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pPrevalence and Characterisation of *Bacillus Cereus* group Isolates from Fish and Fishery based Products and their Role in Food Safety

Pinkey Lhamu Lepcha[1] and Sarita Kumari[2]

[1]Department of Microbiology, Darjeeling Government College
[2]Department of Microbiology, North Bengal University
E-mail: [1]lepchapinkey15@gmail.com, [2]saritamicro@gmail.com

Abstract—*Bacillus cereus group, associated with foodborne outbreaks and spoilage of food products is an indicator of poor hygiene, and high numbers are unacceptable.*

In the present study, the prevalence of B. cereus group in fish and fishery products was investigated. All the samples of dry fish and frozen fish and fish products showed high Bacillus cereus group contamination. The contamination level ranged from 10^5-10^6cfu/g and 10^5-10^7cfu/g in dry and frozen fish product, respectively.

Hundred percent isolates were capable of producing protease, lipase, and amylase, respectively, indicating spoilage potentiality of the isolates.

An antibiogram of isolates of B. cereus group was obtained using different antibiotics commonly used against foodborne diseases. All isolates were resistant to penicillin and metronidazole, 60% of isolates were resistant to vancomycin, 20% and10% of isolates were resistant to tetracycline and kanamycin, respectively. B. cereus group isolates did not show any resistance towards polymyxin B. The emergence of multidrug resistance among foodborne bacterial pathogens can be a major health concern.

All the isolates were biofilm former with (51. 5%) intermediate and (45. 4%) strong biofilm former. As bacteria within biofilms are more resistant to antimicrobial agents and cleaning, it is more difficult to eliminate biofilm than planktonic cells. Hence, their presence in fish and fishery products can be a matter of concern. Since all the isolates in the present study were biofilm former, biofilm formed by them can be responsible for recurrent contamination and spoilage of products or facilitate transmission of diseases.

Cluster analysis performed based on studied characters, showed 33 isolates belonging to Bacillus cereus group were grouped into six different major clusters.

The present study will help to better assess the health and spoilage risk associated with B. cereus group in fishery products and to incorporate adequate preventive measures.

Keywords*: Fish product, B. cereus group, Biofilm, Cluster analysis.*

1. Introduction

Bacilluscereus group is gram positive, facultative anaerobe, spore forming bacteria . It is an opportunistic pathogens and ubiquitous in nature. It has been referred to as *B. cereus* group because *Bacillus species* and its related genera were found to be closely related and has genetic similarity. The existence of high polymorphism in the genes of *B cereus* is responsible for its diverse nature ranging from probiotic as well as pathogenic strains [5].

The contamination of foods with *B cereus* group leads to food poisoning which usually occur under two types of syndromes, the emetic, and the diarrheal syndromes [19]. An emetics train of *B. cereus* secretes highly toxic and heat-stable non ribosomalcyclicpeptide which can with stand cooking temperatures and induce vomiting symptoms, when ingested [6].

The occurrence of diarrheal infection due to consumption of fish contaminated with high amount of *B. cereus*has been reported [10]. Therefore we can assume that seafood can act as a vehicle for food borne illness. The contamination of sea food occurs either naturally from the environment where fish were harvested, or during processing and food preparation. As the processing of fish requires mild cooking, i. e. 130˚C for 50 minutes, the heat treatment are liable to inhibit the growth of competitive bacteria but creates favourable condition for germination of spore produced by *B cereus* group [10].

The study aims at detailed study made on microbial quality with species referred to food safety of fish and fishery product present in the market. Special stress has been given to monitor the incidence of *B. cereus* group in these products and their key

factor responsible for pathogenicity such as resistance to antibiotic, production of enzymes, biofilm formation and survival in low refrigerated temperature.

2. Materials and methods.

2. 1. Collection of sample

Sample (n=10) comprising of dry and frozen products were collected from retail outlets in Siliguri, West Bengal. The dry fish sample used in the study were Kachki fish, Pony fish, Stock fish, Tuna fish and Tilapia fish while frozen fish product was raw and frozen Pabda fish, Bata fish, fish finger, fish fillets All samples were transported to the laboratory immediately and analyzed within 24h of sample collection.

2. 2. Presumptive isolation and enumeration of *B. cereus* group

10g of food samples were homogenized with 90mL sterile peptone-physiological saline(1g neutral peptoneL^{-1}, 8. 5 NaCl L^{-1}, pH 7. 2) using Stomacher lab blender 400 (Seward medical London, UK) at normal speed for 1min. Appropriately diluted suspension(0. 1ml) was spread plated on *Bacillus cereus* selective agar (BCSA) base (M833, Hi Media laboratories Pvt. Limited) supplemented with sterile egg yolk(Himedia FD045), and Polymyxin B sulphate (100U. mL^{-1}; Himedia FD003), and incubated at 35˚C for 24-48 hrs. Characterstic turquoise to peacock blue colonies surrounded by a zone of precipitate of the same colour were regarded as presumptive *B. cereus* group.

2. 3. Confirmation of presumptive isolate

The presumptive isolates were confirmed on the basis of motility, glucose fermentation, acetylmethylcarbinol production and nitrate reduction[2].

2. 4. Antibiotic susceptibility test

Disc agar diffusion method (Himedia 1988) was carried out to develop antibiogram of the *B. cereus* group isolates against 6 commonly used antibiotics (per disc penicillin G, 10 U; vancomycin, 10 µg; kanamycin, 30 µg; tetracycline, 30 µg; polymyxin B, 300 U; metronidazole, 5 µg). 24h bacterial culture was applied with sterile cotton swab (HiMedia PW005) on plates. After drying for 15 minutes, different antibiotic susceptibility test discs (HiMedia) were placed aseptically and the plates were incubated at 37°C for 14-19h. The results were interpreted as sensitive or resistant based on interpretive standard given Himedia instruction sheet.

2. 5. Production of extracellular enzymes

Assay for production of protease, lipase and amylase by each *B. cereus* isolate was carried out using specified medium skim milk agar (HiMedia M163), tributyrin agar base (HiMedia M157) supplemented with 1. 0% v. v^{-1}tributyrin (HiMedia FD081) and starch agar (HiMedia M107) respectively, by following standard procedure.

2. 6. Assay of biofilm formation

The ability of biofilm formation was assessed by method described in [11]. Biofilm was allowed to develop by inoculating overnight culture of an isolate grown on nutrient agar into microtiter wells (initial total cell count, 105. $well^{-1}$) containing 150µL of nutrient broth. The plates were incubated at 30 °C for 24 h. The medium from the wells was drained out. The wells were washed three times with distilled water to remove non-biofilm cells, allowed to dry for 30 min at 30 °C, added with 1% w. v−1of crystal violet, and held at 20 °C. After 45 min, excess crystal violet was removed and the wells were washed thrice with distilled water and airdried at 30 °C for 30 min. Each well was added with100µL of 95% v. v^{-1}ethanol and left for 30 min to elude the stain. Intensity of the stain was measured by taking optical density (OD) readings at 595 nm (micro-plate reader iMark, Bio-Rad, Tokyo, Japan). To correct background staining, the mean OD value obtained for control (without biofilm) was subtracted from the OD value obtained from each condition. Biofilm formation assay was carried out in triplicate for all the 33 isolates belonging to the *B. cereus* group

2. 7. Growth at low temperature

Growth of isolates (n=33) in refrigerated condition was studied by inoculating culture in microtiter plate containing nutrient broth. The spectrophometeric analysis (optical density at 600nm) was carried out after incubation for 24h at 7ºC.

2. 8. Statistical analysis

Agglomerative hierarchical clustering (AHC) was applied to data set to cluster different isolates of the *B. cereus* group based on studied characters by XLSTAT v. 14.

3. Results

3. 1. Prevalence of *B. cereus* group in various dry and frozen fishery products

Prevalence of *B. cereus* group in various dry and frozen fishery products is given in Table1. In total 50 representative presumptive isolate from dry and frozen fishery products were taken. Confirmatory test was carried out for presumptive colonies, from which 17 (80. 95%) and 16 (69. 56%) isolate from dry fish and frozen fish products respectively gave positive result for all 4 biochemical test (nitrate reduction, VP, glucose fermentation and motility test)and were confirmed as *B. cereus* group. In dry fish the contamination level range from 10^5-10^6cfu/g while in frozen fish was 10^5- 10^7cfu/g product.

Table 1: Incidence of *B. cereus* group in different dry and frozen fishery product

Samples	*B. cereus* load (cfu/g)
Dry fish product Kachki fish	1. 2×10^6
Pony fish	1. 17×10^6
Stock fish	2. 9×10^6
Tuna fish	9. 1×10^6
Tilapia	4. 39×10^5
Frozen fish product Fish finger	1. 3×10^7
Fish finger	1. 0×10^7
Fish fillets	5. 2×10^5
Pabda (raw, frozen)	5. 0×10^6
Bata (raw, frozen)	1. 0×10^7

3. 2. Susceptibility to antibiotics

The misuse of antibiotic has led to increase in antibiotic resistance among bacteria. The results for susceptibility of the confirmed isolate of *B. cereus* group are shown in Table 2.

100% isolates were resistant to penicillin and 60% were resistant to vancomycin. For protein synthesis inhibitors 10% isolates showed resistance to kanamycin and 20% isolates showed resistant to tetracycline. *B. cereus* group isolates did not show any resistance towards polymyxin B while 100% isolates were resistant to metronidazole. The emergence of multidrug resistance among foodborne bacterial pathogens can be a major health concern.

Table 2: Antibiogram of *B. cereus* groupisolates from various fishery products

Mode of action	Antibiotic. disc^{-1}	Percentscore[a]		
		Sensitive	Intermediate	Resistant
Inhibition of cell wall synthesis	Penicillin (P;10 U)	-	-	100
	Vancomycn (Va;10 µg)	0	40	60
Inhibition of protein synthesis	Kanamycin (K;30 µg)	80	10	10
	Tetracycline (T;30 µg)	60	20	20
Damage to cell membrane	Polymyxin B (Pb;300 U)	80	20	0
Damage to cell membrane	Metronidazole (Mt;5 µg)	0	0	100

[a]The inhibition zone size (diameter in mm) interpretation was based on HiMedia instruction sheet (the following values are upper and lower cut-off lines for resistant and sensitive respectively); P, 19 and 28; Va, 14 and 17; K, 13 and 18; T, 14 and 19; Pb, 8 and 12; Mt, 8 and 13.

3. 3. Enzyme Production

The results of the production of extracellular enzyme, viz. protease, amylase and lipase are shown in Table 3.

All isolates from dry and frozen fish products showed 100% proteolytic, amylolytic and lipolytic activity. The production of enzyme by the isolate shows its spoilage potentiality.

Table 3: Production of extracellular enzyme by *B. Cereus* isolates from different fishery products

Sample	No of isolate	% of positive isolate		
		Protease	Lipase	Amylase
Dry fish	17	100	100	100
Frozen fish	16	100	100	100

3. 4 Biofilm formation

The result of biofilm formation assay by the isolates of *B. cereus* group is given in Table 4.

Most of the isolates from dry and frozen fishery products were biofilm formers. Out of 33 isolates, 1(3. 03%) was weak biofilm former; 17(51. 5%) were assessed as moderate; and 15 (45. 4%) as strong biofilm former.

Evaluation of biofilm activity of isolate showed its probability to take part in food borne diseases by increasing its resistance towards cleaning agents and acting as recurrent source of contamination of finished products.

Table 4: Clustering of isolates of *B. cereus* group from different fish samples on the basis of biofilm forming ability

Group[a]	% of isolates
Non biofilm former	0
Weak biofilm former	3. 03
Moderate biofilm former	51. 5
Strong biofilm former	45. 4

[a]Isolates were designated as non-biofilm former (<0. 2), weak (0. 2-0. 6), moderate (>0. 6-1. 2) and strong(>1. 2) biofilm formers, according to OD_{595} readings.

3. 5. Growth at low temperature:

Most of the isolate has ability to grow at refrigerated temperature. Out of 33 isolates, 8 isolates (24%) showed increase in growth upto 7 days of incubation. The increase in growth till 5 days and 3 days was also observed in 8 isolate (24%) and 7isolate (21%), and then decrease slowly growth. The reduction in growth may be depletion of nutrients.

3. 6. Relationship among characteristics

The results obtained from AHC areshown in Figure 1**.** Isolate from dry and frozen fish belonging to *B. cereus* group were grouped in 6 clusters as given Table 5. Thus, four clusters were heterogeneous and remaining two was homogenous in nature.

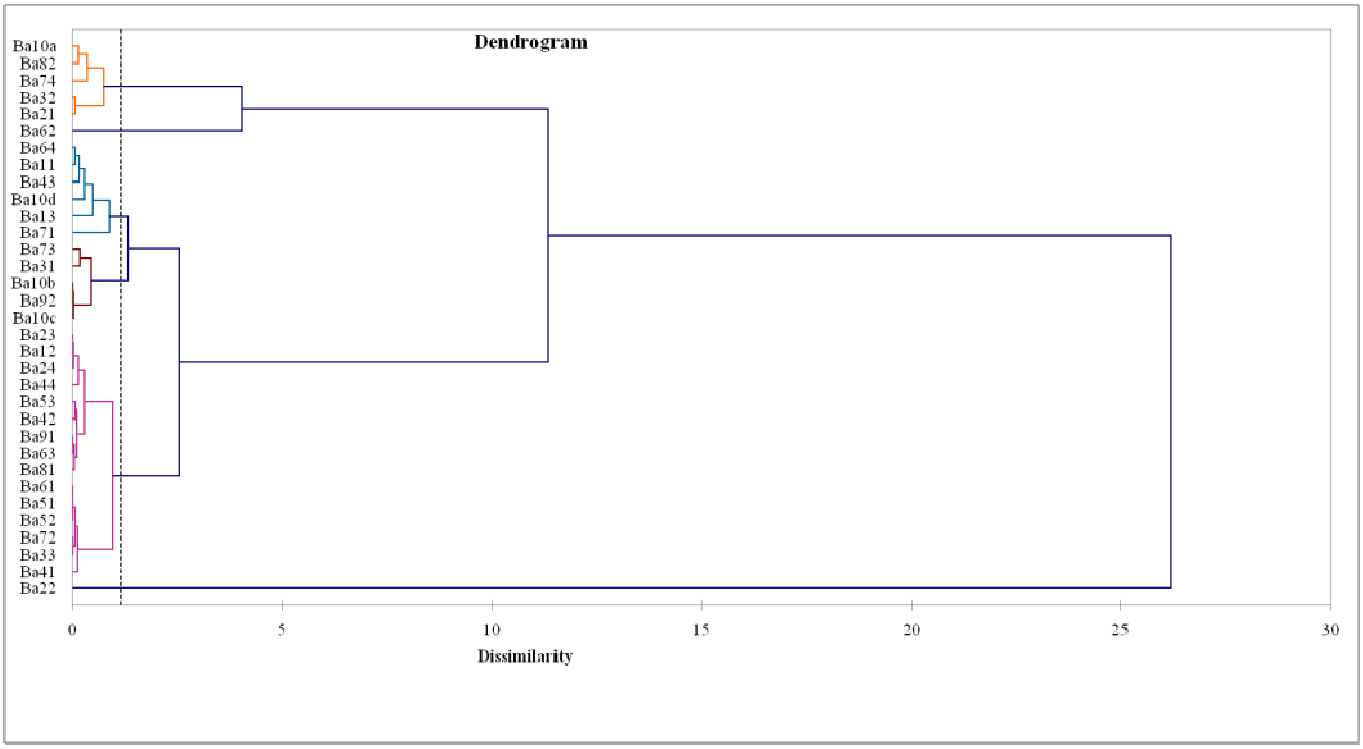

Figure 1: Simplified dendrogram based on wards clustering of dissimilarity coefficient generated by agglomerative hierarchical clustering. Based on studied characters (production of protease, amylase, lipase, and biofilm).

Table 5: Distribution of 33 isolates of *B.* group from various fishery products among the clusters generated through Fig 1

Cluster	Percent of isolates	
	Dry fish	Frozen fish
A	9. 09	9. 09
B	21. 2	24. 2
C	6. 06	9. 09
D	3. 03	Dis
E	3. 03	12. 1
F		3. 03

4. Discussions

From samples analyzed, it was found that the contamination level ranged from 10^5-10^6cfu/g for dry fish and 10^5-10^7cfu/g for frozen fish which tends to falls in region of contamination which is in accordance to report where 10^4 -10^{11}cfu /g is high number of microbial population to cause disease in food [10, 13]. There has been recent report of contamination of sea food at level ranging from 36 cfu/g to 1. 1 10 ^{3}cfu/g [6]. Thus fish can act as vehicle for food borne pathogen as also mentioned by other author [14]. However, range of contamination level varies which may be due to difference in handling, processing and storage time.

In seafood bacteria may exist in the form of spore in dry fish while in frozen fish, remaining viable cell which escape cold stress proliferate and increase in number when subjected to favourable condition. Moreover, adherent *Bacillus* spores exhibit a greater resistance to high temperature and disinfectant than spores in suspension[10]. In conjunction to previous study our study also supports possibility of contamination of fish contaminated by *B. cereus* group

The susceptibility of randomly selected isolates of *B. cereus* group showed that hundred percent of isolates were resistant to beta lactam antibiotic i. e. antibiotic inhibiting cell wall synthesis like penicillin and 80% were resistant against vancomycin, this may be due to production of beta lactamase enzyme by bacteria that hydrolyse beta lactam ring inactivating antimicrobial agent.

Twenty percent of isolates were resistant to tetracycline and 10% were resistant to kanamycin. None of the isolates were found to be resistant against higher concentration of polymyxin B (300U). An earlier study reported susceptibility of only 8% of the 84 isolates of *B. cereus* group from spices to this higher concentration of polymyxin B [3]. However, all the 48 *B. cereus* group isolates from legume-based fermented food products were resistant against this higher concentration of polymyxin B [16]**.** The variations in the percentages may occur due to the differences in the concentrations of antibiotics used, source of isolates, drug resistance transfer [8].

The production of enzymes can lead to detoriation in quality of finished food products. From the study it was found that 100% of the *B. cereus* group isolates from dry and frozen fish showed production of all three enzymes (protease, lipase and amylase). The enzymatic reaction occurs in frozen fish even at temperature of -30˚C [9] Thus it can be concluded these isolate of *B. cereus* group have the potential to cause spoilage of fish.

From the result of biofilm forming ability of isolate, it showed all the isolates were biofilm former with (51. 5%) intermediate and (45. 4%) strong biofilm former. The formation of microbial biofilm on the surface of fish processing equipment increase threat of crossover contamination of product. *B. cereus* produce biofilms which stick to abiotic surface or living tissues and together with spores, it shows high resistance to various stress and high adhesive property on various substrates, including stainless steel, a material widely used in the food processing unit[15]. Inaddition, sporulationoccurs withinbiofilmsonfoodcontact surfaces [20] sometimes at very high levels [7], suggesting a potentially significant role for biofilm-deriveds pores in contamination of food with *Bacillus* species [17]Thus biofilm formation can attribute to increase in pathogenicity of *B. cereus* group.

B. cereus isolates from fish and fishery product had ability to grow at low temperature. Fish storage even at low temperature will not prevent growth of these cold tolerant stains thus, can cause spoilage of fish and fishery product as the enzymatic reaction though slow but continues even at refrigerated temperature as stated by author [1] thereby producing undesirable changes. It has also been reported that the psychotropic bacteria, including *Bacillus* sp caused spoilage and reduced product qualitywhich incurred losses of up to 30%to dairy industry [18].

5. Conclusion

Fishery product used in the study showed higher incidence of *Bacillus cereus* group. An appropriate control measures such as removal of biofilm, proper dosage use of preservatives, shorter shelf life etc. should be taken into consideration for food safety

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