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MICROBIOLOGICAL STABILITY OF SOFT DRINK: AN APPROACH FOR ANNIHILATION OF SPOILAGE BACTERIA

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Abstract— **A soft drink is a non alcoholic beverage typically containing water often carbonated and a flavoring agent. Microbial contamination of soft drinks usually originates during the production process. Orange based soft drink was evaluated for microbial contamination. The aim of the present project was to evaluate the microbiological stability of soft drinks for annihilation of spoilage bacteria. It was found that at 0.06% citral concentration the growth of *Escherichia coli* and *Pseudomonas aeruginosa* was totally inhibited after thermal treatment for 15 minutes. On the other hand *Enterobacter aerogenes* and *Klebsiella* spp. were inhibited at 0.09% citral concentration after thermal treatment for 5 minutes and 10 minutes respectively. The addition of the citral in combination with the thermal treatment progressively increased the frequency of not spoiled test tubes. Finally, citral strengthened the antimicrobial action when used in combination.**

Index Terms— **Soft Drink, Citral, Spoilage Bacteria**

I. INTRODUCTION

Soft drinks are enormously popular beverages consisting primarily of carbonated water, sugar, and flavorings. Nearly 200 nations enjoy the sweet, sparkling soda with an annual consumption of more than 34 billion gallons. Soft drinks rank as America's favorite beverage segment, representing 25% of the total beverage market. In the early 1990s per capita consumption of soft drinks in the U.S. was 49 gallons, 15 gallons more than the next most popular beverage, water.

The roots of soft drinks extend to ancient times. Two thousand years ago Greeks and Romans recognized the medicinal value of mineral water. In the late 1700s Europeans and Americans began drinking the sparkling mineral water for its reputed therapeutic benefits. The first imitation mineral water in the U.S. was patented in 1809. It was called "soda water" and consisted of water and sodium bicarbonate mixed with acid to add effervescence. As flavored carbonated beverages gained popularity, manufacturers struggled to find an appropriate name for the drinks like "marble water," "syrup water," and "aerated water." The most appealing name, however, was "soft drink," adapted in the hopes that soft drinks would ultimately supplant the "hard liquor" market [1]. Until the 1890s soft drinks were produced manually, from blowing bottles individually to filling and packaging. During the following two decades automated machinery greatly increased the productivity of soft drink plants. Probably the most important development in bottling technology occurred with the invention of the "crown cap" in 1892, which successfully contained the carbon dioxide gas in glass bottles.

A soft drink is a non alcoholic beverage typically containing water often carbonated and a flavoring agent. The chemicophysical and composite characteristics of soft drinks make them susceptible to microbial spoilage. They are usually characterized by high C/N ratio and low pH, which allows the growth of specific microbial groups, such as acetic and lactic acid bacteria, moulds and yeast. Soft drinks typically contain water, sweetener (8-12%, w/v), carbon dioxide (0.3- 0.6% w/v), acidulates (0.05-0.3% w/v), flavorings (0.1-0.5% w/v), colorings (0-70 ppm), chemical preservatives (lawful limits), antioxidants (<100 ppm), and/or foaming agents (e.g., saponins up to 200mg/ml). Some types of soft drink use sugar substitutes. However, certain ingredients may be hazardous to health if consumed in large quantities, and there is widespread concern generally with regard to preservatives and sweeteners. Therefore, while on the one hand there is a trend to produce ever wider ranges of more specialist soft drinks, there is also pressure to minimize the use of, in particular, artificial and synthetic additives and ingredients. Modern soft drinks constitute a diverse group of products. They can be classified in several ways, for example on the basis of their sugar and fruit juice content, flavoring, carbonation level, main non water ingredients, and functionality. Apart from drinking water, the most popular types of soft drinks are as follows: (I) ready-to-drink essence-flavored beverages; (ii) ready-to-drink beverages containing fruits or fruit juice; (iii) beverages ready-to-drink after dilution [2].

Functional drinks are a rapidly growing subsector of the market and include drinks enriched with juices, vitamins, and minerals; sports and energy drinks. The target markets for functional beverages are diverse, and products are often tailored towards particular target markets, for instance, according to age and gender, with a growing focus on children, women, and seniors. Many functional drinks have been developed to provide specific medical or health benefits, such as promoting heart health, improving immunity and digestion, and helping to boost energy [3].

Microbial contamination of soft drinks usually originates during the production process. The raw materials, factory environment, microbiological state of the equipment and packages, and lack of hygiene are all possible factors [4]. Packaging materials such as cans and bottles can also be sources of contamination. Yeasts are considered to be the primary spoilage microbes in carbonated products, mainly due to their ability to resist high carbonation and low pH levels. Some yeast can tolerate moderately high carbonation and is also able to grow at refrigerator temperatures. Molds grow as white, delicate, fluffy, cottony masses suspended in soft drinks. Fungal spores or conidia and mycelium fragments can contaminate beverages at any stage of the production process. Certain lactic acid bacteria (LAB) can grow in soft drinks [5]. Beverage-related outbreaks were caused by various enteric pathogens including bacteria, viruses and protozoans. However, in many cases the causative agent remained unknown [6].

Citral or 3,7-dimethyl-2,6-octadienal or lemonal, is either a pair, or a mixture of terpenoids with the molecular formula $C_{10}H_{16}O$ naturally occurring in many essential oils from citrus fruits or other herbs or spices [7-9]. The two compounds are double bond isomers. The E-isomer is known as geranial or citral A. The Z-isomer is known as neral or citral B. Citral is present in the oils of several plants, including orange. Geranial has a strong lemon odor. Neral's lemon odor is less intense, but sweeter. Citral is therefore an aroma compound used as a flavor and has strong antimicrobial qualities. The antimicrobial action exerted by citral against yeasts and moulds in different conditions has already been demonstrated [10][11]. In this work the microbial stability of an uncarbonated orange based beverage has been evaluated. The results obtained with citral were particularly interesting because it is commonly used as flavouring agent in soft drinks. The aim of the present project was to evaluate the microbiological stability of soft drinks for annihilation of spoilage bacteria.

II. MATERIALS AND METHODS

A. Collection of Bacterial Cultures: The following bacteria were obtained from National Chemical Laboratory (NCL), Pune which includes Escherichia coli NCIM-2207, Enterobacter aerogenes NCIM 2065, Klebsiella spp. NCIM 2079 and Pseudomonas aeruginosa NCIM 2036. The cultures were maintained on nutrient agar slants for further use. The suspension was prepared by inoculating a loopful of each bacterial culture in nutrient broth and incubating the tubes at 37^0C for 24 hours.

B. Experimental Design: An experimental design with two variables which includes citral concentration and length of thermal treatment at 55°C at five levels was used.

C. Preparation of beverages: An orange based soft drink was used for testing the inhibitory activity of citral. In first step the test tubes were sterilized with a diluted hydrogen peroxide (3% H_2O_2). Then the soft drink was diluted in 1:6 proportions using sterilized distilled water (1.5 ml soft drink +9 ml distilled water). This citral was poured in previously steriliized test tubes.

D. Method for Evaluation of Citral: A total of 20 test tubes were arranged on the test tube stand and labeled as per the concentration of citral addition and the time duration of thermal treatment. Thus, five sets were prepared. Each set containing four test tubes. The first set (A) had of 0% citral, set (B) had 0.03% citral, Set (C) had 0.06% citral, set (C) had 0.09% citral solution and set (E) had 0.12% citral.

Independently of the amount of citral added, the final concentration of ethanol was 0.5% (vol/vol). The same ethanol amount was added to the samples not supplemented with citral. Subsequently, the test tubes were inoculated with 2.8 µl of the organism. Finally, the tubes were immediately closed with screw caps and treated at 55^0C in the water bath. Each set contains 4 tubes. From all the sets, first tube was thermally treated for 5 minutes, likewise second tube (10 minutes), third tube (15 minutes) while fourth tube for (20 minutes) time duration.

After the heat treatment, samples were rapidly cooled at room temperature in a water/ice bath. The tubes were stored at room temperature and observed periodically over a 30-day period for the presence of cloudiness, cell sediment on the bottom due to bacterial growth. After 30 days, all the no-growth results were confirmed by plating 0.1 ml of each tube on MacConkey agar, Eosine Methylene Blue Agar and Pseudomonas Isolation Agar and incubating the plates at 37^0C for 24 hours. Likewise the inoculum from the cloudy tubes also inoculated on the respective media plates for the confirmation of occurrence of bacteria. On the basis of growth observed on the plates, spoilage or no spoilage of soft drink was determined.

E. Identification of Isolated Bacteria: The isolated spoilage bacteria were identified for the presence of Escherichia coli, Enterobacter aerogenes, Klebsiella spp. and Pseudomonas aeruginosa on the basis of morphological, cultural and biochemical characteristics [12].

III. RESULTS AND DISCUSSION

The present project was carried out to evaluate orange based soft drink for the inhibition of spoilage bacteria. The citral prepared was allowed to treat for the thermal treatment at 55^0C for different time duration i.e. 5 minutes, 10 minutes, 15 minutes and 20 minutes. A total of 20 test tubes were arranged on the test tube stand and labeled as per the concentration of citral addition and the time duration of thermal treatment. Thus, five sets were prepared. Each set containing four test tubes. The first set (A) was of 0% citral, set (B) had 0.03% citral, Set (C) had 0.06% citral, set (C) had 0.09% citral solution and set (E) had 0.12% citral. The tubes were monitored for 30 days for the occurrence of spoilage bacteria (Table 1).

Soft drinks are the most widely used food product. We often drink soft drinks without thinking that what it contain ant this has cause tremendous harm to us. It contain microorganism which can often cause diseases. It also contain antimicrobial agent to prevent the growth of these microorganism which is also the reason to worry. So does it mean we should not drink soft drinks? The answer is no we should drink soft drinks which do not use such a product which has antimicrobial activity as that of preservatives but do not harm us like preservative. Soft drinks due to their high C/N ratio are susceptible for microbial spoilage. Preservative are generally used to stabilize soft drinks. It was noted that traditional preservatives are associated with different hazards such as increase risk of heart disease while some preservatives also acts as carcinogen. This information gave rise to need for finding out new strategies. In this scenario, aroma compound can be interesting alternative. Their antimicrobial potential is well known [13]. Role of citral in orange based soft drink for spoilage bacteria has been described by some researchers [14]. In the 1st instance, the stability of these beverages depends on thermal treatments to which ingredients, intermediate, and final products, can be subjected. Although the beverages filled in glass bottles and in cans are usually pasteurized at temperatures able to kill yeast cells, the products packaged in plastic in most cases polyethylene terephtalate [PET] bottles are not thermally treated. For this reason, their stability relies on the addition of preservatives, belonging to the weak acid group, such as sorbic and benzoic acid. The effectiveness of these antimicrobials depends on several factors, among which the most important are pH, microbial cell concentration, and the intrinsic resistances to weak acids of the species present [15] [16].

Table 1: Evaluation of Orange Based Soft Drink for Microbial Spoilage

Sr.No.	Set	Test Tube No.	Citral Concentration	Time of Thermal Treatmen	Spoilage on the Basis of growth on Culture Media			
					Escherichia coli	*Klebsiella* spp.	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*
1	Set A	1	0%	5	+	+	+	+
	set A	2	0%	10	+	+	+	+
	Set A	3	0%	15	+	+	+	+
	Set A	4	0%	20	+	+	+	+
2	Set B	1	0.03%	5	+	+	+	+
	Set B	2	0.03%	10	+	+	+	+
	Set B	3	0.03%	15	+	+	+	+
	Set B	4	0.03%	20	+	+	+	+
3	Set C	1	0.06%	5	+	+	+	+
	Set C	2	0.06%	10	+	+	+	+
	Set C	3	0.06%	15	-	+	-	+
	Set C	4	0.06%	20	-	+	-	+
4	Ste D	1	0.09%	5	-	+	-	-
	Set D	2	0.09%	10	-	-	-	-
	Set D	3	0.09%	15	-	-	-	-
	Set D	4	0.09%	20	-	-	-	-
5	Set E	1	0.12%	5	-	-	-	-
	Set E	2	0.12%	10	-	-	-	-
	Set E	3	0.12%	15	-	-	-	-
	Set E	4	0.12%	20	-	-	-	-

Where, + = Cloudiness/spoilage observed, - = No Cloudiness/spoilage not observed

Another important factor influencing the microbial stability of soft drink is their composition, including the presence of substances able to support microbial growth (carbon and nitrogen sources, vitamins etc.) as well as to inhibit microorganism proliferation. Among these latter substances, a particular role can be played by the flavoring ingredients, which are often mixtures of molecules with a potential antimicrobial activity. This is particularly true for citrus based soft drinks. In fact, the antimicrobial activity of different citrus essential oils is well documented, although the mechanisms of action and the interactive effects between the constituents are not completely understood [17].

Table 2: Growth Inhibition of spoilage Bacteria in Orange Based Soft Drink

Citral Concentration	**Time**	**Inhibition of Spoilage Bacteria**
0.06%	15 minutes	*Escherichia coli*
0.06%	15 minutes	*Pseudomonas aeruginosa*
0.09%	10 minutes	*Klebsiella* spp.
0.09%	5 minutes	*Enterobacter aerogenes*

It was found that at 0.06% citral concentration the growth of *E. coli* and *Pseudomonas aeruginosa* was totally inhibited after thermal treatment for 15 minutes. On the other hand *Enterobacter aerogenes* and *Klebsiella* spp. were inhibited at 0.09% citral concentration after thermal treatment for 5 minutes and 10 minutes respectively (Table 2). *Escherichia coli* are the most likely known microbial threat in juice-rich beverages due to extreme acid-tolerance and the low infective dose of this organism. Low-acid products are able to support the growth of various food pathogens which needs to be taken into consideration in the product development and preservation. Spoilage microbes must tolerate an acidic environment that is low in oxygen and nutrients and usually rich in CO_2. As microbes differ in their growth requirements, different beverages support different spoilage microbes [18]. So-called specific spoilage microbes can grow even in products produced under good manufacturing practices. In case of production failures, less specialized opportunistic species are often involved, as they are more common in the production environment. New ingredients or new applications of established ingredients could introduce new spoilage species and growth factors in beverages, thereby expanding the spoilage microbe range beyond the well-known species. Soft drinks are very popular among the people of all ages around the world. Also in India, soft drinks are becoming more and more popular as they are usually tastier. Advantage of packed soft drinks is that these are convenient to carry and can be kept for a considerable amount of time [19] [20].

Typical representatives of Gram-negative water microbes are *Pseudomonads, Flavobacteria, Gluconobacter* and *Alcaligenes,* Gram positive Micrococci, Spirilla, Vibrios and the photosynthetic Cyanobacteria. Commonly encountered microbes that contaminate or affect the taste of beverages are Enterobacteriaceae. However this study showed that the antimicrobial activity of citral can be notably potentiated throughout the combination of appropriate concentrations with that of the time duration for thermal treatment, focusing the attention on the cross approach. Thus, this study confirmed the relationship between the temperature treatment and the antimicrobial efficacy of the molecules. In fact, neither the thermal treatment alone nor the presence of the citral at their maximum concentrations in the absence of the thermal treatment was able to guarantee the microbial stability of the beverages. According to previous study, these effects can be defined as synergistic, and are probably the result of the combination of the effects on membrane integrity and cytoplasm systems caused by molecules which can be characterized by different mode of action [11]. In this case, the antimicrobial activity of citral has been explained by the fact that it is a member of the α,β unsaturated aldehydes which act as alkylating agent towards nucleophilic groups of essential cellular constituents [21]. Synergism refers to an enhancement of antimicrobial activity of a compound because of the presence of a second compound [11]. It was demonstrated that the synergistic antimicrobial effect was shown between citral and other phenolic compounds (vanillin, thymol, carvacrol, eugenol) [22-24].

In this scenario, the search for new strategies and new antimicrobials for beverage (and other products) stabilization becomes a central goal for producers. Aroma compounds and essential oils can be an interesting alternative. Their antimicrobial potential is well known [25] [26]. A key role of orange essential oil in the microbial stability of orange based drink as well as the function of vanillin in inhibiting microbial degradation of beverages has been reported [14] [27]. In addition, the possibility to prevent microbial growth in uncarbonated beverages combining a mild thermal treatment with an essential oil or aroma compound was also reported [28]. The microbiological quality of processed soft drinks is the most important aspect to be taken care of by the manufacturer. Negligence in this area may result in serious contamination that ultimately represents a low quality product to the consumers. As these unwanted unhygienic conditions are usually due to the lack of knowledge and unawareness to the fundamental sanitary principles, it is preventable by proper training and monitoring. The government authorized institutes should take intensive investigation and public awareness to control the microbial and chemical quality of the soft drinks.

IV. CONCLUSION

In conclusion, the thermal treatment alone and the presence of the citral alone were unable to avoid the spoilage of the soft drink. It was found that at 0.06% citral concentration the growth of *E. coli* and *Pseudomonas aeruginosa* was totally inhibited after thermal treatment for 15 minutes. On the other hand *Enterobacter aerogenes* and *Klebsiella* spp. were inhibited at 0.09% citral concentration after thermal treatment for 5 minutes and 10 minutes respectively. The addition of the citral in combination with the thermal treatment progressively increased the frequency of not spoiled test tubes. Finally, citral strengthened the antimicrobial action when used in combination. The case of spoilage of soft drinks studied in this work stresses the need for deeper investigations on the role that certain substances can have the potential for the control of microbial proliferation. The failure of the traditional antimicrobials, such as weak acids, can be counteracted by the presence of specific molecules compatible with the overall characteristics of the products. In addition, such strategy can meet the consumer demand for absence of chemical preservatives, which can be substituted by a more accurate and balanced addition of aroma compounds.

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