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Mineral Composition and Microbial Association of a Local Condiment-'Beat Laban' (Black Salt)

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Abstract: Six black salt samples were studied for their morphology, composition (the presence of sodium, potassium, chloride, sulfur, calcium, magnesium, iron, manganese and phosphorus) and microbial association. The samples showed heterogeneity in their composition and also variation in color. After dissolution and recrystallization, the crystals showed defined shape but lost their characteristic colour which indicated that the heterogeneity of the crystals as well as the colour could be due to the presence of impurities. Eight bacterial strains *viz.* *Halomonas* sp (B7), *B. alcalophilus* (B1), *B. pantothenticus* (B2), *B. stearothermophilus* (B3), *B. laterosporus* (B4), *B. pumilus* (B5), *B. circulans* (B6) and *B. marinus* (B8) and only one actinomycetes member *Streptomyces anulatus* (B7/1) were isolated from the black salts. Among these organisms, *B. pantothenticus*, *B. circulans* and *B. stearothermophilus* showed β-hemolysis. *Enterobacter amnigenes, Enterobacter intermedium, E. coli, Alcaligenes* sp., *Salmonella paratyphi* and *Plesiomonas* sp. grown at different concentrations of black salt and NaCl demonstrated higher salt tolerance.

Key words: Association · Black salt · Composition · Microorganism · β-hemolysis

INTRODUCTION

In the biological world, mineral elements are indispensable for several biological processes. About 56% of the adult human body is fluid in which minerals are present as electrolytes acting at the cell membrane which allows transmission of electrochemical impulses in nerve and muscle fibers and also controls the activity of different enzymatically catalyzed reactions that are necessary for cellular metabolism. Balanced amounts of carbohydrates, proteins as well as salts present in the daily food menu are necessary for the homeostasis. The daily requirements of minerals of a human body are Na 3.0 g, K 1.0 g, Cl 3.5 g, Ca 1.2 g, P 1.2 g, Fe 18.0 mg and the requirement of Mg, Co and Cu are unknown [1]. Human beings receive essential minerals through their daily food intake especially from edible salts of different origin e.g., sea, rocks, mines, etc. Varieties of edible salts are used for culinary purposes, such as Table salt, Black salt, Celtic salt, Coarse Salt, Flake Salt, Fleur de Sel, French Sea Salt, Grey Salt, Grinder Salt, Hawaiian Sea Salt, Kosher Salt, etc.

Sodium chloride (NaCl) or table salt is a common requisite in daily cuisines. In modern civilization, excess amount of sodium intake is a common phenomenon which contributes to certain cardiovascular diseases. In contrast, salt appetite is stimulated by the decrease of extracellular sodium concentration, pressure and volume of blood and these are the major stimuli that elicit thirst [1]. In case of human being, salt craving is also observed in Addison's disease [2]. Consecutively, chloride, another ion of table salt is also ingested in a large amount. Chloride, in addition to potassium and sodium, assists in the conduction of electrical impulses when dissolved in body fluid and maintains serum osmolarity, produces HCl, a powerful digestive agent. The exchange of chloride and bicarbonate between red blood cells and plasma helps to prevent accumulation of base and transport of CO_2 [1]. Excess intake of chloride have toxic effects such as fluid retention and high blood pressure which are attributed to the high sodium and potassium levels [4] and impaired sodium chloride metabolism [5]. Deficiency of chloride, in contrast,

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results in severe hypokalemic metabolic alkalosis, low urinary chloride concentrations (<10mEq/liter) and erythrocyturia [6].

Black salt; commonly known as 'Beat laban', 'Kala nemak' and 'Sanchal', is being used in Indian cuisine as a condiment for a very long time. It is obtained from rock deposits and used as a raw material without further processing. It is commonly sprinkled on raw fruits like cucumber, carrot, apples and also consumed with local snack foods like chatpati, singara, shamucha, etc. Even though, the use of this condiment is gaining popularity; no report is available on its mineral composition and also on the microbial association. Generally, halophiles are reported to have high tolerance of salts (NaCl) up to 30% (w/w). Their tolerance reflects the adaptive responses towards adverse environmental condition. The present work was undertaken to study the morphology and mineral composition of six black salts and also explores microbial association to assess any possible health risk.

MATERIALS AND METHODS

Six black salt samples were collected from local supermarkets in the form of lumps (aggregate of crystals). Samples were brought into the laboratory and were subjected to various studies.

Microscopic Observation: General appearance and colour of the crystals of the black salts were examined under scanning electron microscope (SEM- XL 30 Phillips, Netherlands) and research microscope (Nikon Optiphot, Japan). The samples were gently crushed and powdery crystals were brushed off from the freshly exposed surface and collected on dry slides for microscopic studies. Concentrated solution of salts were heated on the deem flame without stirring and after recrystallization, observed under microscope.

Analysis of Physical and Chemical Properties of the Black Salt: Moisture content of the salt samples were measured by gravimetric method; pH of 1% solution was measured by electric pH meter (Jenway 3310 pH meter, U.K.). The amount of sodium and potassium were determined by flame photometer; phosphorus, iron and manganese were determined colorimetrically as described by Jackson [7], Olson and Roscoe [8] and Cambrell and Patrick [9], respectively. Sulphur was measured by the turbidimetric method of Hunt [10] and EDTA-Complexmetric titration methods were applied to determine calcium and magnesium [11]. While, chloride was determined by titrimetric method using silver nitrate as described by Hesse [12]. From 10% black salt solutions, precipitated portions were separated and dissolved in concentrated HCl acid and tested for the presence of iron [8].

Growth Index of Microbes on Black Salt and Sodium Chloride Enriched Media: The effects of black salt and NaCl (1, 2, 3, 4, 6 and 8%) on the growth of selected pathogenic bacterial strains (*Enterobacter amnigenes, Enterobacter intermedium, E.coli, Alcaligenes* sp., *Salmonella paratyphi* and *Plesiomonas* sp.) were tested. Nutrient agar was used as basal medium. Inoculated media were incubated at 37°C for 48 hrs and growth of the organisms were recorded. The test strains were collected from the laboratory of Microbiology, Department of Botany, Dhaka University.

Microbial Association: Nutrient agar medium and Inorganic salt medium [13] supplemented with 1% black salt were used at pH 8.5 to determine microbial loads of black salt samples. Direct inoculation and scrub techniques were applied for the isolation of organisms. The ground salt crystals were directly inoculated in the above media and incubated at 37°C for 48 hrs. Physiological and biochemical tests, as prescribed in the Bergey's Manual vol 1[14], vol 2 [15] and vol. 4 [16], were carried out for provisional identification of the isolated organisms. The organisms which were growing only in media amended with 1% black salt were selected and studied. In search of the existence of microorganisms in the core of black salt, crystals samples were burned in flame for surface sterilization, broken apart and a little amount from the core was inoculated aseptically in the broth medium. The inoculated media were incubated at 37°C for 15 days and then kept at room temperature for further 45 days. Inorganic salt medium and sulfur oxidizing medium [13] were used for this purpose. The whole procedure was carried out in a laminar air flow cabinet sterilized with UV radiation.

Hemolytic Test: Black salt associated microorganisms were inoculated on the blood agar medium supplemented with 5% sheep blood for testing their hemolytic activity [17].

RESULTS AND DISCUSSION

Collected black salt crystals were irregular in shape associated with some non-crystalline materials. The crystals varied in colour from reddish gray, coral pink and pinkish gray (Figure 1A) whereas all salts when crushed

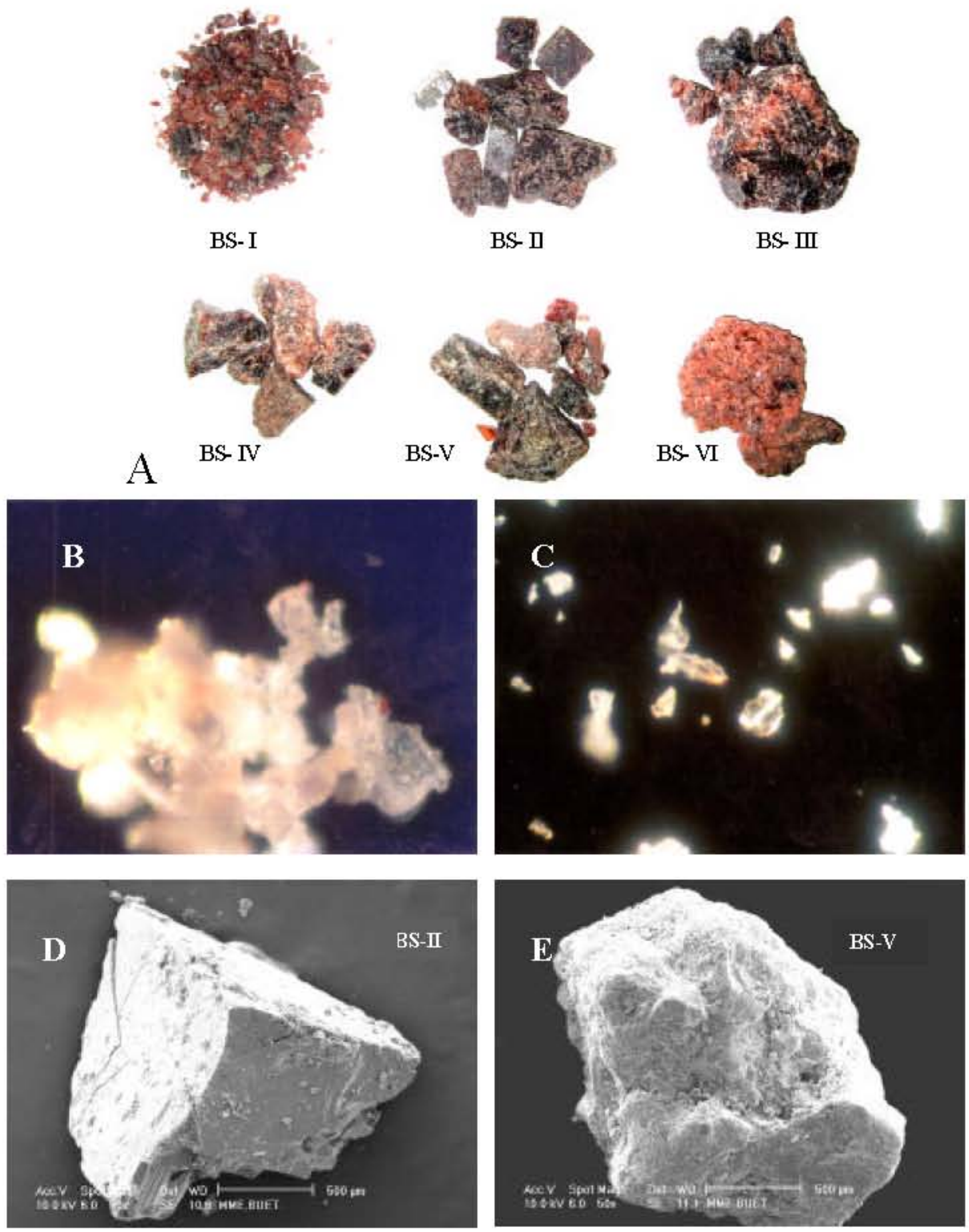

Fig. 1: Different black salt samples. A- Photograph of six black salt samples, B and C –BS-I before and after crystallization under Dark field microscopy, D and E- Two Black salt crystals under SEM.

were light reddish in color. Under SEM, the BS-II and BS-V samples revealed irregular crystalline structure (Figure 1D and 1E). During recrystallization of BS-I, impurities precipitated in the solution and colourless crystals were formed which were observed under dark field microscope (Figure 1B and 1C). According to Phalen [18], pure halite is colourless and impurities i.e. other mineral elements (most commonly iron oxides) or tiny organisms are responsible for different shades of red, brown, yellow, orange or pink. During preparation of solution, impurities were precipitated out which resulted in the colourless salt crystals in this study and this was quite in agreement with Phalen [18]. The pH of the salt samples was found to vary between 9.12-10.56.

Table 1 shows the elemental analyses of six black salts studied. Analysis of variance (S^2) and co-efficient of variation (CV %) indicates that among these six black salt samples, maximum variations were obtained in iron and potassium composition. Comparatively, lesser variations were observed in the amount of sodium and chloride in

Table 1: Mineral composition of six black salt samples

Sample No.	Sodium (Na) g / g of black salt	Chloride (Cl) g /g of black salt	Potassium (K) g / 100 g of black salt	Sulfur(S) g per 100 g of black salt	Iron (Fe) mg/g of black salt
BS-I	0.36	0.59	0.085	0.41	0.425
BS-II	0.34	0.61	0.14	0.3	0.3
BS-III	0.37	0.61	0.08	0.52	0.275
BS-IV	0.42	0.60	0.11	0.41	1.01
BS-V	0.41	0.61	0.08	0.40	0.4
BS-VI	0.37	0.60	0.15	0.65	0.175
Range	0.34-0.42	0.59-0.61	0.08-0.15	0.3-.-0.65	0.175-1.01
Mean	0.3783	0.603	0.087	0.45	0.431
variance	9.4×10^{-4}	6×10^{-5}	2×10^{-3}	0.014	0.09
Standard deviation	0.031	7.75×10^{-3}	0.045	0.12	0.3
CV%	8.1	1.28	51.40	26.3	69.3

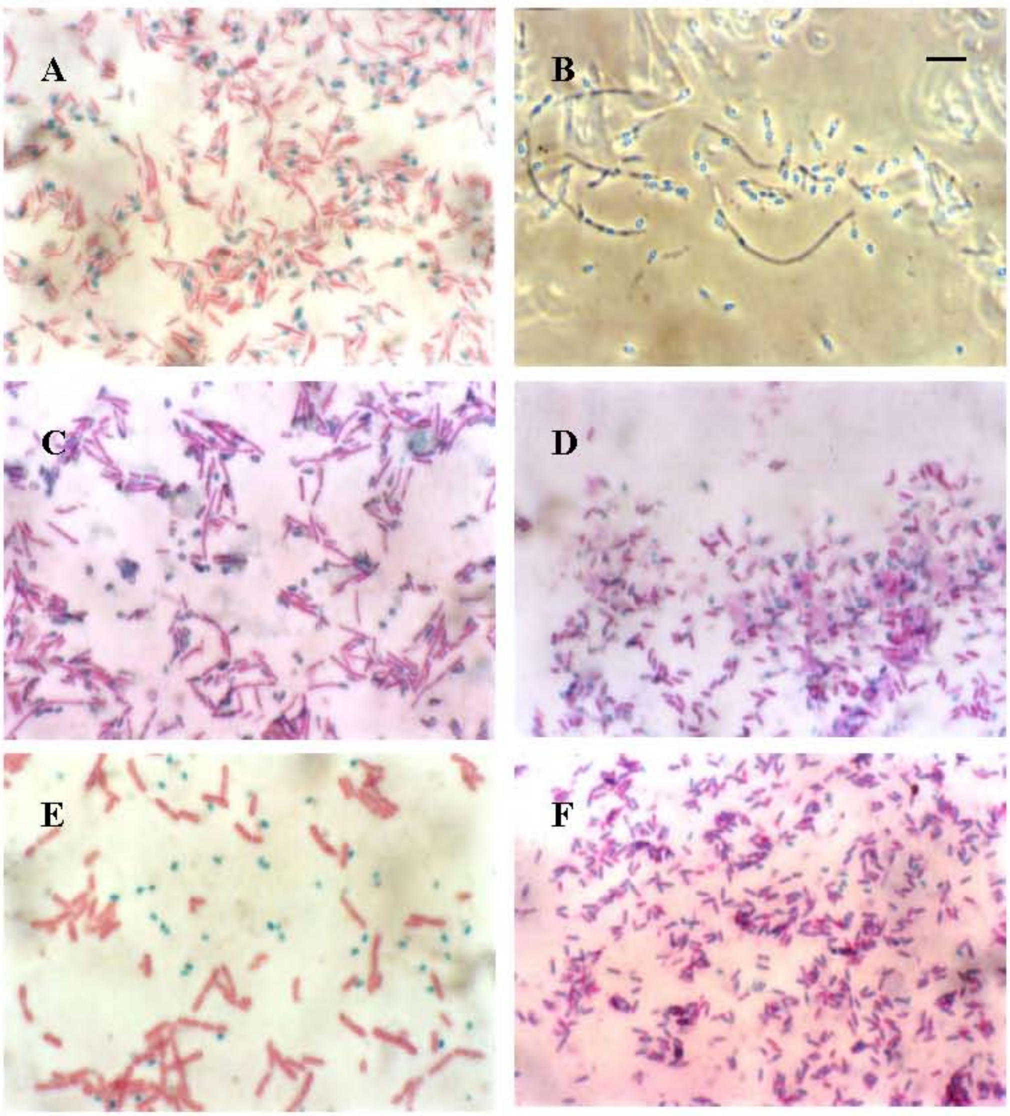

Fig. 2: Photomicrographs of six microorganisms A- *Bacillus alcalophilus* **(B1), B-** *B. pantothenticus* **(B2), C-** *B. stearothermophilus* **(B3), D-** *B. laterosporus* **(B4),E-** *B. pumilus* **(B5) and F-** *B. circulans* **(B6) were found from black salt samples (bar = 5µm)**

Table 2: Characteristics of bacterial isolates from black salt

	Bacterial strains							
Test	Bl	B2	B3	B4	B5	B6	B7	B8
Gram reaction	+	+	+	+	+	+	-	+
Sporangium Swollen	+	+	+	+	-	+	Non Spore former	-
Voges-Proskaure test	-	-	-	-	+	-	-	-
Hydrolysis of Casein	+	+	+	+	+	+	-	-
Starch	+	+	+	+	-	+	+	-
Esculine	+	+	+	+	+	+	-	+
Utilization of Citrate	-	-	+	-	-	+	+	-
Propionate	-	+	+	-	-	+	+	-
Egg yolk Lecithinase	+	+	+	+	-	-	-	-
Nitrate reduction test	-	+	+	+	+	+	+	-
Growth at pH values								
6.5	+	-	+	-	+	+	+	+
8.5	+	+	+	-	+	+	+	+
10.5	+	+	+	+	+	+	+	+
11.5	-	+	+	+	-	+	+	+
12.5	-	+	+	+	-	+	+	+
13.0	-	+	-	-	-	+	-	-
Growth in NaCl concentrations								
2%	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+
7%	+	+	+	+	-	+	-	+
10%	-	+	+	+	-	+	-	+
Growth at								
30°C	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+
50°C	-	+	+	+	+	+	-	-
55°C	-	-	+	-	-	+	-	-
60°C	-	-	+	-	-	+	-	-
Acid formation from								
Glucose	+	+	+	+	+	+	+	+
Arabinose	-	-	+	-	-	-	-	-
Mannitol	-	-	+	-	+	+	+	-
Adonitol	-	-	-	-	-	+	-	-
Cellobiose	-	+	+	-	+	+	-	-
Fructose	-	-	+	-	+	+	-	-
Inositol	+	-	+	-	+	-	-	-
Maltose	-	-	-	-	-	+	+	-
Raffinose	-	-	+	-	+	+	-	-
Salicin	-	+	+	-	+	+	-	-
Sorbose	-	-	-	+	-	-	-	+
Sorbitol	-	-	+	-	-	-	-	-
Sucrose	+	-	+	-	+	+	-	-
Trehalose	-	-	+	-	-	+	-	-

'+' indicates positive results and '-'indicates negative result.

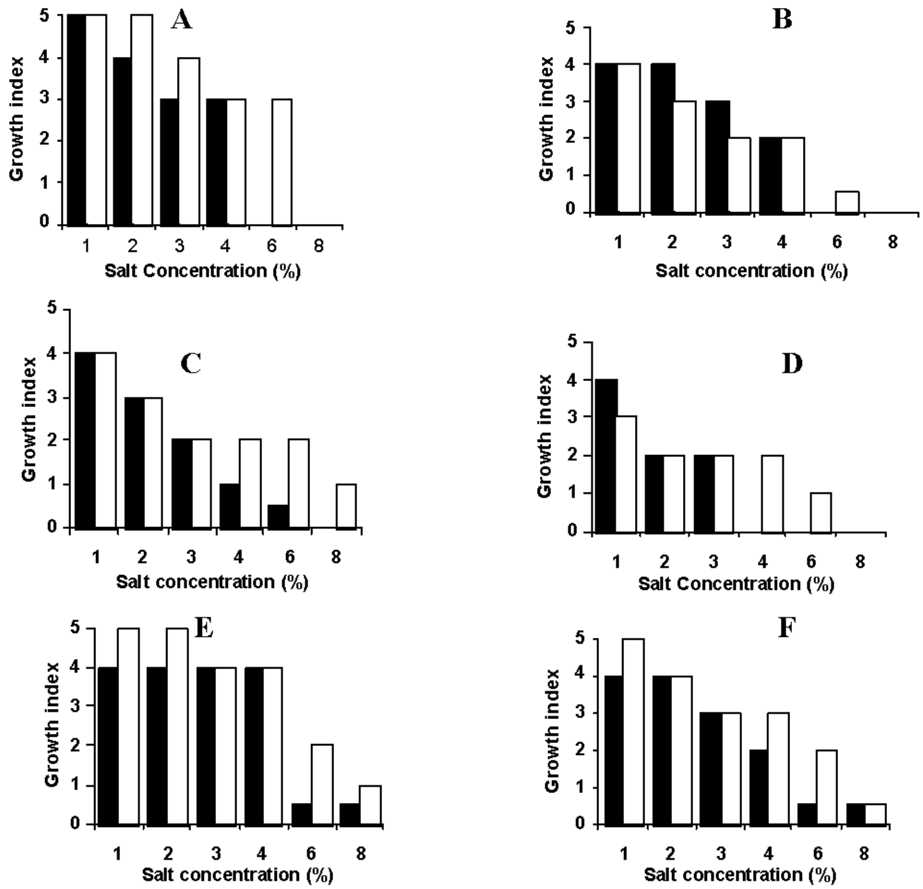

Fig. 3: Growth response of four different test organisms at different concentrations (%) of black salt (BS-I) and common salt (NaCl). (A)*E. coli*, (B) *S. paratyphi*, (C) *E. amnigenus*, (D) *Plesiomonas sp*., (E) *Alcaligenes* sp. and (F) *E. intermedium* Black Salt ■ NaCl □

these samples. The amounts of calcium and magnesium were very low and could not be estimated by the EDTA-Complexmetric titration. Phosphorus and manganese could not be detected by the methods used. In black salt solution, only trace amounts of water soluble iron (ranged from 0.0052-0.0075%) were detected whereas in the precipitate, its amount was found approximately 0.0175-1.01 % (Table 1). In the precipitated portion, a small quantity of acid insoluble material and sand particles were also found. The percentage of moisture content of the six samples were tested and found to vary between 0.33%-0.68%.

Eight bacterial isolates and one actinomycetes strain were isolated from black salt samples (Figure 2) during this study. Characteristics of the bacterial isolates are shown in Table 2. Among eight bacterial isolates, seven were Gram (+) ve and the remaining one was Gram (-) ve. Comparing the results with standard description in the Bergey's Manual of Systematic Bacteriology Vol. 1 [14], the Gram (-) ve bacterial isolate was provisionally identified as *Halomonas* sp (B7). The Gram (+) ve bacterial isolates were found to be the members of the genus *Bacillus viz. B. alcalophilus* (B1), *B. pantothenticus* (B2), *B. stearothermophilus* (B3), *B. laterosporus* (B4), *B. pumilus* (B5), *B. circulans* (B6) and *B. marinus* (B8). Actinomycetes strain was provisionally identified as *Streptomyces anulatus*. The optimum growth of this actinomycetes was found at 30°C and at pH 11.5. Among these isolates, *B. pantothenticus, B. stearothermophilus, B. laterosporus, B. marinus* and *Streptomyces anulatus* showed better growth in 10% NaCl and maximum growth at alkaline conditions. It was interesting to note that *B. pantothenticus* and *B. circulans* were able to grow even at pH 13.0.

The antibacterial, antiviral and anticancer activities of more than 25 inorganic compounds with metallic ions as potentially significant part of the molecule were studied and reported by Schubert [19] and Stanley *et al.* [20].

In the present study, black salt inhibited the growth of the test organisms in comparison to sodium chloride. The growth of *E. coli, Enterobacter intermedium, Enterobacter amnigenus* and *Salmonella paratyphi* was reduced at 4% black salt concentration whereas profuse growth occurred at 6% NaCl. In case of *Alcaligenes* sp., trace amount of growth was observed even at 8% black salt concentration, on the contrary profuse growth of the same organism was found in the same concentration of NaCl. The growth of *Plesiomonas* sp. was inhibited by 3% black salt and 6% NaCl concentrations (Fig. 3). Attempts to culture bacteria from the core of black salt crystals yielded no growth.

Among the isolated eight bacterial strains, *B. pantothenticus*, *B. stearothermophilus* and *B. circulans* showed β-hemolytic reactions which indicated that the indiscriminate massive use of black salt over a period of time could exert adverse reactions on the consumer's health and this needs animal and chemical experiments.

CONCLUSION

The six black salts showed heterogeneity in their elemental composition and also in crystal structure. Among the eight bacterial strains isolated from the surfaces of black salt samples, three bacterial strains showed β hemolysis which pose threat to consumers. However, the cores of the samples were free of microbes. So, it can be inferred that the improper handling and storage under unhygienic conditions cause the surfaces to be infested with potential pathogenic bacteria with higher degree of salt tolerance. Black salt concentration above 8% (w/w) was found to inhibit the growth of pathogens tested.

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