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## Research Paper

# Isolation and identification of bacteria from four different frozen snacks of gazipur district of Bangladesh

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## Abstract

The study was designed to isolate and identify microorganisms from frozen snacks from local market of Gazipur district. For this, four different kinds of snacks (French fry 10, deshi porotha 9, dal-puri 8 and alo-puri 8) were tested for the isolation of microorganisms. For the isolation of bacterial species each food sample was inoculated onto EMB agar, Nutrient agar and MacConkey agar media. The isolated bacteria of each sample were identified by cultural, biochemical (Oxidase, catalase, indole, MR, VP, citrate, TSI, nitrogen reduction, gelatin, lactose, sucrose, glucose, fructose, maltose, mannitol, arabinose, sorbitol) and staining characteristics. The results obtained from the microorganisms associated with the spoilage of frozen snacks showed that the total aerobic counts for French Fry ranged from  $1.10 \times 10^6$  to  $2.20 \times 10^6$  cfu/g where Deshi Porotha ranged from  $1.56 \times 10^6$  to  $2.58 \times 10^6$  cfu/g, Aloo Puri ranged from  $1.68 \times 10^6$  to  $2.36 \times 10^6$  cfu/g, Dal Puri ranged from  $1.88 \times 10^6$  to  $2.56 \times 10^6$  cfu/g. Out of the total 35 samples the prevalence of *Staphylococcus aureus* was found highest following *Bacillus cereus*, *Klebsiella aerogenes* and *Proteus mirabilis* in all the four kinds of frozen food tested in this study. Where *S. aureus* were found 35%, *B. cereus* 31%, *K. aerogenes* 20%, *P. mirabilis* 14%. In this study *S. aureus* was found frequently from all food samples but *K. aerogenes* and *P. mirabilis* were mostly isolated from Dal Puri and Alu Puri. Frozen snacks has to be stable in the local market for a certain period of time, it is considered to be free from spoilage causing and pathogenic microorganisms and during consumption should not cause any discomfort to taste and health. Results of this study clearly indicates that all the four different isolates of bacterial species are responsible for potential cause of spoilage in frozen foods if there is any variation in storage temperature. Furthermore, the presence of the potential spoilage causing bacteria in the selected frozen foods also indicate that the food got contamination during processing, manipulation, packaging and storing.

**Keywords:** Frozen Snacks, Spoilage, Bacterial Interaction, Viable count, Specific Spoilage Bacteria.

## Introduction

The export market for frozen snacks has opened up in the past couple of years. In the past three years, export earnings from frozen snacks, such as deshi paratha, french fry singara, samosa, dal puri, and alu puri, have grown more than seven times and also in cash \$2.38 million according to Bangladesh Agro Processor's Association<sup>[1]</sup>. Exporters linked the rise to growing demand among Bangalees and the entry of some new firms in Europe, North America, Australia and the Middle East.

The demand for frozen snacks is very high as people staying abroad want to taste local snacks. The frozen snacks account about 40 percent of the country's export basket. Demand for frozen snacks is rapidly increasing among ethnic people. The opportunity to enter the cross cultural food markets is now opening<sup>[2]</sup>. Economist presumes that the frozen snacks of Bangladesh has a trillion dollar market worldwide<sup>[1]</sup>. Now it is also becoming popular in the urban areas of Bangladesh. Peoples of big cities and towns of the country are rapidly entering in the market of frozen snakes. But the unexpected spoilage of frozen snacks by microorganisms is interrupting the industrial production and growth by reducing its economic value.

Food quality and safety is an important issue of public health. Nowadays, food quality and food safety are very fruity and two serious issues of food are great concern because of globalization of food supply and increased complexity in the food chain. The consumers are focusing on the nutrient value as well as safety value of food before purchasing which will not be the threat of human health. The aim of food safety is to eliminate the peril to health from different hazards, such as microbial hazards, insecticide residues, abuse of food additives and contaminants, such as chemicals, biological toxins and adulteration. On the other hand, food quality includes all influence that enhance the value of a food product, this includes negative attributes such as spoilage, contamination with grime, discoloration and positive attributes such as the origin, color, flavor, texture and processing method of the food<sup>[3]</sup>. The contamination of food products with bacteria may create a great problem of global health concern. The growth and metabolism of bacteria can cause serious food borne infection and intoxications which may lead rapid spoilage of the food products. It is necessary to mention that a food product naturally holds some indigenous bacteria that can include spoilage and pathogenicity. Across the manufacturing process these species of bacteria can proliferate and affect the shelf life of the product. Actually most bacterial contamination happens during processing and manipulation of the food products<sup>[3]</sup>.

Spoilage causing bacteria deteriorate the frozen food product and develop unpleasant odors, tastes, and textures. A perished food product is deficient of the original nutritional value, texture or flavor and can become unsuitable to eat. The bacterial spoilage of frozen food products are consisting significant economic problem which results in high economic losses for the food industry, especially improper storage under refrigeration conditions. Thus, spoilage causing bacteria can grow in large number in frozen food product, perish the food and cause changes to sour taste an bad smell, which interfere with the quality of the products. Usually non spoilage bacteria normally do not responsible for serious illness, but when consumed a large amount, they can make some discomfort in gastrointestinal tract<sup>[4]</sup>. There are a large variety of bacterial species (*Bacillus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Proteus* spp.) that can cause deterioration of foods.

Food borne infections are caused by bacteria that invade the body through the consumption of spoiled food. Food can serve as a growth medium for bacteria and their metabolic product can cause food poisoning. According to WHO at 2013 alone 1.7 million people died from diarrheal diseases by consuming complete and partial spoilage food. In developed countries, percentage of the population affected by food borne illness in each year has been reported up to 30%<sup>[3]</sup>. Food-borne intoxication is caused by consumption of toxins which is produced in food as a by-product of bacterial multiplication. In such condition the toxins but not bacteria cause illness. Toxins of different species of bacteria may not be responsible for altering the texture, odor or flavor of food<sup>[3]</sup>.

There is potential for a wide range of frozen snack products to become contaminated with microorganisms. The range of microorganisms associated with spoilage linked to fresh produce encompasses bacteria. The spoilage causing bacteria of frozen snakes depends on the endogenous bacterial population present on the products, with post processing, packaging method and storage temperature can be the most important parameters<sup>[5]</sup>. This situation has lead to a growing awareness on the necessity to enumerate and evaluate bacterial population associated with the spoilage of prepared frozen snacks. The main objective of this study was to determine the total viable count of bacteria in frozen snacks as well as to isolate and biochemically characterize the specific bacteria present in different frozen snacks from a commercial and industrial area of Bangladesh.

## Materials and Methods

### Sample Collection

A total of thirty five samples which include the French Fry(10), Deshi Porotha(9), Aloo Puri(8), Dal Puri(8) were collected from different premium grocery shop of Gazipur district, Bangladesh from July to December 2014. Every sample was taken separately into sterile plastic bag and that bags were put into sterile ice bag chamber for transportation to the Microbiology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University. Then every sample was subjected to quantitative and qualitative microbial analysis.

### Enumeration of bacterial load

The spoiled part of each sample was crashed and grinded by sterile mortar pestle. Then the sample was weighed in grams. One gram of each sample was dispensed into a prepared 10 ml of peptone water contained in the McCartney bottles. The suspension was shaken gently for homogenous mixture. Ten fold serial dilutions ( $10^{-1}$  -  $10^{-5}$ ) of the samples was made and plated on the Plate Count Agar, Nutrient Agar and MacConkey Agar for total aerobic and anaerobic counts. These were incubated at 37°C temperature for 24 hours in the incubator. The streaking techniques were done on the Nutrient agar for the purification of bacteria from different colonies as single colony for single bacteria.

### Morphological Characterization

The isolated microbes were morphologically characterized on the basis of size and shape of the colony and gram staining technique [6, 7, 8].

### Biochemical Characterization

The discrete colonies from subculture plates were taken and series of biochemical tests were done for identification of bacterial species. The bacterial isolates were characterized by different biochemical reactions i.e. Oxidase, Catalase, Indole, MR, VP, Citrate, TSI, Nitrogen Reduction, Gelatin, Lactose, Sucrose, Glucose, Fructose, Maltose, Mannitol, Arabinose, Sorbitol for the identification of bacterial species, standard methods were followed in described by Holt [6]; Sherman [7]; Oyeleke [9]; Ashok [8]; Adebayo [10].

### Results and discussion

There is different spoilage causing bacteria which can grow well at relatively low temperature. A large number of bacteria and their products can cause the objectionable changes in odor, taste and texture of frozen snacks. In the present study different samples of spoiled frozen snacks were collected from different grocery shop of Gazipur district, Bangladesh and four different species of bacteria were isolated and identified.

### Total viable count

The results obtained from the microorganisms associated with the spoilage of frozen snacks showed that the total aerobic counts for French Fry ranged from  $1.10 \times 10^6$  to  $2.20 \times 10^6$  cfu/g where Deshi Porotha from  $1.56 \times 10^6$  to  $2.58 \times 10^6$  cfu/g, Aloo Puri from  $1.68 \times 10^6$  to  $2.36 \times 10^6$  cfu/g, Dal Puri from  $1.88 \times 10^6$  to  $2.56 \times 10^6$  cfu/g.

### Morphological characteristics

Four different species of bacteria were isolated from the total thirty five samples of frozen snacks. They are *Bacillus cereus* (31%), *Staphylococcus aureus* (35%), *Klebsiella aerogenes* (20%), *Proteus mirabilis* (14%). *Bacillus cereus* showed Large whitish, irregular, opaque colonies on nutrient agar plate. They are gram positive rod, non capsulated and motile. The bacilli tend to occur in chain. *Staphylococcus aureus* showed entire colony, rough appearance and dry texture, smooth and golden yellow color on nutrient agar. They are gram positive cocci, appearing grape like cluster under the microscope. *Klebsiella aerogenes* showed good growth of brown, dark centered, mucoid colonies on EMB agar. They are gram negative straight rod, non motile and non capsulated. *Proteus mirabilis* showed swarming growth in the form of a uniform film, which spreads over the whole surface of

nutrient agar plate, under the microscope they are gram negative rod, often showed motility in hanging drop preparation.

### Biochemical Characteristics

The four different isolates were characterized on the basis of biochemical tests. *Bacillus cereus* showed catalase, citrate, voges proskauer, nitrate reduction, gelatin, starch, glucose, fructose, maltose and glycerol positive while oxidase, indole, ornithin decarboxylase, mannitol, lactose, galactose, sorbitol, dulcitol and arabinose negative. *Staphylococcus aureus* showed positive reaction with catalase, citrate, urease, lactose, sucrose, glucose, mannitol and lipid hydrolysis but oxidase, indole, methyl red, voges proskauer, H<sub>2</sub>S production, starch hydrolysis, gelatin liquefaction negative. *Klebsiella aerogenes* showed positive reaction for catalase, nitrate reduction, esculin hydrolysis, glucose, arabinose, mannitol, sorbitol, dulcitol, inositol, lactose, sucrose and maltose but negative reaction for oxidase, indole, gelatin hydrolysis, H<sub>2</sub>S production, arginin dihydrolase and lipase. *Proteus mirabilis* showed positive reaction for catalase, methyl red, urease, nitrate reduction, H<sub>2</sub>S production, glucose and ornithin decarboxylase but negative reaction for oxidase, voges proskauer, citrate, indole, starch and lysine decarboxylase (Table 1).

**Table 1: Biochemical Characteristics of the isolated bacteria**

Biochemical	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>	<i>Klebsiella aerogenes</i>	<i>Proteus mirabilis</i>
Oxidase	-	-	-	-
Catalase	+	+	+	+
Citrate	+	+	-	-
Indole	-	-	-	-
MR	-	-	+	+
VP	+	-	-	-
Nitrate reduction	+	+	+	+
H <sub>2</sub> S production	+	-	-	+
Gelatin lequification	+	-	-	
Ornithin decarboxylase	-	-	+	+
Starch	+	-	-	
Glucose	+	+	+	+
Sucrose		+	+	+
Fructose	+	+	-	+
Maltose	+	+	+	-
Lactose	-	+	+	-
Arabinose	-	+	+	-
Glycerol	+	-	-	+
Mannitol	-	-	+	-
Lactose	-	-	-	-
Galactose	-	-	+	+
Sorbitol	-	-	+	+
Dulcitol	-	+	+	+
Inositol	+	-	+	-
Urease	-	+	-	+

**Note:** In this table + sign indicates positive and – sign indicates negative.

The total viable counts of bacteria obtained from French Fry in this study is related to the finding was reported by Adebayo <sup>[10]</sup> and was much lower than the findings reported by Mumtaz Begum <sup>[11]</sup>. The bacterial load counted from Deshi Porotha, Alu Puri, Dal Puri is quite similar with Moshood <sup>[12]</sup> and

very dissimilar with Sabrina<sup>[5]</sup>. The bacterial population in these frozen snacks may have been derived from contamination of soil, air, flies, water, content of manufacture, workers, harvesting and transportation equipments<sup>[10]</sup>. The bacterial isolates found in this study were different from those identified by Ashok<sup>[8]</sup> and Olunlade<sup>[13]</sup> but the isolates were almost similar with those that identified by Moshood<sup>[12]</sup>, Adebayo<sup>[10]</sup> and Mumtaz Begum<sup>[11]</sup>. The microbial population levels found in this study were in agreement with data reported for spoilage of stored vegetable and storage biscuit in other studies of Adebayo<sup>[10]</sup> and Olunlade<sup>[13]</sup>. The bacterial isolates identified in this experiment include *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus mirabilis*. These findings are almost consistent with the interpretation of previous studies.

Bacteria most commonly found in frozen foods are generally involved *Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus* spp. (P. Saranraj<sup>[14]</sup>; Moshood<sup>[12]</sup>). Abusive temperature and improper maintenance during storage and transportation always lead to increase the bacterial population. Bacteria such as *Bacillus cereus*, *Klebsiella* spp. and *Escherichia coli* are naturally present in soil and water and they can easily get entrance into the food and cause spoilage<sup>[8, 10]</sup>. The genus *Proteus* and *Klebsiella* belongs to the family Enterobacteriaceae. They are all associated with fecal contamination where they are known to cause food poisoning<sup>[10]</sup>. Food processing unit can play as a source of microbial access to cause microbial food poisoning, food intoxication and food spoilage. Due to a little careless about good manufacturing practice, the shelf life of the product can be severely affected due to microbial contamination which results in huge economic losses to the manufacturer<sup>[14]</sup>. The isolates found in this study to cause spoilage in frozen snacks is quite different from that reported for isolation of bacteria from spoiled raw salmon fillets stored under modified atmosphere packaging<sup>[5]</sup>.

The biochemical properties of *Bacillus cereus* found in this study highly agreed with previous studies of Gordon<sup>[15]</sup> and Bergey's Manual of Determinative Bacteriology by Buchanan<sup>[16]</sup>. *Staphylococcus aureus* showed positive reaction with catalase, citrate, urease, lactose, sucrose, glucose, mannitol and lipid hydrolysis but negative reaction with oxidase, indole, methyl red, voges proskauer, H<sub>2</sub>S production, starch hydrolysis, gelatin liquefaction which are quite similar to the result of Sushma Konuku<sup>[17]</sup> and Boerema<sup>[18]</sup> but slightly dissimilar to that reported by Balaban<sup>[19]</sup>. The biochemical characteristics represented by *Klebsiella aerogenes* also agree with the report of Don J. Brenner<sup>[20]</sup> but slightly dissimilar with Carter J.S.<sup>[21]</sup>. The biochemical behavior of *Proteus mirabilis* closely agree with the findings of Milagro Fernandez Delgado<sup>[22]</sup> and Korneva<sup>[23]</sup> but slightly variable with the findings of Muller<sup>[24]</sup> and Ajayi<sup>[25]</sup>.

Previously it was found that the *Bacillus* and *Klebsiella* were the most responsible species in the spoilage of every categories of food material. In fruits, vegetables and meat *Klebsiella* was the most potent spoiling bacteria. *Staphylococcus* spp. was found to cause spoilage in sugar cane and papaya<sup>[8]</sup>. *Proteus mirabilis* was reported previously to cause spoilage in smoked fish in Malaysia<sup>[12]</sup>. Ashok<sup>[8]</sup> reported that the bacteria especially gram negative such as *Klebsiella*, *Proteus*, *Pseudomonas* were found as key responsible for a wide variety of food spoilage. P. Saranraj<sup>[14]</sup> reported that the *Bacillus* spp. is most responsible for the spoilage of many kinds of bakery product. V. Singh<sup>[26]</sup> isolated *Staphylococcus* spp., *Proteus* spp., *Bacillus* spp. and observed that these bacteria are responsible for the spoilage of milk and milk products.

Now a days frozen snacks are becoming very popular in urban areas of Bangladesh. In cities and towns people leading a very busy life, that is why they are looking for ready-made frozen snacks for easy cooking and consumption. So the diet of many people is supplemented with frozen snack items preserved by chilling and available in a variety of conditions and stages of preparation. Such food may be frozen, canned or dehydrated. It may be partly baked or partially fried, ready for heating and serving. During preparation, some microorganisms can get access from raw water, processing materials or by improper handling for meeting their nutritional requirement and attack such snacks. However improper industrial storage and preservation can allow microorganisms to grow on snacks and affect the shelf life of the products. Proper transportation is also important, if any condition lead to raise the temperature of vehicle from -18°C to 3-4°C for certain duration of time will facilitate the bacteria to grow on packaged frozen snacks. The improper maintenance of product within recommended temperature (-18°C) in the retail shop can also lead the spoilage. The unrestricted growth and multiplication of such bacterial species in food may turn it unfit for consumption and can result in spoilage or deterioration.

Frozen snacks has to be stable in the local market for a certain period of time, it is considered to be free from spoilage causing and pathogenic microorganisms and during consumption it should not cause any discomfort for taste and health. Safe frozen snacks always have low bacterial counts which should not affect the shelf life. Snakes suspected to be perished and unfit for consumption always showed higher bacterial counts ranging from one million to ten million per gram <sup>[27]</sup>.

## Conclusion

The main purpose of this study was to isolate and identify spoilage causing and pathogenic bacteria from four different kinds of frozen snacks of Gazipur district, Bangladesh. It is therefore very necessary and important that workers, regulatory bodies of the industry, store keepers, transporters, retail sellers and customers have to develop consciousness to avoid contamination of harmful bacteria in the foodstuffs. Results of this study showed that, within four bacterial species associated with snacks under frozen condition, two species *Bacillus cereus* and *Staphylococcus aureus* were determined as rapid and strong spoiler. *Staphylococcus aureus* is most frequently found in all types of frozen snacks. *Klebsiella aerogenes* and *Proteus mirabilis* were found less frequently than *Bacillus cereus* and *Staphylococcus aureus*. Maintaining proper hygiene of the workers, developing adequate cleaning system of the production floor, fumigating production and storage area in a certain interval, proper cleaning and sterilization of manufacturing equipments and machineries, controlling insect and rodent access into the production area, maintaining appropriate storage temperature, maintaining proper temperature during transportation, in retail shops according to recommended temperature (-18°C) will reduce the bacterial load thus avoid spoilage of the product and minimize the health risks to the consumers. Further experiments and investigations are required to understand the process involved in these interactions to have a better interpretation about natural spoilage of readymade frozen snacks and, in the future, to develop techniques to reduce product losses and health risks.

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