Introduction

Bacillus cereus group consists of five closely related species viz., B. cereus, B. anthracis, B. thuringiensis, B. mycoides and B. weihenstephanensis. The organisms of this group are very closely related to each other and there is proposal to group them in a single species (Helgason et al., 2000). B. cereus is a Gram positive spore forming organism, which causes two distinct type of food poisoning syndromes viz., diarrhoeal type and emetic type, caused by two different types of toxins viz., a heat-labile diarrhoeal toxin and a heat-resistant emetic toxin (Dierick et al., 2005). In some B. cereus outbreaks, there appears to be an overlap of diarrhoeal and emetic syndrome (Kramer and Gilbert, 1989). Apart from these, the involvement of B. cereus has also been reported to be associated with opthalmitis, respiratory tract infection and central nervous system disorder (Bekemeyer and Zimmerman, 1985; Beer et al., 1990; Barrie et al., 1992).

Unlike other species of Bacillus, this pathogen can grow even under anaerobic condition. Food poisoning caused by enterotoxigenic B. cereus is very common in many European countries and accounts for 47, 33 and 22% of the total food poisoning cases reported in Iceland, Norway and Finland, respectively (Granum and Lund, 1997). Although, a major food poisoning outbreak due to this organism has not been reported in India, enterotoxigenic B. cereus has been isolated from variety of food items in the country (Kamat et al., 1989; Agarwal et al., 1997; Das et al., 2009). This organism was also isolated from the stool specimen of 3.5% of the patients suffering from diarrhea in a study carried out in Kolkata, India (Banerjee et al., 2011). B. cereus can produce biofilm, which is generally very resistant to various sanitisers (Oosthuizen et al., 2002). Biofilm production on different food contact surfaces remains a constant source of contamination and a potential public health hazard. The conventional isolation and identification method of B. cereus involves isolation on selective agar containing polymixin B and egg yolk, followed by a battery of biochemical tests. As per biochemical tests, generally organisms under Bacillus cereus group are positive to Voges-Proskauer (VP) test (Rhodehamel and Harmon, 1998). In the present study, two atypical isolates of B. cereus have been reported with negative VP reaction.

Materials and methods

Isolation and identification of bacteria

Isolation of Bacillus cereus was done by plating on polymixin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA) (Szabo et al., 1984). Identification of the isolates was carried out by performing motility test, catalase test, nitrate reduction test, lysozyme resistance, lecithinase reaction, mannitol fermentation test, glucose fermentation test etc. as mentioned in Bacteriological Analytical Manual (Rhodehamel and Harmon, 1998). Two of the isolates recovered from white shrimp (Fenneropenaeus indicus) procured from local market in Kochi were found to be negative for VP reaction and these
two VP negative atypical isolates were used in this study for further characterisation.

**Screening for diarrhoeal enterotoxin production and haemolytic activity**

Loopful of culture was inoculated into brain heart infusion broth and incubated overnight at 37°C with shaking. One milliliter of the culture was centrifuged at 5000 g at 4°C for 5 min and the supernatant was passed through 0.22µ membrane filter. The ability to produce diarrhoeal enterotoxin production was judged from the filtrate by reverse passive latex agglutination test using BCET-RPLA kit (Oxoid, U.K.) following manufacturer’s instructions.

For checking β-haemolytic activity, loopful of culture was stab inoculated onto blood agar containing 5% sheep blood.

**Screening for starch hydrolytic property**

The starch hydrolysing property of both the isolates was screened by inoculation on starch agar to find a clear zone around the colony in case of positive reaction following exposure to the iodine vapour (Collins et al., 2001).

**Test for psychrotrophic growth**

Both the strains were streaked on tryptic soya agar plate and incubated at 7°C for 7 days. The plates were observed daily for growth by visual examination up to 7 days.

**Isolation of genomic DNA from the isolates**

Isolation of genomic DNA was carried out by following the protocol of Mantynen and Lindstrom (1998) with slight modification. Briefly, 1.5 ml overnight grown culture in BHI broth with 1% (w/v) glucose was observed daily for growth by visual examination up to 7 days. The plates were screened by inoculation on starch agar to find a clear zone around the colony in case of positive reaction following exposure to the iodine vapour (Collins et al., 2001).

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**Analysis of DNA sequence using BLAST**

The deduced DNA sequences were compared with nucleotide resources of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) accessible at blast.ncbi.nlm.nih.gov.
Table 1. Details of primers used in the study

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Target / Specificity and PCR cycling condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BalF 5'- TGCAACTGTATTAGCACAAGC T -3'</td>
<td>Bacillus cereus group (Chang et al., 2003) 4°C for 45 seconds (denaturation), 55°C for 45 seconds (annealing) and 72°C for 45 seconds (extension).</td>
</tr>
<tr>
<td>BalR 5'- TACCCAGAAGTTTGTTCACTACT -3'</td>
<td></td>
</tr>
<tr>
<td>HblA1 5'- GCTATTGATTTCTCATGCAAC- 3'</td>
<td>hbla gene (Mantynen and Lindstrom, 1998) 94°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 min.</td>
</tr>
<tr>
<td>HblA2 5'- AATCATGCCACTGGACTGGACATAA- 3'</td>
<td></td>
</tr>
<tr>
<td>ENTA 5'- ATGAAAAAGTAATTTCGAG- 3'</td>
<td>entFM gene (Asano et al., 1997) 94°C for 45 seconds, 52°C for 45 seconds and 72°C for 1 min.</td>
</tr>
<tr>
<td>ENTB 5'- TTGATGCTTTTGTGGTACC- 3'</td>
<td>bceT gene (Mantynen and Lindstrom, 1998) 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds.</td>
</tr>
<tr>
<td>BceT1 5'- GAATTCTAAACTGCGACATCTC G- 3'</td>
<td></td>
</tr>
<tr>
<td>BceT2 5'- CTGCGTAATCGTGAATGTCAAT- 3'</td>
<td></td>
</tr>
<tr>
<td>27 F 5'- GAGTM'GATCCTTGCTCAG - 3'</td>
<td>For sequencing of 16S rRNA gene (Rainey et al., 1996) 94°C for 30 seconds; 52°C for 45 seconds and 72°C for 1 min 10 seconds.</td>
</tr>
<tr>
<td>1525R 5'- AGAAAGGAGGTATCCAGGC - 3'</td>
<td></td>
</tr>
</tbody>
</table>

BLAST comparison was done for both the sequences separately using megablast option of nucleotide BLAST (blastn).

Results and discussion

In biochemical tests, both the isolates were found to reduce nitrate, liquefy gelatin, motile, resistant to lysozyme, catalase positive and showed lecithinase reaction in media containing egg yolk (i.e., PEMPA and PEMBA). Both the isolates utilised glucose anaerobically and possessed starch hydrolytic property as evident by the presence of clear zone around the colony following exposure to the iodine vapour.

The result of RPLA indicated that both the isolates were enterotoxigenic. Both of them were β-haemolytic on sheep blood agar, but none of them was found psychrotrophic as no growth was observed even after 7 days when incubated at 7°C.

In B. cereus group specific PCR, both the isolates yielded 533 bp amplified product. The hbla gene specific PCR using primers HBLA1/HBLA2 yielded 834 bp product in both the isolates. The primer pair ENTA/ENTB, which is specific for the entFM gene, showed 1.3 kb amplified product in both the isolates (Fig. 1). No amplified product was obtained in the bceT gene specific PCR using BceT1/BceT2 primers.

On PCR amplification of 16S rRNA gene using the universal primers 27F and 1525R, approximately 1.5 kb PCR product was obtained for both the isolates. The sequences of both the isolates (BC-shrimp-19 and BC-shrimp-39) are depicted in Fig. 2. The sequences have been submitted to NCBI and are accessible at www.ncbi.nlm.nih.gov through the accession numbers JN676164 and JN676165 for BC-shrimp-19 and BC-shrimp-39, respectively.

On BLAST analysis, 16S rRNA gene of both the isolates showed almost 99% similarity with sequences of many B. cereus isolates. Apart from the organisms of Bacillus spp., the sequences of BC-shrimp-39 (JN676165) showed similarity with Clostridium acetobutylicum, while the sequences of BC-shrimp-19 showed similarity with Enterobacter cloacae and Clostridium acetobutylicum.

Enterotoxigenic B. cereus causes serious food poisoning and hence the prompt identification of this organism is of utmost importance. Like most of the species of Bacillus, B. cereus is also a spore forming
The isolates resembled Enterobacter cloaceae gene sequence similarity. Similarity was also found with current study, two enterotoxigenic isolates of tests for identification of this organism. But in this organism. VP test is considered as one of the important followed by a series of biochemical tests (Rhodehamel reaction on organism is observation of typical colony with lecithinase conventional methods of identification of this pathogenic leading (b) BC-shrimp-39 (Accession No. JN676164) (a) BC-shrimp-19 (Accession No. JN676164) Bacillus cereus isolates from white shrimp. Therefore, it is essential to perform other biochemical tests, PCR assay (e.g., PEMPA or PEBA), followed by a series of biochemical tests (Rhodehamel and Harmon, 1998).

The pathogen B. cereus is generally a VP positive organism. VP test is considered as one of the important tests for identification of this organism. But in this current study, two enterotoxigenic isolates of B. cereus with negative VP reaction have been reported. Both Enterobacter cloaceae and Clostridium acetobutylicum. But, Enterobacter cloaceae is Gram negative organism and Clostridium acetobutylicum is an anaerobic organism and thus, both of them can be ruled out. The results of the present study indicate that VP negative enterotoxigenic B. cereus is present in tropical aquatic system of Cochin and the criteria of VP negative cannot rule out the identification of B. cereus. Previously, VP negative B. cereus has also been reported from pasteurised milk and dairy products (Te Giffel et al., 1997; Wong et al., 1998). Te Giffel et al. (1997) observed that one out of 143 isolates of B. cereus from seafood. Therefore, it is essential to perform other biochemical tests, PCR assay and test for enterotoxin production (e.g., RPLA) even for VP negative suspected B. cereus isolates from white shrimp.

Acknowledgements

The authors are thankful to the Director, Central Institute of Fisheries Technology for providing necessary facilities for conducting this research work.

References

Atypical enterotoxigenic *Bacillus cereus* from shrimp


