STUDY ON THE EFFECTIVENESS OF PRESERVATIVES AT DIFFERENT CONCENTRATIONS IN CONTROLLING MICROBIAL GROWTH IN SOFT DRINKS

by

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DECLARATION

The work describe in the thesis was carried out by me at Ceylon Cold Stores Ltd and Faculty of Applied Sciences under the supervisors Mr. D.A.M. Arsecularathna and Mrs. K.M. Somawathi. A report on this has not been submitted to any other university for another degree.

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Dedicated to my parents, brothers and sister

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ABSTRACT

Soft drinks are the carbonated non-alcoholic beverages, which contain water and carbon dioxide as major ingredients. Sugar, colors, flavors, acids, liquid heading, essence and preservatives are also used as ingredients.

Soft drinks do not normally susceptible for the microbial spoilage because of their acidity and carbonation. However during processing and storing soft drinks can be contaminated by bacteria, yeast and moulds and it will be spoiled. So it can impact taste and flavor. For this reason a small amount of preservatives are added. There are two permitted preservatives mainly used in soft drinks, benzoic acid and SO_2 in different concentrations. The present study was carried out to determine the best concentrations of preservative to incorporate in carbonated soft drinks.

When studying on the effectiveness of preservatives of different concentration in soft drinks, microorganisms were enumerated aseptically using sterilized equipments and media. Nutrient media was used to enumerate total colony counts and yeast extraction agar media was used to enumerate yeast and moulds counts. Benzoic acid and SO_2 were added as sodium benzoate and potassium metabisulphaite (KMS) respectively in order to get preservative action. The effect of benzoic acid and SO_2 individually and as a mixture were studied. Total colonies were incubated at 30-35 °C for 48 hours and yeast and moulds were incubated at room temperature for 72 hours.

For the preservation of soft drinks the suitable optimum concentration of benzoic acid was 45 ppm and it was 49 ppm for SO_2 . The best combination of concentrations were 39/95 ppm of benzoic acid and SO_2 respectively when they were used as a mixture

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CHAPTER 1

1.1 Introduction

Carbonated non-alcoholic beverages are generally sweetened, flavored, acidified, colored, carbonated and chemically preserved. Their origin goes back to Greek and Romen time at 1767. Artificial incorporation of carbon dioxide to water was first done by Joseph Priestly a British chemist (Potter and Hotchkiss,1996). Carbon dioxide was produced by $NaHCO_3$ / $NaCO_3$. Carbonated water improves flavor. Soft drinks are usually prepared from carbonated syrup that contain sugar, flavor, essence, citric acid, water ,CO_2 and preservatives. Artificial coloring matter is added to some drinks and products such as ginger beer contain a foaming agent of the flavor syrup. In the manufacture of a soft drinks definite volume of the syrup is measured into bottles. The bottle is then filled with a mixture of water and liquid carbon dioxide and closed. The degree of carbonation employed very according to the products.

Soft drinks in common with other carbonated beverages are susceptible to microbial spoilage, which is undesirable from an economical stand point. Microbiological contamination may be happened in raw materials and finished products during processing and manufacturing by personal and equipments. Mainly bacteria, yeast and moulds are found in soft drinks. Therefore microorganisms deteriorate the soft drinks. So to reduce deterioration in soft drinks, preservatives are used. Preservatives are capable of inhibition, retarding, arresting the growth of microorganism of any food .There are two main preservatives used in soft drinks that are SO_2 and benzoic acid. The study was carried out to determine the best concentration of preservatives incorporation in finished soft drinks.

1.2 Objective

To determine the best concentration of preservative incorporation in carbonated soft drinks.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Ingredients

The major ingredients of carbonated beverages of soft drinks in addition to water and CO_2 .The other ingredients are sugar, color, flavor, citric acid, liquid heading and essences.

2.1.1 Sugar

In the UK the 1964 soft drinks regulation define sugar as "any soluble carbohydrate sweetening matter". This definition includes glucose; fructose and other sugar as well as sucrose (Potter and Hotchkiss, 1996) .The most common sugar used in soft drinks is high fructose corn syrup or related corn sugar. Initially sucrose as a pure colorless syrup from the manufactures or made into syrup at a beverages plan from high purity crystalline sugar was most commonly and widely used. Sugar will decompose on heating therefore it should never be boiled.

Finished beverages contain about 8%-14% sugar. The sugar not only contributes sweetness and calories to the drinks but also added to body and mouth feel. Sugar such as sucrose exert their processing effect in microorganism, some yeast and moulds can grow in the presence of as mush as 60% sucrose. Organisms that have ability to grow in high concentration of sugar are some yeast such as *Sacchromyces rouxii* (Srivastava and Singhal, 1995)

Sugar improve preservation and increases the taste acceptability. Carbohydrate sugar used in carbonated soft drinks can be divided into granule sugar and liquid sugar. Granule sugar is a dry, crystallized disaccharide extracted from sugar beet and sugar cane called sucrose. Liquid sugar is an aqoues solution of sucrose at a saturated concentration of 67% w/w (67 brix) at a 20 ^{0}C. Liquid sugar will not support to the growth of most organism due to its low water activity. Liquid sugar can be sterilized by filtering material through 8 micrometer pour size.

2.1.2 Flavors

Synthetic flavor compounds, natural flavors like ginger extracts and fruit juice concentrates are used in soft drinks. These flavors must be stable at an aseptic condition of the beverages. The flavors do not have to be stable to heat much over 38^0C since beverages are not commonly heat sterilized or pasteurized. The most popular taste in soft drinks are still the citrus flavor, orange, lemon lime and grape fruit .The flavor of these products are mostly based on the essential oil from the peel of fruit, e.g. orange oils. These are water insoluble. They contain more than 90% hydrocarbons (mainly limonene), which contribute little or nothing to taste. This 90% can be regarded as solvent or carrier not contributing to the final taste of soft drinks and leaning us with only 0.0015% for the factor, which will flavor soft drinks. Two methods of incorporating this 0.0015% in to soft drinks are possible. The first is to make a water-soluble flavoring and the second is emulsifying the oil with the aid of weight agents and emulsifiers. (Green, 1978)

2.1.3 Color

Some Important coloring agents used for soft drinks are the synthetic colors. Most impart a little color to a drink and this is enhanced in comminuted products. The synthetic coloring matters are still the subjects of intense scratinly for carcinogenicity. Synthetic coloring matter in a drink may be only 5-15 ppm but the quantity drunk by one person may some time constitute a hazard over a long period. Experience has show that a number of colors both synthetic and natural are satisfactory in soft drink carbonated beverages. To trace recent and possible further development in color for soft drinks it is usefull to consider the legislative aspect of color use as well the technical development. (Green,1978)

Most of colors used have a responsible all round performance but the only reliable guide for stability under normal storage condition. Synthetic color is alternative color both synthetic and natural interest to the soft drinks industry. The technical features of synthetic colors are widely known and alternative synthetic formulation is in many causes already in use where a delisting has occurred. The cost of such replacement is of some order as that of replace order. Major colors are synthetic such as tartazine

2.1.4 Acid

Carbon dioxide in solution contributes to acidity, but this is a supplemented with additional acid in most carbonated drinks. The main reason for acidification are to enhance beverages flavors and to act as preservatives against microbial growth. The principal acid used are citric acid, tartaric acid etc. Citric acid and tartaric acid are important natural acid of fruits. Citric acid is the most widely employed. In addition to flavor enhancement acid act as a preservative in non-heated beverages. It generally is used act a level to yield a pH range of 2.5-3.5 in finished drinks. Citric acid is colorless or white powder odorless and taste strongly acid, slightly hydroscopic in most air slightly efflorescent in warm dry air. It has 210.1g/mol molecular weight. (General medical council publish, 1968)

2.1.5 Carbon dioxide

The sparkle and zest carbonated beverages stems from the carbon dioxide gas. Carbon dioxide can be obtained from carbonates lime store the burning of organic fuel, and industrial process. Soft drinks bottles buy carbon dioxide in high-pressure cylinder from manufacture that produces the gas to comply with food purity regulations. In the cylinder the gas under pressure exist as a liquid.

Carbon dioxide improves flavors contributes acidic preservatives action produces tingling, mouth feel and sparkling effervescent appearance to the beverages. The amount of carbon dioxide in beverages is measured in volume of gas per volumes of liquid. A volume of gas is the volume occupied by the gas under standard temperature and pressure. The amount of CO_2 gas used in beverages depends on their particular flavor and brand. The carbonation parameter depends on temperature and pressure of gas.

2.1.6. Water

The major ingredient in carbonated soft drinks accounting for as much as 93% by volumes is water. It is essential that the water be as nearly chemical pure as is commonly feasible since trace of impurities react with other constituents of the drinks .In this respect municipal drinking water although satisfactory from a bacteriological standpoint, generally is not chemically pure enough for used in soft drinks. (Green, 1978)

The objectives of this water treatments as follows

Uniform water at all season of the year

Removal of colloidal and suspended mater

Removal of color

Removal of undesirable off testes and orders.

Reduction in alkalinity to test level.

Free from microorganisms.

There are two main source of water that are Surface water Ground water.

Water treatment could be include Softening Coagulation ,Filtering, Filtering chlorination, Sedimentation, Flotation sludge treatment and pH adjustments.

Treated water for beverages should be tasteless, odorless, colorless and free for suspended matter. Mustry and earthy, off-taste and smell is not uncommon with certain sources of supply due to algal growth or break down in water supplies and also to certain organism such as *Actinomycetaes* and these can cause unpleasant order or off-taste development in beverages during storage in the trade. Similarly, suspended matter can be provided unsightly deposited in clear drinks such as tonic water with high color levels the beverages loosing their desirable, sparking bright quality. The soft drinks and most manufactures install further water treatment plant in their own factories.

2.1.7 *Heading* liquids

A number of natural plant extract are used as heading liquids or formally agent in the beverage industries mainly in ginger beer. (Green, 1978) Due to these agent soft drinks has permanent gas bubble that is a character of soft drinks.

2.1.8 Preservatives

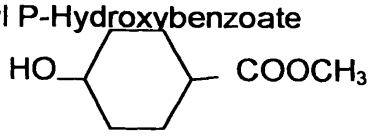
A chemical preservatives may be defined as "any chemical which when added to a food, tend to prevent or retard determination thereof but does not include common salt, sugar, vinegar, spice or substances added by wood smoke. The food some preserved in various ways. Chemicals play important role in the preservation of food. When we add food preservatives in the foods, it has to show anti-microbial activity (Srivastava and Singhal, 1995). There are actually only two permitted preservatives that are SO_2 and benzoic acid. The amount of preservatives present must be calculated as weight/ weight. SO_2 may be incorporated into the as sulphite but must be calculated as SO_2 .Sodium benzoate to be calculated as the acid. Preservative effect is greatly increased by a corresponding decrease in pH.

2.1.8.1 Benzoic acid

Sodium benzoate was the first and oldest chemical preservative permitted in the food Benzoic acid [C_6H_5COOH] and it's sodium salt [$C_7H_5NaO_2$] along with the esters of P-hydroxybenzoic acid (parabens) are considered by food and Drug Administration and it continues in wide use today in the large number of food there are structural formulas as this,

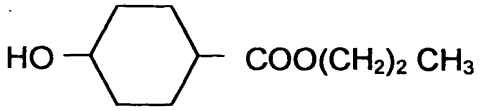
Methyl paraben

Methyl P-Hydroxybenzoate



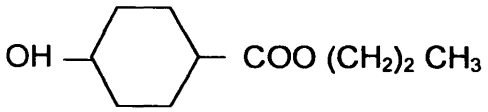
Methyl paraben

Propyl hydroxy benzoate



Hydroxy parabean

n-Heptyl-p-hydroxybenzoate



The anti-microbial activity of benzoic acid is related to pH, the greatest activity being of low pH values. The anti-microbial activity resides in the undissociated molecules. Theses compounds are most activated at lowest pH valve of food and essentially in effective at neutral values. The pK of benzoate is 4.20 and at pH 4.0,60% of compound is undissociated. While at a pH of 6.0 only 1.5% is undissociated this results restriction of benzoic acid products such as soft drinks (Pearson,1999).

High acidity alone is generally suffient to prevent growth of bacteria is theses food but not that of certain mould and yeasts. As used in acidity food benzoate acts essentially as a mould and yeasts inhibitor although it is effective against some bacteria 10-100ppm effectively complete inhibition of some gram positive and gram negative bacteria. The benzoate have been show to act against microorganism by inhibition the cellular uptake of substrate molecules .Benzoic acid is a precipitate in water it i does not dissolve in water and dissolve in ether. Benzoic molecular weight is 122.01g/mol. It may be prepared by hydrolysis of benzortichlorid. It contains not less than 99.05% of $C_7H_6O_2$. Colorless, light, feathery crystals or a white powder, order slight and characteristic. It is melting point 121.0

to 123.5. Sodium benzoate [$C_7H_5NaO_2$] contains no less than 99.0% of $C_7H_5NaO_2$ and molecular weight is 144.1g/mol (Srivastava. and Singhal , 1995)

2.1.8.2 Sulphur dioxide

Sulphur dioxide (SO_2) and the sodium and potassium salt of sulfite (SO_3), bisulfite (HSO_3) and metabisulfite (S_2O_3) all appear to act similarly and are here treated together. Sulphur dioxide is a gas or liquid , in the form of one or more of it is neutral or acid salt on dried fruits in lemon juice, molasses, wines and fruit juices . The parent compound has been used as a preservatives since ancient time with regard to it's effect on microorganisms. SO_2 is bacteriostatic agent *Acetobactor spp* and lactiacid bacteria at low pH being effective in beverages. The source of SO_2 was sodium metabisulphite(Tressler and Joslyn, 1961).

Sulphur acid at level of 0.2-20ppm was effective agent some yeast including *Sacchoromyces* and Candida. SO_2 and bislphite can be used to destroy afflotoxin. It has anti-microbial activity. Although the actual mechanism of action of SO_2 is not known, several possibilities have been suggested each supported by some experiment evidence. One suggestion is that the undissosiated. Sulphur acid or molecular SO_2 is responsible for the anti-microbial activity. It's greater effectiveness at low pH tend to support this. Vas and Ingram suggested the lowering of pH of certain food by addition of acid as a means of obtaining greater preservation with SO_2. It has been suggested that antimicrobial action is due to the strong reducing power that allows these compound to reduces O_2 tension to a point below that at which aerobic organism can grow or by direct action upon some enzyme system. SO_2 is also though to be an enzyme poison, inhibition growth of microorganism by inhibiting enzymes. SO_2 is being make up KMS tend to retard corrosion of metal surface SO_2 is mixed with water easily and it is a heavy gas (Srivastava and Singhal, 1995)

2.2 Spoilage of carbonated soft drinks

Soft drinks are more susceptible to microbial spoilage than other food products, due to both intrinsic and extrinsic factors. The natural flavorings contribute to undergo a gradual change. The flavor changes that occur during storage often result in an off flavor. In addition to aging or off flavor characteristics of the product, chemical changes, detected in either loss of flavor or conversion of one compound to another. They are actually due to three different kind of microorganism like yeast, mould and bacteria.

2.3 Microbiological examination of carbonated soft drinks

When microorganisms are enumerated, aseptic techniques are used. Sterilized equipments and solutions prevent their concentration while in used .The microbiologist used a range of special techniques and apparatus which are designed to prevent contamination of nutrient media. Special laboratories are need for routine microbiological works. These will have easily cleaned surface and special enclosed benches, which receive filter, sterile air e.g. laminar flow should be used. Bacteria, yeast and moulds are cultured in cultured media that is the nutrient environment in which microorganisms are grown in the laboratory. Culture media can be dispending in either liquid form or in a solid or semisolid form gel. Liquid culture media are normally referred to as agar. In combination with the Petri dish another important innovation was introduced by Petri in 1887.

pH is effective on microbial growth. Sterilization of the media is essential to remove contamination, including bacteria container, in media originated from the water other used to make up the medium. Media can be sterilized by filtration if they contain components that are broken down by autoclaving or boiling. Sterilized supplements are frequently added to a base medium after it has been autoclaved and cooled to 40 ^{0}C. There are actually two permitted media are used, that are nutrient agar which is used to inoculation of the total colony and yeast extraction agar which is used the inoculation of the only yeast and moulds.

2.4 Culture media

The type of culture media recognized are,

General purpose (non-selective)
Selective
Enrichment
Differential
Selective and differential.
Chemical defined
Elective
Living

2.4.1 General-purpose media (non selective)

General-purpose media have a nutritional content that will allow the growth or a wide range of either bacteria or yeast and moulds. Media of this type are often complex, prepared from natural products such as meat, yeast and vegetable extracted and hydrolysis products of meat. (Garbutt,1997)

2.4.2 Selective media

These media contain ingredients that will inhibit the growth of certain organisms but allowed to other to grow A wide variety of different chemicals can be added to media to select group of organism or specific organisms. The media will be selective for yeast and moulds that are not affected by the antibiotic at the concentration used. The media can also be considered a general-purpose medium as far as yeast and moulds are concerned. (Garbutt,1997)

2.4.3 Enrichment media

Enrichment media are broths that contain selective ingredients and designed to shift the growth of mixed population of bacteria in direction of a specific organism or group of organisms. (Garbutt,1997)

2.4.4 Differential media

Differential media contain ingredients that are change as a result of microbial metabolism. (Garbutt,1997)

2.4.5 Selective and Differential media

It is used extensively for the isolation of specific organism. (Garbutt,1997)

2.4.6 Elective media

Elective media are designed to promote the growth of specific organisms that have special nutritional requirements by adding particular ingredients to the medium so that their growth is improved. (Garbutt,1997)

2.4.7 Living media

Some microorganisms e.g. virus will only grow in the living cells of their host (Garbutt ,1997).

The plate count is a traditional method and have the colonies are spread through the medium instead of growing on the surface. To estimate the number of yeast and moulds and other microorganisms in soft drinks sample is based on the fact that living microorganism cell will grow and increase in number in the surface of a suitable agar media to give visible colonies that can be counted.

Colonies that are growing on the agar plate can originate from one to several cells. The countable plate should have 30-300 colonies. The fewer the colonies counted the wider the 95% confidence interval and account bellow 30 is no considered acceptable. The statistical error reduced more colonies that are counted but with counts exceeding 300 on the petri dish the numbers become depressed to an unknown degree by competition for nutrient and microbial anti-organisms between developing colonies.30-300 rule apples to mixed populations growing on general purpose media (Garbutt, 1997). The two most commonly used plating techniques are the pour plate technique and the spread plate technique. The medium used has a major effect on the information obtained from a plate count. Any medium used will be to some extent selective, but special selective and differential media or elective media can be used to count special organisms or group of organisms. The pour plate method involves the use of agar poured at 45°C which may kill some damaged cells and reduces the counts. The techniques give both surface and sub surface colonies. After inoculation plate are incubated aerobically at 30°C for 48hrs for total colony counts, but yeast and mould counted inoculation plates are incubate at room temperature for 72hrs (Garbutt, 1997). After incubation microorganisms are counted by viable counts. It may be import to know the number of living cells, for example the effectiveness of preservatives in carbonated beverages in controlling certain yeast and moulds could be measured by making viable counts before and after added preservatives. Two types of cells counts are possible, mainly viable counts and total counts. The viable counts are the total of living cells. The total count is the total number of cell, living and dead

cells. When measuring the growth of population of bacteria or yeast, it can be done directly by counting the number of cells or indirectly by measuring some indication of the number of cells such as the cloudiness of a solution or production of a gas. It is usual to inoculate a small sample of the microorganism in to a sterilized nutrient medium and to place the culture in an incubator at the optimum preservatives concentration for growth. Other condition should be as close as possible to optimum growth can be measured from the time of inoculation. Control sample that is not added preservatives test whether technique is generally aseptic.

2.5 The effect of pH on the microbial growth.

pH is one of the main factors affecting the growth and survival of the microorganism in the culture media and in soft drinks. All microorganisms have a pH range in which there can grow and optimum pH at which they grow best. *Sachoromyces cerevisiaea* for example has pH range 2.35-8.60 with and optimum at pH 4.5. It is possible to generalize regarding the influence of pH on the growth rate of microorganism .The pH range and optima for the majority of bacteria, yeast and moulds.

Bacteria generally have a minimum pH for growth around 4.0-4.5 and the optimum pH between 6-8 and 7.2 that is more or less natural and maximum between 8.0-9.0.Some bacteria are exception to this generalization e.g. *Lactobacillus spp* grow within range 3.8-7.2 with optima around pH 5.0 and *Acitobacter spp* grow between 2.8 and 4.3 with an optimum around 3.0. Yeast and moulds are generally less sensitive to pH than bacteria and capable of growing over wide pH range e.g. *Fusarium spp* are capable of growing over the pH range 1.8-11.1 yeast have optima between 4.0 4.5 and moulds between pH 3.0-3.5 When the microbial cell is subjected to extreme pH, cell membrane become damaged. H^+ and OH^- ions can then leak in to cell where enzymes are denatured leading to cell death. Adjusting the pH of food using organic acid is an important method of food preservation, controlling the growth of microorganisms that cause food spoilage or food poisoning. The pH of food process are often modified using organic acids. Such as citric acid and a number of food preservatives are weak organic acids. Most manufactured products to varying degrees are prone to microbial contamination and this respect soft drinks or no exception. Infact they are relatively more susceptible to microbial spoilage than other food product due to both intrinsic and extrinsic factors. Although their effect on public health can not be completely excluded yeast, mould and bacteria found in soft drinks are manly important from a spoilage or economic stand point. The microbiology of soft drinks must be start from the basis such as the origin of contamination microbes, their distribution the intrinsic and extrinsic factor influencing their effect on these product and the methods and material for controlling microbial contamination.

Extrinsic factors.

Nature of raw material.

Raw materials vary in their susceptibility to microbial spoilage which initially depend on their origin and nutritional properties.

Initial microbial growth.

Success of anti microbial treatment will be low if initial microbial load is high.

Handling.

Poor or unhygienic handling of raw material may increase microbial counts

Processing and treatment.

Under processed raw materials and inadequate physical or chemical treatment will means more organism survival.

Packaging.

Raw material packed in unsuitable container may acquire large microbial numbers during storage.

Storage condition.

Due to humidity and other unsuitable condition will deteriorate soft drinks.

Intrinsic factor of raw materials are not easily controllable or alterable as this will change the basic characteristic for which there were originally chosen. These factors are important because of their effect on the growth of bacteria, yeast and moulds.

These factors are,

pH or acidity.

Hydrogen ions concentration or buffering power of a raw material is one of the important factors influencing the growth of microorganism.

Water activity.

Most food bacteria has an a_w range of 0.88-0.96, where as favorable a_w range for yeast is 0.88-0.94 and that of moulds is 0.93-0.99.

Osmotic condition.

Most microorganisms do not thrive in condition of high osmotic pressure.

Minerals content.

Mineral salt are essential for the growth of bacteria, yeast and moulds and their presence at optimum level.

Organic content.

Raw material contain higher amount of organic contents (Green, 1978).

2.6 Carbonation

A fundamental statement for the solution of any gas in any liquid is for 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 temperate high amount of CO_2 gas is dissolved in water (Green, 1978).

2.7 Packaging

It is well worth pausing before considering the various materials and system available for the packaging of the soft drinks. To examine the requirements for the soft drinks packaging which indeed are no different from those for many other food products. The package must ensure that the product reaches, the ultimate consumer in prime condition and satisfy the legal requirements for the sale of the goods. This means there must be,

No pick up of foreign flavors.

No deterioration due to action of light.

No loss of carbonation.

No oxidation contents due to ingress of oxygen.

It must also be economic and safe to manufacture and distribute through the entire length of the manufacture to consumer chain. It must prevent a hazards or a problem to user in the purchase, transport, opening and consumption of the products (Green, 1978).

2.8 *Yeast*

Most dangerous and usual spoilage inducing organism with which the mineral water manufacture has to control. Yeast is a single cell organism, which are supposed to have oringated from the submerged threads of a multicellular. The size of the cells of yeast is of the order 10^{-3} micrometer. Yeast that reproduce by budding or fission. Yeast is base purely on biochemical reaction. Yeast identification involves both structural characteristics. Water activity of yeast is 0.85 (Pyramid ,1978).

2.9 Mould

The moulds are multicelluler organisms consisting of two type of cells. Moulds are almost susceptible to heat treatment and oxygen elimination. The term mould is applied to any mycelial fungus that is important industrially e.g. in food spoilage, food fermentation, industrial fermentation and process of biodegradation. The group of which has no taxonomic significance and is used purely for convenience, includes the *Zygomycetes* mycelial fungi imperfective and some members of the *Ascomycetes* The spore bearing structures produced by some common moulds are associated with food spoilage and food fermentation. Moulds identification involve critical observation using the optical light microscope of structure feature mainly asexual spore bearing statures, any sexual reproductive structures and the spores themselves. Colony charactraristics may also be significant. Some attempt have been made to identify fungi on the basis of any extra cellular enzymes produced. (Pyramid ,1978).

2.10 Bacteria

The size of the cells bacteria is of the around 10^{-4} mm. Bacteria are relatively simple , single cell organism and are the smallest free living organism known. Most bacteria have cell dimension within the range 0.2-3.0 millimeter in diameter and 0.5 -10.0mm in length. This is well-below the limit that can be observed with the unaided eye, which means that bacteria structure can only be revealed with aid of some form of microscopy. (Pyramid ,1978).

CHAPTER 3

MATERIALS AND METHOD

3.1 MATERIALS

Equipments.

Petridishes.

1ml pipettes.

Incubator.

Colony counter.

Conical flasks.

Cotton plug.

Balance.

Bunsen flame.

Measuring cylinder.

pH meter.

Glass bottles.

Ingredients.

Distilled water.

Preservatives (Benzoic acid and SO_2).

Yeast Extraction Agar.

Acid (citric acid).

Colors (permitted).

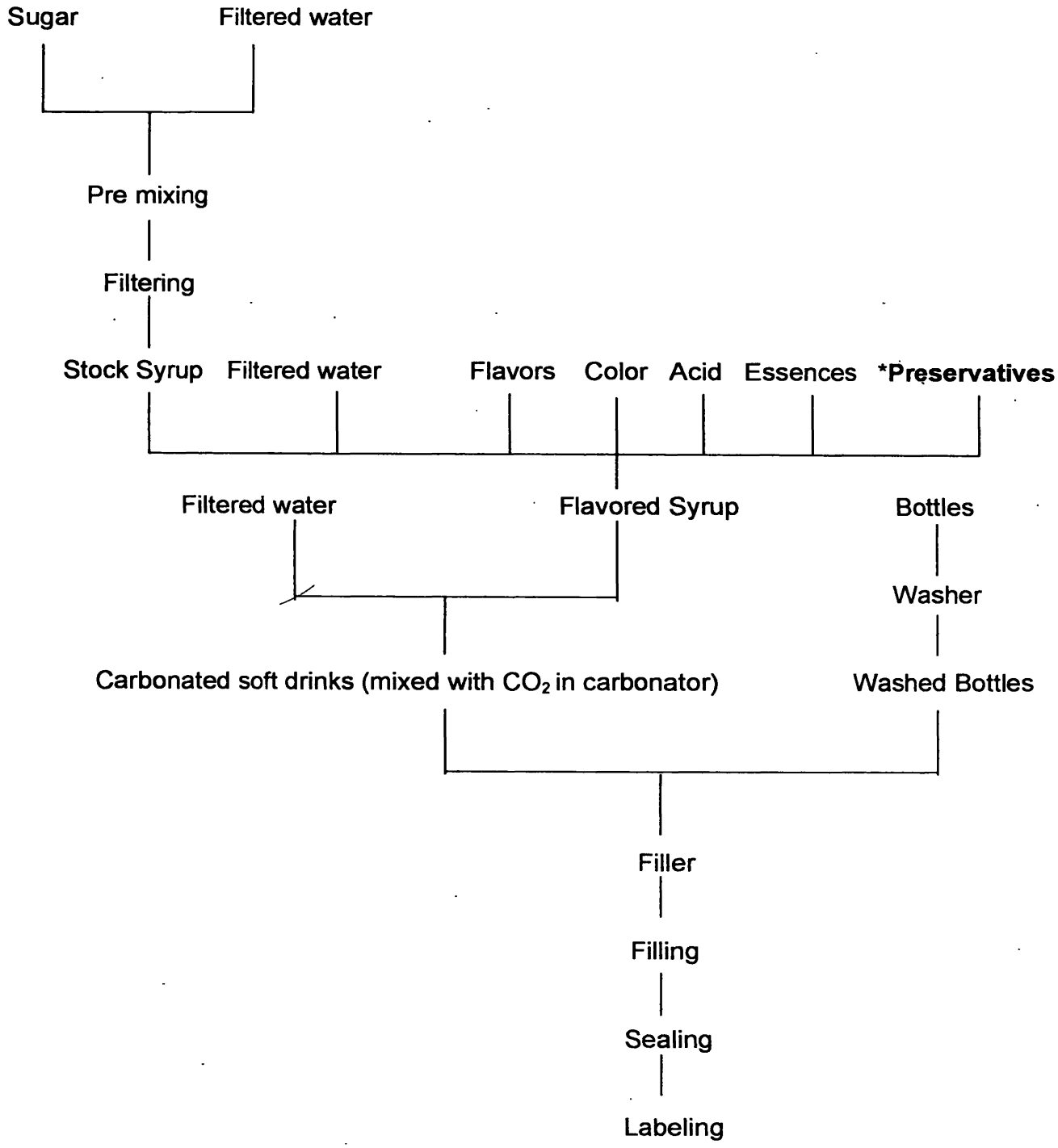
Essences (permitted).

Flavors (permitted).

Liquid Heading.

3.2 METHOD

3.2.1 Preparation of soft drinks samples



*Preservatives concentrations were changed.

Soft drinks samples were prepared by changing preservatives

Treatment(preservatives)	Minimum level (ppm)	Maximum level (ppm)
Benzoic acid	45	150
SO_2	9	59
Benzoic acid and SO_2	65(benzoic) / 9 (SO_2)	115(benzoic) / 59(SO_2)
Without preservatives	-	-

Table 3.1 Use of preservatives in soft drinks

3.2.2 Method of Microbiological Examination

3.2.2.1 Preparation of the Nutrient Agar media.

Nutrient agar and Distilled water were mixed together and the mixture was boiled until the agar was dissolved.

The media was dispensed in to cultured flasks.

Mouth of the flask was closed with a cotton plug and the medium was autoclaved under 15 lb.psi at 121^{0}C for 20 mints.

The media was allowed to cool up to about 45^{0}C

3.2.2.2 Preparation of the Yeast Extract Agar

Yeast Extract Agar and distilled water were mixed together and the mixture was boiled.

The media was dispensed in to cultured flasks.

Mouth of the flask was closed with a cotton plug and the medium was autoclaved under 15 lb.psi at 121^{0}C for 20 mints.

The media was allowed to cool up to about 45^{0}C and an antibiotic was added to the yeast extract agar at a ratio of 1:50 respectively.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Microbiological results and discussion

4.1.1 Use of benzoic acid as a preservative

Benzoic acid is generally considered to exhibit an inhibitory effect on microbial growth. It is a white powdery solid and soluble in water at normal temperature. It is added to the soft drinks as the sodium salt. Total colony counts under different concentrations of benzoic acid were as follows (Table 4.1).

Table 4.1 Total colony counts in Benzoic acid.

Concentration (ppm)	2days	1week	3weeks	5weeks	7weeks
45	1	4	4	4	4
55	2	4	3	3	0
65	1	2	5	3	4
75	6	3	2	4	1
85	1	4	5	6	1
95	4	3	2	1	1
105	2	4	6	4	1
115	5	4	0	0	1
125	2	3	5	1	1
150	1	6	3	1	1

According to the above results bacteria, yeast and moulds counts were changed, when preservative concentration was changed. Some readings can be deviated due to contamination. So when producing soft drinks contamination should be minimized by using sterilized materials.

4.6 Table Density of yeast and moulds in both preservatives

Concentration (ppm)	2 weeks	5 weeks	9 weeks
9/65	0	5	1
19/75	1	4	0
29/85	0	1	0
39/95	0	5	0
49/105	0	3	0
59/115	0	2	0

When adding both preservatives, effectiveness is higher than adding either only benzoic acid or SO_2 as a preservative.

According to statistical analysis (SAS) P value is 0.0001(See Appendix iii) and it was less than 0.05 So there was a significant difference between concentrations at 95% confidence level . Then Z cal value was calculated between each and every concentrations and compared with Z table value, that is 1.96. If Z cal < Z table, there was no significance difference between two concentrations and if Z cal >Z table , there is a significance different between two concentrations . If there is a significance difference between considered two concentrations, lower counts has more effectiveness concentration than the other. Above procedure was done for each and every concentration. According to final results after 39/95 ppm levels of SO_2 and benzoic acid respectively the concentrations have same effect that means there was no significant difference in microbial counts according to the changes preservatives concentrations. So 39/95 ppm levels of SO_2 and benzoic acid respectively were more effective than other concentrations. Above readings were taken one readings.

Both benzoic acid and SO_2 show antimicrobial activity. They inhibit or kill microorganisms by interfering with the permeability of the microbial cell membrane and inhibition of membrane transport of amino acid occur resulting in starvation of cell. In addition, benzoic acid or sodium benzoate can also inhibit specific enzyme system within the cell and inactivate the genetic materials.

4.1.2 Sulphur dioxide as a preservative

When adding SO_2 to the soft drinks it undergoes association and dissociation reactions in the aqueous phase and equilibrium is set up between SO_2 , H_2SO_3 , HSO_3 and SO_3 . The equilibrium depends both on the pH of the product and other reactive species present in soft drinks.

Table 4.3 Total colony counts in SO_2 .

Concentration (ppm)	1 day	2 weeks	6 weeks	9 weeks	12 weeks
9	7	14	10	12	14
19	5	8	9	1	10
29	15	8	8	9	20
39	5	1	7	7	2
49	15	4	3	3	5
59	5	21	4	2	4

Higher total colony counts (Table 4.3) and a high density of yeast and moulds (Table 4.4) were observed in soft drinks sample containing SO_2 than the sample containing benzoic acid. This is due to the more effectiveness of benzoic acid on the microbial growth inhibition than that of SO_2.

Symptoms of allergic reactions can be developed in some people when potassium metabisulphite (KMS) is used as a preservatives. Hence when adding KMS to the soft drinks a narrow range than benzoic acid is used.

Table 4.4 Growth of yeast and moulds counts in SO_2 .

Concentration (ppm)	1 day	2 weeks	6 weeks	9 weeks	12 weeks
9	1	2	0	0	0
19	1	7	0	1	0
29	0	1	1	1	0
39	0	1	1	0	0
49	1	3	4	0	0
59	2	1	0	0	0

According to statistical analysis (SAS) P value is 0.0230 and it is less than 0.05 (See Appendix ii). So there is a significant difference between SO_2 concentrations at 95% confidence level. Then Z cal value was calculated between each and every concentrations and compared with Z table value, that is 1.96. If Z cal < Z table, there is no significant difference between two concentrations and if Z cal >Z table, there is a significance difference between two concentrations. If there is a significance difference between considered two concentrations the sample with lower counts has more effective concentration than the other. Above procedure was done for each and every SO_2 concentrations. According to the final results the concentrations after 49 ppm level had the same effect that means there is no significant difference in microbial counts when changing the SO_2 concentrations. So 49 ppm value of SO_2 was more effective than others.

4.1.3. Both benzoic acid and SO_2 as preservatives

Total colony counts and yeast and moulds counts observed in sample containing both preservatives (SO_2 and benzoic acid) were shown in the table 4.5 and table 4.6 respectively.

4.5 Table Total colony counts in both preservatives

Concentration (ppm)	2 weeks	5 weeks	9 weeks
9/65	3	8	12
19/75	3	8	34
29/85	5	7	51
39/95	3	7	1
49/105	3	6	8
59/115	3	7	2

4.1.4 With no preservatives

Microbial growth was rapid when there was no preservatives used during processing of soft drinks (Table 4.7). In this experiment some samples had unlimited microorganism density, because it may be contaminated during processing.

Table 4.7 Development of total colonies count and yeast and moulds

Concentrations	Total colony counts			Yeast and moulds counts		
(ppm)	2 weeks	5 weeks	9 weeks	2 weeks	5 weeks	9 weeks
0	6	Unlimited	34	4	Unlimited	Unlimited

Soft drinks provide suitable media for the growth of microorganisms hence easily susceptible to microbial spoilage and microbial contamination. Raw material and finish products can be contaminated during processing and manufacturing. Bacteria, yeast and moulds found in soft drinks are mainly important from a spoilage economic standpoint. Due to that reason, the intrinsic and extrinsic factors of the microbes and the method or material used in controlling microbial contamination are important .In nature microbes, bacteria, yeast and moulds are very widely distributed. Therefore manufacturing environment should be cleaned. Microbial contamination occurs through raw materials, personal and plant equipments, processing water and dust or contaminated air.

Sugar and water major ingredients used in soft drinks provide the best media for the growth of microorganism i.e. yeast and moulds. Therefore to minimize the microbial density in soft drinks use of uncontaminated ingredients, equipments and preservatives is important. Due to added preservatives microorganisms will be destroyed or controlled but few amount of microorganisms can be remained in the soft drinks. No preservatives are completely effective against all microorganisms present in a given foodstuff. One should be able to combine various preservatives having different mode of actions in order to compensate for this gap. According to this experiment combination of benzoic acid and SO_2 inhibited the several yeast, moulds and bacteria than either only benzoic acid or only SO_2 alone. Benzoic acid and potassium metabisulphite (K.M.S) are widely used preservatives in soft drinks. When the preservative concentration was increased microbial density was decreased.

In soft drinks small amount of microorganisms are found. The effect of low preservative concentration and high preservative concentration is same when destroying few amount of microorganisms. On the other hand different preservatives have different effectiveness on the microorganisms because they have different reactions with microorganisms.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The total colony counts were higher in soft drinks than the yeast and moulds counts. The best concentration of benzoic acid and SO_2 which can be used in soft drinks were 45 ppm and 49 ppm respectively. When both SO_2 and benzoic acid were used as combination, the best concentration was 39/95 ppm respectively .Microbial growth was high in soft drinks processed with no preservatives. Addition of preservatives is necessary in soft drinks in minimizing microbial growth

5.2 Recommendations

1. Different types of soft drinks having low pH and high pH should be tested to study effectiveness of preservatives.
2. The experiment should be carried out for about 6 months.

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APPENDIX I

The SAS System
The CATMOD Procedure

Response	con	Response Levels	9
Weight Variable	cou	Populations	1
Data Set	BEN	Total Frequency	15
Frequency Missing	0	Observations	9

Maximum Likelihood Analysis of Variance

Source	DF	Chi-Square	Pr > ChiSq
Con	8	7.17	**0.5179**

The SAS System
The CATMOD Procedure

Maximum Likelihood Predicted Values for Frequencies

		-------Observed-------		-------Predicted------	
	Function		Standard		Standard
Con	Number	Frequency	Error	Frequency	Error
1	F1	17	3.860803	16.9999999	3.860884
2	F2	12	3.310064	11.9999999	3.310076
3	F3	15	3.656442	15	3.656336
4	F4	16	3.760974	15.9999999	3.761019
5	F5	17	3.860803	16.9999999	3.860884
6	F6	11	3.181696	11	3.181763
7	F7	17	3.860803	16.9999999	3.860884
8	F8	10	3.045548	10.0000005	3.045103
9	F9	12	3.310064	11.9999999	3.310076
10	F10	11	3.181696	11	3.181763

APPENDIX II

The SAS System
The CATMOD Procedure

Response	con	Response Levels	6
Weight Variable	cou	Populations	1
Data Set	SO2	Total Frequency	34
Frequency Missing	0	Observations	6

Maximum Likelihood Analysis of Variance

Source	DF	Chi-Square	Pr > ChiSq
con	5	13.05	**0.0230**

The SAS System
The CATMOD Procedure

Maximum Likelihood Predicted Values for Frequencies

		Observed		Predicted	
Function			Standard		Standard
con	Number	Frequency	Error	Frequency	Error
1	F1	12	2.786522	12	2.786523
2	F2	1	0.985184	1	0.985152
3	F3	9	2.572479	9	2.57248
4	F4	7	2.357716	7	2.357718
5	F5	3	1.653872	3	1.653874
6	F6	2	1.371989	2	1.37199

Appendix iii

The SAS System
The CATMOD Procedure

Response	con	Response Levels	6
Weight Variable	cou	Populations	1
Data Set	SO2	Total Frequency	108
Frequency Missing	0	Observations	6

Maximum Likelihood Analysis of Variance

Source	DF	Chi-Square	Pr > ChiSq
Con	5	65.17	**<.0001**

The SAS System
The CATMOD Procedure

		-------Observed-------		------Predicted------	
Function			Standard		Standard
Con	Number	Frequency	Error	Frequency	Error
1	F1	12	3.265986	12	3.265987
2	F2	34	4.826624	34	4.826624
3	F3	51	5.188127	51	5.188128
4	F4	1	0.99536	1	0.995346
5	F5	8	2.721655	8	2.721656
6	F6	2	1.401058	2	1.401058

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