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Annals of Biological Research, 2016, 7 (2):1-6
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Biofilm formation potentiality of some antibiotic resistant *Bacillus cereus* isolates from fresh raw chhana in Kolkata, India

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ABSTRACT

Bacillus cereus is a highly underestimated endospore forming food borne bacterial pathogen that is ubiquitous in nature. Though reported from milk and various milk products marketed in India, no study was there to characterize isolates from fresh raw chhana (coagulated milk separated from whey) which is used to produce various sweets. Its presence in milk and dairy products is a matter of concern in food industry because of its spoilage and pathogenic potential. Two different types of toxins are produced by B. cereus causing diarrhoeal and emetic syndrome. In this study B. cereus was isolated from fresh raw chhana from sweet meat shops in and around Kolkata. Seven of the isolates were studied for their extracellular enzyme production, antibiotic resistance pattern and biofilm forming potentiality. All the isolates produced amylase and caseinase but none produced lipase. All the isolates were multiple-drug-resistant. Three of the isolates showed positive result for biofilm production by three different methods. Presence of multiple-drug-resistant bacteria with biofilm forming potentiality poses threat to the public health.

Keywords: *B. cereus*, chhana, spoilage, antibiotic resistance, biofilm

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INTRODUCTION

Sporeforming microorganisms are ubiquitous in nature because of the resistance of their endospores to heat and various physical and chemical factors. Most aerobic sporeformers are therefore reported from various sources including foods where heat and chemical preservatives kill vegetative cells and the competition-free environment now becomes favourablefor germination of spores [1].Milk and dairy products contain various microorganisms including spore-formers,some of which may be pathogenic to humans.Persistence of these pathogens forming biofilm in food processing establishments and their subsequent transfer to dairy products pose greater problem to manufacturer and consumers [2]. The various microorganisms present in raw milk issued from dairy cows come from teat interior, udder exterior and the surroundings including feed, soil, faeces, grass and milking environment and establishments including teat cup, hands, storage tanks, water, pipe lines etc[3]. While cattle is mostly reared in dairy farms and good dairy farm practices are followed in developed countries, farmers in developing countries rear animals by non-intensive method for various purposes including ploughing of agricultural fields, pulling of cart etc. In West Bengal, milk is produced from cattle and buffalo in individual households or very small dairy establishments primarily for subsistence. Nutrition deficiency and poor management affect cattle health. In winter, used beddings act as an important source of contamination. In developed countries the factors correlating with high spore concentration in milk includes degree of contamination of teat from soil, high water content of soil, low evaporation of water and dirty access alloys. In dairy farms the major contaminating sources has been found to be soils and faeces[4].Adulteration ofmilk with various inferior and toxic substances is another source of microorganisms in raw milk[5].

B. cereus is widespread in the environment and has been isolated from fruits, vegetables, nuts, cereals, milk and dairy products, meat, dried foods and spices. *B. cereus* is an important cause of foodborne illness and is frequently involved in foodborne outbreaks in heat treated foods because of their endospores and absence of competitive flora [6]. It causes food spoilage and two distinct types of food poisoning: the diarrhoeal type and the emetic type. Diarrhoeal syndrome is caused by complex enterotoxins produced during vegetative growth of the organism in the small intestine, which act on the epithelial cells, causing massive secretion of fluid into the intestinal lumen leading to diarrhoea. The emetic syndrome is caused by emetic toxin produced by the bacteria during their growth in the food that affects vagus nerve[7, 8].

Milk is heat treated and coagulated to separate chhana from whey that is the principal raw material for production of various sweets in West Bengal. Whey is removed from chhana by hanging over cheese-cloth for 30-60 min. Chhana plays an important role in human nutrition but could represent a risk to human health, due to poor hygiene throughout the production chain and marketing. The degree of heat treatment may not be sufficient to kill heat resistant endospores that survive in the chhana.Moreover, high water activity of curd and post-preparation contamination during wrapping with clothes, storing in open container and transportation via crowded vehicles to sweet shops provide ample scopes for contamination with other bacterial pathogens and germination of endospores.Processing with bare hands and storing at ambient temperature increase the chance of multiplication of these microorganisms in the finished product.Presence of *B. cereus*resistant to various antibiotics, synthesizing extracellular enzymes and producing biofilm is agreat concern in sweet industry. The objective of this study is to isolate and characterize *B. cereus* present in raw chhana used in sweet shopsin and around Kolkata.

MATERIALS AND METHODS

2.1. Collection of sample

A total of eleven samples of raw chhana used to prepare various sweets were purchased from sweet shops in and around Kolkata (capital of West Bengal, a state in India) and brought to the laboratory in ice box. Analysis of samples was performed within 2h of collection.

2.2. Presumptive isolation of *B. cereus*

Ten gram of representative food sample was homogenized with 90ml sterile peptone-physiological saline (0.1% w v^{-1} neutral peptone, 0.85% w v^{-1} sodium chloride, pH 7.2) by shaking in an orbital rotary shaker at 150 revolutions per minute for 2 min. Serial dilutions were prepared using the same diluent. 0.1ml of appropriate dilutions was spread plated on sterile dried plates containing *Bacillus cereus* agar base supplemented with polymyxin B selective supplement and egg yolk emulsion. The plates were examined after 24h of incubation at 35°C and again after 24h of incubation at room temperature. Typical *B. cereus* colonies of bluish-green-blue colour with zones of egg yolk precipitate on the medium were counted and calculated as colony forming units (cfu) per gram fresh weight sample. Representative colonies were randomly selected from each sample and purified by streaking out on freshly prepared agar plates. Purified colonies were grown on nutrient agar slants and stored at 4°C.

2.3. Confirmation of presumptive *B. cereus*

Presumptive isolates were confirmed by morphological, biochemical and physiological characteristics. Gram positive, rod shaped, motile, endospore forming cells were confirmed as *B. cereus* when produced acid and gas from glucose, acetyl methylcarbinol from glucose phosphate broth and reduced nitrate.

2.4. Phenotypic characterization of the confirmed isolates

Confirmed *B. cereus* isolates were tested for production of indole from tryptone broth, utilization of citrate as sole carbon source, production of oxidase, urease, DNase and hydrolysis of gelatin.

2.5. Production of extracellular enzymes

Confirmed isolates were tested for the production of enzymes viz. amylase, caseinase and lipase on starch agar, standard method caseinate agar and tributyrin agar base supplemented with 1.0% vv^{-1}tributyrin, respectively. Briefly one loopful of 18h old culture in nutrient broth was streaked singly on the surface of respective medium and incubated for 72h at 37°C. Amylase production was tested by flooding Lugol's iodine solution on incubated starch agar plates to check formation of clear zones surrounding the colony. Colonies surrounded by clear zones indicated production of caseinase and lipase.

2.6. Antibiotic resistance pattern

An antibiogram was developed using the disc agar diffusion method. Three colonies, grown on tryptone soya agar at 37°C for 24h, were transferred to about 5ml tryptone soya broth (TSB) and incubated at the same temperature for 4-6h until the broth became moderately turbid. A sterile cotton swab was dipped into the inoculum and applied evenly

onto Mueller-Hinton agar plate (4mm thick). After drying for 15min, various antibiotic susceptibility test discs were applied aseptically keeping a distance of at least 3cm between their centers. The plates were be incubated at 37°C for 18h. The zones showing complete inhibition were measured and designated as sensitive, intermediate or resistant based on manufacturers zone-size chart.

2.7. Detection of biofilm formation

Formation of biofilm was tested by congo red agar (CRA) plate method, standard tube method and tissue culture plate method.

2.7.1. CRA plate method

Brain heart infusion (BHI) broth was supplemented with 8% glucose (wv^{-1}) and 1% (wv^{-1}) agar before sterilization. Solutions of congo red stains prepared and autoclaved separately were added to the supplemented BHI at a final concentration of 0.08% (vv^{-1}) in molten condition to prepare CRA. Dried plates of CRA were inoculated with the *B. cereus* isolates and incubated aerobically for 24 to 48h at 37°C. Positive result was indicated by dark red colonies. Weak biofilm producers usually remained pink, though occasional darkening at the centers of colonies was observed. Biofilm negative strains produced white or very light pink coloured colonies. The experiment was performed in triplicate.

2.7.2. Standard tube method

The method described by [9] was followed to assess biofilm formation. Briefly 10ml tryptone soya broth was inoculated with loopful of 18h old *B. cereus* grown in nutrient agar plates and incubated for 24h at 37°C. Then the culture was decanted from the tube, washed with phosphate buffer saline (PBS) of pH 7.4 and dried. Dried tubes were stained with 0.1% (wv^{-1}) crystal violet solution. Excess stain was decanted and washed with distilled water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation.

2.7.3. Tissue culture plate method

In this method 2.9ml of sterile TSB was dispensed in each well of polystyrene tissue culture plate. An aliquot of 100µl culture grown in TSB for 18h was added to each well and incubated overnight at 37°C. After incubation content of each well was gently pipetted out and washed two times with sterile PBS (pH 7.4) to remove free floating planktonic bacteria. The remaining attached bacteria were fixed with 1 ml methanol per well for 15 min and dried. Biofilms formed with adherent sessile bacteria in plate was stained with 0.1% crystal violet solution for 5 min. Excess stain was removed with distilled water, dried and then 3ml of 33% glacial acetic acid was added to each well. Optical density of the content (A) in each well was then recorded at wavelength of 560nm (OD_{560}) against a control (Ac) containing only glacial acetic acid. These OD values were considered as an index (A=Ac, no biofilm; Ac<A=2Ac, Weak; 2Ac<A=4Ac, Moderate; 4Ac<A, Strong) of biofilm formation.

RESULTS AND DISCUSSION

B. cereus is a highly underestimated food borne bacterial pathogen that is ubiquitous in nature [10]. Presence of *B. cereus* in foods is of significant concern because of the organism being responsible in a number of food poisoning outbreaks. *B. cereus* causes food spoilage and two distinct types of food poisoning: the diarrhoeal type and the emetic type. Diarrhoeal syndrome is caused by complex enterotoxins produced during vegetative growth of the organism in the small intestine, which act on the epithelial cells, causing massive secretion of fluid into the intestinal lumen leading to diarrhoea.Diarrhoeal syndrome is usually associated with proteinaceous foods, vegetables, sauces and puddings. In contrast the emetic syndrome is almost exclusively associated with farinaceous foods and is characterized by rapid onset of nausea, vomiting and malise, in some cases followed by diarrhoea[6].The contamination of milk and dairy products by *B. cereus*is a common problem because of its effect on shelf life of the finished products prepared from contaminated milk as well as its impact on consumer's health [11]. Analysis of the eleven samples of raw chhana for the presence of *B. cereus* is presented in Table 1. Presence of *B. cereus* based on bluish-green-blue colour of the colony with zones of egg yolk precipitate was presumed in five of the samples. On further analysis of the representative isolates to confirm their identity, four of the samples were found to be contaminated with *B. cereus*. Overall 36% of the samples contained *B. cereus* in a range from 1.0×10^2 to3.0×10^3cfu g^{-1}. However, none of the samples crossed threshold limit (10^4cfu g^{-1}) of the pathogen denoting them safe from *B. cereus*toxicoinfection. Contamination of raw milk with *B. cereus* is very common and its presence in various dairy products marketed in India has been reported by various workers [12, 13]. Antimicrobial activity of indigenous lactic acid bacteria in chhana may have profound effect on the growth of contaminating bacteria. However, no work onthe presence of *B. cereus*in raw chhana has been found by our literature survey. Altogether seven isolates from four of these samples were selected for further characterization.

Result of the phenotypic characterization of the seven *B. cereus* isolates is presented in Table 2. None of the isolates produced indole. Five isolates could grow and utilize citrate as sole carbon source. All of the isolates produced oxidase, urease, gelatinase and DNase. Gelatinase is an extracellular metallo-endopeptidase or metalloproteinase which is able to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen [14]. In humans, gelatinase may cause breakdown of extracellular matrix inhibiting embryonic development, cell movement, as well as diseases, such as arthritis and metastasis. Gelatinase and DNase production increase spoilage potential.

All of the seven *B. cereus* isolates tested for extracellular enzyme production were found to produce amylase and caseinase. However, none of the isolates produced lipase (Table 3). Production of extracellular enzymes like amylase, protease and lipase is indicator of spoilage potential of producer strains. *B. cereus* has been frequently found in pasteurized milk, causing spoilage due to production of protease [15, 16]. Presence of *B. cereus* isolates producing amylase and caseinase increase the risk of spoilage of raw chhana causing textural problem and off-flavour. When the initial concentration of contaminating bacteria is low in raw chhana there may not be obvious spoilage indication. Ready-to-eat sweets produced from this type of contaminatedchhana without heat treatment may become spoiled within short time causing economic loss. *B. cereus* is also a matter of concern as spoilage causing organism in sweets produced after processing raw chhana with heat because of heat-resistant endospores.

Over the last 60 years, due to widespread and irrational use of antimicrobial drugs, bacteria have evolved towards antibiotic resistance posing a great concern to public health especially in developing countries. Unless used judiciously, the day is not far-away that most of the strains may become pan-resistant with no or new antibiotics left to combat them.Result of the antibiotic sensitivity assay performed against twelve antibiotics is presented in Table 4. All the isolates were multiple-drug-resistant against a minimum of five antibiotics. Five of the isolates were resistant against five antibiotics. One each isolate was resistant against six and seven antibiotics. All the isolates were resistant against four of the five antibiotics inhibiting synthesis of cell wall. Five of the isolates were sensitive to polymyxin-B. 100% of the isolates were sensitive to nucleic acid synthesis inhibitors. The results are in accordance with various workers [17, 18]who reported >95% % sensitivity of *B. cereus* isolates to chloramphenicol and ciprofloxacin. All the isolates were resistant to trimethoprim. Overall the antibiotics viz. azithromycin, chloramphenicol, ciprofloxacin, levofloxacin, norfloxacin and vanomycin were available to effectively treat possible *B. cereus* infection.

Biofilms are complex microbial communities formed on a wide range of surfaces that are generally encapsulatedby an extracellular protective matrix consisting of various types of biopolymers. Biofilm forming bacteria differ from their planktonic counterparts in their surface attachment ability, high population density, extracellular polymeric substances (EPS), slime and a wide range of physical, metabolic and chemical heterogeneities [19]. Due to their strong adhesion to the substrate biofilms are very difficult to eradicate.Production of biofilm by the isolates was tested by three different methods to compare their sensitivity. Five isolates (71%) each showed positive result by both thequalitative detection methods vizCRA method and standard tube method. Four of them were the same isolates. One of the seven isolates was negative in both methods. Thus the CRA method showed 71% correlation with the standard tube method.Four of these isolates also showed biofilm production when studied by quantitative tissue culture plate method. 43% of all the isolates showed positive result for biofilm production by all the three methods. Three isolates were negative when studied quantitatively. Overall similarity of the three detection methods was for 57% of the isolates. Presence of biofilm forming bacteria in raw chhana may act as a persistent source of (re)contamination to the finished sweet meats.

Table 1. Isolation of *B. cereus* from chhana

Sample No.	Presumptive count (cfu g^{-1})	Confirmed count (cfu g^{-1})	Confirmed isolate
1	<DL	<DL	nil
2	1.0×10^2	<DL	nil
3	5.0×10^3	3.0×10^3	S3B1, S3B2, S3B4
4	<DL	<DL	nil
5	<DL	<DL	nil
6	<DL	<DL	nil
7	1.0×10^2	1.0×10^2	S7B1
8	5.0×10^2	2.0×10^2	S8B1, S8B2
9	2.0×10^2	1.0×10^2	S9B2
10	<DL	<DL	nil
11	<DL	<DL	nil

Table 2. Phenotypic characterization of *B. cereus* isolates from chhana

Isolate	Production of					
	Indole	Citrate-permease	Oxidase	Urease	Gelatinase	DNase
S3B1	-	+	+	+	+	+
S3B2	-	-	+	+	+	+
S3B4	-	+	+	+	+	+
S7B1	-	+	+	+	+	+
S8B1	-	-	+	+	+	+
S8B2	-	+	+	+	+	+
S9B2	-	+	+	+	+	+

Table 3. Production of extracellular enzymes by *B. cereus* isolates from chhana

Isolate	Zone diameter (mm)		
	Amylase	Caseinase	Lipase
S3B1	36	30	0
S3B2	40	42	0
S3B4	42	33	0
S7B1	44	32	0
S8B1	42	44	0
S8B2	40	38	0
S9B2	44	40	0

Table 4. Antibiotic resistance of *B. cereus* isolates from chhana

Antibiotic	Diameter of inhibition zone (mm)						
(µg/units disc^{-1})	S3B1	S3B2	S3B4	S7B1	S8B1	S8B2	S9B2
Cell wall synthesis inhibitor							
Amoxyclav (30 µg)	0-R*	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R
Ampicillin (10 µg)	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R
Penicillin G (10 units)	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R
Piperacillin/Tazobactam (100/10 µg)	12.33 -R	15.67 -R	11 -R	15.33 -R	15 -R	16 -R	14.33 -R
Vancomycin (30 µg)	18.67 -S*	17.67 -S	19 -S	17.67 -S	17 -S	30 -S	18.67 -S
Cell membrane synthesis inhibitor							
Polymyxin-B (300 units)	12 -S	12 -S	12 -S	12 -S	10 -R	11 -R	12 -S
Protein synthesis inhibitor							
Azithromycin (15 µg)	16.67 -I*	24.33 -S	18.67 -S	17.67 -S	16.33 I	7 -R	18 -S
Chloramphenicol (30 µg)	21.67 -S	25.33 -S	23.67 -S	21.67 -S	26.33 -S	25.33 -S	26 -S
Nucleic acid synthesis inhibitor							
Ciprofloxacin (5 µg)	28.67 -S	26.67 -S	26.33 -S	27.67 -S	23.67 -S	26.33 -S	26.33 -S
Levofloxacin (5 µg)	30.67 -S	27.33 -S	25.33 -S	27.33 -S	27.33 -S	25.67-S	25.67 -S
Norfloxacin (10 µg)	26.33 -S	25.33 -S	22.33 -S	26 -S	22.67 -S	25 -S	24.33 -S
Folic acid synthesis inhibitor							
Trimethoprim (5 µg)	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R	0 -R

**(S), Sensitive; (R), Resistant; (I), Intermediate*

Table 5. Detection of biofilm formation by *B. cereus* isolates from chhana

Isolate	Formation of biofilm		
	CRA method	Standard tube method	Tissue culture plate method
S3B1	negative	positive	negative
S3B2	negative	negative	negative
S3B4	positive	positive	negative
S7B1	positive	positive	strong
S8B1	positive	positive	weak
S8B2	positive	negative	weak
S9B2	positive	positive	weak

CONCLUSION

Chhana is the coagulated milk separated from whey that is the raw materials for various sweets produced in West Bengal, India. Raw chhana may contain various pathogenic bacteria indigenous to milk or contaminants during preparation, storing and transport. Heating during chhana preparation may kill most of the vegetative cells making the substrate favourable for germination of endospores to produce vegetative cell and their subsequent multiplication. *B. cereus* is the most prevalent spore-former that causes diarrhoeal and emetic syndrome. In this study 36% of raw chhanasamples collected from sweet shops were found to be contaminated with *B. cereus* at a level of 1.0 x 10^2 to3.0 x 10^3cfu g^{-1}.On subsequent study, all the seven isolates were found to produce gelatinase, DNase, amylase and caseinase posing a risk of spoilage potentiality. Antibiotic sensitivity study reveals that all the isolates were multiple-drug-resistant. Antibiotics targeting protein and nucleic acid synthesis were more effective than others. Three isolates showed positive result on detection of biofilm formation by three different techniques.

Presence of multiple drug resistant strains with spoilage potential and capable of biofilm formation is a great concern to the dairy industry. However, antimicrobial activity by lactic acid bacteria present inchhana may have profound effect to control their growth within safe level which aspect requires rigorous investigation.

Acknowledgement
Authors are thankful to the Principal, Ramakrishna Mission Vidyamandira for providing financial and infrastructural assistance, constant encouragement and generous support.

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