and Amber, two capacities, 400 ml and 250 ml.

Table 2.3 Specification of glass bottle (400ml)

Characteristic	Value
Fulfill Capacity	400 ml
Filling Level	1 cm above TM
Brimful capacity	408 – 422 ml
Colour	Clear/Amber
Height	235 ± 1.6 mm

Source: (Elephant House Quality Manual, 2003).

Table 2.3 Specification of glass bottle (250ml)

Characteristic	Value
Fulfill Capacity	250 ml
Filling Level	1 cm above TM
Brimful capacity	263.7 – 274.3 ml
Colour	Clear/Amber
Height	233 ± 1.6 mm
Weight	425 ± 07 g

Source: (Elephant House Quality Manual, 2003).

PET bottles

There are three types according to colour (clear, amber, green.). Benefits of the pet bottles are,

Light weight
Space saving
High quality finish
Shatterproof
Environmentally acceptable
Modern image
Cost competitive

Table 2.4 Specification of PET bottle (1500ml)

Characteristic	Value
Meterials	Poly Ethylene Terephthalate
Fulfill Capacity	1.5 Ltr.
Brimful Capacity	1531 ml
Colour	Clear
Height	328.5 – 331.5 mm
Weight	48.5 – 51.5 g

Source: (Elephant House Quality Manual, 2003)

Crowns

Table 2.5 Specification for crowns

Characteristic	Value
Height	6.48 – 6.88 mm
Diameter	31.85 – 32.25 mm
Weight of Crown Cork	2.55 – 2.60 g
Weight of wadding	0.28 – 0.30 g

Source: (Elephant House Quality Manual, 2003).

2.2.3. Process steps

Syrup filtering

After the adding preservative the stock syrup should filter to remove foreign matters.

Decrating

Empty bottles are unload from crates to conveyer belt which goes to bottle washer

Bottle washing

Returnable bottles are washed. Main four steps are involved.

a) Rinse with water and remove labels and foreign matters.
b) Dipped and washed with hot Coastic (60^0C). Then again washed by hot caustic (80^0C).
c) Rinse by water

Pet bottles are use after the rinse by treated water.

Intermixing

The water and flavoured syrup are carefully combined by sophisticated machine called proportionater, which regulates the flow rate and ratios of the liquids.

Filling and Crowning or craping

Finished product is transferred into bottles (Glass or PET) or cans at extremely high flow rates. The containers are immediately sealed with pressure-resistant closures, either tinplate or steel crown with corrugated edges, twist offs, or pull tabs.

Labeling

After the filling, labels are affixed to bottles to provide information about the brand, ingredients, shelf life, and safe used of the product. Before to labeling, the containers are cooled with warm water and drying them. Labeling is done by labeling machine which is in different brand name.

Crating

After the labeling Full bottles are put in to crates or packed in cartons.

Storing

Full bottle crates or cartons are stack in the pallets to storing or distribution

2.3. Chemical parameters

2.3.1. pH

The symbol, pH has been adopted for the logarithm of the reciprocal of the hydrogen ion concentration. If the hydrogen ion concentration (represent by $[H^+]$) of a solution is known, the corresponding pH of the solution may be calculated from the formula:

$$pH = \log 1/[H^+]$$

Different type of calibrated pH meters can be use measure the pH of the content (Jacobs, 1959).

2.3.2. Benzoic acid

Undissociate molecules are important to prevent growth of yeast and moulds.

2.3.3. Sulpher dioxide

Precursor of SO_2 is SMS. Used as preservative.

Carbonation

A fundamental statement for the solution of any gas in any liquid is given condition of pressure and temperature; there is a maximum quantity of gas, which may be dissolved in a liquid. At constant temperature the volume of dissolved gas is proportional to the absolute pressure. At the lower temperature high amount of CO_2 gas is dissolved in water (Green, 1978).

2.3.4. Brix

Since the amount of sucrose dissolve in water is important industrially, a whole series of measuring indices were developed to indicate the relative proportion of the two materials. The brix scale is also used to measure solutions other than pure sucrose and water. Thus, a Brix reading will normally be used to obtain the corresponding specific gravity or refractive index of a solution that, by practice, is agreed to correspond to a solution of pure sucrose and water at a set reference temperature, usually 20^0C. The instrument to used to measure this variable are called refractometers (Jacobs, 1959).

2.4 The Microbiology of the carbonated soft drinks

2.4.1 Introduction

Soft drinks are more susceptible to microbial spoilage than other food products, due to both intrinsic and extrinsic factors. The natural flavourings contribute to undergo gradual change. The flavour changes that occur during storage often result in an off flavour. In addition to aging or off flavour or conversion of one compound to another. They are actually due to three different kind of microorganisum like yeast, mould and bacteria.

2.4.2 Microbial problem associated with soft drink

Effect of microbes in soft drink cannot be completely excluded of public health and economic stand-point. Microbiological problems associated with soft dink are almost entirely those of spoilage; Spoilage may occur as,

Off odor
Off flavour
Sediments or large clusters
Turbidity

Excess gas production (Kees, 1999)

2.4.3 Sources of Microbial contamination in soft drinks

Of the raw materials utilized in the manufacture of soft drinks, water, sugars, fruit juices, plant extracts, flavouring and colouring agents are the major source of microbial contamination, whilst other raw materials such as washed bottle may contains few microorganisms which may survive treatment during processing and manufacturing (Green, 1978).

2.4.4 Micro organism responsible for spoilage of Soft drink

The problem microorganisms associated with the soft drink industry are usually confined to some yeast, acid tolerant bactria, a few non acid tolerant bacteria and a selection of moulds (Ashurst, P.R, 1998).

Bacteria

Some bacteria can also survive in finished beverage and, in addition to causing off taste, off odor, and gas production and a slimy texture in beverage. E.g. *lactobacillus*, *Leuconostoc* and *Gluconobacter* are types of soft drink spoilage bacteria.

Yeast and Moulds

Yeasts are the dominant spoilage organisums in soft drinks. Spoilage patterana typically involve film formation, fermentation with gas production, turbidity and sediments and "fruity" flavours. Mould spoilage involves visible growth together with bitter flavour and discoloration (Varnnam, 1994).

2.4.5 Yeast and moulds species in non-alcoholic beverages

Yeast species

Taxonomy

Division: Ascomycotina
Family: Saccharomicetaceae (ascospors and arthrospores formed; vevegetative reproduction by fission or budding)
Subfamily:Nadsonioideae

Genus:Hanseniaspora

Subfamily:saccharomycotoideae

Genus: *Debaryomyces*

Issatchenkia

Kluyveromyces

Pichia

Saccharomyces

Torulaspora

Zygosaccharomyces

Division:Deuteromycotina

Family:Cryptococcaceae(the"imperfects"; reproduce by budding)

Genus: *Brettanomyces*

Candida

Brettanomyces

These sporogenous yeasts from ogival cells, terminal budding, and produce acetic from glucose only under arobic conditions. *Brettanomyces intermedius* is the most prevalent, and it can grow at a pH as low as 1.8. They cause spoilage of beer; wine, soft drinks and some are involved in after-fermentation of some beers and ales.

Candida

This genus was erected in1923 by Berkhout and has since undergone many changes in definition and composition. It is regarded as being a heterogeneous taxon that can be divided into 40 segments comprising three main groups, based mainly on fatty acid composition and eleectrophoretic karotyping. The generic name means "shining white," and cells contain no carotenoid pigments. Some species found in soft drinks. Such as,

Candida boidinii

Candida guilliermonii

Candida intermidia

Pichia

This is the largest genus of true yeasts. They reproduce by multilateral budding, and the asci usually contain four spheroidal, Hat- or saturan shaped spores. Pseudomycelia and arthrospores may be formed. Typically form films on liquid media and are known to be important in producing

indigenous foods in various part of the world. *Pichia anomala* and *Pichia membranaefaciens* are responsible for spoilage of soft drink.

Saccharomyces

These ascosphorogenous yeasts multiply by multilateral budding and produce spherical spores in asci. They are diploid and do not ferment lactose. *Saccharomyces cerevisiae* is cause to ferment soft drinks. It is resistance to benzoate and sorbate preservatives.

Torulaspora

Multilateral budding is the method of reproduction with spherical spores in asci. Three haploid species formerly in the genus saccharomyces are now in this genus. They are strong fermentors of sugars, and contain enzyme Q-6. *Torulaspora debrueckii* is the most prevalent speasis and spilage soft drinks.

Zygosaccharomyces

Multilateral budding is the method of reproduction, and the bean- shaped ascospores formed are generally free in asci. Most are haploid and they are strong fermenters sugars. Some are common spoilers of mayonnaise, salad dressing and soft drinks, especially *Zygosaccharomyces baili* , which can grow at pH 1.8.

Moulds species

Division:Zygomycota
- Class:Zygomycetes (nonseptate mycelium,reproduction by sporangiospores,rapid growth)
  - Order:Mucorales
    - Family:Mucoraceae
      - Genus:Rhizopus

Division:Deuteromycota (the "imperfects",anamorphs;perfect stages are unknown)
- Class: Hypomycetes (hyphae give rise to conidia)
  - Order:Hyphomycetales
    - Family:Moniliaceae
      - Genus:*Aspergillus*
        - *Botrytis*
        - *Cladosporium*
        - *Fusarium*

Geotrichum

Penicillium

Aspergillus

Chains of conidia are produced. Where cleistothecia with ascospores are developed, the perfect state of those found in foods. Produses bright- yellow cleeistothecia, and all species are xerophilic. Two species (*A.flaves* and *A. niger*) produce aflatoxin, and others produce ochratoxin A and stregmatocystin.

Botrytis

Long, slender, and often pigmented conidiophores are produced. Mycelium is septate; conidia are borne on apical cells and are gray in colour, although black, irregular sclerotia are sometimes produced. B.cinerea is the most common in foods. They are notable as the cause of gray mould rot of apples, pears, raspberries, strawberries, grapes and some stone fruits.

Cladosporium

Septate hyphae with dark, treelike budding conidia variously branched, characterize this genus. In culture, growth is velvety and olive colored to black. Some conidia are lemon shaped.

Feusarium

Extensive mycelium is produced that is cottony with tinges of pink, red purple, or brown. Septate fusiform to sickle-shaped conidia (macro conidia) are produced. They cause brown rot of citrus fruit and pineapples and soft rot of figs.

Giotrichum

This yeasts like fungi are usually white. The hypae are septate, and reproduction occurs by formation of arthroconidia from vegetative hyphae. The arthroconidia have flattened ends. *G. candidum*, the anamorph of *Dipodascus geotrichum*, is the most important species of foods. It is variously referred to as machinery mould, since it builds up on food-contact equipment in food processing plants.

Penicillium

When conidiophores and conidia are the only reproductive structures present, this genus is placed in the Deuteromycota. They are placed with the ascomycetes when cleistochecia with ascospores are formed as either *Talaromyces* or *Eupenicillium*. Of the two-

telemorphic genera, *Talaromyces* is the more important in foods. *T. flavus* is the telemorph of *P. dengerdii,* and it has been involved in the spoilage of fruit juice concentrates. It produces heat resistant spores. When conidia are formed in the penicillius, they pinch off from phialides. Typicaly colours on foods are blue to blue-green. Some species produced citrinin, ochratoxin A, rubratoxin B and other mycotoxin.

Rhizopus

Nonseptate hyphae are produced that give rise to stolons and rhizpids. Sporongiophores typically developed in clusters from ends of stolons at the point of origin of rhizoids (Jay, J.M, 1996).

2.4.6 Growth conditions for yeast and mould

Both intrinsic factors and extrinsic factors are affecting to the growth of yeast and moulds.

- Water activity (a_w)

  Water activity is differs to genus to genus and species to species.

Aspergillus	0.68 - 0.88 (a_w)
Botrytis	0.93 (a_w)
Penicillium	0.78 -0.98 (a_w)

- pH

Yeast and moulds have narrow pH rang.

Yeast: pH 1.5 -8.5

Moulds: pH 0 - 11

- Temperature

Many moulds are able to grow at refrigerator temperatures, notably some strain of *Aspergillus, Cladosporium.* Yeast grows over the psychotropic and mesophilic temperature ranges but generally not within the thermopilic range.

- Nutrient content

To grow, yeast and molds need foods which contain a carbon source. Especially sucrose medium has high susceptibility to yeast and mould contaminations. Many yeasts can grow in the presence of 55 -60 % sucrose (Banwart, G.J, 1998).

2.4.7 Aspects and effectiveness of product quality

According to the SLS yeast and moulds count should be nil per 1ml of finished product (SLS, 183:1997). So if yeasts and moulds are present it should affect quality of the product.

2.4.7.1 Appearance

Appearance may decrease due to the production of sediments, pellicles, gasses, turbidity and clusters.

2.47.1 Consumer acceptability

Consumer acceptability may reduce due to the production of off taste, off flavours, off odors.

2.4.7.3 Effectiveness of consumer health

Occasionally allegations of food borne disease associated with soft drinks. The vast majority of these are with out substances, although a small number of cases are known where mild sickness has followed consumption of soft drink containing large numbers of yeast or visible mould pellicles (Varnnam, 1994).

2.4.8 Microbiological analysis

2.4.8.1 Sample collection

A convinient way to do microbiological analysis by examining samples of syrup, water and product taken aseptically from key points during manufacturing. The sample should include water from the treatment plant, simple syrup before and after filteration, Final syrup from the proportioniater, carbonated water, washed bottle and finished product (Green, 1978).

2.4.8.2 Routine microbiological test methods in the soft drink industry

Pour plate technique

In this method agar medium is inoculated while it is still liquid (but cool, at about 45^0C) and therefore colonies develop throughout the medium and not only on the surface. Better distributions of colonies are obtained in a well made pour plate and isolations are more easily made (Coomaraswamy. U, 1997).

Dry film technique

A dry film method consisting of two plastic films attached together on the side and coated with culture media ingredients. When using selective culture media ingredients certain specific groups can be detected. Use of this method to date indicates that it is an acceptable alternative to standerd plte count methods that employ Petri dish, and it has been approved AOAC (Jay, J.M, 1996).

Swab testing

Swabbing is the oldest and most widely used method for the microbiological examination of surfaces. This method was developed in 1917 by W. A Manheiner and T. Yabaanez. Either cotton or calcium alginates swabs are used. It gives microbial count in a limited area (E.g. cm^2 or $inch^{2)}$. This method remains a rapid, simple, and inexpensive way to asses the microbiological flora on food surface and tensile (Jay, J.M, 1996).

Spread plate method

This method used in microbiological examination in washed bottles. Pre determine amount of sterilize Ringer was added and roll in flat surface. Assume that microorganism, which is in inner surface of bottle come to the Ringer solution (EHQM, 2003).

Slide culture technique

The best method for preserving and observing the structure of fungi and their sporulation characteristics is the slide culture technique. It is not a rapid method, but is unsurpassed as a routine means of studying the finer points of the microscopic morphology of fungi. When making slide cultures, sterile conditions are very important. The work must be done under a sterilized Petri dish lid on a surface cleaned with alcohol. All instruments, slides and cover-glass should be carefully sterilized (Coomaraswamy.U, 1997).

2.5 Maintenance of Factory hygiene

2.5.1 Clean-In-Place (CIP) Applications

CIP is the method used in sanitary processing plants to clean tanks, piping and even workspaces between production batches by automatically recirculating detergent and rinse solutions. The washing process consists of several cycles in which rinsing material is recycled through the vessels, pumps, valves and other process equipment in the flow system. Benefits of CIP application are,

- Reduced Water Usage
- Reduced Sanitizer Consumption
- Reduced Operating Costs
- Reduced Wastewater Costs
- Increased Efficiency - Reduced Cycle Times
- Increase Available Process Time

2.5.2 CIP of production line

For plant hygiene, CIP scheme should follow procedures such as,

- Cold water rinse to remove syrup and product residues,
- Circulation of detergent,
- Cold water flushed to ditergent,
- Sterilisation by circulation of cold chemical sterilant (Usually halogen based),
- Cold water flushes to remove sterilant.

Much modification to this scheme can be made. E.g. If the equipment can withstand high temperatures, hot water (Grater than $80^{0}C$) can be used as a sterilent or the detergent and steriliosation step could be combind in one step by circulating hot ditergent solution (Green, 1978).

2.5.3 CIP of production environment

Production environment should be clean and dry. Dirt bottles, dirt crates are stack in separate places. Production area covered from external environment to prevent migration of air born contaminations. Production floor and tables are rinse with hot water or pressurized water or pressurized air every day (Green, 1978).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Preliminary Study

The ongoing manufacturing process of carbonated soft drinks was studied, firstly in order to identify possible sources of contamination. Previous counts of yeasts and moulds for the finished products were analyzed to identify the occurrences unacceptable conditions. Then the process flow diagram was constructed to identify process steps, raw materials and process conditions which may render the contamination by yeasts and moulds favourable.

3.1.1 Analysis of past data

Yeast and mould results of the soft drinks (finished product) for last 9 months i.e. from August 2005 to March 2006 were analyzed.

3.2 Microbiological analysis

3.2.1 General methods

Yeast extracts agar media preparation

23g (OXOID yeast extract agar) of sample was suspended in 1 liter of distilled water and boiled to dissolve the content completely. Then it was sterilized by autoclaving at 121^0C for 15 minutes.

Ringer solution preparation

45g NaCl, 2.25g KCl, and 1.25g $CaCl_2$ were added in to 2000 ml of distilled water to prepare the stock solution. Then mixed well and put into containers after diluting 10 times. Then it was sterilized by autoclaving at 121^0C for 15minutes.

Antibiotic solution preparation

50 mg of tetracycline and 50mg of chlorumphenicol were dissolved in 100 ml of phosphate buffer.

Glassware sterilization

Well cleaned Petri dishes, pipettes and all other glass ware were sterilized by autoclaving at 121^0C for 15 minutes.

Dilution preparation

- **First dilution**

10ml of sample was measured and add into 90ml of Ringer solution and the mixed thoroughly.

- **Second dilution**

1ml of sample was measured and added in to the 99ml of Ringer solution and the solution was mixed thoroughly.

- **Third dilution**

1 ml of sample was taken in first dilution and added in to the 99ml of Ringer solution and the solution was mixed thoroughly.

3.2.2 Sample testing

3.2.2.1 Determination of yeast and moulds in water.

Materials

Sterilized sample bottles and pipettes
Samples - Purified water (1ml)
Carbonated water (1ml)
Final rinse water (1ml)
3M™ petrifilm plate
Sterilized yeast extracts agar media (15 ml per Petri dish)
Antibiotics (1ml for 50 ml prepared yeast extract agar)
Sterilized Petri dishes
Colony counter

Methodology

Pour plate techniques and 3M™ Petrifilm were used.

Pour plate method

- 1ml of sample was aseptically incubated into sterilize plate and approximately 15-25ml of yeast extract agar with antibiotic was poured (50ml of yeast extract agar was prepared by adding 1ml of antibiotics).
- The temperature of yeast extract agar was 45^0C. Then the lid was closed and shaken gently for even distribution of media in Petri dish.
- After that it was kept in few minutes at the room temperature for solidifying the yeast extract agar.
- The solidified plates were placed upside down in the sterilized plate in room temperature for 72 hours.
- Colonies appearing in the media were counted by using the colony counter and the results were recorded.

3M™ Petrifilm method

- The Petrifilm Y& M plate was placed on a flat, level surface.
- Top film was lifted and dispensed 1ml of sample suspension onto the center of the bottom film.
- Top film was droped down onto the sample.
- Plastic Petrifilm Y& M spreader was placed on the center of the plate and pressed gently on the center of the spreader to distribute the sample evenly.
- The inoculum was spread over the entire Petrifilm plate growth area before the gel is formed.
- The spreader was removed and the plate was undisturbed for at least one minute to permit the gel to form.
- Petrifilm plates were incubated in a horizontal position with the clear side up in stacks of no more than 20 plates.
- Colonies were counted on Petrifilm Y& M plates by using colony counter.

3.2.2.2 Determination of yeast and moulds in Stock syrup and Flavored syrup.

Materials

Samples - Stock syrup and flavourerd syrup (1ml)
Sterilized sample bottles and pipettes
3M^{TM} petrifilm plate
Sterilized yeast extracts agar media (15 ml per Petri dish)
Antibiotics (1ml for 50 ml prepared yeast extract agar)
Sterilized Petri dishes
Colony counter

Method

- Dilution series (1st, 2nd, 3rd) was prepared from both stock syrup and flavoured syrup.
- 1ml was taken as sample from each dilution and analyzed for yeasts and moulds using above methods.

3.2.2.3 Determination of yeast and moulds in mixed product

Materials

Mixed product - syrup which pump to filler (1ml)
Sterilized sample bottles and pipettes
3M^{TM} petrifilm plate
Sterilized yeast extracts agar media (15 ml per Petri dish)
Antibiotics (1ml for 50 ml prepared yeast extract agar)
Sterilized Petri dishes
Colony counter

Method

- Sample was degassed and taken 1ml and followed analyzed for yeasts and moulds using above methods.

3.2.2.4 Determination of yeast and moulds in mouthpieces in filler

Materials

Sterile swabs (cotton wool) and test tubes
Sterile Ringer solution (20 ml)
Sterilized pipettes
3M™ petrifilm plate
Colony counter
Sterile measuring cylinder (100ml)

Method

- Sterile cotton swab was dipped in sterile Ringer solution and mouthpieces of filler were wiped and samples were taken into test tubes.
- 20ml sterile ringer solution was added in to test tubes and roll twenty times by hands.
- 1ml was taken as a sample and followed the above 3M™ Petrifilm method for detecting yeasts and moulds.

3.2.2.5 Determination of yeast and moulds in washed bottles

Materials

Sterile cotton plugs
Washed bottle
Sterile Ringer solution (20 ml)
Sterilized pipettes
3M™ petrifilm plate
Colony counter
Sterile measuring cylinder (100ml)

Method

- Washed bottles were collected from Just after the washing and just before the filling.
- Washed bottle mouth was closed by sterile cotton plug by just after taking it for further sampling.
- 20 ml of sterile Ringer solution was added to each bottle and then rolled 20 times on a flat surface.
- 1ml was taken as sample and followed above 3M™ Petrifilm method.

3.2.2.6 Determination of yeast and moulds in finished products.

Materials

Finished product (1 ml)
3M™ petrifilm plate
Sterilized yeast extracts agar media (15 ml per Petri dish)
Antibiotics (1ml for 50 ml prepared yeast extract agar)
Sterilized Petri dishes
Colony counter

Method

- Sample was degassed by shaking and taken 1ml and analyzed for yeasts and moulds using above methods.

3.2.2.7 Determination of yeast and moulds in air (production environment and Syrup room)

Material

Sterile Petri dishes with solidified yeast extract agar. (15 ml per Petri dish)

Method

- After opening lid and solidified agar plates were placed in the production environment.
- Solidified agar plates were exposed to air for about 30 minutes.
- Then closed the agar plate placed upside down in the sterilized plate in room temperature for 72 hours.
- Colonies appearing in the media were counted by using the colony counter and the results were recorded.

3.2.3 Isolation of yeasts and Moulds genera in soft drink environment.

3.2.3.1 Identification of colony characteristic

Materials

Sterilized solidified agar plates mixed with antibiotics (1ml for 50 ml prepared yeast extract agar)
Sterilized wire loop

Method

- Inoculation loop was sterilized until red hot.
- A small portion of sample (one loop full) was taken to the wire loop and streaked in zig zag manner on solidified agar medium.
- The streaked plates were placed in room temperature for 72 hours.
- Pure colony was observed by naked eye.

3.2.3.2 Identification of morphological characteristics.

Materials

Slide and coverslips
Bent glass rod (U shaped)
Agar plates with squares cut
Inoculation needle
Sterile Petri dish
Sterile Water
Microscope

Method

- A Bent glass Rod (U Shaped) was placed on a filter paper at the bottom of a petri dish. A clean glass slide was placed on the glass rod. Petri dish was covered and sterilized.
- Sterile water was used for moistering and the filter paper was introduced.
- A small block of agar (about 5-6mm square, cut from an agar layer obtained by pouring 10-12ml appropriate agar in a sterile dish) was placed on the center of the glass slide.
- The fungus was inoculated onto the center of the four sides of the agar block by using inoculation needle.

- A cover slip (from a 70% alcohol jar) was flamed and placed centrally over the black and slight pressure is applied to ensure adherence.
- The set up was incubated under room temperature and light conditions suitable for sporulation of the microorganism for 2-5 days.
- With in 2-5 days the slide was examined by using microscope.

3.3 Testing for chemical parameters for finished product

3.3.1 pH value of finished product

Material

Bench type pH meter
Distilled water
Buffer solutions (4.0, 9.0 and 7.0) pH
Beaker (50ml)

Method

- 25ml of sample taken into a cleaned 50ml beaker and pH meter was switched on.
- Electrodes was placed in buffer 7 and temperature was adjusted to buffer temperature
- pH meter was calibrated to buffer 7 using 'Cal's 1 adjestment.
- Electrode was rinsed with distilled water and placed in second buffer (Buffer 4 or buffer 9).
- pH meter was calibrated to buffer 4 or 9 using 'Cal 2' adjustments.
- Electrode was rinsed with distilled water.
- Electrode was placed in the sample and temperature was adjusted to sample temperature.
- pH reading was taken.

3.3.2 Determining the concentration of benzoic acid in the finished product by HPLC.

Materials

HPLC machine

Mobile phase: Acitic acid / Sodium acitate buffer with acetonitrile

Benzoic acid (AR grade)

Ethanol (HPLC grade)

Volumetric flask (10, 500, 1000) ml

HPLC condition: Coloumn: ODS Hypersil, C_{18} (280*4.6mm)

Flow rate: 1ml/minute

Inject volume: 20μl

Detector: UV detector at 245nm

Minimum detection level: 10 ppm

Method

3.3.2.1 Sample preparation

Standard solution

- For stock solution, 0.5g A.R grade benzoic acid and 2ml at 98% HPLC grade ethanol were added to 1000ml volumetric flask and top up with distilled water.
- For preparation of four standard samples 2ml, 4ml, 6ml, 8ml at benzoic acid were taken from stock solution.

Soft drink sample

- A soft drink sample was degassed by using ultrasonic bath with in 5 minutes.
- 10 ml was taken and dilute to 100ml.

3.3.2.2. Sample inject to HPLC machine

- First 20μl were injected from each slandered sample.
- Then 20μl were injected from sample.

Calculation

- Calculate area under the peak of the chromatogram of benzoic acid solution.
- Construct calibration curve: Peak area vs. concentration using the computer soft ware STATGARPHIC (Version. 2.6).
- If the co-relation coefficient is less than 0.95 review the standered an exclude any standered that are obvious outliers. If less than three value for strandered are remaining after elimination of suspect points.
- Prepare a new series of standered and repeat the calibration.
- If the correlation coefficient is grater than 0.95 calculate the benzoic content of the sample using the peak area of the sample chromatogram.

Benzoic acid content (ppm)

$$\frac{C_1V_1}{V_0(M)}$$

C_1 Benzoic acid in sample solution (ppm)

V_1 - Sample volume (ml)

V_0 – Final sample volume (ml)

M- Mass of the sample (g)

3.3.3 SO_2 concentration in the finished product

Materials

25 ml Measuring cylinder

Conical flask

50ml Burette

Porcelain dish

N/50 Iodine solution

Method

- 25ml of degassed sample was taken in to conical flask.
- Two drops of starch was added as indicator.
- Sample was titrated against N/50 Iodine solution.
- In the end point Burette reading was recorded and the SO_2 concentration was calculated by using reference chart. (Appendix 1I)

3.3.4 Carbonation of finished product

Materials

Pressure gage

Thermometer

Carbonation chart

Method

- Pressure gage was attached to crowned bottle and twisted to seal and hole is made in the crown.
- Dial was showing the pressure.
- Then bottle was opened and measured the temperature.
- Carbonation valve was determined by analyzing reference carbonation chart (Appendix 11).

3.4 Evaluation of CIP parameters

3.4.1 Temperature and time combination of the CIP processes

Temperature and time were recorded at the hot water tank and filler.

3.5 Implementation of corrective actions and preventive measures

The best concentration of the chlorine solutions used for washing mouthpieces was selected by performing a one way Analysis of Variance (ANOVA) for yeast and mould counts obtained for different chlorine concentrations viz. 175 ppm, 250 ppm, 400 and 500 ppm.

3.5.1 Preparation of 400ppm chlorine solution for washing of mouthpieces.

Materials

NaOCl (1.53g)
Neslerrizer
Spray gun
Purified water (50 ml)
Nestler tubes
Orthotoluine (0.5 ml)

Method

- 1.53g of sodium oxichloride wsa dissolved in 1L of distilled water.

To analyze residual chlorine in prepared solution

- 0.05 ml of chlorine solution was put in to Nestler tube from stock chlorine solution.
- 0.5ml Ortotoludine was added and top up to 50ml with distilled water.
- Another Nestler tube was filled with 50ml of distilled water and it placed left side in the nestlerizer.
- Sample solution was pleased in the right side of the nestlerizer.
- Col'our was compared by using Nesler disk and taken the value.

Calculation

Chlorine concentration = Dilution factor* disk reading

- Mouthpieces were washed from prepared chlorine solution by using spray gun.

3.5.2 Evaluation of the effectiveness of corrective and preventive measures

Yeast and moulds count were determined before and after the treatment with Cl_2 i.e. washing and finished product as described in swab testing and petrifilm methods respectively. Effectiveness was proven by performing a Paired t Test using the MINITAB statistical package.

CHAPTER 4

RESULT AND DISCUTION

4.1 Identification of the places of production line that are contaminated with yeasts and moulds

4.1.1 Construction of process flow diagram

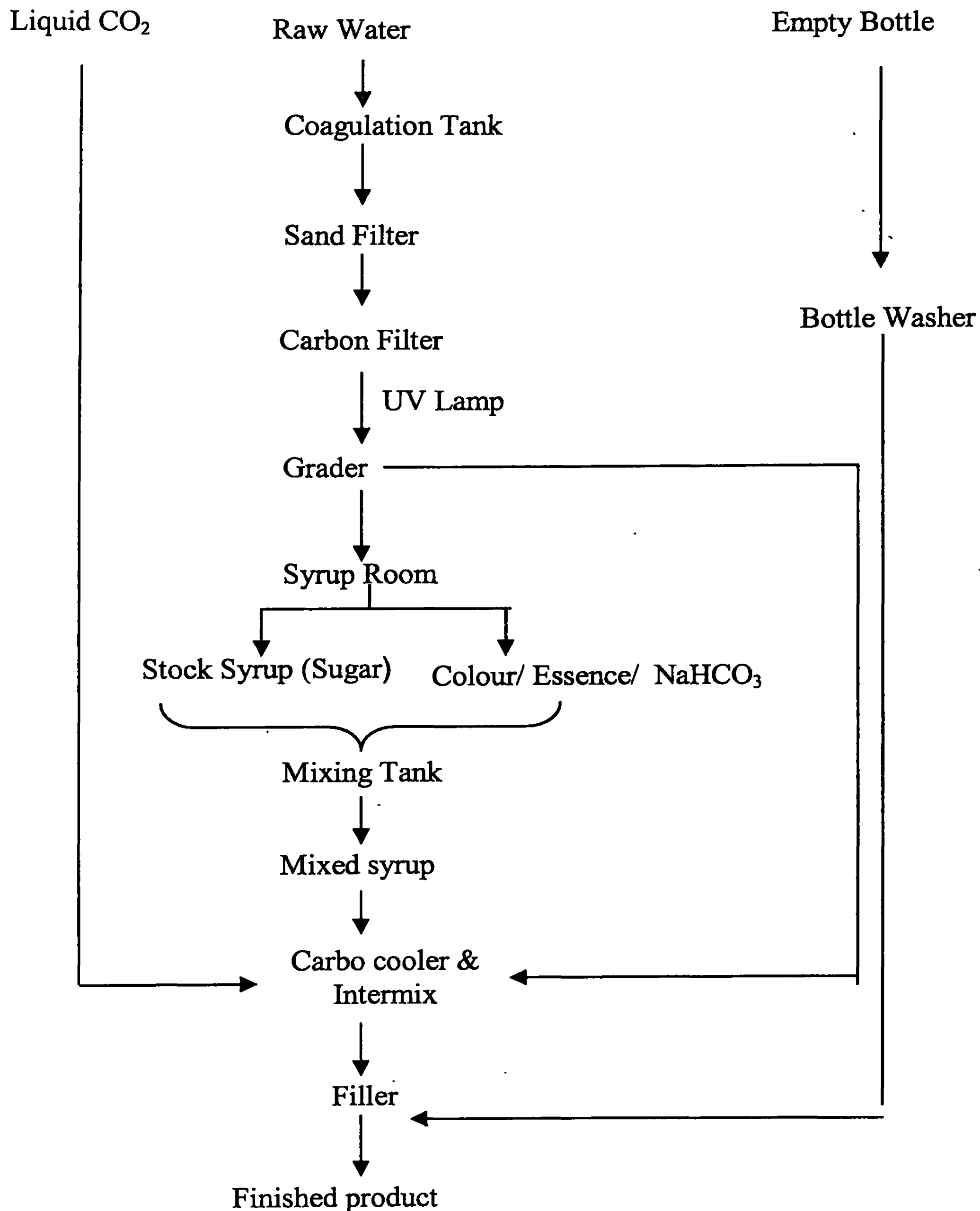

Fig 4.1 Production flow of the soft drink production line.

Construction flow has been designs for determining raw materials, which are required for the production process and to identify the steps, which are prone to microbial contamination.

4.1.2 Past data analysis

Yeasts and Moulds count in Carbonated soft drink

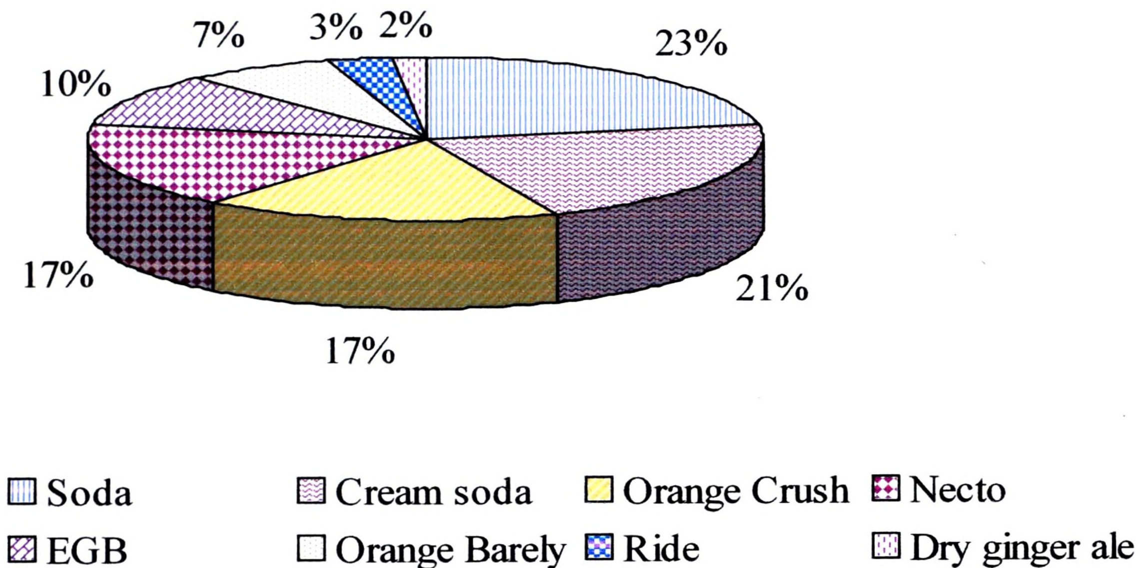

Fig.4.2 Past data of yeasts and moulds recorded in finished products from Aug 2005 to March 2006.

During the sample collection period dated from August 2005 to March 2006 it could be observed that Soda was contaminated 39 times by yeast and moulds. Followed by Cream soda with 37 times and Orange Crush with 30 times. Even though Soda showed a count of 39 for yeast and mould it doesn't contain any carbon source and no preservatives are used during the production. Necto, EGB, Orange Barely, Ride and Dry ginger ale were counted respectively 29, 18, 13, 05 and 03 times for yeasts and moulds. All the other products except Soda are added with sugar, which adds sucrose to the product, and also they contain preservatives such as benzoic acid and sulphur dioxide. And colours and flavours as well.

4.2. Results of microbiological analysis.

4.2.1 Determination of yeast and moulds in water.

4.2.1.1 Determination of yeast and moulds in treated water.

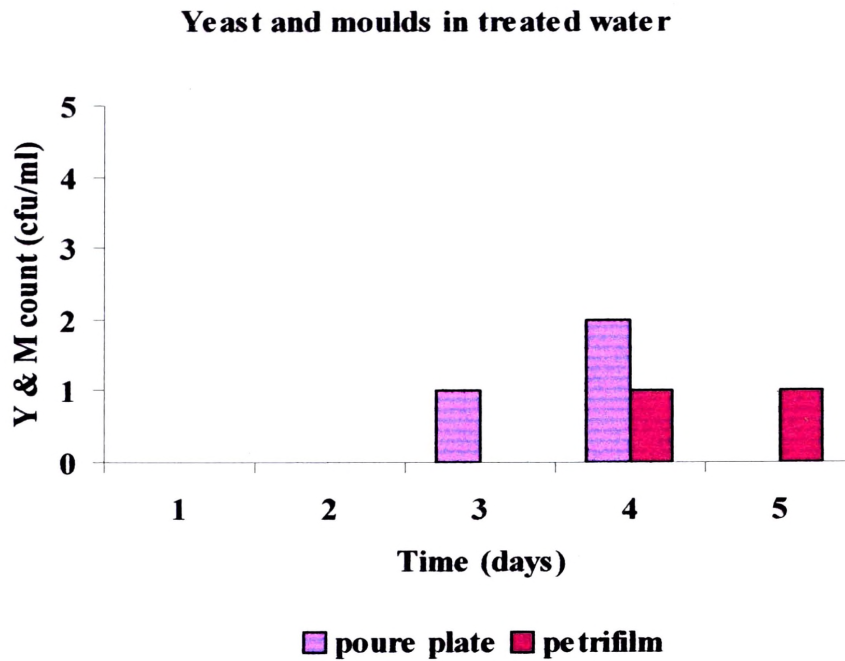

Fig. 4.3 yeasts and moulds count in treated water

Yeast and moulds has been detected in purified water (Table 4.2), which has gone through coagulation tank, Sand filter, Carbon filter and UV sterilizer. It was identified that UV sterilizer was not working properly.

4.2.1.2 Determination of yeast and moulds in Final rinse water.

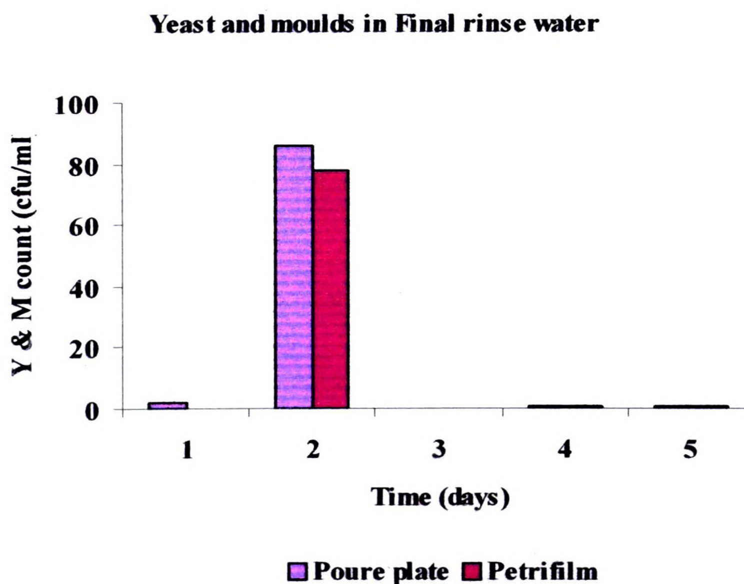

Fig 4.4 yeasts and moulds count in Final rinse water

Raw water, used as final rinse water, which is not either purified or sterilize, has been detected for yeasts and moulds (Table 4.3). Raw water is used for the final stage of washing bottles and to wash mouthpieces, which are damaged due to the blasting of bottles.

4.2.2 Determination of yeast and moulds in stock syrup and flavored syrup

4.2.1.1 Determination of yeast and moulds in stock syrup

Table 4.4 yeasts and moulds count in Stock syrup

Date	Yeast extract agar	3M™ Petrifilm
1	Uncountable	Uncountable
2	Uncountable	Uncountable
3	143	178
4	90	62
5	104	90

Table 4.5 yeasts and moulds count in stock syrup before and after filtering

Date	Yeast extract agar	
	Before filtering	After filtering
1	0	Un Countable
2	0	197
3	52	46
4	0	106
5	76	98

Cane sugar is the main sweetening ingredient in the soft drink production accepts Soda. Sucrose is the main component of sugar. It is easily contaminated with yeast and moulds and is further grown rapidly in medium of sugar. So, it shows high count of yeast and moulds.
If not sterilized or partially sterilized filter papers, where the stock syrup is traveling through, even uncontaminated syrup will get contaminated yeasts and moulds.

4.2.2.2 Determination of yeast and moulds in flavoured syrup

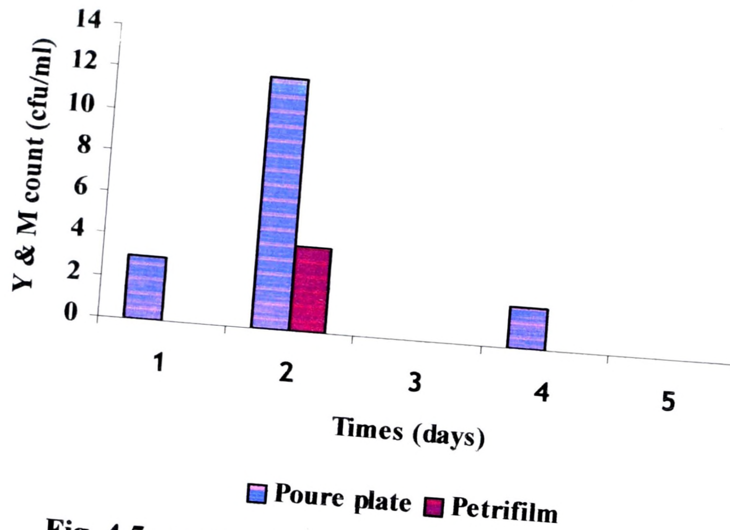

Fig. 4.5 yeasts and moulds count in flavoured syrup

Contaminated stock syrup which is added with flavors, colours and accidulants does not give yeast and mould count due to the increase in pH by acidulants.

4.2.3 Determination of yeast and moulds in mixed product.

4.2.3.1 Determination of yeast and moulds in mixed product (for sweet drinks)

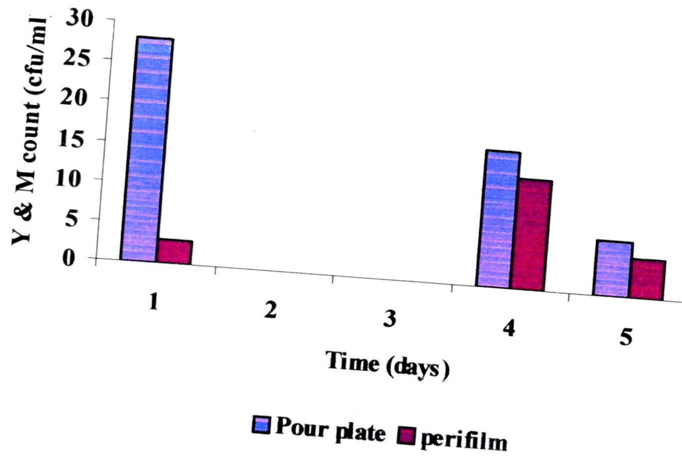

Fig. 4.6 yeasts and moulds count in mixed product (for sweet drinks)

Mixed product of sweet drinks, which is the mixture of flavoured syrup and carbonated water, is contaminated with yeasts and moulds mainly due to the contaminated flavour syrup. Moulds and yeasts count is further decreased due to high carbonation pressure and acidification by carbon dioxide.

4.2.3.2 Determination of yeast and moulds in mixed product (for soda drink)

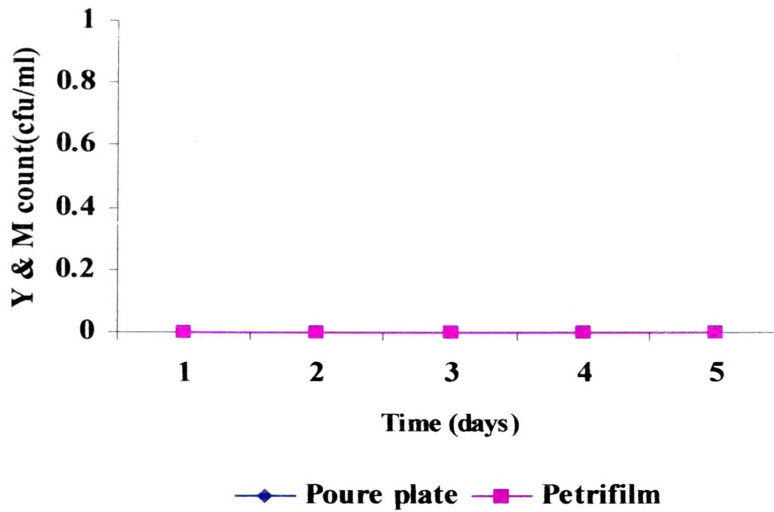

Fig. 4.7 yeasts and moulds count in mixed product (Soda drink)

Since raw material of Soda ingredient of Sodium bicarbonate or mixed product mixture of Sodium bicarbonate and carbonated water it is not contaminated, mixed product of Soda does not show any yeasts and moulds count.

4.2.4 Determination of yeast and moulds in mouthpieces in filler

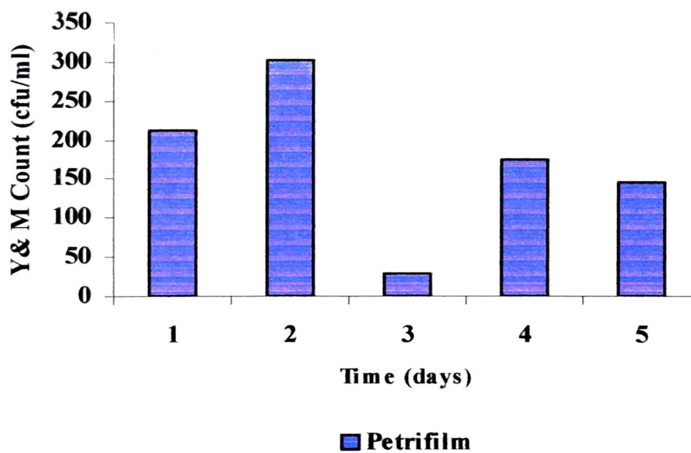

Fig. 4.8 yeasts and moulds count in mouthpieces in filler

During the process of CIP, mouthpieces of filler are not washed with hot water. So Yeasts and Moulds are not destroyed. And also, damaged filler mouthpieces due to blasted bottle particles produce a suitable medium for the growth of yeasts and moulds by depositing sucrose in damaged spaces.

4.2.5 Determination of yeast and moulds in washed bottles

4.3.5.1 Determination of yeast and moulds in washed bottles (just after washing).

Bottles, just after washing are contaminated with yeasts and moulds due to raw water, the material using to wash bottles.

4.3.5.2 Determination of yeast and moulds in washed bottles (just before filling)

Bottles, which are washed and coming to the filler through conveyer belt are contaminated with yeasts and moulds, because they are exposed to the environment spores.

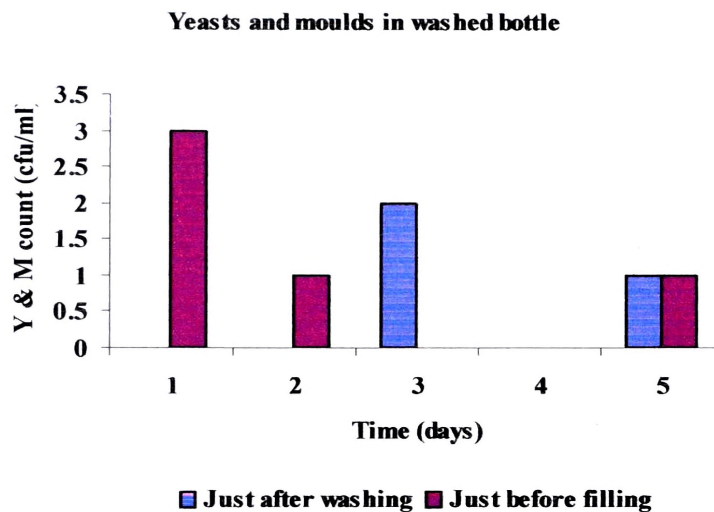

Fig. 4.9 yeasts and moulds count in washed bottles

4.2.6 Determination of yeast and moulds in production and syrup room environment

4.2.6.1 Determination of yeast and moulds in production environment

Table 4.12 yeasts and moulds count in production environment

Date	3M^{TM} Petrifilm
1	Uncountable
2	Uncountable
3	Uncountable
4	Uncountable
5	Uncountable

4.2.6.2 Determination of yeast and moulds in syrup room environment

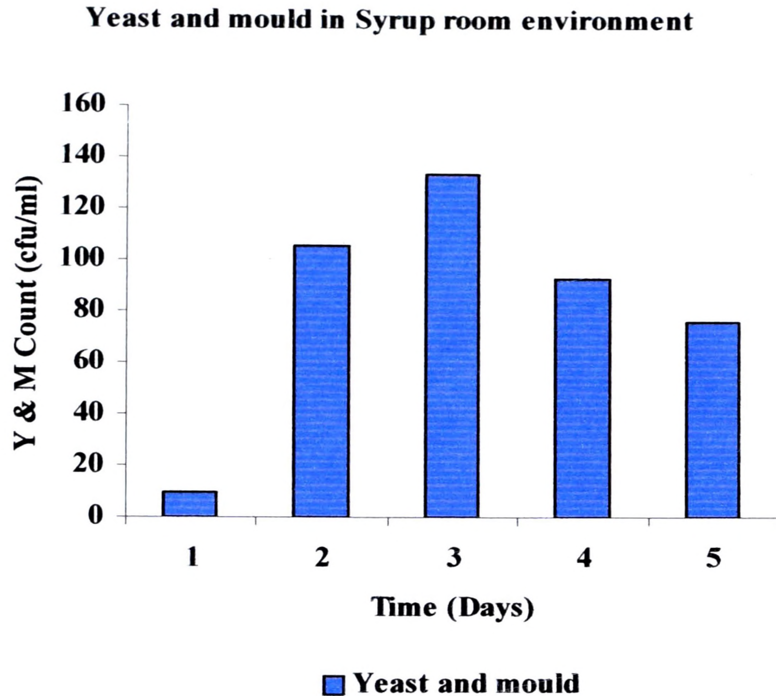

Fig. 4.10 yeasts and moulds count in syrup room environment

Production area is highly contaminated with yeasts and moulds because the production area is not squarely separated from the environment. Production area is also contaminated due to the mixed syrup deposited on the floor due to the braking of bottles during the filling process. The deposited mixed syrup is not evacuated properly and acts as a growth media for yeasts and moulds.

4.3 Isolation of yeasts and moulds genera in soft drink environment

4.3.1 Identification of colony characteristic

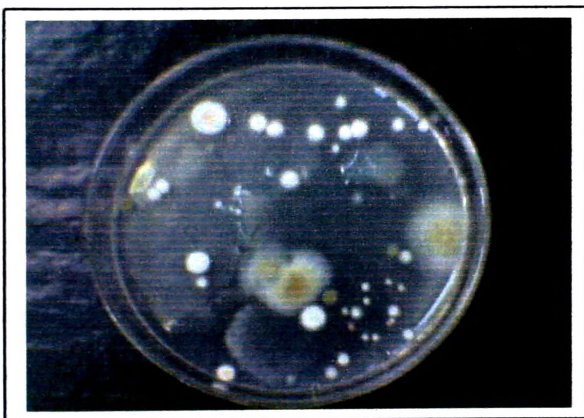

Fig. 4.11 Yeasts and moulds in production environment

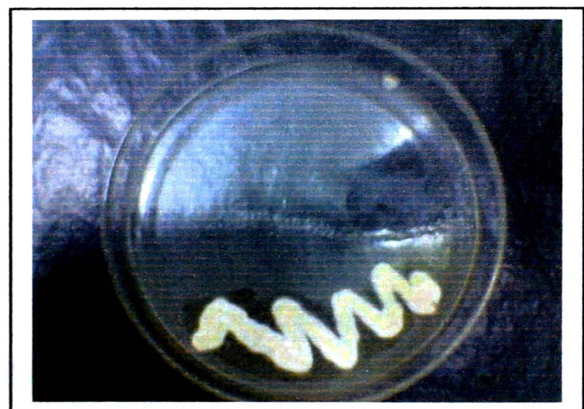

Fig. 4.12 A colony of genus *Aspergillus*

4.3.2 Identification of morphological characteristic

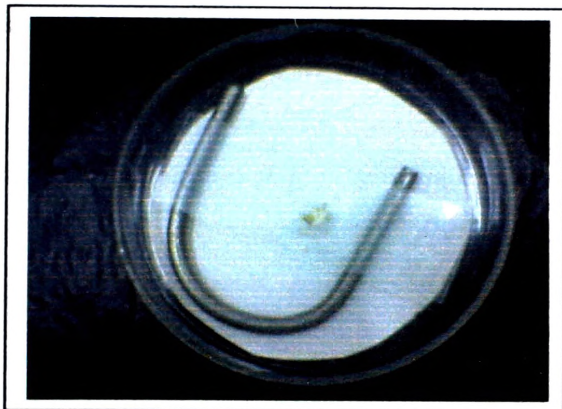

Fig. 4.13. Inoculum Agar cube on slide

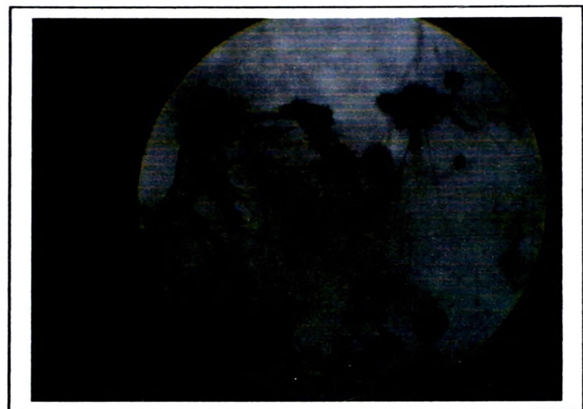

Fig. 4.14 Genus *Aspergillus* (40X10)

According to the literature review genus *Aspergillus* has been identified to be common in the soft drink production environment. In this study by using streak plate method colony characteristics of genus *Asperghillus* were identified.

It could be confirmed by using the slide culture technique to observe morphological characteristics of genus *Aspergillus* under microscope (40 X 10).

4.4 Identification of favorable condition for yeast and moulds

4.4.1 pH value of finished product

Table 4.14 pH values of finished products.

Varity	pH Value	Temperature (^{0}F)
Soda	5.06	73
Lemonade	3.62	62
Cream Soda	3.38	72
EGB	3.20	65
Orange Crush	3.02	72
Necto	2.86	75
Orange Barely	2.78	74
Ride	2.76	74
Tonic	2.63	75

In terms of pH, acidity is one of the most important single intrinsic factors in soft drink spoilage. Soft drinks generally have pH values between 2.0 and 4.5. Yeast and moulds have an optimum pH range of 4.0 to 6.0 but several yeasts grow in soft drinks at pH 3.0 to 4.0 and some can grow in the range 1.5 to 3.0.

4.4.2 Benzoic acid concentration of the finished product.

Benzoic acid is a preservative, which is responsible for preventing yeasts and moulds growth. Undissociated molecule of benzoic acid is responsible for antimicrobial activity, which inhibit or kill microorganisms by interfering with the permeability of the microbial cell membrane. As well as benzoic acid or sodium benzoate can also inhibit specific enzyme system within cells e.g. inhibit enzymes which are responsible for acetic acid metabolism. Undissociated molecules of benzoic acid are increase when decreasing pH. Soft drinks have pH 2 to 6. Therefore Undissociated molecule percentage is high and preservative action is in optimum level (Chipley, J.R, 1989). HPLC recorder gives following type of Chromatograms to benzoic acid content of Standard solution, Orange crush, Cream Soda and Necto.

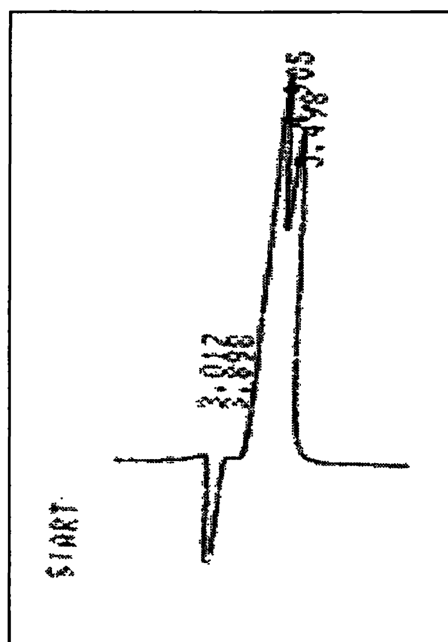

Fig 4.15 Chromatogram of standard (20ppm) solution.

Standard solutions of benzoic acid (20ppm) give 170190 areas in its retention time of 4.905 minutes (Appendix IV).

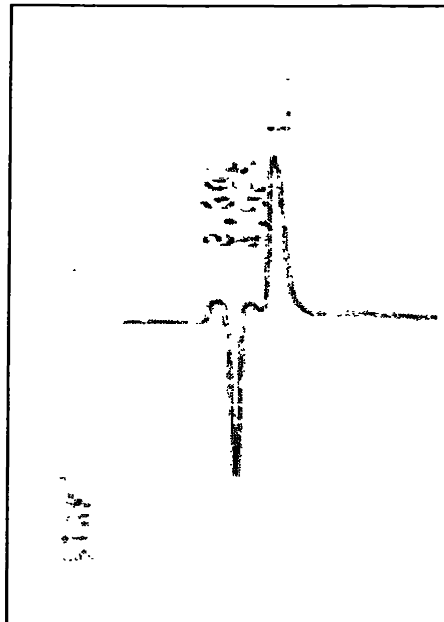

Fig4.16 Chromatogram of standard Orange crush sample.

During 4.87 minutes retention time, 90666 areas were indicated in Orange crush analysis Chromatogram (Appendix III). According to the calculation finished product has 113 ppm benzoic acid.

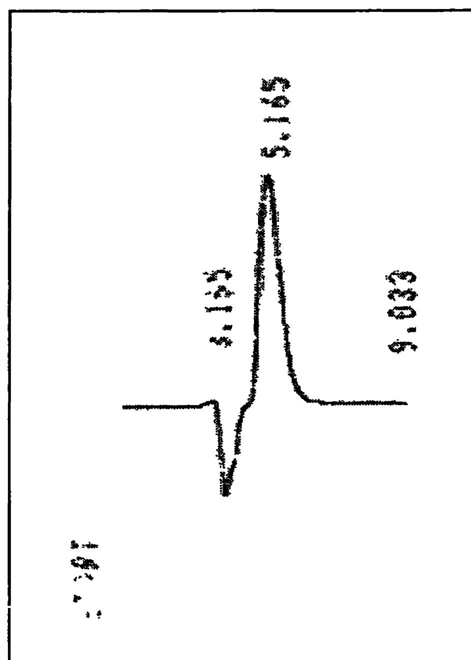

Fig4.17 Chromatogram of Cream Soda sample.

During the 5.156 minutes retention time, 177457 areas were observed in the Cream Soda analysis Chromatogram (Appendix VI). According to the calculation finished product has 263 ppm benzoic acid.

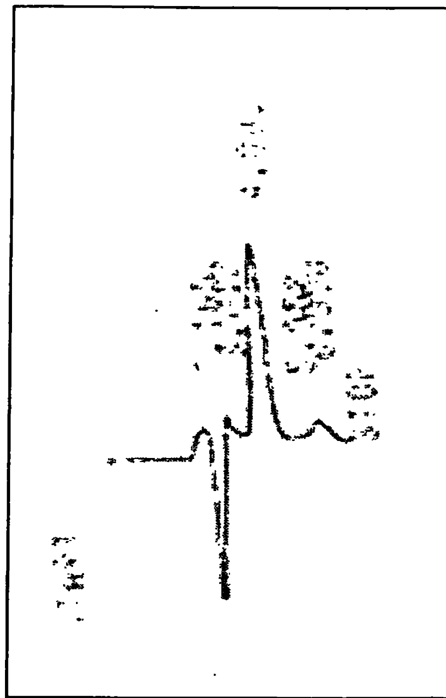

Fig4.18 Chromatogram of Necto sample.

During 4.803 minutes retention times, 117317 areas were indicated in Necto analysis Chromatogram (Appendix V). According to the calculation finished produut has 156 ppm benzoic acid.

4.4.3 SO_2 Concentration of finished product

Table 4.16 SO_2 concentration of finished products.

Varity	SO_2 (ppm)
Cream soda (Export)	Nil
Necto	28.1
Orange crush	25.6

SO_2 can cause conformational changes in disulfide bonds in proteins, which cause growth inhibition of bacteria, yeasts and moulds. Specially used to prvent growth of *Clostridium botulinum.* It inhibits ATP hydrolyzing enzymes in yeasts. *Torulopsis* and *Saccaromyces* were the most tolerant to sulphur dioxide (Ough, C.S, 1989).

4.4.4 Carbonation of finished product

Table 4.17 Carbonation of finished products.

Varity (400ml & 250ml)	Carbonation (Volume)
Cream soda (Export)	2.0 ± 0.2
Necto	2.0 ± 0.2
Orange crush	2.0 ± 0.2
Soda	4.0 ± 0.2

In carbonated soft drinks, the elevated concentration of dissolved CO_2, resulting from an over pressure grater than 0.1 Mpa is an important factor in controlling the growth of microorganisms. Yeasts are the most resistant members of the spoilage microflora, *Brtanomyces spp.* being able to grow in a medium containing 4.45 volumes CO_2 (Varnam, A. H, 1994).

4.5 Evaluation of CIP parameters

4.5.1 Temperature and time of the CIP processes (hot water)

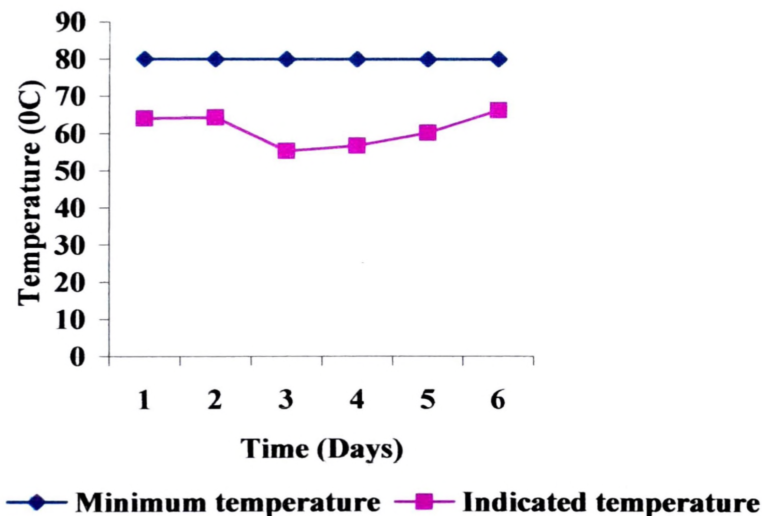

Fig. 4.19 Hot water temperature variation in CIP process

A hot water temperature of CIP processes is not sufficient to sterilize the Syrup line, Valve and Filler. All the time the temperature of hot water goes to the filler is below 80^0C. It positively effect on the growth microorganisms, specially yeast and moulds.

4.6 Implementation of corrective actions and preventive measures.

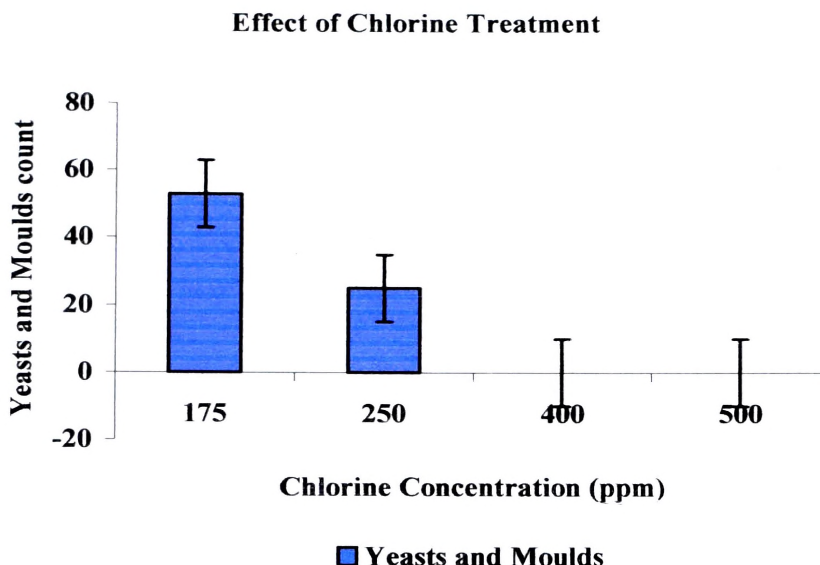

Fig. 4.20 Effect of Chlorine treatment on elimination of yeasts and moulds

After treating the mouth piece (which is the part that the mouth of the filling bottle and touches once it is taken to the filler) with varying concentrations of chlorine solutions at levels of 400ppm and 500ppm no colonies were appeared. This implies that there is a significant effect on controlling/eliminating yeast and moulds when different concentrations of chlorine were used for this task (Since $p < 0.05$). Since this is a routine commercial activity, the sanitizer selected as the most effective one must be cost effective at the same time. Therefore, average numbers of colonies were compared and these are illustrated in table 4.21. According to these results it could be concluded that 400ppm concentration is more profitable than 500ppm, although both of them have similar effects against yeast and mould.

Table 4.21 Mean values chlorine treatment

Chlorine concentration, ppm	Mean value (colony count)
175	53.33
250	26.67
400	0.00
500	0.00

3.6.1 Evaluation of the effectiveness of corrective and preventive measures

Chlorine is a sanitizer, which has antimicromial activity. It affects cell membrane and sulfhydral enzymes and enzymes involved in glucose metabolism. Chlorine is capable of producing leathal raction at or near the cell membrane, as well as affecting DNA. It has also been suggested that NaOCl reacts with the DNA of living cells, causing mutation of purine and pyrimidine bases. The sporocidal effect of chlorine have attributed to spores coat disruption by chlorine combining with the removing protein (Cords, B.R, 1989).

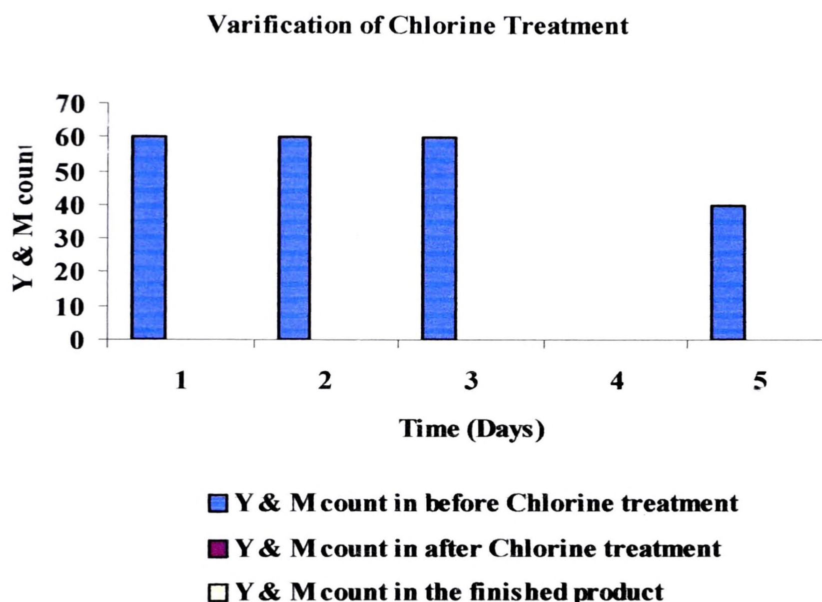

Fig. 4.21 Effect of Chlorine treatment for Yeasts and moulds

The results obtained for test samples before and after the chlorination process reveals that there is a significant effect of the chlorination process with a concentration of 400 ppm (since $p>0.05$).

In this study yeasts and moulds counts of pour plate method and 3M^{TM} Petrifilm technique are not equal to each other. Some reasons may affect to this variation. Because pour plate method have high probability of cross contamination than Prtrifilm method. Sometimes sample was distribute unevenly. As well as liquid yeast extract agar may solidified when it pour in to the sample and it difficult to spread evenly through sample.

CHAPTER 5

Conclusion and recommendations

5.1 Conclusion

- Such places as, syrup room, production environment, stock syrup filter are highly contaminated from yeasts and moulds.
- According to slide culture technique results, genus *Aspergillus* was present in the environment.
- During the CIP process mouthpieces in filler are not properly sterile.
- Soda was contaminated from yeasts and moulds due to mouthpieces.
- To eliminate yeasts and moulds in mouthpieces it should be washed with 400ppm chlorine solution.

5.2 Recommendations

- Regular and frequent check of UV sterilizer water treatment plant for its prompt should be conducted.
- Any washing propos of the production line (bottle Washer and filler) should use purified water.
- After the production Filter papers should be washed with hot water (above 80^0C) or steam. Filter papers should change at least once in two weeks.
- To eliminate yeasts and moulds from stock syrup UV sterilizers can be used.
- During CIP process hot water temperature should be above 80^0C when it drains out from filler.
- During CIP process and before production Mouthpieces should be rinsed with 400ppm chlorine solution and check weather residual chlorine is present or not by using Orthotoluidine.
- Production environment (bottle washer to filler) should be separate from Syrup room, stores (full bottle and empty bottle) and prevent direct exposure to the outdoor environment.
- flavoured syrup vats should be arrange with UV sterilizers.

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Appendix I

SULPHUR – DIOXIDE LEVELS

I_2 Volume. (ml)	SO_2 (ppm)	I_2 Volume. (ml)	SO_2 (ppm)
0.05	1.2	1.05	26.8
0.10	2.5	1.10	28.1
0.15	3.8	1.15	29.4
0.20	5.1	1.20	30.7
0.25	6.4	1.25	32.0
0.30	7.6	1.30	33.2
0.35	8.9	1.35	34.5
0.40	10.2	1.40	35.8
0.45	11.5	1.45	37.1
0.50	12.8	1.50	38.4
0.55	14.0	1.55	39.6
0.60	15.3	1.60	40.9
0.65	16.6	1.65	42.2
0.70	17.9	1.70	43.5
0.75	19.2	1.75	44.8
0.80	20.4	1.80	46.0
0.85	21.7	1.85	47.3
0.90	23.0	1.90	48.0
0.95	24.3	1.95	49.9
1.00	25.6	2.00	51.8

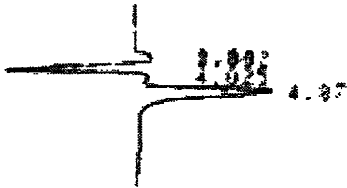
Appendix II

CARBONATION CHART

°C	12.8°	14°	15°	16°	17°	18°	19°	20.6°	22°	24°	25°	26°	27°	28°	29°	30.6°
°F	55°	57°	59°	61°	63°	65°	67°	69°	71°	75°	77°	79°	81°	83°	85°	87°
02	1.2	1.2	1.2	1.1	1.1	1.1	1.0	1.0	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.7
04	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.1	1.1	1.0	1.0	0.9	0.9	0.9	0.9	0.8
06	1.5	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.1	1.0	1.0	1.0	0.9	0.9
08	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.4	1.3	1.2	1.2	1.1	1.1	1.1	1.0	1.0
10	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.1
12	2.0	1.9	1.8	1.8	1.7	1.7	1.6	1.5	1.5	1.4	1.4	1.3	1.3	1.3	1.2	1.2
14	2.1	2.0	2.0	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.4	1.3	1.3
16	2.3	2.2	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3
18	2.4	2.3	2.2	2.2	2.1	2.0	2.0	1.9	1.8	1.7	1.7	1.6	1.6	1.5	1.5	1.4
20	2.6	2.5	2.4	2.3	2.2	2.2	2.1	2.0	1.9	1.8	1.8	1.7	1.7	1.6	1.6	1.5
22	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.1	1.9	1.9	1.8	1.8	1.7	1.7	1.6
24	2.9	2.7	2.7	2.6	2.5	2.4	2.3	2.3	2.2	2.0	2.0	1.9	1.9	1.8	1.8	1.7
26	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.0	2.0	1.9	1.9	1.8
28	3.1	3.0	2.9	2.8	2.7	2.6	2.6	2.5	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9
30	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.2	2.1	2.0	2.0
32	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.3	2.2	2.1	2.1
34	3.6	3.5	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.2	2.1
36	3.7	3.6	3.5	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.3	2.2
38	3.9	3.7	3.6	3.5	3.4	3.3	3.2	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.4	2.3
40	4.0	3.9	3.7	3.6	3.5	3.4	3.3	3.2	3.1	2.9	2.8	2.7	2.6	2.5	2.5	2.4
42	4.1	4.0	3.9	3.7	3.6	3.5	3.4	3.3	3.2	3.0	2.9	2.8	2.7	2.6	2.6	2.5
44	4.3	4.1	4.0	3.9	3.8	3.6	3.5	3.4	3.3	3.1	3.0	2.9	2.8	2.7	2.7	2.6
46	4.4	4.3	4.2	4.0	3.9	3.8	3.6	3.5	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.7
48	4.6	4.4	4.3	4.1	4.0	3.9	3.7	3.6	3.5	3.3	3.2	3.1	3.0	2.9	2.8	2.8
50	4.7	4.5	4.4	4.3	4.2	4.0	3.8	3.8	3.6	3.4	3.3	3.2	3.1	3.0	2.9	2.8
52	4.9	4.7	4.6	4.4	4.3	4.1	4.0	3.9	3.7	3.5	3.4	3.3	3.2	3.1	3.0	2.9
54	5.1	4.9	4.7	4.6	4.4	4.2	4.1	4.0	3.9	3.6	3.5	3.4	3.3	3.1	3.1	3.0
56	5.2	5.0	4.9	4.7	4.5	4.3	4.2	4.1	4.0	3.7	3.6	3.5	3.4	3.2	3.2	3.1
58	5.3	5.2	5.0	4.8	4.6	4.5	4.3	4.2	4.1	3.8	3.7	3.6	3.5	3.3	3.3	3.2
60	5.5	5.3	5.1	4.9	4.7	4.6	4.4	4.3	4.2	3.9	3.8	3.7	3.6	3.4	3.4	3.3
62	5.6	5.4	5.3	5.1	4.9	4.7	4.6	4.4	4.3	4.0	3.9	3.8	3.7	3.5	3.5	3.4
64	5.8	5.6	5.4	5.2	5.0	4.8	4.7	4.5	4.4	4.1	4.0	3.9	3.8	3.6	3.6	3.5
66	5.9	5.7	5.6	5.3	5.2	5.0	4.8	4.7	4.6	4.2	4.1	4.0	3.9	3.7	3.7	3.6
68	6.1	5.9	5.7	5.4	5.3	5.1	4.9	4.8	4.6	4.3	4.2	4.1	4.0	3.9	3.7	3.6
70	6.2	6.0	5.8	5.6	5.4	5.2	5.1	4.9	4.7	4.4	4.3	4.2	4.1	4.0	3.8	3.7
72	6.3	6.1	5.9	5.7	5.5	5.4	5.2	5.0	4.9	4.5	4.4	4.3	4.2	4.1	3.9	3.8
74	6.5	6.3	6.1	5.9	5.7	5.5	5.3	5.1	5.0	4.7	4.5	4.4	4.3	4.1	4.0	3.9
76	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.3	5.1	4.8	4.6	4.5	4.3	4.2	4.1	4.0
78	6.8	6.6	6.3	6.1	5.9	5.7	5.6	5.4	5.2	4.9	4.7	4.6	4.4	4.3	4.2	4.1
80	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.5	5.3	5.0	4.8	4.7	4.6	4.4	4.3	4.2
82	7.2	6.9	6.7	6.4	6.2	6.0	5.8	5.7	5.5	5.1	5.0	4.9	4.7	4.5	4.4	4.3
84	7.4	7.1	6.8	6.6	6.4	6.2	6.0	5.8	5.6	5.2	5.1	5.0	4.8	4.6	4.5	4.3
86	7.5	7.3	7.0	6.7	6.5	6.3	6.1	5.9	5.7	5.3	5.2	5.1	4.9	4.7	4.6	4.4
88	7.7	7.4	7.1	6.9	6.7	6.4	6.2	6.0	5.8	5.4	5.3	5.2	5.1	4.8	4.7	4.5
90	7.8	7.5	7.3	7.0	6.8	6.5	6.3	6.1	6.0	5.5	5.4	5.3	5.2	4.9	4.8	4.6
92	8.0	7.7	7.4	7.1	6.9	6.7	6.4	6.3	6.0	5.6	5.5	5.4	5.3	5.0	4.9	4.7

Appendix III

Orange crush



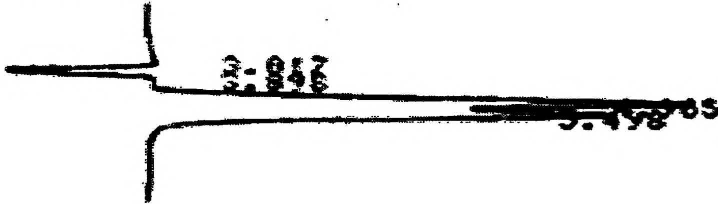
CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 672

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.94	14208			8.4659	
2	3.082	17662	V		10.5241	
3	4.925	45289			26.9858	
4	4.87	90666	V		54.0242	
	TOTAL	167825			100	

Appendix IV

START



CHROMATOPAC C-R6A　　FILE 0
SAMPLE NO 0　　METHOD 41
REPORT NO 664

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.017	27021			6.9727	
2	3.898	34808			8.98	
3	4.905	170190	V		43.917	
4	5.498	155515	V		40.1303	
	TOTAL	387526			100	
	TOTAL	826294			100	

Appendix V

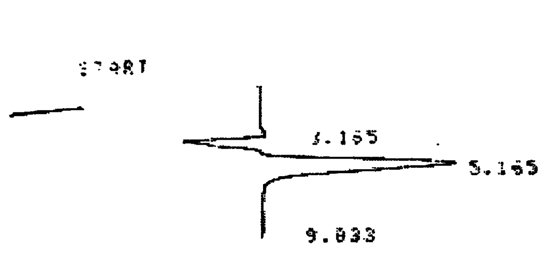
CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 691

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.105	37542			13.9836	
2	3.817	65509			24.4007	
3	4.303	117317	V		43.6979	
4	5.958	17845	V		6.6467	
5	6.558	30260	V		11.2711	
	TOTAL	268473			100	

15.79.

Appendix VI



CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO. 670

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.165	16576			8.5428	
2	5.165	177457			91.4572	
	TOTAL	194033			100	

Appendix VII

Table 4.1 Past data of yeasts and moulds recorded in finished products August 2005 to March 2006

Varity	Number of time detected of Yeast and moulds
Soda	39
Cream soda	37
Orange Crush	30
Necto	29
EGB	18
Orange Barely	13
Ride	05
Dry ginger ale	03

Table 4.2 yeasts and moulds count in treated water

Date	Yeast extract agar	3M^{TM} Petrifilm
1	0	0
2	0	0
3	1	0
4	2	1
5	0	1

Table 4.3 yeasts and moulds count in Final rinse water

Date	Yeast extract agar	3M^{TM} Petrifilm
1	2	0
2	86	78
3	0	0
4	1	1
5	1	1

Appendix VIII

Table 4.6 yeasts and moulds count in flavoured syrup

Date	Yeast extract agar	3M^{TM} Petrifilm
1	3	0
2	12	4
3	0	0
4	2	0
5	0	0

Table 4.7 yeasts and moulds count in mixed product (For sweet drink)

Date	Yeast extract agar	3M^{TM} Petrifilm
1	28	3
2	0	0
3	0	0
4	17	14
5	7	5

Table 4.8 yeasts and moulds count in mixed product (For Soda drink)

Date	Yeast extract agar	3M^{TM} Petrifilm
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0

Appendix IX

Table 4.9 yeasts and moulds count in mouthpieces in filler

Date	3M™ Petrifilm	Number of Y & M in finished product (Soda)
1	213	1
2	301	1
3	30	0
4	176	2
5	147	2

Table 4.10 yeasts and moulds count in washed bottles of just after washing

Date	3M™ Petrifilm
1	0
2	0
3	2
4	0
5	1

Table 4.11 yeasts and moulds count in washed bottles of just before filling

Date	3M™ Petrifilm
1	3
2	1
3	0
4	0
5	1

Appendix X

Table 4.13 yeasts and moulds count in syrup room environment

Date	$3M^{TM}$ Petrifilm
1	10
2	106
3	134
4	93
5	76

Table 4.15 Benzoic acid of finished products.

Varity	Benzoic acid (ppm)
Cream soda (Export)	256
Necto	156
Orange crush	113

Table 4.18 Temperature and time of the CIP processes (Hot water)

Date	Hot water Tank Temp. (^{0}C)	Filler Temp. (^{0}C)	Time Duration (Min.)
1	73	64.1	15
2	73	64.3	17
3	62	55.3	15
4	65	56.8	15
5	69	60.2	16
6	76	66.3	15

Appendix XI

Table 4.19 Identification of suitable Chlorine concentration for mouthpiece washing

Date	Chlorine concentration (ppm)	Yeast and mould count
1	175	53
2	250	25
3	400	0
4	500	0

Table 4.20 Evaluation of chlorine treatment

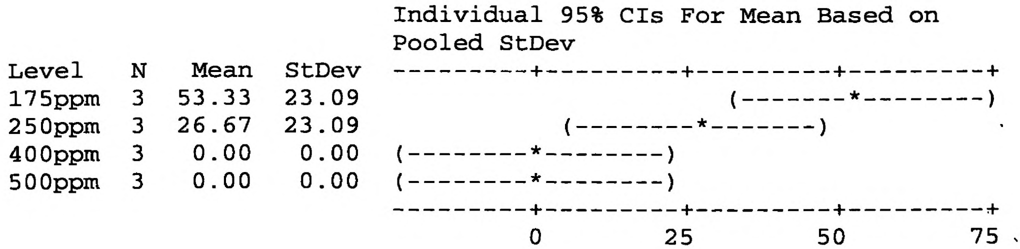
Date	**YM count in before Chlorine treatment**	**YM count in After Chlorine treatment**	**YM count in finished product**
1	60	0	0
2	60	0	0
3	60	0	0
4	0	0	0
5	40	0	0

Appendix XIII

One-way ANOVA: 175ppm, 250ppm, 400ppm, 500ppm

Source	DF	SS	MS	F	P
Factor	3	5867	1956	7.33	0.011
Error	8	2133	267		
Total	11	8000			

S = 16.33   R-Sq = 73.33%   R-Sq(adj) = 63.33%



Level	N	Mean	StDev
175ppm	3	53.33	23.09
250ppm	3	26.67	23.09
400ppm	3	0.00	0.00
500ppm	3	0.00	0.00

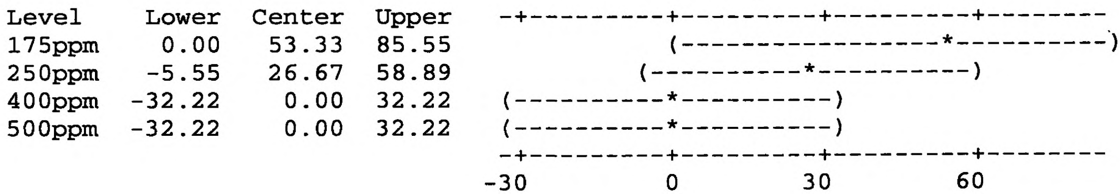
```
Individual 95% CIs For Mean Based on
Pooled StDev
---------+---------+---------+---------+
                    (-------*--------)
          (--------*-------)
(--------*--------)
(--------*--------)
---------+---------+---------+---------+
         0        25        50        75
```

Pooled StDev = 16.33

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.42

Intervals for level mean minus smallest of other level means



Level	Lower	Center	Upper
175ppm	0.00	53.33	85.55
250ppm	-5.55	26.67	58.89
400ppm	-32.22	0.00	32.22
500ppm	-32.22	0.00	32.22

```
-+---------+---------+---------+--------
           (----------------*----------)
         (----------*----------)
(----------*----------)
(----------*----------)
-+---------+---------+---------+--------
-30        0        30        60
```

H0:All the treatments are the same
H1: All the treatments are not same

P<0.05

Since reject H0 at 95% significant level. That means all the treatments are not same.

Appendix XIV

Paired T-Test and CI: Before, After

Paired T for Before - After

	N	Mean	StDev	SE Mean
Before	15	48.0000	43.2930	11.1782
After	15	0.0000	0.0000	0.0000
Difference	15	48.0000	43.2930	11.1782

95% upper bound for mean difference: 67.6883
T-Test of mean difference = 0 (vs < 0): T-Value = 4.29 P-Value = 1.000

H0: There's no significant effect before and after the chlorination.

H1: There's a significant effect before and after the chlorination.

P value: 1.000

P>0.05

Since reject H0 at 95% significant level. That means there's a significant effect before and after the chlorination.

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