academicJournals

Vol. 7(19), pp. 2050-2056, 7 May, 2013
DOI: 10.5897/AJMR12.1428
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Influence of incubation temperature, ph and food substrate on production of *Bacillus cereus* extracellular proteins and toxins

Ifeoma M. Ezeonu* and Onyekachi Okonkwo

Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Accepted 26 April, 2013

A total of 422 isolates of *Bacillus cereus*, isolated from fresh vegetables were characterized alongside a reference strain (NRRL-B14726) and conditions influencing thier extracellular protein and toxin production were evaluated. Optimal temperature and pH conditions for growth of the isolates were 28 to 37°C and pH 6 to 7, respectively. Under these conditions, permissive for growth, the isolates produced extracellular proteins, with molecular weights ranging from 14 to 150 kDa, including those consistent with the 39, 45 and 105 kDa proteins of the Non-Haemolytic Enterotoxin (NHE) complex. There was no growth or extracellular protein detected at 4°C and pH 2 and 4. All isolates grew on rice medium, but only B24 produced an 8 kDa protein band which is presumed to represent the emetic toxin. Results from this study re-establish the great diversity in morphological and metabolic characteristics of *B. cereus* strains and the importance of food-storage conditions for prevention of *B. cereus* foodborne illnesses. Furthermore, it shows that food substrate may be an important determinant of toxin production.

Key words: *Bacillus cereus*, food poisoning, diarrhoeal toxin, emetic toxin, vegetables.

INTRODUCTION

Bacillus cereus is the causative agent of two kinds of foodborne disease: an emetic (vomiting) intoxication due to the ingestion of a peptide toxin pre-formed in the food and a diarrhoeal infection due to the ingestion of bacterial cells/spores, which produce enterotoxins in the small intestine (Valero et al., 2002; EFSA, 2005; Granum, 2007). The organism is ubiquitous and low numbers of its spores can be found in a wide range of foodstuffs. Consequently, almost all kinds of foods have been implicated in *B. cereus* foodborne poisoning. A majority of reported outbreaks have been linked to the consumption of heat treated foods and frequently occurred in restaurant and catering establishments. Desserts, meat dishes and dairy products are most frequently associated with the diarrhoeal disease, whereas, rice and pasta are most commonly implicated in emetic intoxications (Kramer and Gilbert, 1989; EFSA, 2005). There is a paucity of information on the involvement of vegetable dishes in *B. cereus* outbreaks, but recent reports have shown that fresh vegetables are increasingly being implicated in foodborne disease outbreaks. Generally, food-borne illnesses caused by *B. cereus* are relatively mild and do not last more than 24 h. However, the emetic syndrome can have a fulminant and fatal outcome (Mahler et al., 1997; Musa et al., 1998).

B. cereus constitutes an ever present threat to public health because of its ubiquity. The organism produces spores that are highly resistant to heat, drying, toxic chemicals, UV radiation, gamma radiation and other adverse environmental factors and these spores are capable of surviving most procedures applied in the cooking of food.

*Corresponding author. E-mail: ifeoma.ezeonu@unn.edu.ng, ifyezeonu@yahoo.com.

The spores subsequently return to active growth through the process of germination either in already processed and stored food or in the intestinal tract (Kramer and Gilbert, 1989). Therefore, for prevention of illness due to *B. cereus*, emphasis should be, not on elimination of the organism, but control of environmental factors allowing growth of the organism and elaboration of its toxins. The diarrhoeal disease has been characterized on a molecular level and shown to be associated with the production of two peptide-based toxins: the tripartite hemolysin BL (HBL), with proteins of molecular weight 25, 40 and 100 kDa and non-hemolytic enterotoxin (NHE), consisting of a complex of three proteins with molecular weights of 39, 45 and 105 kDa (Beecher et al., 1994; Granum et al., 1996; Lund and Granum, 1996; Kashid and Ghosh, 2010). The emetic disease, on the other hand, has been linked to a single toxin known as the emetic toxin or cereulide (Agata et al., 1995, 1996). While the production of diarrhoeal toxins appears to be widely distributed among the members of the *B. cereus* group; it has been suggested that the strains producing cereulide are restricted to what appears to be a relatively clonal group of *B. cereus* isolates (Agata et al., 1995; Carlin et al., 2006). However, recent studies have revealed that there may be more diversity within this group of pathogens than previously thought (Rasko et al., 2007).

In this study, *B. cereus* strains were isolated from fresh vegetables. The isolates were characterized morphologically and biochemically and some factors, such as temperature, pH and food substrate were evaluated for their effects on production of *B. cereus* extracellular pro-teins and toxins.

MATERIALS AND METHODS

Sample collection

A total of 30 fresh vegetable samples, comprising 10 *Amaranthus retroflexus*, 10 *Telfairia occidentalis* and 10 *Talinum triangulate* were purchased at different times from the local market at Nsukka. Each batch of fresh vegetables was wrapped in a clean plastic bag and returned to the laboratory for analysis. All samples were processed promptly.

Isolation and identification of organisms

One gram of each vegetable sample was washed with 100 ml of sterile distilled water. *B. cereus* strains were isolated by inoculating 0.1 ml of the vegetable wash-water onto *B. cereus* selective medium comprising (^{w}/v): Agar, 2%; D-mannitol, 1%; ammonium phosphate, 0.1%; potassium chloride, 0.02%; magnesium sulphate, 0.02%; yeast extract, 0.02%; bromocresol purple, 0.004% and egg yolk emulsion, 20% (Ranald, 1996). Plates were incubated for 24 h at 37°C. Resulting colonies were characterized in terms of Gram stain, spore production, catalase reaction, haemolysis and starch hydrolysis as outlined in District Laboratory Practice in Tropical Countries (Cheesbrough, 2004). Presumed *B. cereus* colonies were stored in nutrient agar slants at 4°C until further use.

Reference *B. cereus* strain

The reference *B. cereus* strain (NRRL-B14726) used in the study was generously provided by Dr. Alejandro P. Rooney of Microbial Genomics and Bioprocessing Research Unit, U.S.A.

Extraction and separation of extracellular proteins

The isolates were grown in tryptone soy broth (TSB) and incubated at 37°C for 72 h. A 1 ml aliquot of each culture was placed in a microcentrifuge tube. The tubes were centrifuged at 3,000 rpm for 5 min and the cell-free culture supernatants were collected. Proteins were precipitated from the supernatants with ice-cold acetone (4X sample volume). Thereafter, the tubes were vortexed and incubated for 60 mins at -20°C followed by centrifugation at 13,000 rpm for 10 min. The supernatants were decanted and acetone was allowed to evaporate from the uncapped tubes at room temperature for 30 min. The proteins were separated by SDS-PAGE (12% acrylamide gel), using a Bio-Rad Mini-Protean II Dual Slab Cell. The protein bands were visualised by Coomasie blue staining and the molecular weights of the proteins were calculated using Sigma SDS-PAGE molecular weight standards.

Effect of temperature on growth and production of extracellular proteins

Three laboratory isolates (BB, B10, B24), and a type strain (NRRL-B14726, hereafter referred to as BTC) were used for this study. These isolates were tested for the effect of temperature (4, 28 and 37°C) on growth and toxin production. Four test tubes containing 5 ml each of TSB were inoculated with 0.1 ml of standard inoculum (cultures adjusted to 0.5 MacFarland) and incubated at different temperature for 3 to 4 days. At 24 h intervals, 1 ml was taken from each tube for serial dilution and determination of viable cell counts and after 72 h, 1 ml was taken for extraction and separation of extracellular protein products as described previously.

Effect of pH on growth and production of extracellular proteins

Flasks of TSB medium were adjusted to pH of 2, 4, 6 and 8, using either 1N HCl or 1N NaOH. The four isolates (BB, B10, B24 and BTC) were inoculated separately into tubes containing the broth media. The tubes were incubated at 37°C for 3 to 4 days. Determination of viable cell counts and extracellular protein production were done as described previously.

Effect of food substrate on growth and production of extracellular proteins

In order to determine the effect of food substrate, rice was used. A standardized inoculum of each isolate was inoculated into rice medium (a sterilized mixture of 5 g of rice in 100 ml of distilled water) and incubated for 3 to 4 days. Viable cell counts and extracellular protein production were determined as described previously.

Statistical analysis

Data from all growth experiments were analyzed by one-way ANOVA and least significant difference (LSD) at the 95% confidence limit.

RESULTS

Isolation and characterization of *B. cereus*

All the vegetable samples (100%) examined yielded *B. cereus* as determined by growth on *B. cereus* selective

Table 1. Incidence of *Bacillus cereus* in different vegetable samples.

Vegetable sample	Number of sample	Number positive for *B. cereus* (%)	Number of *B. Cereus* isolates	*B. cereus* load (cfu/g)
Amaranthus retroflexus	10	10 (100)	127	1.27×10^9
Telfairia occidentalis	10	10 (100)	141	1.08×10^9
Talinum triangulate	10	10 (100)	154	2.08×10^8
Total	30	30 (100)	422	

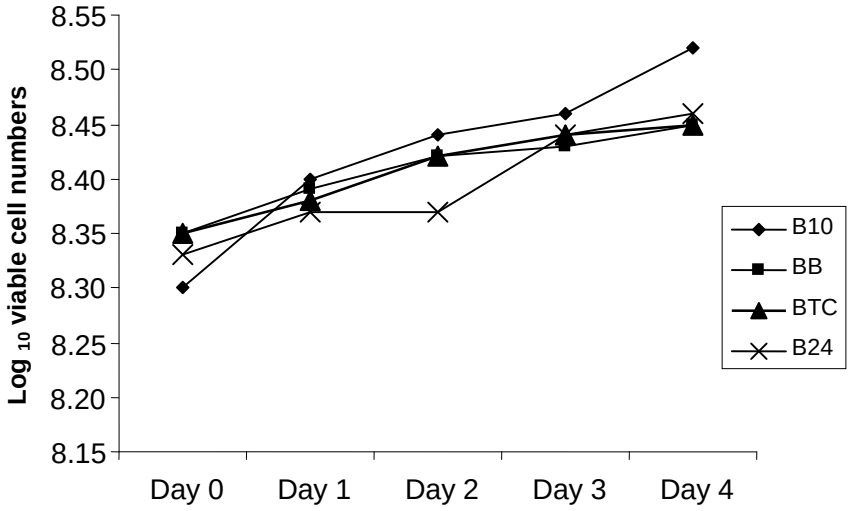

Figure 1. Growth of *B. cereus* strains at 37°C and pH 7 over a period of four days.

media. A total of 422 isolates were recovered, comprising 127 isolates from *A. retroflexus*, 141 isolates from *T. occidentalis* and 154 isolates from *T. triangulate*. The *B. cereus* loads on the vegetable samples were in the order of 10^8 to 10^9 colony forming units per gram of vegetable (Table 1).

Growth and extracellular protein production of *B. cereus* at different temperatures

The four isolates (BB, B10, BTC and B24) selected for the studies grew at 28 and 37°C, but not at 4°C on TSB medium. B10 had the best growth with cell numbers increasing steadily up to 96 h. B24, on the other hand, had a prolonged lag period, before cell numbers increased significantly by 72 h (Figure 1). Proteins from the cell-free culture supernatants were precipitated with cold acetone. SDS-PAGE with Coomasie blue staining revealed four to six protein bands for each isolate, but with different patterns (Figure 2). The proteins had estimated molecular weights ranging from 14 to 150 kDa.

Growth and extracellular protein production of *B. cereus* at different culture pH

All isolates grew at pH 6, 7 and 8, but not at pH 2 and 4. B24 grew best at pH 6 (Figure 3). SDS-PAGE separation of extracellular proteins precipitated by acetone also revealed more protein bands from B24 than other isolates (Figure 4).

Growth and extracellular protein production on rice medium

Only BTC and B24 showed significant ($p < 0.05$) growth on rice medium. Cell numbers of the two strains increased steadily until the third day of culture, before dropping slightly. BB and B10, on the other hand, had prolonged lag periods and only achieved significant increases in cell number after the third day of culture (Figure 5). SDS-PAGE of the precipitated extracellular proteins showed that only B24 produced extracellular proteins on this medium (Figure 6).

DISCUSSION

B. cereus is found in a wide range of habitats from soil, skin and fur of animals to food condiments and spices (Beattie and Williams, 2000). Since this bacterium is widespread in the environment, it is introduced into the food chain through raw materials. It is the major problem in convenience foods and mass catering (EFSA, 2005). The high resistance of the spores allows *B. cereus* to survive

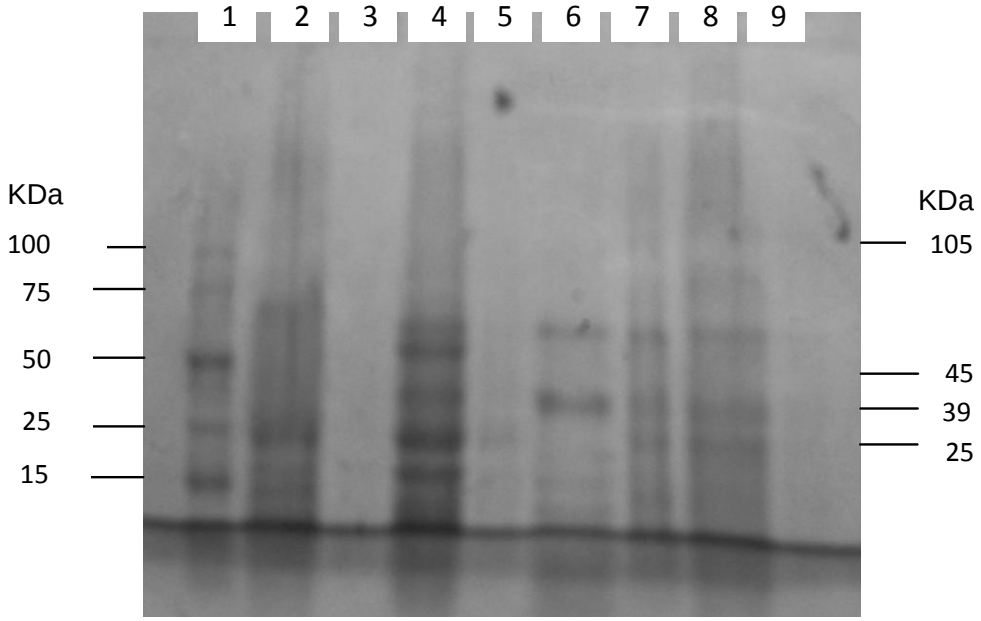

Figure 2. SDS-PAGE of extracellular and intracellular proteins of *B. cereus* isolates grown at 37°C and pH 7: Lane 1, molecular weight standard; Lane 2, BB-extracellular; Lane 3, BB-intracellular; Lane 4, B24-extracellular; Lane 5, B24-intracellular; Lane 6, BTC-extracellular; Lane 7, BTC-intracellular; Lane 8, B10-extracellular; and Lane 9, B10-intracellular.

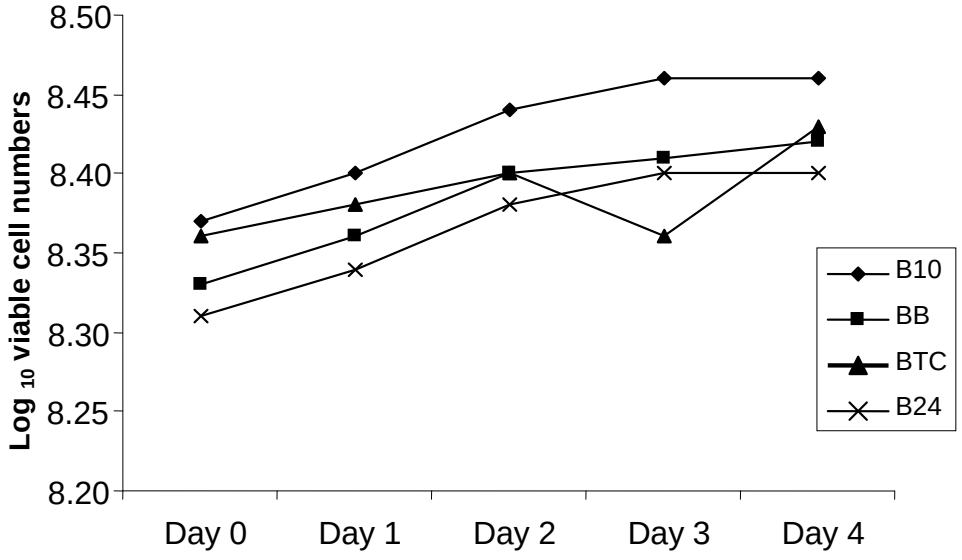

Figure 3. Growth of *B. cereus* strains at 37°C and pH 6 over a period of four days.

most drying and cooking processes and the organism grows well in cooked food because of the lack of a competing microbiota. Outbreaks of *B. cereus* food poisoning due to cooked foods, particularly starchy foods such as rice and pasta, have been widely reported (EFSA, 2005; Granum, 2007). Outbreaks due to other foods such as dairy products have also been reported (EFSA, 2005). There are scant reports of outbreaks of *B. cereus* poisoning from vegetables, but it has been suggested that the organism can get to the vegetables from the soil on which the vegetables are grown (Guinebretière and Nguyen-The, 2003). This study investigated the occurrence of *B. cereus* in fresh vegetables. The results showed that all samples examined were highly contaminated with the organism (Table 1). This is in line with the reports of EFSA (2005) that all kinds of food have been implicated in *B. cereus* food poisoning. However, the bacterial loads recorded from the vegetables in this study (10^8 to 10^9)

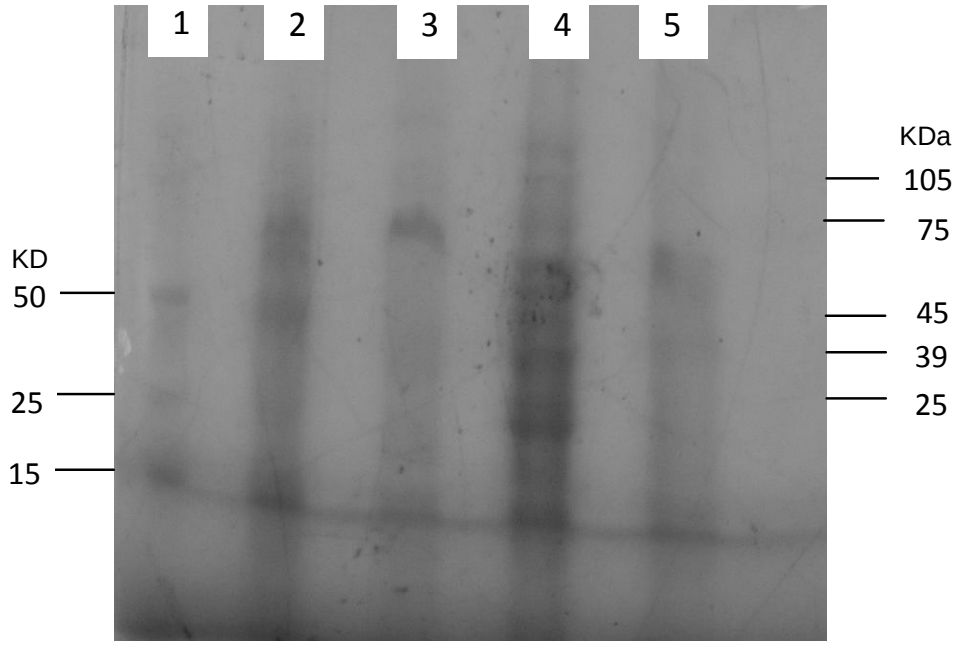

Figure 4. SDS-PAGE of acetone-precipitated extracellular proteins of *B. cereus* isolates grown at pH6: Lane 1, molecular weight standard; Lane 2, BB; Lane 3, BTC; Lane 4, B24; and Lane 5, B10.

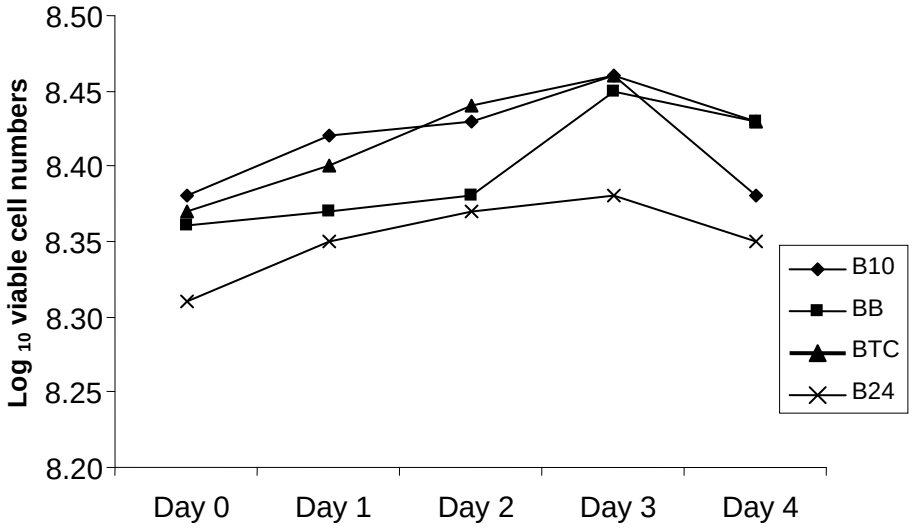

Figure 5. Growth of *B. cereus* strains on rice medium at 37°C and pH 7, over a period of four days.

are very much higher than those (10^2 to 10^3) previously reported by other authors (Valero et al., 2002; EFSA, 2005). This could be attributed to the fact that in the Nigerian setting, it is not uncommon for farmers to irrigate their farms with sewage water or even use manure consisting of both animal and human excrement.

It is easy to come up with a list of possible sources of *B. cereus* contamination for vegetables growing in the field: organic manure added to the soil, domestic and wild animals, birds, water contaminated with animal or human faecal material and poor hygiene by agricultural workers. It is therefore clear that almost every food can be associated with *B. cereus*, including fresh vegetables. Thus, the area of emphasis for control of *B. cereus* food poisoning must be on control of growth and toxin production, rather than complete elimination of the organism from foods.

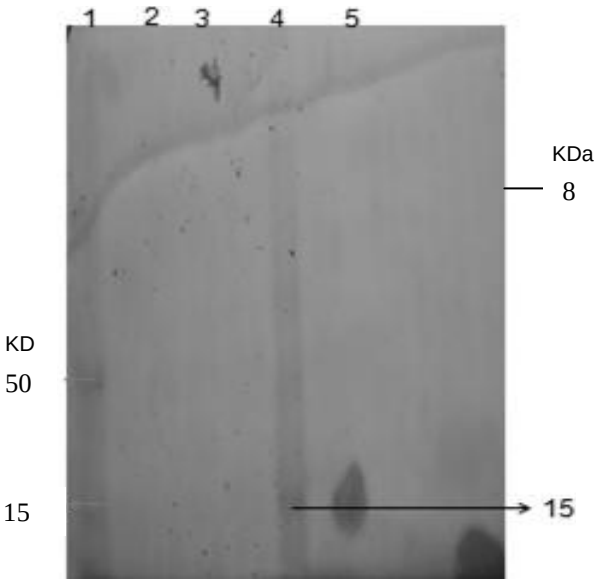

Figure 6. SDS-PAGE of acetone-precipitated extracellular proteins of *B. cereus* isolates grown on rice medium: Lane 1, molecular weight standard; Lane 2, BB; Lane 3, BTC; Lane 4, B24; and Lane 5, B10.

The *B. cereus* isolates from fresh vegetables were characterized in comparison with a reference strain (NRRL B14726). The results showed a great variability in their morphological and metabolic characteristics. This observation is in line with results from a previous study on isolates from rice and other cooked foods (Ezeonu and Ugwu, 2009). Evaluation of the effect of temperature on the growth and extracellular protein production of *B. cereus* showed that all the *B. cereus* isolates examined grew best at 37°C and produced extracellular proteins with molecular weight ranging from 14 to 150 kDa. About seven protein bands were detected by SDS PAGE, including 25, 39, 45 and 105 kDa proteins believed to be diarrhoeal toxins. This agrees with the report of other authors that the optimum temperature for growth of *B. cereus* is between 30 and 37°C and only a few isolates can grow at temperatures as low as 4°C or as high as 55°C (EFSA, 2005).

The result is also in line with the reports of Ezeonu and Ugwu (2009) that growth of *B. cereus* isolates occurred from 28 to 37°C but not at 4°C. This inability of the strains to grow and release extracellular proteins at low temperature confirms the importance of refrigeration of foods in preventing foodborne illnesses due to *B. cereus*. The evaluation of the effect of pH on the growth and extracellular protein production of *B. cereus* revealed that most of the isolates tested grew best and produced extracellular proteins from pH 6 to 8. However, at pH 2 and 4, growth and extracellular protein production were inhibited. This finding again suggests a potential area of investigation for protection of foods. It is worthy of mention, however, that *B. cereus* is able to multiply in the gastrointestinal tract where the stomach has a pH of 2 to 3, and cause diarrhoeal illness. This has been attributed to the fact that while vegetative cells do not survive the low pH of the stomach, the spores survive and are able to reach the small intestine where they germinate and produce enterotoxins leading to diarrhoeal illness.

B. cereus is responsible for two types of food poisoning; a diarrhoeal illness caused by enterotoxins with molecular weights from 25 to 105 kDa and an emetic illness caused by a 5 to 10 kDa peptide. It is worthy of note that in this study, extracellular proteins with molecular weights within the size range of these known toxins were detected by SDS-PAGE (Figures 2 and 4). This is in line with the reports of Lund and Granum (1997) which showed 39, 45 and 105 kDa protein bands on SDS-PAGE of the non-haemolytic enterotoxin and Kashid and Ghosh (2010) in which 25, 40 and 100 kDa protein bands were observed for the haemolytic toxin of *B. cereus*. The implication is that regardless of the food concerned, storage of food at temperatures permissive for growth of *B. cereus* would result in production on both the haemolytic (HBL) and non-haemolytic (NHE) enterotoxins. Cooked foods containing rice or pasta have been the main foods implicated in emetic intoxication.

Results from evaluation of growth and protein production in rice medium showed that one of the isolates (B24) produced an 8 kDa protein consistent with the molecular weight of the emetic toxin. This is in accordance with the suggestion of Graham and Paul (2006), that there could be a relationship between substrate and emetic toxin production. They suggested that rice is one of the few food substrates that contains all the necessary amino acids for incorporation into the emetic toxin. It has been reported that *B. cereus* strains producing emetic toxin are unable to hydrolyze starch and therefore the incidence of starch-negative *B. cereus* could provide an estimate of emetic *B. cereus* (Christiansson et al., 1999). Based on this, the production of the 8 kDa protein, which is within the size range of the emetic toxin, by isolate B24, which was also starch-negative, suggests that this isolate is an emetic strain.

The results from this study therefore show that there is a high rate of contamination of vegetables with both diarrhoeal and emetic strains of *B. cereus* and this poses a health risk. However, control of food storage conditions such as pH and temperature, where possible, along with consideration of the food substrate would help reduce the risk of *B. cereus* poisoning associated with foods.

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