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# Characterization of Bacteria Found in Soft-Drink through Biochemical Analysis and 16s rDNA PCR

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**Abstract:** ***Western culture brings us soft drinks as a part of our daily meal. It is aerated beverages which is very easily replacing water from our drinking habits. Ingredients of soft drinks help bacteria to survive in very odd conditions also. Here, efforts are being made to identify if any bacteria is found in our collected samples from open market. To characterize bacteria found we used various techniques like biochemical tests and 16s PCR. We used universal primer to complete PCR. Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities. 16sr PCR is a technique to identify conserved sequence of bacteria. 16sr PCR gel electrophoresis image is analyzed using TotalLab Quant version 12.4 software. This software analyses gel image with respect to ladder run along with samples and generates reports in term of molecular weight of each electrophoresis band.***

**Keywords:** 16s rDNA, Biochemical test, Total Lab Quant, Soft drink, Universal primer

## 1. Introduction

Soft drinks are a type of beverages categorized under non-alcoholic, carbonated drinks [1]. Soft drinks include juices, nectars and carbonated drinks. Nutritional significance of soft drinks includes plenty of water that prevents body from dehydration [1].

These drinks are often brewed using yeast. A little amount of alcohol is added in some soft drinks where alcohol is used in the preparation of the flavoring extracts such as vanilla extract or at home. Soft drinks can be made at home by mixing either a syrup or dry ingredients with carbonated.

Frequent or over consumption of these soft drinks may result into obesity [2], diabetes [3], low nutrient levels [4] and dental carries etc. Sugar sweetened drinks include drinks having high fructose corn syrup and sucrose. In some brands of soft drinks caffeine is also added. Caffeine added soft drinks may result into anxiety and sleep disruption [4]. Soft drinks may also contain sodium benzoate that may cause DNA damage and hyperactivity [6]. Chief components of all type of soft drinks are glucose, fructose, sucrose and other simple sugar. These carbohydrates may dissolve tooth enamel that lead to tooth decay. Soft drinks are acidic in nature with pH of nearly 3.0 or less [7]. Drinking acidic rinks may erase tooth decay.

## 2. Literature Survey

Soft Drinks are carbonated drinks containing water, sweetener and natural or artificial flavoring. Concentration of artificial additives like flavoring and sweetener should be according to government regulation [1]. Soft drinks are called soft due to less percentage of alcohol in contrast to hard drinks or alcoholic beverages. Alcoholic percentage in soft drinks should not exceed 0.5% of total volume [2] [8]. Soft Drinks are most consumed beverages in India. Soft drinks represent an important market in food industry. There are number of products of soft drinks with different brand available in market.

Microbial contaminations in soft drinks are either due to deterioration or contamination of products by general organism or pathogens to produce spoilage or food posing [9]. Soft drinks can be spoiled by variety of microorganisms like yeast, acid tolerant bacteria and fungi. Generally yeast and most commonly *Zygosaccharomyces bailii* act as main spoilage organisms due to its physiology and resistivity to acid preservatives [4] [10]. Many microbes can survive the acidic and low oxygen environment of soft drinks [11]. Spoilage of soft drinks is due to growth and metabolic byproducts like CO2, acid and tanning compounds [11]. Spoilage organisms of soft drinks are divided into 3 groups. Group1 organisms are basically spoilage/hygiene types that spoil the soft drinks only if any mistake during manufacturing [12]. Group 2 organism are common contamination of industries but can be restricted. Group 3 organisms are poor hygiene indicators [12].

There are number of bacteria that cause spoilage of soft drinks like Acetobactor, Alicyclobacillus, Leuconostoc, Sacanobacter, Zymobacter etc. [13]

## 3. Problem Definition

Although common bacteria hardly survive at low pH and low temperature, but if they do, it is very important to characterize them. Western culture brings soft drinks to our daily meal whether it lunch, dinner or party. As we know, to

preserve real taste of soft drink, it adds co2 gas which can be used by many bacteria as a source of carbon. Some quantity of sugar is also responsible for the same. If soft drink is slowly replacing water in urban life style, we should be fully aware consequences of using soft drinks casually. Clean distilled water does not carry any nutrients for bacteria, so even though it gets contaminants, bacteria struggle to survive for long time but soft drinks have all needed ingredients for bacterial survival.

## 4. Methodology

Diluted soft drink was used to spread in Nutrient Agar plate and very next day bacterial colonies were found. After making culture for all kinds of colony. Only one colony which was quite common in plate was used for biochemical tests. Composition of Nutrient Agar is as follows:

**Nutrient Agar:**
**Media Composition: 50ml**

| | |
|---|---|
| Peptone | 0.025g |
| NaCl | 0.25g |
| Beef Extract | 0.015g |
| Yeast Extract | 0.015g |
| Agar | 0.15g |

Prepared nutrient agar and sterilized it at $121^0$C for 15 min. Pourd media into the plate and let them solidify, and pour 100 µl of sample and spreaded on plate.

**Biochemical test:** Bacterial physiology differs from one species to the other. These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, ability to utilize a particular compound etc. help them to be identified by the biochemical tests.

**Table 1:** Biochemical Test

| Sample | Indole test | Methyl red test | VP test | Citrate test | $H_2S$ test | Urea test | Lysine test |
|---|---|---|---|---|---|---|---|
| **Sample 1** | -ve | +ve | -ve | +ve | +ve | -ve | +ve |
| **Sample 2** | -ve | -ve | +ve | +ve | +ve | -ve | +ve |
| **Sample 3** | -ve | +ve | -ve | +ve | +ve | -ve | +ve |
| **Sample 4** | -ve | -ve | +ve | -ve | +ve | -ve | +ve |
| **Sample 5** | -ve | -ve | +ve | +ve | -ve | -ve | +ve |
| **Sample 6** | -ve | -ve | +ve | -ve | -ve | -ve | -ve |
| **Sample** | **Arginine test** | **Motility test** | **Lactose test** | **Sucrose test** | **Mannitol test** | **Starch test** | **Skim milk** |
| **Sample 1** | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| **Sample 2** | +ve | -ve | -ve | -ve | +ve | +ve | +ve |
| **Sample 3** | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| **Sample 4** | +ve | -ve | -ve | -ve | -ve | +ve | +ve |
| **Sample 5** | +ve | +ve | -ve | -ve | +ve | +ve | +ve |
| **Sample 6** | -ve | -ve | -ve | -ve | +ve | -ve | +ve |

Biochemical tests only tells about species not strain, this is the reason why a microbiologist look towards molecular biology techniques. In this techniques, pcr (16s rDNA) is very strong technique to identify bacterial strain. Therefore, this technique is extremely vital for bacterial identification.

**DNA Isolation**

DNA Isolation using C-TAB method, Bacterial culture, Micro centrifuge tube, Pipette, Centrifuge (SPECTRAFUGE-24D), B.O.D Incubator (THERMOTECH), Micropipettes, Tips, Vortex mixer (TETA-2BIOCOM) ,heating block (THERMO 24-15 Termoctat) ,burner/hotplate.

C-TAB buffer ,Beta-mercaptoehthanol, Chloroform:Isoamyl alcohol(24:1),chilled Isopropanol, TE buffer.

1) Take 1.5ml of bacterial culture in micro-centrifuge tube(1.5ml).
2) Centrifuge it at 10,000 rpm for 5 min.
3) Discard the supernatant, add 500 µl C-tab buffer and 15 µl of beta-mercapto-ethanol to the pellet and dissolved it properly. Incubate it for 45 min at $42^0$C.
4) Add 200 µl of C-tab buffer and 200 µl of chilled CIA (Chloroform:Iisoamyl alcohol, 24:1) to the homogenate. Invert and mixed it properly. Centrifuge at 10,000 rpm for 10 min.
5) Transfer upper aqueous layer carefully without touching the middle protein layer in another micro centrifuge tube. Add equal amount of chilled Iso-propanol to it. Invert mixed slowly to see any thread like structure. Centrifuge at 10,000rmp for 10 min.
6) Discard supernatant. Wash pellet (if thick) with 70% ethanol. Dissolve pellet in ethanol properly (Do not vertex at any step). Centrifuge at 10,000 rpm for min.
7) Discard supernatant.
8) Air dry the pellet and add 50 µl of TE buffer and stored it at $4^0$C.

**Quantitative analysis of DNA**

**Table 2:** Spectrophotometer reading

| *Sample* | *Absorbance AT 260* | *Absorbance AT 280* | *CONC. OF DNA(ug/ml)* | *A260/A280* |
|---|---|---|---|---|
| Sample1 | 0.114 | 0.064 | 570 | 1.78 |
| Sample2 | 0.028 | 0.016 | 140 | 1.75 |
| Sample3 | 0.176 | 0.099 | 880 | 1.77 |
| Sample4 | 0.164 | 0.093 | 820 | 1.76 |
| Sample5 | 0.112 | 0.063 | 560 | 1.77 |
| Sample6 | 0.53 | 0.032 | 265 | 1.65 |

**16s rDNA;**

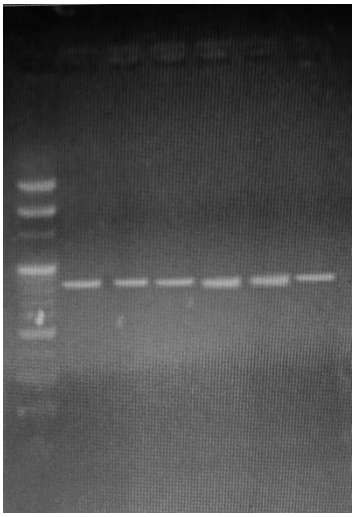
**16sr PCR Conditions**: Following are the cocktail for 16sr reaction : PCR grade water 20 ul, Taq Ready Mix- 25 ul,

Forward primer 1.0 ul, Reverse primer 1.0 ul, $Mgcl_2$ 1.0 ul ,Template DNA 2.0ul,

**PCR PROGRAM** : Initial denaturing temperature $93^0$C for 3 minutes, 36 times loop include $95^0$C temperature for 30 seconds, Annealing temperature $50^0$C for 30 second , Elongation temperature $72^0$C for 50 seconds and final extension temperature 72 $^o$C for 3 minutes including holding temperature $4^o$C for 1 hour.

**Agarose Electrophoresis**: 2.5% Agarose gel prepared in 1X TBE buffer to run pcr samples at 95V for 45 minutes along with 100bp ladder.

## 5. Result and Discussion



**16s rDNA pcr electrophoresis image**
**(Ladder from bottom reads 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1300, 1500) bp**

| Lane1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane 7 |
|---|---|---|---|---|---|---|
| Ladder | Sample1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 12 bands | ~ 834 | ~ 834 | ~ 834 | ~ 834 | ~ 834 | ~ 834 |

## 6. Conclusion

16s rdna pcr result and biochemical tests confirms presence of bacteria pseudomonas sp. in sample. A very important question is raised how and from where these bacteria are becoming part of soft drinks? What conditions are allowing to grow them in soft drinks even though they are stored at very low temperature. Is it mismanagement of store or packaging or something else?

Although we are getting same bacteria in all kind of samples, therefore, it is matter of concern that why this bacteria has selected soft drink to grow in.

## 7. Future Scope

This paper may challenge safety measures taken by companies who prepare soft drinks and will bring precautions to consumers regarding use of soft drink.

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