# The Occurrence of *Bacillus Cereus* in White Pepper from Bogor, Indonesia



# The Occurrence of *Bacillus Cereus* in White Pepper from Bogor, Indonesia

**Hasifa Nanteza*, Ratih Dewanti-Hariyadi, Siti Nurjanah**

Bogor Agricultural University, Bogor, Indonesia

*hasifananteza@apps.ipb.ac.id

**Abstract**. White pepper (*Piper nigrum* L.) is a spice with earthy heat and an intense floral aroma widely produced and consumed in Asia. In Indonesia, it is commonly used to flavor dishes that require a spicy taste. However, white pepper can be contaminated by pathogenic microorganisms, such as *Bacillus cereus*, an endospore-forming bacterium widely grounded in soil and dust. The bacterium causes emetic and diarrheal syndromes and has been implicated in various foodborne disease outbreaks in different parts of the world. Currently, data pertaining to the occurrence of *Bacillus cereus* in white pepper in Indonesia is not available. This study aimed to isolate and assess the occurrence of *Bacillus cereus* in white pepper obtained from markets in Bogor, Indonesia. The study consisted of sample collection and preparation, isolation and enumeration of *Bacillus cereus* using Mannitol egg yolk polymyxin (MYP) agar, and confirmation by biochemical tests and Polymerase Chain Reaction to detect the gene encoding for16S rRNA. Of the 20 samples analyzed, 18 of them (90%) were contaminated with presumptive *Bacillus cereus*, and the highest concentration was 5.77 $\log_{10}$ cfu/g. The high counts of *Bacillus cereus* were probably attributed to the postharvest processing operations that the spice encountered. All the 15 tested isolates showed a clear band at the expected length of around 1686bp after being separated from PCR products on ethidium bromide-stained 2% agarose gel. This result confirmed the existence of *Bacillus cereus* in white pepper samples.

***Keywords***: *Bacillus cereus;* Occurrence; Polymerase Chain Reaction; White pepper.

## 1. Introduction

White pepper (Piper ningrum) is a member of the Piperaceae family which is usually cultivated for its fruits, known as peppercorns. It is the most consumed spice in the world with Asia as the largest producer and consumer. In 2017, world annual white pepper production was 102,900 MT, and Indonesia was the largest producer with 42,000MT [1]. The spice has earthy heat and an intense floral aroma [2] which encourage its use in a variety of processed and unprocessed foods.

The production of white pepper involves several postharvest operations which present bacteriological risk [2], predominantly for spore-formers, chiefly molds (including *Penicillium spp.,* and *Aspergillus spp.*), bacterial pathogens (including *C. perfringens, C. botulinum*, and *Bacillus cereus/B. cereus*) [2], and an endospore, forming bacterium widely grounded in soil dust, water, and surfaces of raw and cooked foods [3]. The organism has been reported to contaminate cereals like rice, pasta, meat and its products, infant formula, milk and its products, vegetables [4], [5], [6], [7]-[8], ready-to-eat foods [9], and spices like white pepper [10].

White pepper is often contaminated by *B. cereus*. Several studies by [6], [10]-[11] isolated *B. cereus* from white pepper at the concentrations ranging from >3cfu/g to >$10^4$cfu/g. Untreated black and white pepper were reported to contain 3.0-5.0×$10^5$ cfu/g *B. cereus* [8]. The existence of *B. cereus* in

white pepper may be attributed to the wide distribution of the organism in the environment, especially in the soil where this spice grows or get in contact with bacterium during other processing stages [3]. According to [2], the soil is a vital reservoir for *B. cereus* spores and prolonged contact with food can contaminate the food with spores. Water, dust, air, and microorganisms, such as insects, animal waste, or human waste that get into contact with white pepper at different stages, are other sources of *B. cereus* contamination.

The bacterium produces spores that can survive in harsh environmental conditions, such as low water activity and heat [12]-[13], and can be passed on to food when white pepper is added. Given favorable conditions, the spores germinate and produce the emetic toxin that causes the emetic syndrome or vegetative cells producing enterotoxin after ingestion of contaminated food causing diarrheal syndrome and subsequent foodborne disease outbreaks. For example, in 2010, a stew was prepared using *B. cereus* contaminated white pepper; as a result, 112 people in Denmark were poisoned [14]-[15].

Since white pepper is associated with *B. cereus* contamination and is widely used in almost all Indonesian dishes, white pepper from markets in Bogor is probably contaminated with this bacterium. To date, there is no data on white pepper contaminated with *B. cereus* in Indonesia. The purpose of this study was to determine the occurrence of *B. Cereus* in white pepper from Bogor, Indonesia.

## 2. Methodology

### Sample collection and preparation

Whole dry white pepper fruits and powder were purchased from several retailers in markets around Bogor. Fifty grams of each sample was weighed, diluted with 450mL $KH_2PO_4$ buffer, and homogenized for 120s using a Bag Mixer 400 (Interscience). Serial dilutions of $10^{-1}$ to $10^{-4}$ were made by aseptically adding 1mL of each sample to 9mL of diluent followed by vertexing for 8 seconds.

### Enumeration and isolation of presumptive *B. cereus* in white pepper

Inoculation of Mannitol egg yolk polymyxin (MYP) agar (Oxoid Ltd. UK) plates was done with $10^{-1}$ to $10^{-4}$ serial dilutions in duplicates by spreading 0.1 mL onto the surface of each plate followed by incubation for 18 to 24 h at a temperature of 30°C [16]. Plates with about 15-150 pink color colonies in a precipitate zone were counted. The number of *B. cereus* was calculated as cfu/g according to [16]. A lecithinase-positive colony was streaked onto Nutrient agar media proceeded by incubation for 18 to 24 hours to obtain pure colonies that were then slanted on nutrient agar and incubated for a period of 24 h at a temperature of 30°C.

### Confirmation of presumptive *B. cereus* isolates

Forty-nine isolates were observed to discover the morphology of colonies, lecithinase production, catalase production (using 3% hydrogen peroxide), mannitol fermentation, and microscopic observation (Gram and spore staining) with an electronic microscope (Olympus CX21) at x1000 magnification. Fifteen of the positive isolates were subjected to molecular confirmation by the use of PCR for the detection of the gene encoding for 16S rRNA.

DNA was extracted from the 15 isolates using Presto™ Mini gDNA bacteria kit (Gene aid) according to the user manual. Briefly, a loopful of *B. cereus* culture was transferred into a 10mL sterile Brain Heart Infusion Broth (BHIB), vortexed, and incubated at 30°C. 1.5mL culture, transferred to 2mL microcentrifuge tubes, and centrifuged at 14000 x g, 60sec. A dissolved mixture of Lysozyme and Gram+ Buffer was added to each sample as well as vortexed and incubated at 37ºC for 30 minutes. Proteinase K was added followed by incubation at 60ºC for 10 minutes. Lysis of cells was achieved by adding GB Buffer and being incubated at 70ºC for at least 10 minutes. This was followed by adding 5 µL of RNase with vigorous shaking and incubating at $25^0$C for 5 minutes, as well as adding absolute ethanol. The mixture was transferred to a GD Column and centrifuged at 14000 x g for 2 minutes. The DNA was washed with W1 and Wash Buffers and eluted by adding a pre-heated Elution Buffer. DNA purity and concentration were determined using Nanodrop 2000 spectrophotometer (Thermo scientific) at the absorbance of 260 and 280nm.

### Detection of 16S rRNA gene with Polymerase Chain Reaction

PCR primers 67-F: TGA AAA CTG AAC GAA ACA AAC and 1671-R: CTC TCA AAA CTG AAC AAA ACG AAA 3´[17] that targeted rRNA of conserved regions were used to amplify the 16S rRNA gene. The gene was detected to confirm the presence of *B. cereus* at an annealing temperature of $51^0$C. Reaction components in Table 1 (a) were mixed to constitute a final reaction volume of 20 µL

under running conditions in Table 1 (b) for 30 cycles [17] with modification.

**Table 1.** PCR components (a) and PCR running conditions (b) for 16S rRNA gene amplification of *B. cereus* isolates

(a)

| *B. cereus* target gene | Components | Volumes (µL) |
|---|---|---|
| 16S rRNA | 2x master mix | 10 |
| | Primer 67F (0.4 µM) | 1 |
| | Primer 1671R (0.4 µM) | 1 |
| | NFW | 7 |
| | Template DNA | 1 |
| | Total | 20 |

(b)

| PCR Steps | Temperature (°C) | Time (s) |
|---|---|---|
| Pre denaturation | 95 | 180 |
| Denaturation | 94 | 30 |
| Annealing | 51 | 45 |
| Extension | 72 | 60 |
| Final extension | 72 | 300 |

**Visualization of PCR products on agarose gel**

PCR outputs were separated on 2% agarose gel followed by visualizing DNA bands using ethidium bromide (EtBr). The gel was electrophoresed for 45 minutes at a voltage of 90V, followed by staining with EtBr for 30 minutes then being soaked in distilled water for 15 minutes. EtBr could bind the double strands of DNA, and it caused fluorescence since it contained a fluorescent substance [18]. The resulting bands on the gel were viewed under a transilluminator (Bio-Rad). The amplicon size of the DNA product was confirmed by comparing the intensity of the bands to bands of known intensity on a 1kb Hyper Ladder Marker, (Geneaid Biotech Ltd).

**Data analysis**

Plate counts inoculated from the 20 samples were enumerated as average colony counts of each that were duplicated and computed to cfu/g. The cfu/g was transformed to base-10 logarithms.

## 3. Results and discussion

**Occurrence and concentration of *B. cereus* in white pepper samples**

This study analyzed 20 white pepper samples obtained from markets in Bogor. The result showed that 18 of them (90%) were contaminated with presumed *B. cereus* (Figure 1). The total number of presumptive *B. cereus* in the white pepper varied from 1.70 to 5.77 $\log_{10}$ cfu/g. Based on [15], 9 of the 20 samples (45%) showed unsatisfactory status (>4 $\log_{10}$ cfu/g), 7 samples (35%) had acceptable status (<4 $\log_{10}$ cfu/g), and only 4 samples (20%) had satisfactory status (<3$\log_{10}$cfu/g). These results are summarized in Figure 1 (b).

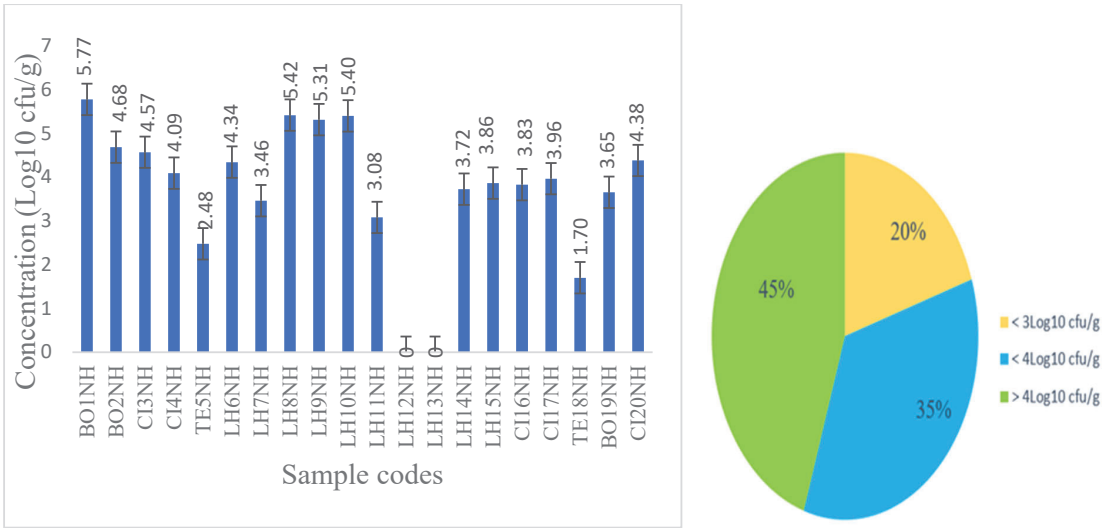


**Figure 1.** (a) The occurrence and concentration of *B. cereus* in white pepper samples; (b) the classification of white pepper samples based on a load of presumptive *B. cereus*

Our results were in line with those of other researchers. For example, [11] conducted a study in the Netherlands and discovered that white pepper contained $>10^4$cfu/g counts of *B. cereus*. An equal case also occurs to ginger and mixed spices. IA study by [10] found that white pepper in Turkey contained $10^2$ cfu/g of *B. cereus*, and [8]reported that untreated black and white pepper in Korea contained $3.0\text{-}5.0\times10^5$ cfu/g of *B. cereus*.

Although white pepper is a Low Moisture Food (LMF) and will therefore not be considered ideal for the growth of *B. cereus* whose minimum water activity is 0.93[12], the bacterium producing spores, which can survive in harsh environmental conditions, requires low water activity, pH, and heat [12]. Moreover, this bacterium can be passed to food to which white pepper is added. Favorable conditions enable the spores to germinate and multiply into vegetative cells that produce the emetic toxin which causes the emetic syndrome or enterotoxin when the food is consumed; such a condition results in food-borne disease and outbreaks [3], [7]. Furthermore, postharvest processes cause further contamination of the spice. White pepper is dried on soil and retted in water from contaminated sources, such as rivers and swamps [2]. Moreover, even when boiled water is used, blanching leads to recontamination by *B. cereus* spores. Further processing stages, such as drying, grinding, blending, packaging, and storage are carried out under unhygienic conditions [15], which further contribute to the emergence of *B. cereus* into the spice.

Most white pepper samples (45%) showed unsatisfactory status (*B. cereus* concentration > 4 $\log_{10}$ cfu/g). These values represent a possible foodborne poisoning risk and should be considered cautiously if found in dried spices and herbs [15].

**Morphological/biochemical characteristics of *B. cereus* colonies from white pepper**

All colonies were observed on MYP supplemented with egg yolk emulsion and polymyxin B exhibited archetypal colony morphology. They were pink in color and showed a zone of precipitation implying production of lecithinase through lecithin hydrolysis. The presence of eosin pink growth and surrounding media also indicated that mannitol was not fermented, and it is the characteristic of *B. cereus.*

Forty-nine isolates were subjected to Gram staining, spore staining, and catalase reaction. All isolates showed positive results (purple color, green color, and rapid release of oxygen bubbles respectively). Gram staining was performed to determine the chemical composition of the cell wall of bacteria analyzed. *Bacillus cereus* was gram-positive implying that a thick peptidoglycan cell wall surrounded its plasma membrane. Thus, the retention was the purple dye. A rapid release of oxygen bubbles implied the presence of catalase which was used to differentiate *Clostridia* aerotolerant strains from *Bacillus* species. *Clostridia* were catalase-negative while *Bacillus* are positive. These results

proved that the organism in the samples was *B. cereus.*

**Confirmation of *B. cereus* using Polymerase Chain Reaction**

The presence of the 16S rRNA gene in *B. cereus* isolates was determined using 67F and 1671R primers at a concentration of 0.4 µM [17]. The 15 tested isolates showed a thick and clear band at the expected length of around 1686 bp (Figure 2) after being separated from 3µL PCR products on ethidium bromide-stained 2% agarose gel. This separation confirms the presence of *B. cereus*. The 16S rRNA gene has been extensively used in bacterial identification because its entire nature was highly conserved [19], but some regions encountered vast disparity.

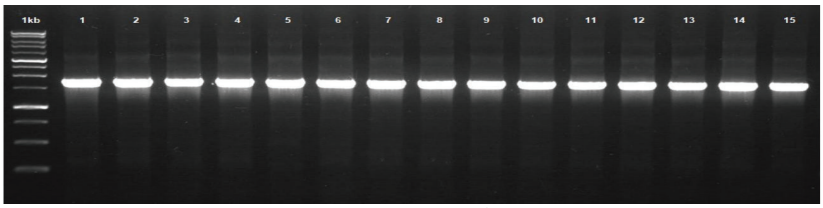


**Figure 2.** Visualization of 16S rRNA gene using PCR (1st bar with 1kb ladder; lanes 1 to 15 referring to isolates 1 to 15)

**4. Conclusion**

This study discovered that white pepper from markets around Bogor area was highly contaminated with *B. cereus* with concentrations of up to 5.77 log10 cfu/g. The majority of the samples (45%) exhibited unsatisfactory microbial status, presenting a possible foodborne poisoning risk. Therefore, the white pepper should be considered cautiously when used, especially for ready-to-eat food.

**Acknowledgment**

We thank the Directorate General for Higher Education, Ministry of Education and Culture - Indonesia for funding this research.

**References**

[1] E. Hartulistiyoso, Y. A. Purwanto, and A. M. A. Mukhlis, “Spouted Bed Drying of White Pepper (Piper nigrum L.) with Microwave Preheating Treatment,” *IOP Conf. Ser. Mater. Sci. Eng.*, vol. 557, no. 1, 2019, doi: 10.1088/1757-899X/557/1/012045.

[2] A. G. Mathot, F. Postollec, and I. Leguerinel, “Bacterial spores in spices and dried herbs: The risks for processed food,” Compr. Rev. Food Sci. Food Saf., vol. 20, no. 1, pp. 840–862, 2021, doi: 10.1111/1541-4337.12690.

[3] A. Tewari and S. Abdullah, “*Bacillus cereus* food poisoning: International and Indian perspective,” *J. Food Sci. Technol.*, vol. 52, no. 5, pp. 2500–2511, Apr. 2015, doi: 10.1007/s13197-014-1344-4.

[4] Pedoman Penerapan Peraturan Badan POM tentang cemaran mikroba dalam pangan olahans, PerBPOM. No. 13/2019, Direktorat Standardisasi Pangan Olahan Deputi Bidang Pengawasan Pangan Olahan Badan POM, Jakarta, Indonesia, July. 2019.

[5] A. Berthold-Pluta, A. Pluta, M. Garbowska, and I. Stefańska, “Prevalence and toxicity characterization of *Bacillus cereus* in food products from Poland,” *Foods. MDPI*, vol. 8, no. 7, p. 269-271, July. 2019, doi: 10.3390/foods8070269.

[6] U. Hariram and R. G. Labbé, “Growth and inhibition by spices of growth from spores of enterotoxigenic *Bacillus cereus* in cooked rice,” Food Control, vol. 64, pp. 60–64, Dec. 2016, doi: 10.1016/j.foodcont.2015.12.024.

[7] M. W. Griffiths and H. Schraft, "*Bacillus cereus Food Poisoning*", in *Foodborne Diseases*. C. E. R Dodd, 3rd ed., Elsevier Inc., 2017. pp. 395-405.

[8] K. Min Park, M. Jeong, K. J. Park, and M. Koo, “Prevalence, enterotoxin genes, and antibiotic resistance of *Bacillus cereus* isolated from raw vegetables in Korea,” *J. Food Prot.*, vol. 81, no. 10, pp. 1590–1597, Aug. 2018, doi: 10.4315/0362-028X.JFP-18-205.

[9] S. Yu *et al.*, "A Study on prevalence and characterization of *Bacillus cereus* in Ready-to-Eat Foods in China," *Front. Microbiol.*, vol. 10, no. 3043, pp. 1–11, Jan. 2020, doi: 10.3389/fmicb.2019.03043.
[10] H. Hampikyan, E. B. Bingol, H. Colak, and A. Aydin, "The evaluation of microbiological profile of some spices used in Turkish meat industry," *J. Food, Agric. Environ.*, vol. 7, no. 3–4, pp. 111–115, 2009.
[11] E. De Boer and E. M. Boot, "Comparison of methods for isolation and confirmation of *Clostridium perfringens* from spices and herbs," *J. Food Prot.*, vol. 46, no. 6, pp. 533–536, 1983, doi: 10.4315/0362-028x-46.6.533.
[12] D. Rodrigo, C. M. Rosell, and A. Martinez, "Risk of *Bacillus cereu*s in relation to rice and derivatives," *Foods*, vol. 10, no. 2, 2021, doi: 10.3390/foods10020302.
[13] A. Bianco et al., "Characterization of *Bacillus cereus* group isolates from human bacteremia by Whole-Genome Sequencing," *Front. Microbiol*., vol. 1-15, no. 599524, 11, pp. Jan. 2021, doi: 10.3389/fmicb.2020.599524.
[14] J. M. Van Doren, K. P. Neil, M. Parish, L. Gieraltowski, L. H. Gould, and K. L. Gombas, "Foodborne illness outbreaks from microbial contaminants in spices," *Food Microbiol.*, vol. 36, no. 2, pp. 456–464, 2013, doi: 10.1016/j.fm.2013.04.014.
[15] European Food Safety Authority, "Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations)," *EFSA J.*, vol. 11, no. 1, 2013, doi: 10.2903/j.efsa.2013.3025.
[16] Bacteriological Analytical Manual on *Bacillus cereus* (2012). Accessed. Apr. 4, 2021. [online]. Available: https://www.fda.gov/food/laboratory-methods-food/bam-*bacillus-cereus*
[17] C. T. Sacchi et al., "Sequencing of 16S rRNA gene: A rapid tool for identification of *Bacillus anthracis*," *Emerg. Infect. Dis.*, vol. 8, no. 10, pp. 1117–1123, 2002, doi: 10.3201/eid0810.020391.
[18] M.K. Fatchiyah, E. L Arumningtyas, S. Widyarti, E. L. Arumningtyas and S. Permana, " Buku Praktikum teknik Analisis Biologi Moleculer," Jurusan Biologi Univ. Brawijaya Malanga, Indonesia. 2012. pp. 1–49.
[19] S. Raza and A. Ameen, "Comparison of 16S rRNA gene of *Bacillus cereus* with different bacterial species," *J. Adv. Biol. Biotechnol*., vol. 7, no. 2, pp. 1–6, 2016, doi: 10.9734/jabb/2016/26524.