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# Evaluation of Growth and Cereulide Production by *Bacillus cereus* Isolated from Cooked Rice

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**Abstract**

Conditions influencing *Bacillus cereus* growth and cereulide production, such as temperature and pH, were evaluated at varying incubation periods. The growth and cereulide production at different temperatures and pH values ranging from 10 to 40 °C and 5.0 to 8.5, respectively showed that the temperature from 20 to 30 °C and at pH from 6.0 to 7.0 gave the optimum growth and cereulide production by *B. cereus* SA105. pH below 6.0 resulted in reduced growth and cereulide production. Cereulide production increased along with the incubation period, and the maximum cereulide titre (ng/mL) of 1219.1±8.90 was obtained after 6 days of incubation at 30 °C and pH 6.5 under static conditions. There was no quantifiable toxin at incubation temperatures of 10 and 40 °C by *B. cereus* SA105. This work further reveals that *B. cereus* growth and cereulide production was significantly affected by temperature and pH in relation to the incubation period. Furthermore, the findings of this study will serve as a means for reducing the diversity of the emetic toxin-producing *B. cereus* population in food and food products, thus preventing food poisoning.

***Keywords:*** *Bacillus cereus*; Growth; Cereulide; pH; Temperature; Incubation period

## 1 Introduction

*Bacillus cereus*, a Gram-positive, rod-shaped, beta haemolytic, endemic soil-dwelling bacterium is a common cause of food poisoning around the world (Turnbull, 1996). Episodes of *B. cereus* food poisoning occur sporadically worldwide, resulting from ingestion of contaminated food containing the bacteria, which multiply in high levels (McKillip, 2000). Basically, *B. cereus* is the etiological agent of two distinct types of gastrointestinal disorders, the diarrhea and emetic syndromes. The two types of food-borne diseases are caused by toxins: the diarrhea type by protein toxins which are thermolabile and formed in the intestinal tract of the host by the growing organism (enterotoxin) and the emetic type by a cyclic peptide (non-protein) toxin that is thermostable and pre-formed in food (emetic toxin) (Agata et al., 1994; Agata, Ohta, Mori, & Isobe, 1995; Granum, 2007; Jay, Loessner, & Golden, 2005).

The emetic syndrome was first identified in the 1970s and was associated with the consumption of fried rice (Kramer & Gilbert, 1989). This syndrome is an intoxication caused by *B.*

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*cereus* emetic toxin, called cereulide which is secreted in foods before ingestion. The toxin is a ring shaped, dodecadepsipeptide consisting of four acids, repeating three times and oxy acids (Granum & Lund, 1997). Jay (1996) reported that the disease is more acute than the diarrhea syndrome with an incubation time of 1-5 hrs, manifesting nausea, vomiting (emesis) and sometimes diarrhea which lasts for 6-24 hrs (Ehling-Schulz, Fricker, & Scherer, 2004). However, for the transmission of this type of *B. cereus* food poisoning, the infective dose of *B. cereus* in implicated food is $10^5$-$10^8$ cell/g of food (Wijnands, Dufrenne, Rombouts, In't Veld, & Van Leusden, 2006).

Furthermore, several studies have indicated that only a minority of *B. cereus* isolates may produce cereulide (Agata, Ohta, & Mori, 1996; Mikami et al., 1994; Pirttijärvi, Andersson, Scoging, & Salkinoja-Salonen, 1999). Yokoyama et al. (1999) suggested that more than 90% of food poisoning caused by *B. cereus* is of the emetic type in countries of the Far East. This may be partly due to the wide consumption of rice, which is a well-known food vehicle for the emetic toxin. Lund, De Buyser, and Granum (2000) reported the toxin produced by B. cereus, cereulide, as the most dangerous toxin to human health responsible for the deaths of young healthy individuals. The prolific growth of *B. cereus* in various food sources and its ability to produce heat stable, non-protein toxin calls for urgency to the microbiological quality and safety of food products. Several studies have been done on the effect of environmental factors on the growth and cereulide production of *B. cereus* (Agata et al., 1996; Agata, Ohta, & Yokoyama, 2002; Finlay, Logan, & Sutherland, 2002a, 2002b). However, it could be of interest to study the effect of environmental factors in relation to time for the growth and cereulide production of emetic toxin-producing *B. cereus*. In this article, we compare and assess the effects of temperature and pH on growth and cereulide production of *B. cereus* at different days of incubation.

## 2 Materials and Methods

### 2.1 Microorganism

The emetic toxin-producing strain of *Bacillus cereus* SA105 isolated from cooked rice in Ibadan, Oyo State, Nigeria was used for this study.

### 2.2 Effect of environmental factors on *B. cereus* growth

#### Effect of temperature on *B. cereus* growth

The effect of temperature on the growth of the toxigenic *B. cereus* strain was determined using the method of From, Pukall, Schumann, Hormazabal, and Granum (2005) and Chorin, Thuault, Cleret, and Bourgeois (1997). 10 mL of sterile Tryptone Soy Broth was inoculated with a suspension of vegetative cells of *B. cereus* to achieve a concentration of $10^3$CFU/mL. The tubes were sealed with paraffin film to avoid evaporation and concentration of the liquid medium at higher temperatures. After inoculation of 3 replica tubes, for each condition tested, media was then incubated for 1, 2, 4, 6, 8 and 10 days at temperatures of 10, 20, 30 and 40 $^o$C.

#### Effect of pH on *B. cereus* growth

The modified method of Chorin et al. (1997) was used to determine the effect of pH on the growth of the toxigenic isolate. Culture of the toxigenic *B. cereus* isolate was performed in Tryptone Soy Broth. 10 mL of sterile media was inoculated with a suspension of vegetative cells of *B. cereus* to achieve a concentration of $10^3$CFU/mL. The media was buffered with M.E.S (2-(N-morpholino) ethane-sulfonic acid, for pH ranging from 5.0 to 6.7 and with M.O.P.S (3-(N-morpholino) propane-conse-sulfonic acid, from 6.5 to 7.9. The pH was adjusted using 0.1 M of HCl or NaOH. Precise volumes of sterile HCl or NaOH were added respectively for pH: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5. The adjusted media from pH 5.0 to 8.5 was inoculated as previously described using 3 replicates for each condition

tested. The different media were later incubated at 30 $^{o}$C for 24 hrs.

## 2.3 Effect of environmental factors on cereulide production

### Effect of *B. cereus* growth and incubation temperature on cereulide production

Tryptone Soy Broth (100 mL) was inoculated with $10^3$ CFU/mL of *B. cereus* SA105 overnight culture. Triplicate cultures were thereafter incubated at temperatures of 10, 20, 30, and 40 $^{o}$C; static condition and agitation speed of 150 rpm. Following incubation, 1 mL was taken from each culture tube for serial dilution and the viable count was determined. Cereulide was extracted with pentane, and analysed using HPLC-MS after 1, 2, 4 and 6 days of incubation (Haggblom, Apetroaie, Andersson, & Salkinoja-Salonen, 2002).

### Effect of pH on cereulide production

The pH (5.0-8.5) of Tryptone Soy Broth was adjusted and inoculated as previously described using 3 replicates for each condition tested. The different media were later incubated at 30 $^{o}$C at 150 rpm. Cereulide was extracted with pentane, and analysed using HPLC-MS after 1, 2, 4 and 6 days of incubation.

## 2.4 Preparation of cell extracts

Incubated cultures were autoclaved (120 $^{o}$C; 19 mins) to destroy heat-labile proteins and other substances. The liquid cultures were extracted twice, each time with an equal volume of pentane for 1 hr with mild agitation (25 rpm) in vertical motion and after shaking the tubes were frozen. Organic phase layer was then separated from the aqueous phase layer in a smaller test tube. The combined pentane phases were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 1 mL of methanol (Andersson, Mikkola, Helin, Andersson, & Salkinoja-Salonen, 1998).

## 2.5 HPLC-MS analysis

High-performance liquid chromatography (HPLC)-MS analysis was performed on a Waters 2695 Separation Module HPLC equipped with a $C_8$ column (250 x 4.6 mm,5 $\mu$m Waters) and a solvent made up to 95 % Acetonitrile, 4.9 % $H_2O$, and 0.1 % Trifluoroacetic acid at a flow rate of 0.15 mL/min, with sample injection monitored with a Diode array detector. A full mass spectrum was recorded from 500 to 1,300 m/z in positive electron spray mode (ESI±). The total ion chromatogram was smoothed with Gaussian function. Valinomycin (Sigma) was used as the standard compound for quantification of cereulide. To quantify cereulide and valinomycin absorbance, Integrated Extracted Ion Current (EIC) chromatograms with ion ranges ($m/z$) of 1,170.5 to 1,193.5 for cereulide and 1,128.5 to 1,151.0 for valinomycin targeting the $NH4^{\pm}$ and $K^{\pm}$ adducts respectively, were used. Calibration curves extrapolated from integrated peak areas were plotted to calculate cereulide amounts via linear regression (Haggblom et al., 2002).

# 3 Results and discussion

## 3.1 Effect of temperature on *B. cereus* growth

he *B. cereus* SA105 growth profile at varying temperatures (10-40 $^{o}$C) is shown in Figure 1, with maximum and minimum growth recorded at 30 and 10 $^{o}$C after day 6 and 10 of incubation, respectively. The optimum temperature (30 $^{o}$C) for *B. cereus* SA105 recorded is in accordance with the report of previous authors who observed the optimum temperature for the growth of *B. cereus* to be between 30-37 $^{o}$C (European Food Safety Agency, 2005; Nguyen-The, Carlin, & Guinebretière, 2003). Similar to the present observation, Pielaat, Fricker, Nauta, and Van Leusden (2005), Carlin et al. (2006) and Wijnands et al. (2006) also reported that emetic toxin-producing *B. cereus* are found to be mesophilic in nature. The implication is that refrigeration will considerably increase the lag time and reduce the growth of emetic toxin-producing strains of *B. cereus.*

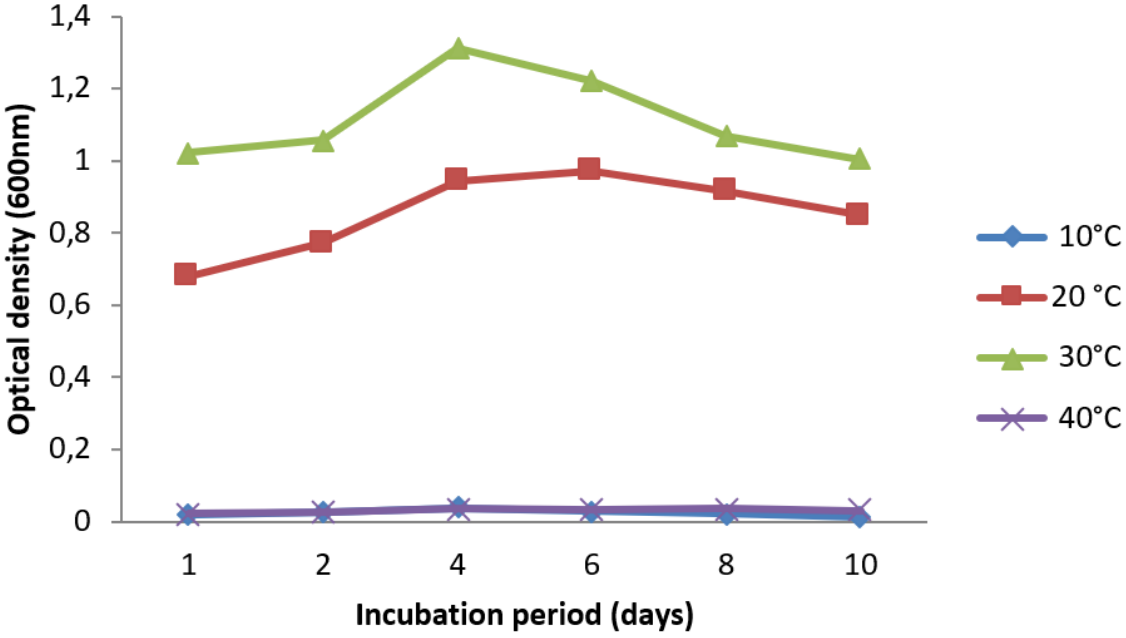


Figure 1: Effect of temperature on the growth of *B. cereus* SA105. Suspension of vegetative cells of the selected strain grown in Tryptone Soy Broth at varying incubation temperature and period (days) under static conditions. The values are presented as the mean ± SEM, n=3

### 3.2 Effect of pH on *B. cereus* growth

Results of the effect of pH on the growth of *B. cereus* SA105 are shown in Figure 2. The optimum pH for growth of *B. cereus* SA105 was pH 6.5. This was followed by pH 6.0 which gave the second highest absorbance while the least growth was observed at pH 5.0. The growth decreased with pH below 6.0 and above 8.5. Evaluation of the effect of pH on the growth rate of the emetic strain of *B. cereus* in this study revealed that it grew best from pH 6.0 to 7.0 and that it is not acid-tolerant. It is important to note that, at this optimum pH (6.0-7.0), the growth rate will be increased and lag time will be shorter (Benedict, Partridge, Wells, & Buchanan, 1993; Martinez, Borrajo, Franco, & Carballo, 2007). From the result obtained, it is indicated that pH below 5.5 was inhibitory to the strain thus, acidification is sufficient to prevent the growth of emetic strains of *B. cereus* during a longer storage period. This is useful in extending the shelf life and safety of processed foods but the significance to food protection is minimal since very few foods have such high acidity (Lindsay, Brozel, Mostert, & von Holy, 2000).

### 3.3 Effect of *B. cereus* growth and incubation temperature on cereulide production

The result (Figure 3) of viable counts and cereulide concentration at different incubation temperatures under static conditions revealed that *B. cereus* SA105 grew at all the incubation temperatures after 4 days of incubation. The highest cereulide concentration of 762.0±2.28 ng/mL and viable count of 6.3±0.23 $\log_{10}$CFU/mL was recorded by *B. cereus* SA105 at 30°C after 4 days. Increased temperature

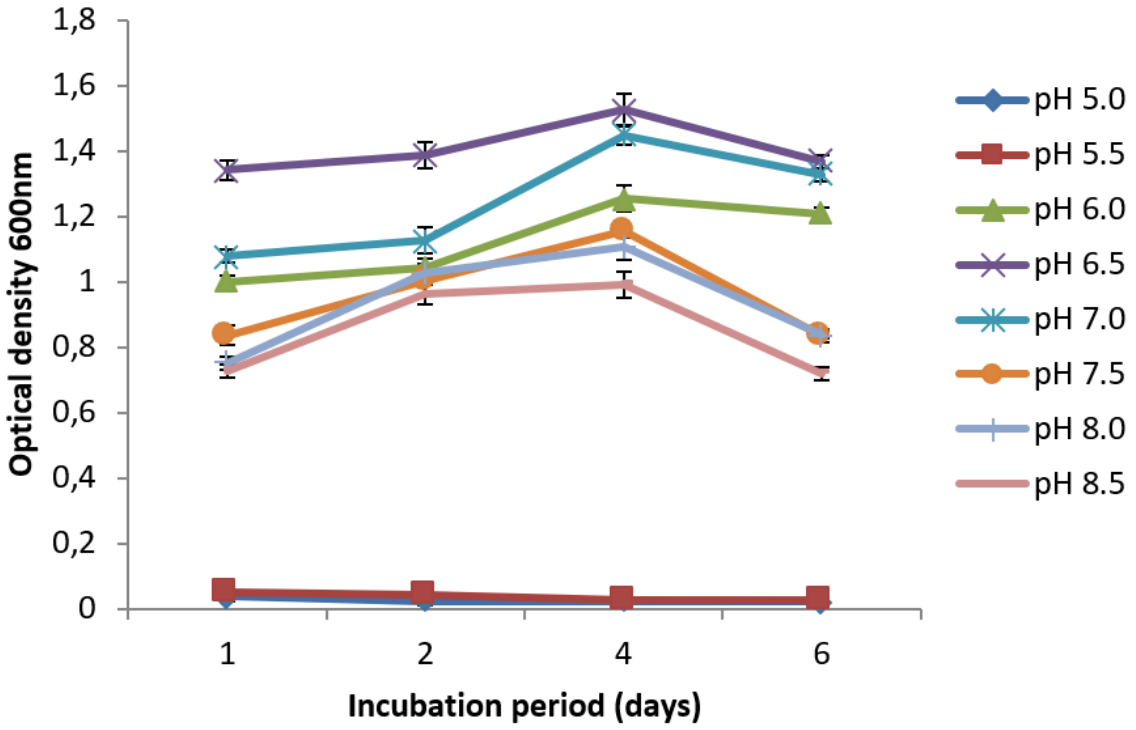


Figure 2: Effects of pH on the growth of *B. cereus* SA105. Suspension of vegetative cells of the selected strain grown in Tryptone Soy Broth, incubated at 30 $^{o}$C and, at varying pH and incubation times (days) under static conditions. The values are presented as the mean± SEM, n=3

(above 10 $^{o}$C) led to an increase in the growth of the test strain which influenced the production of cereulide. However, the mesophilic temperature has been previously observed to favor cereulide production in emetic strains of *B. cereus* (Dommel, Luecking, Scherer, & Ehling-Schulz, 2011). In our study, no quantifiable amount of cereulide was produced at 10 and 40 $^{o}$C although the emetic strain was able to grow at these temperatures. In contrast, quantifiable amount of cereulide was produced in *B. weihenstephanensis* at 8-10 $^{o}$C (Guerin et al., 2017). It has been previously reported that the production of cereulide below 10 $^{o}$C does not seem possible and that temperatures above 37 $^{o}$C also do not permit cereulide production (Finlay, Logan, & Sutherland, 2000, 2002a, 2002b; Jaaskelainen, Haggblom, Andersson, & Salkinoja-Salonen, 2004). The lack of quantifiable cereulide production by *B. cereus* at 10 and 40 $^{o}$C implies that foods held for long periods at $\leq$10 $^{o}$C and at raised temperatures are unlikely to be a risk for emetic food poisoning.

At 30 $^{o}$C under static conditions, the growth curve of the tested strain (Fig. 4) attained its peak after day 4 with a mean viable count ($\log_{10}$CFU/mL) of 6.3±0.23. Cereulide production was first detectable after day 1 of incubation with the mean toxin titre of 117.5±4.45 ng/mL and gradually a maximum mean titre of 1131.7±0.90 ng/mL was attained after 6 days of incubation. In this study, the emetic strain of *B. cereus* analyzed started its exponential growth from day 2 to day 4 with a slight drop after day 4, probably from depletion of nutrients. Interestingly, cereulide was detectable after 24 hrs at 30 $^{o}$C with an amount exceeding the minimum acute toxic level of cereulide (10 ng/g) as quanti-

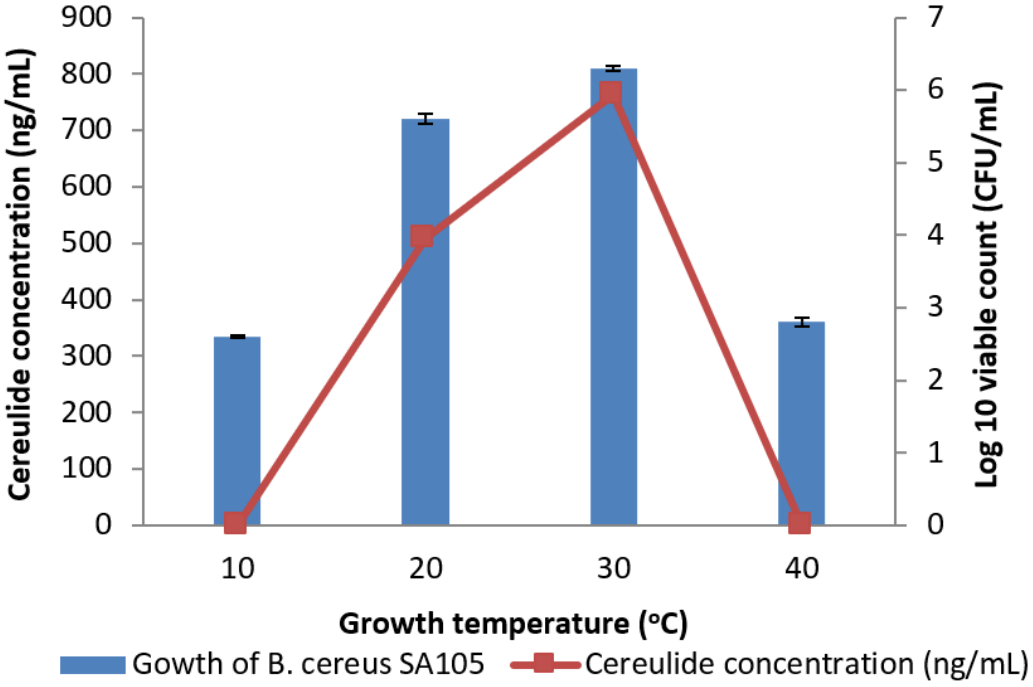


Figure 3: Effects of *B. cereus* growth and incubation temperatures on cereulide (ng/mL) production. The viable count was determined and cereulide was quantified using HPLC-MS after 4 days of incubation under static conditions. The values are presented as the mean± SEM, n=3

fied by Agata et al. (2002) in samples implicated in different outbreaks. Other studies have revealed that 20-30 $^{o}$C is the optimal temperature for cereulide production and this supports the findings of the study (Thorsen, Budde, Henrichsen, Martinussen, & Jakobsen, 2009). The difference in the optimal temperature is influenced mainly by the bacterial growth rate and composition of the media or food (Agata et al., 2002; Rajkovic et al., 2006). Cereulide production in relation to temperature and time was analyzed in this study and it was discovered that the highest cereulide concentration was observed at the end of the growth period, which was between day 6-8. This agrees with the previous work of Haggblom et al. (2002) who reported that cereulide production is growth phase-dependent and that maximum cereulide was recorded at the end of the growth period. Hence, the incubation period (storage time) plays an important role in cereulide production since it was observed that cereulide production increased during the late stationary phase.

The effect of agitation on growth and cereulide production was established at 30 $^{o}$C and 150 rpm for 10 days. The maximum mean viable count ($\log_{10}$CFU/mL) recorded was 8.1±0.31 (day 4) which was higher than the non-agitated broth at 30 $^{o}$C. Cereulide was detectable after day 1 of incubation with a mean toxin titre of 317.5±2.99 ng/mL and the maximum mean toxin titre of 1670.7±7.43 ng/mL (day 8) was recorded (Fig. 5). The role of aeration in cereulide production cannot be underestimated, since the production of cereulide is greatly inhibited by a reduction in atmospheric oxygen. Oxygen acts as a terminal electron acceptor for oxidative reactions in providing energy for all cellular activities. Aeration

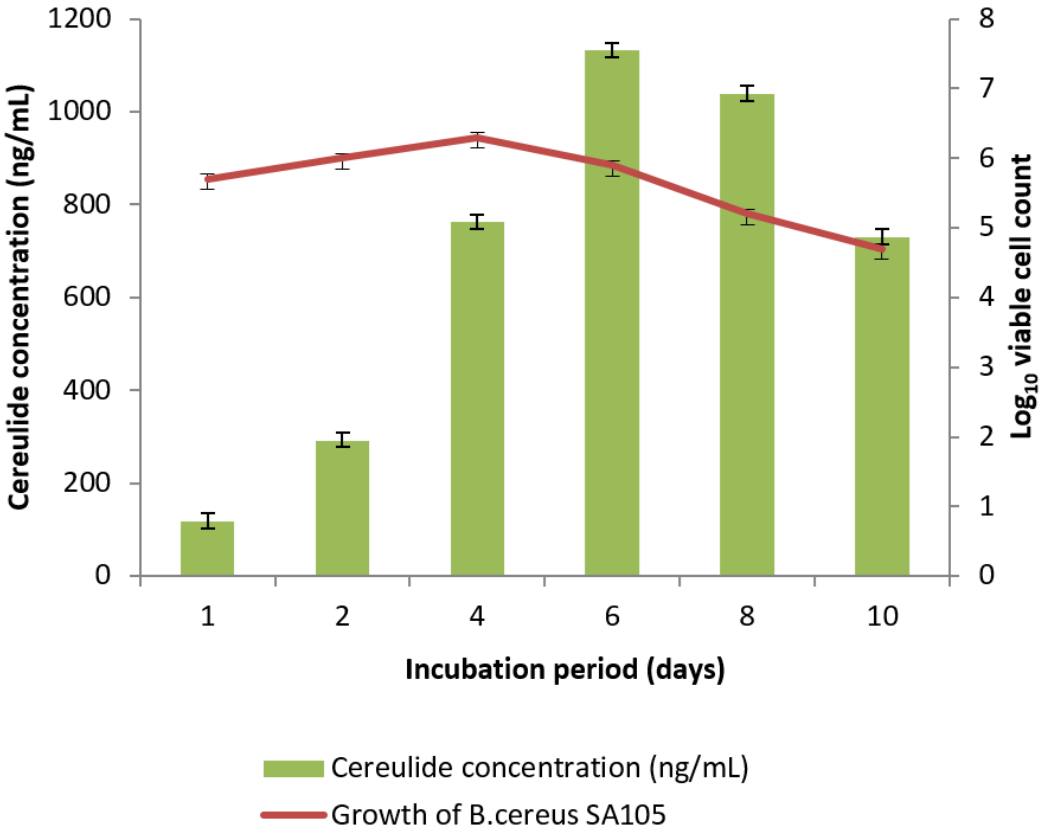


Figure 4: Production of cereulide (ng/mL) by *B. cereus* SA105 at 30 $^{o}$C under static conditions. Vegetative cells of *B. cereus* SA105 were grown in broth and incubated at 30 $^{o}$C under static conditions. The viable count was determined and cereulide was quantified using HPLC-MS after different incubation periods. The values are presented as the mean± SEM, n=3

influences the mixing and nutrient availability in shaking flasks (do Nascimento & Martins, 2004). Thus, oxygen is regarded as a stimulating factor in cereulide production (Agata et al., 2002; Finlay et al., 2002b). In contrast, Rajkovic et al. (2006) and Shaheen et al. (2006) reported an inhibitory effect of oxygen on cereulide production.

## 3.4 Effect of pH on cereulide production

The result (Table 1) of cereulide production in broth inoculated with *B. cereus* SA105 at different pH values incubated for 6 days. At pH 5.0 cereulide was not detected until day 4 while at pH 5.5 cereulide was detected at day 2. Quantifiable cereulide concentration was recorded after day 1 in pH ranging from 6.0-8.5. However, the highest mean toxin titre of 1219±8.9 ng/mL was produced at pH 6.5 followed by pH 6.0 reaching a mean toxin titre of 970.6±1.18 ng/mL after 6 days of incubation. Acidic pH inhibited cereulide production by the emetic strain of *B. cereus* in this study. In a previous study, Agata et al. (2002) reported that the addition of dressings like mayonnaise, vinegar or ketchup to rice for the purpose of acidification decreased *B. cereus* growth in these foods and cereulide produced was

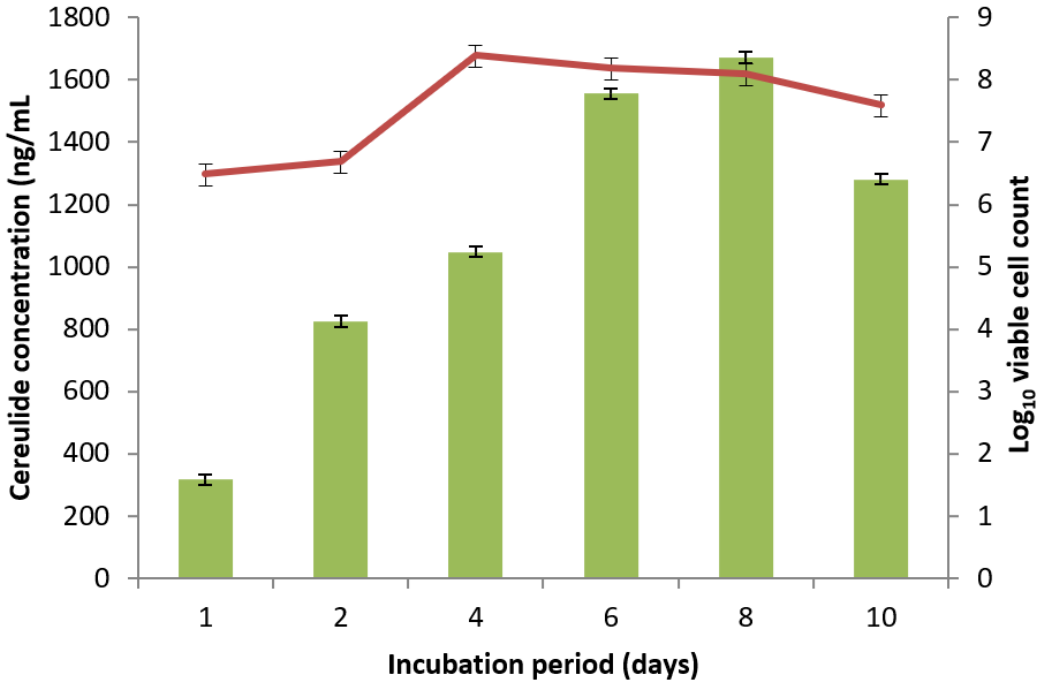


Figure 5: Production of cereulide (ng/mL) by *B. cereus* SA105 at 30 $^{o}$C and 150 rpm. Vegetative cells of *B. cereus* SA105 were grown in broth and incubated at 30 $^{o}$C and 150 rpm. The viable count was determined and cereulide was quantified using HPLC-MS after different incubation periods. The values are presented as the mean± SEM, n=3

Table 1: Effect of pH on cereulide production (ng/mL) by *B. cereus* SA105

| | Incubation Period (days)/ Cereulide production (ng/mL) | | | |
|---|---|---|---|---|
| pH | 1 | 2 | 4 | 6 |
| 5.0 | ND | ND | 15.4±2.28$^{g}$ | 24.4±0.90g$^{\pm}$ |
| 5.5 | ND | 31.7±0.30$^{g}$ | 39.4±4.90$^{f}$ | 44.8±3.50$^{f}$ |
| 6.0 | 115.6±3.55$^{d}$ | 476.2±1.15$^{c}$ | 732.2±4.48$^{b}$ | 1070.6±1.18$^{b}$ |
| 6.5 | 295.3±1.95$^{b}$ | 521.2±9.95$^{a}$ | 828.4±6.25$^{a}$ | 1219.1±8.90$^{a}$ |
| 7.0 | 108.5±4.75$^{a}$ | 431.5±2.75$^{b}$ | 673.3±4.75$^{c}$ | 1028.4±2.70$^{c}$ |
| 7.5 | 94.1±2.75$^{c}$ | 212.5±2.24$^{d}$ | 375.0±4.75$^{d}$ | 653.2±5.03$^{d}$ |
| 8.0 | 89.4±6.20$^{d}$ | 178.3±1.18$^{e}$ | 317±2.50$^{c}$ | 543.5±0.02$^{e}$ |
| 8.5 | 45.0±1.90$^{e}$ | 94.9±9.65$^{f}$ | 124.5±2.80$^{e}$ | 256.7± 0.85$^{e}$ |

The values are presented as the mean ± SEM, n=3. $^{\pm}$Values with different letters in the same column indicate a significant difference (P≤0.05) using Duncan's Multiple Range Test. ND: Not Detected

below 0.01 $\mu$g/g, which is similar to the effect of acidic pH in this study. This agrees with the work of Wong and Chen (1988) who reported the effect of pH on *B. cereus* growth and the experimental result of its growth and emetic toxin production on Brain Heart Infusion (BHI) bouillon and food products. Hence, a possible strategy to prevent growth and cereulide production by emetic strains of *B. cereus* is through the acidification of food.

## 4 Conclusions

This extensive study shows that the various environmental factors analyzed (temperature, pH, aeration, and incubation period) played an important role in influencing growth and cereulide production by the emetic strains of *B. cereus*. Hence, the findings of this study will serve as a means for reducing the diversity of emetic toxin-producing *B. cereus* populations able to multiply in food and food products thus preventing food poisoning.

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