

Culture Dependent Examination of Commercially Available Bar Chocolates

S. Sheik Asraf, N. Shobi, R. Soundarya, M. Udaya Kumar

Abstract: In the year 2018 a report published by TechSci Research entitled “India Chocolate Market Forecast and Opportunities, 2018” reiterated that the chocolate market earnings in India has witnessed the growth rate of 21% from 2013-2018. Indian chocolate market witnessed USD 3.2 billion revenues in 2018. Indian chocolate market is divided into 4 sectors, in which bar chocolates accounts for highest share of 36%. Hence, We had chosen to study the commercially available bar chocolates through culture dependent methods. We had selected 3 commercially available bar chocolates namely Cadbury Dairy milk, Cadbury 5 Star and Cadbury Perk. Bacteria were isolated from these 3 brands of chocolate by streak plate method. Microbiological and Biochemical assays such as Simple staining, Gram staining, Catalase test, Methyl Red test, Oxidase test, Vogus Proskaur test, Indole test, Citrate utilization test and Starch hydrolysis test were performed. The bacterial isolates were found to be Rod shaped and Gram negative. The bacterial isolates were found to be negative for all the above mentioned tests except Methyl Red and Starch hydrolysis test. Thus, these findings highlight the impending health hazard posed by the commercially available bar chocolates.

Keywords : Culture dependent, Bar chocolates, Bacteria

I. INTRODUCTION

During 2018 TechSci Research released a report entitled “India Chocolate Market Forecast and Opportunities, 2018” that confirmed the fact that the revenues of Indian chocolate market showed the annual growth rate of around 21% for the time period between the years of 2013-2018[1]. The chocolate industry is considered as the majority admired product in the food and beverage processing industry in India. In 2018, the chocolate market in India reached the revenues of around 3.2 Billion U.S.\$ due to the ever increasing gifting practice in India and enlargement in the overall income among the working class which has fuelled the demand for chocolate products in the country[1]. Indian chocolate market is divided into four segments in which Bar chocolates segments accounts for maximum revenue of 36% [1]. It was reported that instant chocolate puddings were found to be spoiled by *Pseudomonas* sp. Strain AZR1 and *Klebsiella* sp. Strain AZR2 due to the

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Azoreductase activity of the spoilage organisms [2]. The microbial spoilage of UHT-treated milk chocolate by *Enterobacter* sp. and *Micrococcus* sp has been reported [3]. We had chosen to examine the microbiota of commercially available bar chocolates through microbiological and biochemical methods. We had selected the following bar chocolates namely Cadbury’s Dairy milk, 5 Star and Perk for this study. Streak plate culture method was used to isolate the pure cultures of bacteria from these 3 bar chocolates. Biochemical and Microbiological tests like Starch hydrolysis test, Citrate utilization test, Indole test, Vogus Proskaur test, Methyl Red test, Oxidase test, Catalase test, Simple staining and Gram staining was done for the bacterial isolates. Bacterial isolates were Rod shaped and Gram negative. Except for Methyl Red test and Starch hydrolysis test, the bacterial isolates were found to be negative for all the above mentioned tests. Thus, these findings highlight the impending health hazard due to the commercially available bar chocolates to the Indian consumers

II. MATERIALS, METHODS, RESULT AND DISCUSSION

A. Bacterial isolation from commercially available chocolate bars

Segregation of the bacterial population from commercially available bar chocolates by streaking the culture on agar plate (Table 1, Figure 1)

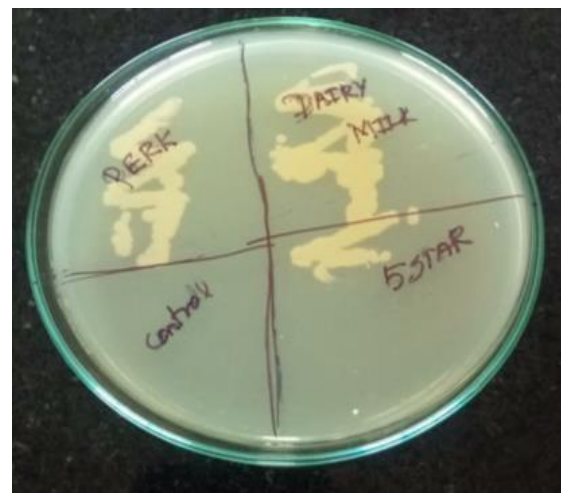


Fig. 1. Isolation of the bacteria from bar chocolates

Table-I. Bar chocolates used for this study

Name of the bar chocolates	Date of manufacturing	Date of expiry
Cadburys' Dairy milk	October, 2018	October, 2019
Cadburys' Five star	November, 2018	July, 2019
Cadburys' Perk	October, 2018	June, 2019

B. Simple staining

A loop packed with suspension of the bacterial isolates of the bar chocolates was taken on a dirt free slide and broadened and later air dried. The bacterial smear was heat fixed. Later, a drop of crystal violet was added for 15-60 S. The slide was rinsed in water, air dried and later observed under microscope. Rod shape of bacteria was observed [4,5].

C. Gram staining

A loop packed with suspension of the bacterial isolates of the bar chocolates was taken on dirt free slide slide, spread and later heat fixed. Crystal violet was added and maintained for 60 S and further the bacterial smear was rinsed in water. Gram's iodine was added and the slide was left untouched for one minute. It was again washed in water and later the decolouring agent was added and it was again washed in water. Safranin was added and incubated for one minute. It was again washed in water and the glass slide was examined under microscope [6]. The bacteria isolated from the bar chocolates were found to be Gram negative (Figure 2).

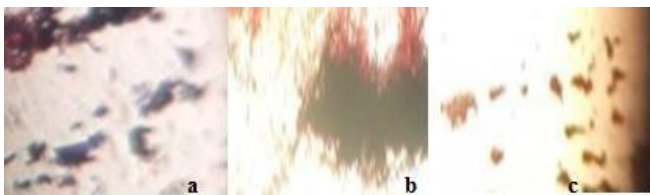


Fig. 2. Gram staining of bacterial isolates. a,b,c are the Gram staining results of bacterial isolates from Cadburys' Dairy milk, Perk and Five star respectively.

D. Catalase test

A dirt free glass slide was taken and few drops of suspension of the bacterial isolates of the bar chocolates were added. Later, few drops of H₂O₂ was added[7]. Evolution of oxygen was not observed indicating negative result for catalase for the bacterial isolates of the bar chocolates (Figure 3).

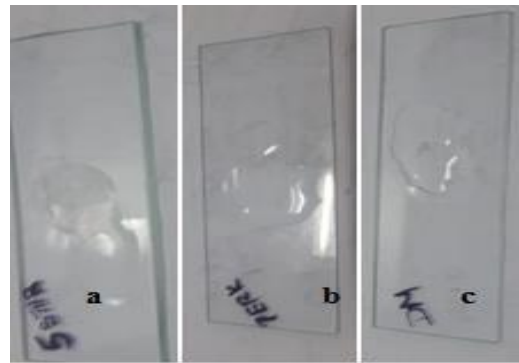


Fig. 3. Catalase test of bacterial isolates.a, b, c are the catalase results of bacterial isolates from Cadburys' Five star, Perk and Dairy milk respectively.

E. Oxidase test

Amino dimethyl aniline oxalate discs were placed on the glass slide. The bacterial isolates of the bar chocolates was added using pipette to the disc [8]. There was no development of Pink/Maroon/Black color formation indicated the negative result for oxidase for the bacterial isolates of the bar chocolates (Figure 4).

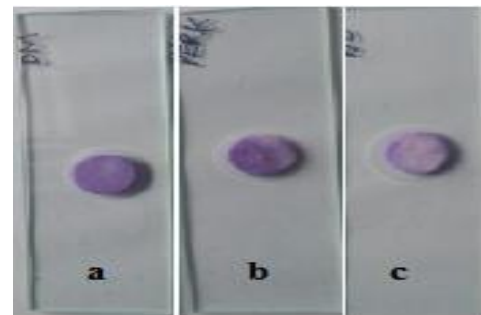


Fig. 4. Oxidase test for bacterial isolates a, b, c are the oxidase test results of bacterial isolates from Cadburys' Dairy milk, Perk and Five star.

F. Indole test

The peptone broth was prepared. After autoclaving at autoclaved at 121°C, 15 lbs for 15 minutes, the bacterial isolates of the bar chocolates was inoculated into the test tubes and later incubated. Later 1ml of Kovac's reagent was introduced and was shaken well [9]. No cherry red formation indicated that the bacterial isolates of the bar chocolates were found to be -ve for indole test (Figure 5).

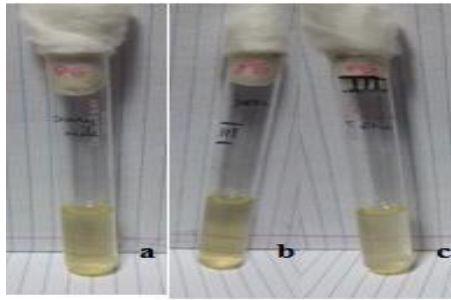


Fig. 5. Indole test for bacterial isolates a, b, c are the Indole test results of bacterial isolates from Cadburys' Dairy milk, Perk and Five star.

G. Methyl red test

MR-VP broth in the test tubes were kept at 121°C for 15 minutes. The MR-VP broth was inoculated with the bacteria isolated from the bar chocolates and later incubated and 5-6 drops of methyl red solution was added [10]. The formation of Red color indicated positive result for methyl Red test for the isolated bacteria of the bar chocolates (Figure 6).

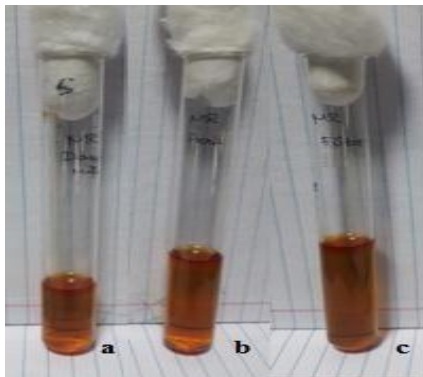


Fig. 6. Methyl Red test of the isolated bacteria. a, b, c are the test results of isolated bacteria from Cadburys' Dairy milk, Perk, and Five star.

H. Vogus proskaur test

MR-VP broth was autoclaved at 121°C, 15 lbs for a period of 15 minutes. After sterilization, the isolates of the bacteria from the bar chocolates was introduced into the medium and were incubated. Barrit's reagent was added and mixed well[11]. The absence of deep rose color indicated the negative result for Vogus Proskaur test for the bacterial isolates of the bar chocolates (Figure 7).

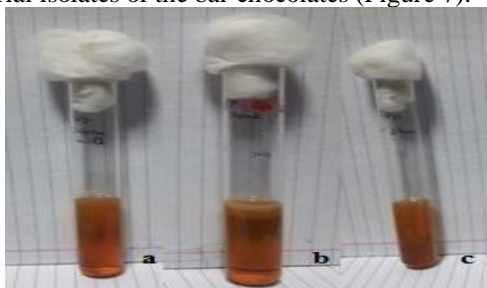


Fig. 7. Vogus Proskaur test for bacterial isolates. a, b, c are the test results of bacterial isolates from Cadburys' Dairy milk, Perk and Five star

I. Citrate utilization test

The simmon's citrate agar medium was prepared and later was autoclaved at 121°C, 15 lbs for 15 minutes. The sterilized simmon's citrate agar medium were made as agar slants. The isolated bacteria of the bar chocolates were inoculated. The test tubes were incubated [12] and there was no Blue color formation. Hence, the isolated bacteria of the bar chocolates was found to be negative for citrate utilization (Figure 8).



Fig. 8. Citrate utilization test of the isolated bacteria. a, b, c are the test results of bacterial isolates of Cadburys' Dairy milk, Perk, Five star.

J. Antibiotic disc test- Kirby bauer method

Using sterile technique, the agar plate was inoculated with the isolated bacteria as follows: A sterile cotton swab was dipped with bacterial culture and using the swab, the entire agar surface was streaked with the bacterial culture in horizontal, vertical and edges of the petri plate to ensure heavy bacterial growth over the complete surface. The petriplate was dried for about 5 minutes. The antibiotic disc such as ampicillin, streptomycin and nitrofurantoin were placed at equal distance with forceps dipped in alcohol and flame. The antibiotic discs were gently pressed down with the wooden edge of the cotton swab [13]. The petriplates were incubated for 24 hours at 37°C. The region of inhibition was observed around all the three antibiotic discs. (Figure 9)

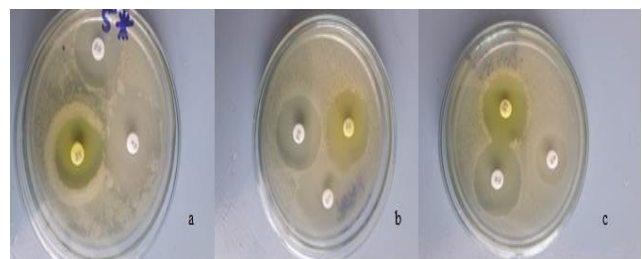


Fig. 9. Antibiotic disc test for bacterial isolates. a, b, c are the test results of bacterial isolates of Cadburys' Dairy milk, Perk, Five star.

K. Starch hydrolysis test

Nutrient starch agar medium was prepared, sterilized at 121°C at 15lbs pressure for 15 minutes and dispersed into petriplates. The bacterial isolates of the bar chocolates was inoculated onto the medium and kept at 37°C for 24 hours. Following the incubation, the plates were flooded with lugol's iodine. The bacterial isolates were found to be positive for starch hydrolysis (Figure 10)

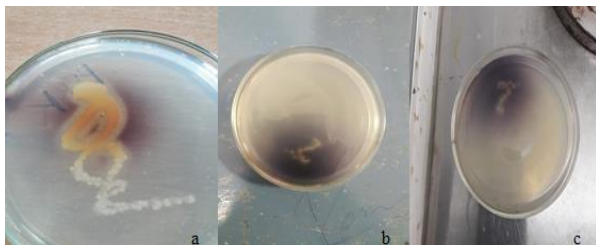


Fig. 10. Starch hydrolysis test for bacterial isolates. a, b, c are the test results of bacterial isolates of Cadbury's Dairy milk, Perk, Five star.

“India Chocolate Market Forecast and Opportunities, 2018” report confirmed the fact that the Indian chocolate market revenues showed the annual growth rate of approximately 21% for between 2013-2018[1]. The chocolate industry is considered as one among the major food and beverage processing industries in India which is evident from the fact that in the year 2018, the Indian chocolate market reached the revenues of approximately 3.2 Billion U.S.\$ due to increase in gifting practice and overall income among the working class in India [1]. Our study is significant to address the issue of quality of the chocolates. Our study correlates with earlier reports of chocolate puddings found to be spoiled by *Pseudomonas* sp. Strain AZR1 and *Klebsiella* sp. Strain AZR2 due to the Azoreductase activity of the spoilage organisms [2]. Our study also emphasizes the earlier report of the microbial spoilage of UHT-treated milk chocolate by *Enterobacter* sp. and *Micrococcus* sp [3].

Thus, our study highlight the impending health hazard posed by the commercially available bar chocolates to the Indian consumers.

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