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RESEARCH ARTICLE

INCIDENCE AND EFFECT OF NUTRITIONAL CONDITIONS ON THE GROWTH RATE OF EMETIC TOXIN- PRODUCING *BACILLUS CEREUS* ISOLATED FROM SOME SELECTED FOOD PRODUCTS IN NIGERIA

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Abstract: The incidence, characteristics, and effect of nutritional factors on the growth rate of emetic toxin-producing *B. cereus* isolated from various food products marketed in Nigeria were examined. A total of one hundred and thirty food products which included processed rice based food fermented milk products, traditional condiments and vegetables were analyzed. Seventy-five of 130 (57.7 %) analyzed samples were found positive for *Bacillus cereus*. The highest incidence (84.0 %) occurred in traditional condiments followed by processed rice based food (67.5 %). Emetic toxin was produced by 7.3 % (6 of 82) of the isolated strains. The growth of three emetic *Bacillus cereus* strains was examined on different carbon and nitrogen sources. All the three emetic *Bacillus cereus* strains had the highest growth rate when using 10-12 g/L of glucose and 1.5-2.5 g/L of yeast extract as carbon and nitrogen sources respectively. The carbon and nitrogen sources had significant effect on the growth of the three emetic *B. cereus* strains. The results of this study highlight the low incidence of emetic *Bacillus cereus* strains within and among various Nigerian food products. However, the presence of emetic *Bacillus cereus* strains in these food products is a concern to public health.

Keywords: *Bacillus cereus*, emetic toxin, cell cytotoxicity assay, carbon source, nitrogen source

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Introduction

Food-borne illness is a significant public health problem which has a negative impact on the entire world (Altekruse and Swedlow, 1996). Worldwide, *B. cereus* is recognized as an important cause of food-borne disease even though it is officially under-reported (Clavel *et al*., 2007; Granum, 2007). However, in European legislation, *B. cereus* has been classified as a Hazard group 2 organism on the basis of its ability to cause infections in humans (European Commission, 1993).

B. cereus is a Gram-positive, mesophilic, aerobic, facultative anaerobic bacterium (Griffths and Schrafts, 2002). It is ubiquitous in nature and commonly found in soil where it lives as a saprophyte (Vilain *et al*., 2006). In relation to food borne illness, it is recognized as an important public health hazard due to its ability to produce heat-resistant endospores and toxins in various food coupled with its ability to thrive in extremely cold environments (Griffith and Schraft, 2002; Dierick *et al*., 2005).

Furthermore, *B. cereus* has been reported to be a distinguished food pathogen that causes two distinct gastrointestinal diseases. The emetic syndrome is caused by a preformed toxin called emetic toxin (cereulide) (Granum and Lund, 1997; Beecher, 2002) and the diarrheal syndrome caused by an enterotoxin (Kramer and Gilbert, 1989; Granum and Lund, 1997). The emetic toxin is a heat stable toxin that has been reported to cause severe intoxication such as liver failure, respiratory distress, and possibly death in young healthy persons when consumed in low doses (Kramer and Gilbert, 1989; Lund, 1990; Mahler *et al*., 1997; Dierick *et al*., 2005). The diarrhea syndrome is characterized by 8-16 h diarrhea after consuming contaminated food while the emetic syndrome is characterized by 1-6 h nausea and vomiting after consumption of food contaminated with emetic toxin (Granum *et al*., 1995; Alouf, 2000). Various food types are associated with *B. cereus* food borne disease: the emetic syndrome is usually associated with farinaceous food products such as cooked rice, pasta, noodles and pastry (Granum and Lund, 1997) while the diarrhea syndrome is mainly associated with consumption of high protein products such as cooked meat, milk products, sauces and vegetables (Granum, 1994; Kotiranta *et al*., 2000; Granum, 2007).

Several studies have been carried out on the incidence of *B. cereus* in various foods from Nigeria (Kolawale and Akinsoji, 2011; Agwa *et al*., 2012; Bello *et al*., 2014) but the isolates' ability to produce emetic toxin was not determined. However, the relevance of *B. cereus* as a food poisoning

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organism and its emetic toxin has called for a need to assess the presence of emetic toxin-producing *B. cereus* in our environment. Thus, this study was carried out in order to evaluate the incidence of the emetic toxin produced by *B. cereus* in various Nigerian food products and also to characterize and examine the effect of the nutritional factors on its growth rate.

Materials and Methods

Isolation and enumeration of B. cereus in food products

A total of 130 food products were purchased from retails in Ibadan Metropolis, Oyo State, Nigeria. The food products were made up of processed rice based food, fermented milk products, traditional condiments and vegetables. All the samples were aseptically collected under sterile conditions and transferred to the laboratory for analysis.

Isolation of *B. cereus* from the various food samples was done according to the modified method of Ghelardi *et al*. (2002). Food samples were plated on a selective medium called mannitol egg yolk polymyxin B (MYP) agar (Sigma). The homogeneous sample was prepared with 10 g of each sample aseptically transferred into 90 mL of sterile distilled water to give an initial dilution of 1:10 and shaken vigorously for 2 min. Then, 10 mL of the homogenous mixture was incubated at 80ºC for 10 min and serial diluted in sterile distilled water to obtain the dilutions from 10^{-1} to 10^{-6} prior to plating on MYP agar. 0.1mL of desired dilution was pipetted onto the center of the surface of the MYP agar plate. After which sterile L-shaped spreader was used to evenly spread the sample over the surface of the agar, and at the same time rotating the Petri dish at an angle of 45ºC. Plates were incubated for 24 to 48 h at 30ºC, and the characteristic colonies (light pink with a white to pink halo) were counted using a colony counter. The pure cultures were stored at 4 ^{0}C for further analysis.

Cultivation and preparation of B. cereus supernatant

In order to culture *B. cereus* and obtain the supernatant, the modified method of Beattie and Williams (1999) was used. *B. cereus* was inoculated into 10 g/L of skim milk medium (SMM) (BBL) and incubated at 30ºC, 150 rpm for 18 h. After incubation, the sample was centrifuged at 4,800 xg, 4º C for 30 min. The supernatant was collected and autoclaved at 121ºC for 15 min to denature enterotoxins and proteases. Thereafter, the supernatant was filtered through a 0.22 µm syringe filter to remove residual cells. Samples were stored at −20°C until analyzed.

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Cell cytotoxicity assay

The ability of the *B. cereus* strain to produce emetic toxin was determined by cell cytotoxicity assay. Hep-2 cells were grown in monolayers in tissue culture flasks (25 cm^2) using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10 % (w/v) fetal bovine serum (Sigma) at 37°C in a humidified 5% CO_2 atmosphere (NAPCO Series 5400 CO_2 incubator). Confluent monolayers of Hep-2 cells grown for 3 - 4 days at 37°C (10% N_2 and 5% CO_2) were further diluted to 10^6 cell/mL and 100 µL portions were used to inoculate the flat bottom of the 96-well microtitre plates. 100 µL of the supernatant from each test strains were then serially diluted (two-fold) in triplicate in the 96- well microtitre plates already inoculated with the suspended Hep-2 cells in DMEM. Valinomycin (Sigma) was used as a standard; it was solubilized in DMSO (Sigma) and then further diluted in DMEM to obtain lower concentration. 100 µL of valinomycin final stock solution was added to the HEp-2 cells and a two-fold dilution of the valinomycin was carried out across the plate. Replicates of the series were tested and a negative control series of DMEM/DMSO was included. The plates were also incubated at 37°C (10 % N_2 and 5% CO_2) for 24 h (Finlay *et al*., 1999).

Afterwards, the cells were examined using an Olympus CK2 inverted microscope (Olympus Optical Ltd, London, UK) at 20X magnification for mitochondrial vacuolation following the 24 h incubation. When vacuolation was observed, 20 µL of aqueous 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Sigma) (2 mg ml^{-1}) was added to each well according to the method described by Hughes *et al.* (1988). The plates were further incubated for 4 h and were read based on two methods (color change and microplate reader). Before reading the plates, the plates were shaken to distribute the proliferation agent uniformly and later the absorbance was taken in a microplate reader (Bio-Tek Systems USA) at 450 nm. The mean end point toxicity titre was recorded as the highest dilution of each sample giving a colorimetric reading less than that of the negative control.

Characterization of emetic toxin-producing B. cereus isolates

Characterization of the toxigenic isolates was done using the morphological, cultural and biochemical characteristics (MacFaddin, 2000; Bennett and Belay, 2001; Eaton *et al.*, 2005).

Effect of carbon sources on the growth rate

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The effect of carbon sources on the growth of emetic strains of *B. cereus* was done according to the modified method of White (1972). Eight carbon sources including monosaccharide (glucose, fructose), disaccharide (sucrose, maltose, lactose) and sugar alcohols (mannitol, xylitol, inositol) were used in this study. The basal medium contained (g/L): Na_2HPO_4. 12 H_20 (11.2); KH_2P0_4 (2.4); $(NH_4)SO_4$ (2.0); $MgSO_4$. 7 H_20 (0.05); $MnCl_2$.4 H_2O (0. 004); $FeS0_4$.7 H_20 (0.0028), glucose, (10), the pH value was 6.5. The basal medium was supplemented with each of the carbon sources at the rate of (g/L) 2.5, 5.0, 7.5, 10.0, and 15.0. Thereafter, the medium was sterilized and inoculated with test organisms in triplicates and incubated at 37^{o}C for 24 h. Growth rates were determined using Thermo Scientific (Multiskan Go) spectrophotometer at wavelength of 600 nm

Effect of nitrogen sources on the growth rate

The effect of nitrogen sources on the growth of emetic strains of *B. cereus* was done according to the modified method of White (1972). Six nitrogen sources investigated were inorganic nitrogen sources (ammonium chloride, ammonium sulphate and ammonium nitrate), complex organic nitrogen source (yeast extract, peptone and tryptone). The basal medium was prepared as described earlier and supplemented with each of the nitrogen sources at the rate of (g/L) 0.5, 1.0, 1.5, 2.0, and 2.5. Thereafter, the culture medium was sterilized and inoculated with test organisms in triplicates and incubated at 37^{o}C for 24 h after which growth rates were measured as described above.

Results and Discussion

Prevalence and mean viable count of B. cereus

The prevalence and mean viable count of *B. cereus* in the analyzed food samples is represented in Table 1. *B. cereus* was isolated from 75 (57.7 %) of the analyzed food samples. The isolation of *B. cereus* from all the food products analyzed in this study is in agreement with the work of Sarrias *et al*. (2002), Valero *et al*. (2002), Bello *et al*. (2014) and Okanlawon *et al*. (2010) who also isolated *B. cereus* from rice, vegetables, fermented milk products and food condiments respectively. The ubiquitous nature of *B. cereus* in the environment makes it widespread in a variety of food thus causing food contamination (Turnbull, 1996; Pirttijärvi *et al*., 1999).

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Table 1. Prevalence and mean viable count of *B. cereus* in food samples

Sample	No of samples analyzed (n)	*B. cereus* positive samples (n)	(%)	Mean viable count of *B. cereus* ± SD (log_{10} CFU g^{-1})
Processed rice based food (PRBF)	40	27	67.5	4.8 ± 0.5
Fermented milk products (FMP)	25	15	60.0	3.3 ± 0.3
Traditional condiments (TC)	25	21	84.0	3.7 ± 1.0
Vegetables	40	12	30.0	5.2 ± 0.6

The number (n) of samples positive for *B. cereus*, prevalence (%) and mean viable count of *B. cereus* in the food samples along with the standard deviation (SD) are indicated

As shown in Table 1, the mean viable counts obtained ranging from a minimum of 3.3 log_{10} CFU/g in fermented milk products to a maximum of 5.2 log_{10} CFU/g in vegetable samples. In this study, the mean of the viable counts of *B. cereus* (5.2 ± 0.6 log_{10} CFU/g) observed are in agreement with the studies of Obuekwe and Ogbimi (1989) and Yusuf *et al*. (1992). The mean of the viable count ranging from 3.3 ± 0.3 - 4.8 ± 0.6 log_{10} CFU/g observed in rice based foods, fermented milk products and food condiments is at variance with their work in which they reported that Nigerian foods and food condiments had high load of *B. cereus* (>5.0 log_{10} CFU/mL or g). However, the results of the mean of the viable counts of *B. cereus* found in all the sampled food products (>10^3 CFU/mL or g) showed that the level of contamination with *B. cereus* was unsatisfactory according to Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods (2009) and Martinez-Blanch *et al.* (2009). Furthermore, Becker *et al.* (1994) reported that the hazardous levels or infective doses of 10^7, 10^5 and 10^3 CFU/mL or g are needed to cause food-borne illness in adults, children and infants respectively. Therefore, the mean counts of *B. cereus* found in these food

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samples were higher than the hazardous levels for children and infants thus posing a great risk on them.

Emetic toxin-producing B. cereus

Figure 1 shows the mean results of cell cytotoxicity assay for emetic toxin-producing *B. cereus* strains.

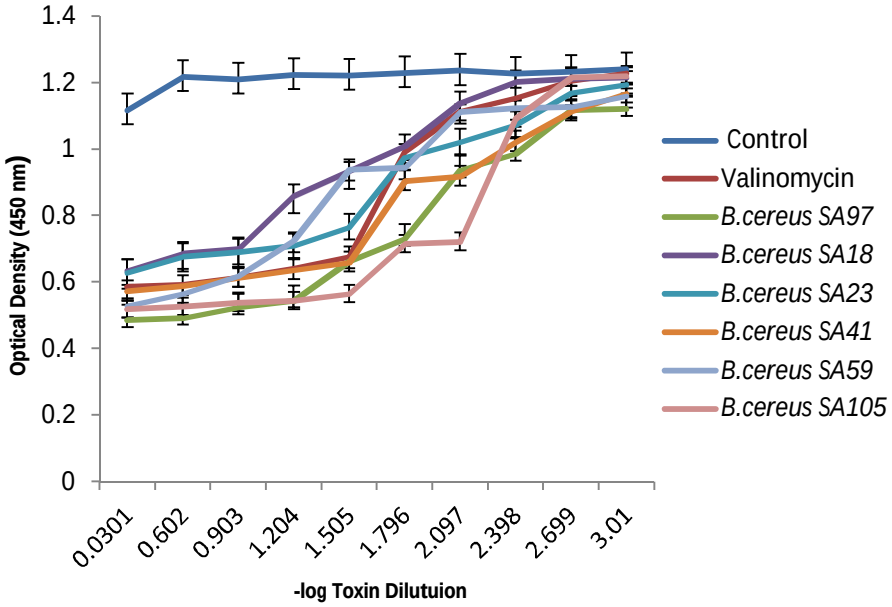

Figure 1. Mean results of cell cytotoxicity assay for emetic *B. cereus* strains

The graph reveals that the supernatant of six *B. cereus* isolates produced cell damage to the Hep-2 cells. The optical density (OD) of the emetic strains (*B. cereus* SA18, SA97, SA23, SA41, SA59 and SA105) were recorded with *B. cereus* SA97 having the highest colorimetric reading at the first dilution followed by *B. cereus* SA105 and *B. cereus* SA18 with the smallest mean colorimetric reading. Six isolates of *B. cereus* (7.3 % of the total 82 *B. cereus* isolates obtained from food products) were emetic toxin producers. The results of this study are in agreement with the findings of Ehling-Schulz *et al*. (2006), Park *et al.* (2009) and Ceuppens *et al*. (2011) who reported a low incidence of emetic strains out of the random *B. cereus* isolates and this implies that emetic strains are rare in the environment. In this study, the emetic toxin strains were isolated from rice, fermented milk and food condiment; and earlier studies had shown that emetic strains of *B. cereus* are present in rice and rice paddy fields (Ueda and Kuwabara, 1993; Wijnands *et al*., 2006; Ceuppens *et al*., 2011), milk (Taylor, 2005) and food condiments (Yim *et al*., 2015). Furthermore,

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fermented milk and food condiments are among the few foods that are capable of supporting the production of emetic toxin since it was reported that emetic food poisoning usually occurs in food products that have high starch contents such as pasta, rice, mashed potatoes, bread and pastries, which stimulate the production and accumulation of stable toxin cereulide (Jaaskelainen *et al.*, 2003; Rajkovic *et al*., 2006).

Results of the morphological and biochemical characteristics of isolated emetic *B. cereus* strains are presented in Table 2.

Table 2. Results of morphological and biochemical characteristics of emetic *B. cereus* strains

Test	Isolate I	Isolate II	Isolate III	Isolate IV	Isolate V	Isolate VI
Gram's reaction	+	+	+	+	+	+
Endospore	+	+	+	+	+	+
Crystal protein	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-
Motility	+	+	+	+	-	+
Methyl red	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+
Salicin fermentation	+	+	-	+	+	+
Lecithin hydrolysis	+	+	+	+	+	+
Hemolysis of blood	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+
Tyrosine decomposition	-	-	-	-	-	-
Lysozyme	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Xylose	-	-	-	-	-	-

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Lactose	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Source of isolate	PRBF	TC	PRBF	TC	PRBF	FMP

Characteristics of six isolated emetic *B. cereus* strains namely: I, *B. cereus* SA23; II, *B. cereus* SA59; III, *B. cereus* SA105; IV, *B. cereus* SA41; V, *B. cereus* SA97; VI, *B. cereus* SA18 are indicated. PRBF = processed rice based food; TC = Traditional condiments; FMP = Fermented milk products; + = positive; - = negative

All the emetic strains were found to be positive for endospore formation, catalase, Vogues-Proskauer, hemolysis, casein hydrolysis and lysozyme tests, and negative for methyl red, tyrosine decomposition, mannitol and starch fermentation tests. They also produced acid from glucose and lactose. The emetic strains showed variable biochemical characteristics results in their motility and salicin fermentation. The variation in the ability of the obtained emetic strains to ferment salicin divides the strains into two groups; the group that could ferment salicin and the group that could not ferment salicin. According to Kim *et al*. (2010), emetic strains are capable of fermenting salicin which is in contrast to the findings of other researchers such as Shinagawa, (1993); Agata *et al*. (1996); Pirttijärvi *et al*. (1999); Andersson *et al*. (2004). Furthermore, the six emetic strains of *B. cereus* isolated in this study were unable to hydrolyze starch and decompose tyrosine. This is consistent with the observation that emetic strains of *B. cereus* are unable to degrade starch (Kramer and Glbert, 1989; Agata *et al*., 1996). These phenotypical traits may explain why they are rare in the environment.

Effect of carbon sources on the growth

The effect of different carbon sources on the growth of emetic toxin-producing *B. cereus* strains after 24 h of incubation is shown in Figure 2 a and b.

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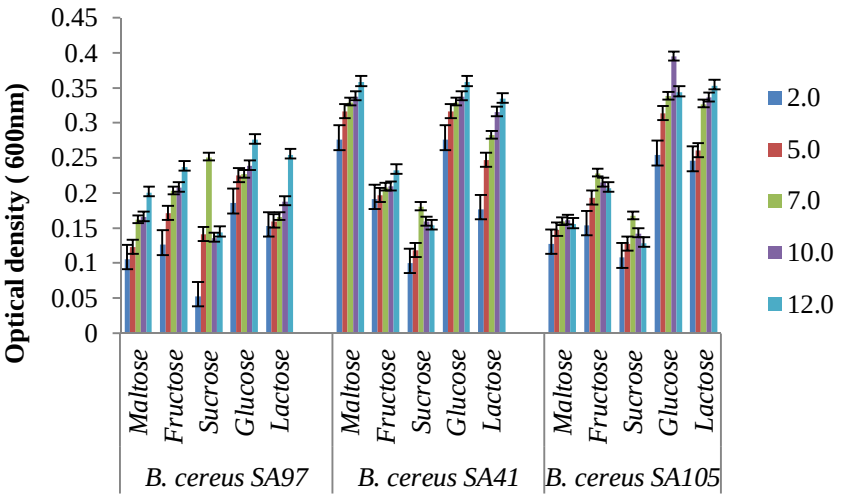
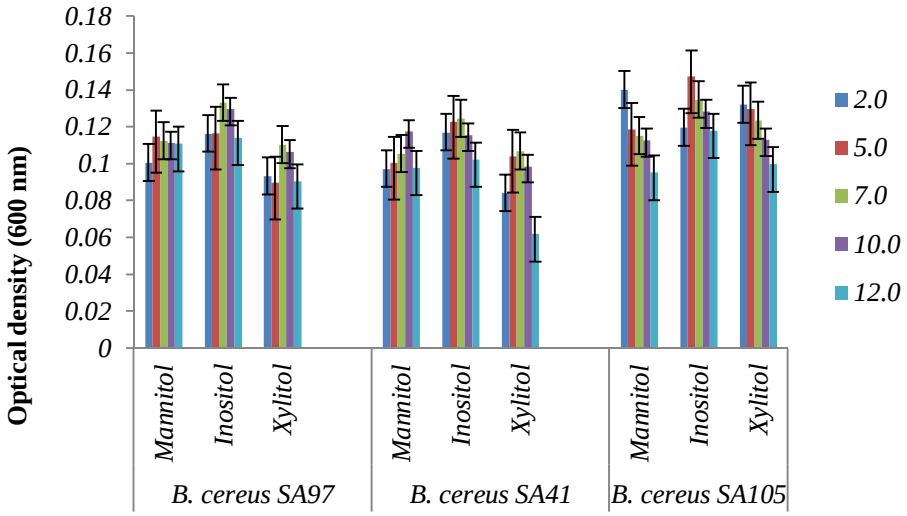


(a)



(b)

Figure 2a, b. Effect of different carbon sources on the growth of emetic *B. cereus* strains

Suspensions of vegetative cells of three selected emetic *B. cereus* strains were grown in basal medium containing 8 different carbon sources. Growth rate was quantified using a spectrophotometer (OD_{600}). The growth rates mean along with the standard deviation (SD) are indicated

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After incubation, the result of this study showed that glucose (12 g/L) was the most suitable carbon source for the growth of *B. cereus* SA97 and SA41 producing the highest growth rate with OD of 0.28 ± 0.09 and 0.36 ± 0.15 respectively. The lowest growth rate was recorded in the two toxigenic isolate (*B. cereus* SA97 and SA41) when xylitol (7 g/L) was used as a carbon source. Whereas, in *B. cereus* SA105, highest growth rate (0.40 ± 0.21) was recorded when glucose (10 g/L) was used as a carbon source in the basal medium. The smallest growth rate (0.13 ± 0.12) was recorded when xylitol (2 g/L) was used. The results showed that higher concentration of carbon sources increased the growth rate of the toxigenic strains.

The highest value observed in glucose could be explained by the fact that glucose is a readily fermentable sugar (Shirai *et al*., 2001) and a carbon source as well as an energy source for microbial growth. Interestingly, cereulide production is dependent on the cell growth (Logan and Rodrigez-Diaz, 2006) and since glucose established the highest growth it will increase cereulide production. This agrees with the reported effect of glucose on the *B. cereus* growth (Garcia-Arribas and Kramer, 1990) and enterotoxin production. A similar study (Lücking, 2009) reported the addition of glucose in Luria-Bertani (LB) media resulting in a delayed but higher cereulide production and this may be attributed to a total increase of RNA- and protein rates in the presence of glucose.

Effect of nitrogen sources on the growth

The result of the study carried out on the effect of nitrogen sources on the growth of three toxigenic isolates of *B cereus* (*B. cereus* SA97, SA41 and SA105) is shown in Figure 3 a and b.

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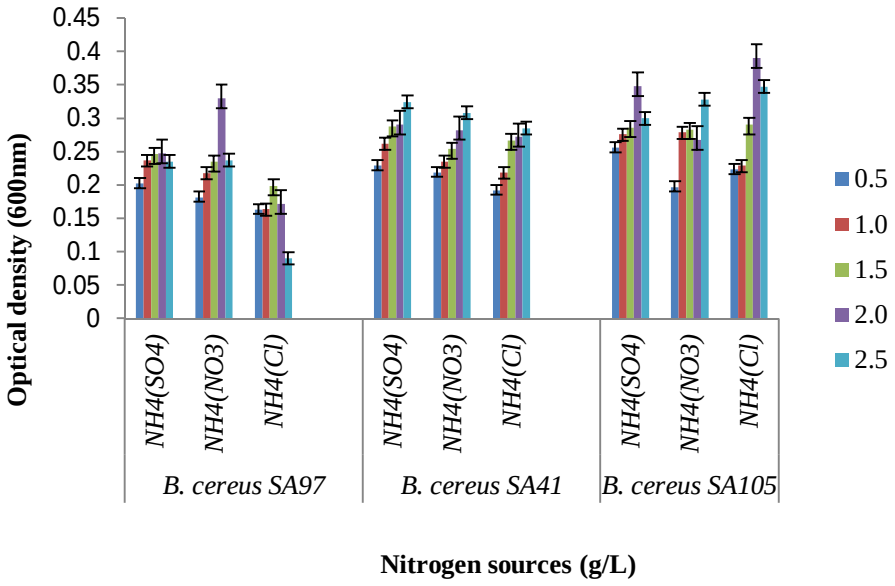

(b)

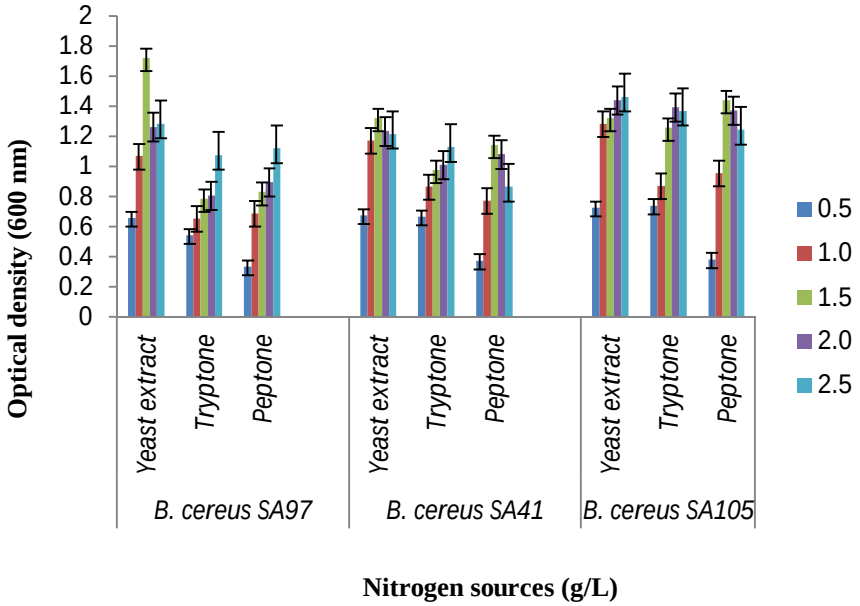

Figure 3 a, b. Effect of different nitrogen sources on the growth of emetic *B. cereus* strains.

From all the *B. cereus* strains, SA97 and SA41 had the highest growth rate with OD values of 1.72 ± 0.32 and 1.32 ± 0.46 respectively using the yeast extract (1.5 g/L) as the nitrogen source, closely followed by peptone (2.5 and 1.5 g/L respectively) with an OD of 1.12 ± 0.77 and 1.15 ± 032 respectively. The same thing was observed, in the case of *B. cereus* SA105 strain. The highest growth rate (1.5 ± 0.23) was recorded also when yeast extract (2.5 g/L) was used as the nitrogen

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source, this was closely followed by peptone (1.5 g/L) which yielded second highest growth with an OD value of 1.4 ± 0.56. However, the smallest growth rate was obtained in the case of *B. cereus* SA97 and SA41 respectively (0.20 ± 0.06 and 0.29 ± 0.13) this was recorded when NH_4Cl was used (2.5 and 0.5 g/L respectively). When $NH_4(NO_3)$ (0.5 g/L) was used, it recorded the smallest growth rate (0.28 ± 0.11) in the case of *B. cereus* SA105 strain.

According to our results, yeast extract gave the highest growth in the case of three emetic strains followed by peptone. Several researchers have also reported the growth-promoting properties of yeast extract based on the growth rate of bacteria (Milton et al. 1991; Jensen and Hamer, 1993; Ibrahim and Bezkororainy, 1994; Olmos-Dichara *et al.*, 1997). The high growth yield observed by using yeast extract is attributed to the high digestibility of the protein component into short chain peptides of about 4 amino acids in length and the presence of vitamin B, sugars and minerals in high concentrations (Bridson and Brecker, 1970). Peptone being the second nitrogen source that gave a maximum growth yield is a complex mixture of peptides and amino acids containing some water-soluble vitamins (Cochrane, 1958) which form its basis for promoting the growth of the strains. Hence, this study has revealed that the growth rate of emetic *B. cereus* strains is influenced by extrinsic factors; and also the availability of nutrients.

Conclusion

This work demonstrated that few of the isolates (7.3 %) were able to produce emetic toxin. Though the emetic strains are rare in our environment, this may impose a public health threat. The germination and proliferation of *B. cereus* in food and food products should be restricted since they proliferate in various carbon and nitrogen sources. This is the first work showing the incidence of emetic toxin-producing *B. cereus* strains in food products in Nigeria. Further work will be done with a larger number of isolates to give us more information about the distribution of emetic strains in Nigeria.

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