

Article Review: Phenotypic and Genotypic Identification of *Bacillus cereus* in Soy Milk

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Abstract.

Bacillus cereus is a Gram-positive, spore-forming bacterium frequently found as a contaminant in various food products, including soy milk, and is recognized as a causative agent of foodborne illnesses. Its pathogenicity mainly arises from the production of toxins such as non-hemolytic enterotoxin (*nhe*), hemolysin BL (*hbl*), and cytotoxin K (*cytK*), which contribute to gastrointestinal disorders. Conventional phenotypic identification methods, including culture, morphological observation, and biochemical assays, are simple but often lack specificity in distinguishing *B. cereus* from the *B. cereus* group. Therefore, genotypic approaches based on molecular techniques such as PCR and Whole Genome Sequencing (WGS) are required to achieve higher sensitivity and precision. The *gyrB* gene serves as a molecular marker for species confirmation, while the *nhe* gene complex acts as a virulence determinant in identifying toxigenic strains. The integration of phenotypic and genotypic methods provides a more comprehensive understanding of *B. cereus* contamination in soymilk, elucidating its genetic diversity and pathogenic potential, which are essential aspects of food safety assessment.

Keywords: *Bacillus cereus*, phenotypic approach, genotypic approach, *gyrB* gene, *nheB* gene and food safety.

I. INTRODUCTION

Soybean can be processed into various food products through two main approaches: fermentation and non-fermentation. Fermented soybean products include tempeh, soy sauce, and tauco, whereas non-fermented products include tofu, soybean curd sheets, soy milk, and soybean flour (Hendriani & Budiarmo, 2020; Mustika et al., 2019). These soybean-based foods are widely consumed in Indonesia as an important source of plant-based protein. However, while serving as a nutritional source, these products are also at risk of bacterial contamination at several stages, including raw material selection, processing, equipment handling, serving, and storage (Hendriani & Budiarmo, 2020).

Traditional processing practices that are still commonly used increase the susceptibility of these products to microbial contamination. Consumption of contaminated products may lead to foodborne diseases. The major bacteria associated with such illnesses include *Escherichia coli*, *Listeria*, *Campylobacter*, *Salmonella spp.*, *Staphylococcus aureus*, and *Bacillus cereus* (Mustika et al., 2019; Arisanti et al., 2018). In particular, soy milk is highly susceptible to contamination due to its rich content of protein, fat, carbohydrates, vitamin B₂, pyridoxine, and isoflavones, which provide a favorable growth medium for microorganisms (Azizah & Octavia, 2023; Cahyani, 2019). Microorganisms commonly found contaminating soy milk include coliform bacteria, *E. coli*, *Salmonella spp.*, *Staphylococcus aureus*, *Bacillus cereus*, and molds (Azizah & Octavia, 2023; Butovskaya et al., 2025).

Bacillus cereus is a Gram-positive, ubiquitous bacterium that can be found in soil, water, and food products, including raw materials used in the food industry (Azizah & Octavia, 2023; Enosi Tuipulotu et al., 2021). This bacterium is capable of forming spores and exhibits heat resistance. The spores of *B. cereus* may survive and proliferate during soy milk processing. Inappropriate storage conditions may allow spores to germinate into vegetative cells, supported by the nutritional content and water activity of soy milk (Enosi Tuipulotu et al., 2021; Hasanah et al., 2022).

Food poisoning cases in Indonesia in 2020 were attributed to *Staphylococcus aureus* (30%), *Bacillus cereus* (26.67%), *Salmonella spp.* (16.67%), *Escherichia coli* (16.67%), and *Clostridium spp.* (6.67%) (Apriliansyah et al., 2022). The high proportion of *B. cereus* indicates its significant role as an agent of food

poisoning. Therefore, a literature review on phenotypic and genotypic approaches for identifying *Bacillus cereus* in soy milk is essential to better understand available detection methods and the pathogenic potential of this bacterium (Enosi Tuipulotu et al., 2021; Yennie et al., 2022).

II. METHODOLOGY

This article was prepared using a literature review approach. Relevant national and international journals related to soybeans, soy milk, *Bacillus cereus*, Identification conventional and molecular were used as sources. Research articles were obtained through keyword-based searches on Google Scholar, ScienceDirect, and PubMed. Only sources published within the last ten years were included in this review.

III. RESULTS AND DISCUSSIONS

This article uses eleven journals related to soybeans, soy milk, *Bacillus cereus*, Identification conventional and molecular. The selection of these journals considered similarity of the bacteria examined, similarity of examination methods, and publication of the journal.

Soybean

Soybeans are one of the most important food commodities widely utilized as a source of plant-based protein. They contain essential nutrients such as plant protein, fats, vitamins, and minerals (Hendriani & Budiarsa, 2020). Fermented soybean products include tempeh, soy sauce, and tauco. In addition to fermented products, soybeans are also extensively processed into non-fermented products such as tofu, soybean curd sheets, soy milk, and soybean flour (Mustika et al., 2019). Soy milk has increasingly gained popularity as an alternative to cow's milk (Azizah & Octavia, 2023). The consumption of soy milk continues to rise in line with the growing adoption of plant-based diets and increasing cases of lactose intolerance and cow's milk allergy. Analysis of the NHANES data shows that the prevalence of soy milk consumption in the United States reached 1.5% during the 2017–2020 period (Storz et al., 2024).

The production of soy milk involves several stages, including soaking, grinding, heating, and filtration (Cahyani, 2019). The soaking process is carried out for 8–12 hours using water to separate the soybean hulls from the seeds. The beans are then soaked again for about 10 minutes to soften them before being ground with the addition of hot water, using a water-to-soybean ratio of 7:1. The resulting slurry is filtered and subsequently heated for approximately 10 minutes after boiling. Following the heating step, the soy milk is filtered again before being packaged into bottles (Cahyani, 2019).

These processing steps must be carried out hygienically, as each stage presents potential entry points for pathogenic microorganisms. Contamination may occur through the water used to clean the material, unsterile equipment, production environment, or post-processing handling, including storage and distribution (Hasanah et al., 2022). Soy milk produced under poor hygienic conditions may be exposed to various contaminating microorganisms that can compromise product quality and safety (Cahyani, 2019). Microorganisms commonly found contaminating soy milk include coliform bacteria, *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus*, *Bacillus cereus*, and molds (Azizah & Octavia, 2023; Butovskaya et al., 2025).

Bacillus cereus

1. General Overview

Bacillus cereus is a Gram-positive, rod-shaped, facultatively aerobic bacterium capable of forming heat-resistant spores. It possesses peritrichous flagella that contribute to its motility. Taxonomically, it belongs to the genus *Bacillus* and the species *Bacillus cereus*. It is ubiquitous in nature and widely distributed in the environment, particularly in soil, water, and plants (Enosi Tuipulotu et al., 2021). Due to its ability to survive in soil, it is frequently detected in soybean seeds (Enosi Tuipulotu et al., 2021).

This bacterium forms spores that can withstand various food processing conditions, including cooking. The spores are elongated in shape and consist of complex structural components, including the core, which is surrounded by the inner membrane, peptidoglycan cortex, inner coat, and outer coat (Enosi Tuipulotu et al., 2021). These components provide high resistance to physical, chemical, and biological stress. The spores are metabolically inactive and highly resistant to heat, freezing, drying, and gamma or ultraviolet radiation. This resilience allows spores to persist in the environment until more favorable conditions support germination.

Additionally, *B. cereus* spores play a role in facilitating adhesion to human epithelial cells (Enosi Tuipulotu et al., 2021).

2. Pathogenesis

The pathogenesis of *Bacillus cereus* is primarily associated with its ability to produce various toxins that contribute to colonization, invasion, and tissue damage (Enosi Tuipulotu et al., 2021). Generally, *B. cereus*-associated illness is categorized into two syndromes: emetic and diarrheal (Ramarao et al., 2020). The emetic syndrome is caused by cereulide toxin, a hydrophobic cyclic depsipeptide synthesized by a non-ribosomal peptide synthetase (NRPS) encoded by the *ces* gene. This toxin is heat-stable and resistant to digestive enzymes. *Cereulide* functions as a potassium ionophore, leading to mitochondrial membrane depolarization. Consequently, it disrupts cellular respiration and energy production, resulting in acute vomiting a few hours after consumption of contaminated food (Enosi Tuipulotu et al., 2021).

Meanwhile, the diarrheal syndrome is caused by enterotoxins secreted during bacterial growth in the small intestine, including *hemolysin BL (hbl)*, *non-hemolytic enterotoxin (nhe)*, and *cytotoxin K (cytK)* (Dietrich et al., 2021). In addition to toxin production, *B. cereus* also produces extracellular enzymes such as phospholipase C, hemolysins, and proteases, which contribute to tissue degradation and facilitate host cell invasion (Enosi Tuipulotu et al., 2021). The bacterium also possesses additional virulence factors, including an S-layer that enhances adhesion to epithelial surfaces and biofilm formation (Enosi Tuipulotu et al., 2021). Furthermore, *B. cereus* toxins can induce macrophage activation and inflammation via the NLRP3 inflammasome pathway, triggering the release of IL-1 β and TNF- α . This inflammatory response contributes to tissue damage and exacerbates gastrointestinal symptoms (Enosi Tuipulotu et al., 2021).

3. Health Impacts

Contamination of food by *Bacillus cereus* may lead to food poisoning characterized by symptoms such as nausea, vomiting, sore throat, abdominal cramps, diarrhea, visual disturbances, fever, and chills (Enosi Tuipulotu et al., 2021). In severe cases, foodborne poisoning may result in foodborne outbreak events (Hasanah et al., 2022). Based on causative agents, reported incidences have identified *Escherichia coli* (20%), *Bacillus cereus* (19.4%), *Staphylococcus spp.* (18.3%), and other bacteria (17.1%) as major contributors (Arisanti et al., 2018).

Beyond compromising food quality, contamination with *B. cereus* directly affects consumer health through the onset of food poisoning symptoms (Enosi Tuipulotu et al., 2021). These cases are often associated with foods stored at room temperature for extended periods, allowing spores to germinate into vegetative cells and produce toxins (Hasanah et al., 2022). Once formed and ingested, these toxins can trigger foodborne illness with various clinical manifestations (Arisanti et al., 2018).

4. Phenotypic Identification

Phenotypic identification is a method used to determine the type or species of microorganisms based on observable external characteristics, including physical features, morphology, and biochemical properties (Ahaotu et al., 2013). This approach involves examination of cell shape, Gram staining results, spore-forming ability, growth characteristics on specific culture media, hemolysis patterns, and reactions in various biochemical tests (Widodo et al., 2022). Phenotypic approaches are commonly used as an initial step to classify bacterial groups because they provide direct visual and metabolic characteristics that can be readily observed (Ahaotu et al., 2013).

Phenotypically, *B. cereus* is a Gram-positive, rod-shaped bacterium that is aerobic or facultatively anaerobic and capable of forming endospores resistant to extreme environmental conditions (Enosi Tuipulotu et al., 2021). Prior to inoculation on selective media, serial dilutions such as 10¹, 10², and 10³ are typically prepared to reduce bacterial density (Dos Anjos et al., 2020). Culture on selective media such as Bacillus Cereus Agar (BCA) or Blood Agar Plate (BAP) plays an essential role in observing colony characteristics (Yennie et al., 2022). Colonies of *B. cereus* generally appear large, dull white to grayish, non-mucoid, and typically exhibit β -hemolysis zones surrounding the colonies on BAP, attributable to the activity of hemolytic toxins such as *hemolysin BL (hbl)* (Enosi Tuipulotu et al., 2021). BAP allows differentiation between hemolytic and non-hemolytic bacteria, identified by the appearance of clear halo zones surrounding colonies, which may indicate *B. cereus* contamination (Azizah & Octavia, 2023).

However, variations exist among studies regarding phenotypic identification of *B. cereus*. In some studies, initial cultivation is performed on general media, followed by selective media as preliminary confirmation before biochemical testing (Azizah & Octavia, 2023). In contrast, other studies perform enumeration using selective Bacillus Cereus Agar (BCA) followed by hemolysis confirmation on BAP when growth is detected (Butovskaya et al., 2025).

Biochemical testing generally involves inoculation onto glucose, lactose, mannitol, maltose, and sucrose media. A positive reaction is indicated by a color change from red to yellow (Yennie et al., 2022). In addition, testing on Sulfide Indole Motility (SIM) medium is used to assess indole production and bacterial motility (Enosi Tuipulotu et al., 2021). Typical biochemical characteristics of *B. cereus* include positive reactions for glucose, maltose, sucrose, sulfur production, motility, and citrate utilization, while negative reactions are usually observed for lactose, mannitol, and indole (Enosi Tuipulotu et al., 2021). Suspected *B. cereus* isolates are further inoculated on BCA, where positive results are indicated by turquoise blue colonies surrounded by an opaque zone (Azizah & Octavia, 2023).

In addition to colony observation on agar media, spore staining serves as a confirmatory technique to determine the location of endospores within vegetative cells (Enosi Tuipulotu et al., 2021). The Shaeffer–Fulton staining method is commonly used to detect spores of *Bacillus sp.*, employing malachite green as the primary stain and safranin as a counterstain (Widodo et al., 2022). Although various spore-staining methods exist, the Shaeffer–Fulton method provides strong contrast between endospores and bacterial cells, is cost-effective, and utilizes easily obtainable reagents (Widodo et al., 2022). The staining results typically show green-colored spores and red vegetative cells against a pale red background (Widodo et al., 2022).

The advantage of phenotypic approaches lies in their ability to provide direct visual results, making them useful as preliminary screening tools for bacterial isolates (Ahaotu et al., 2013). However, their limitation is relatively lower specificity compared to molecular methods such as PCR (Enosi Tuipulotu et al., 2021). Therefore, culture-based observations and spore staining should be complemented with additional biochemical tests and molecular approaches to achieve more specific identification (Yennie et al., 2022). Phenotypic assessment through culture and spore staining plays a crucial role in evaluating morphological characteristics, sporulation ability, and potential virulence prior to molecular confirmation. The combination of phenotypic and genotypic data provides a more accurate and comprehensive microbiological analysis (Enosi Tuipulotu et al., 2021).

5. Genotypic Identification

The genotypic approach is an identification method based on the analysis of genetic material (DNA) to determine species (Ahaotu et al., 2013). This approach is used to complement phenotypic identification based on morphological characteristics and biochemical tests (Enosi Tuipulotu et al., 2021). Genetic analysis enables the detection of specific genes, virulence genes, and phylogenetic relationships among strains that cannot be distinguished using phenotypic methods (Ahaotu et al., 2013). Therefore, the genotypic approach provides higher specificity and sensitivity in microbial identification (Enosi Tuipulotu et al., 2021).

The most commonly used genotypic method is the Polymerase Chain Reaction (PCR), particularly conventional PCR or endpoint PCR, which is an in vitro DNA amplification technique using primers specific to target genes (Yennie et al., 2022). One of the genes frequently used for confirmation of *Bacillus cereus* is the *gyrB* gene, which encodes the B subunit of DNA gyrase (Enosi Tuipulotu et al., 2021). This gene has higher specificity compared to the 16S rRNA gene due to its greater sequence variability, allowing differentiation among closely related species within the *Bacillus* group (Ahaotu et al., 2013). In this method, genomic DNA is isolated from bacterial isolates and amplified using PCR with gene-specific primers. The amplified products are then analyzed using agarose gel electrophoresis and visualized under a UV transilluminator to observe DNA bands corresponding to the target size (Yennie et al., 2022).

Conventional PCR has several advantages, including high sensitivity and specificity, the ability to detect target genes in small quantities, and relatively faster analysis compared to conventional culture-based methods (Enosi Tuipulotu et al., 2021). In addition, this method is not influenced by the physiological condition of the bacteria and can be used as an accurate species confirmation tool (Yennie et al., 2022). However, this method also has limitations, as it cannot distinguish between live and dead bacteria because PCR detects the

presence of DNA rather than biological activity (Enosi Tuipulotu et al., 2021). Furthermore, this method requires high-quality DNA and optimal reaction conditions to ensure efficient amplification (Yennie et al., 2022). Other challenges in PCR application include the presence of PCR inhibitors originating from food matrices, the risk of DNA contamination leading to false-positive results, and genetic variation among strains that may affect amplification efficiency (Ahaotu et al., 2013).

The genotypic approach can also be used to detect virulence genes involved in pathogenicity, such as toxin-encoding genes including *ces*, *nhe*, *hbl*, and *cytK* (Enosi Tuipulotu et al., 2021). Genomic analysis of clinical, food, and environmental strains has shown that the *hbl*, *nhe*, and *cytK* genes are commonly present, with distributions of 40–90%, 95–98%, and 50–80%, respectively, among *Bacillus cereus* isolates (Dietrich et al., 2021). The high prevalence of these genes indicates that most isolates have virulence potential and may cause disease (Dietrich et al., 2021). The *nhe* gene, which encodes the non-hemolytic enterotoxin, is considered one of the major virulence factors of *Bacillus cereus* (Dietrich et al., 2021). Another study reported the distribution of *nheA*, *nheB*, and *nheC* genes at 85–100% among various isolates (Osama et al., 2020). In soybean milk samples, the distribution of *nheA*, *nheB*, and *nheC* genes was reported as 86.9%, 93.4%, and 89.1%, respectively (Osama et al., 2020). The high prevalence of these genes indicates that most *Bacillus cereus* isolates from dairy products have the potential to produce enterotoxins associated with diarrheal symptoms. The *nhe* toxin complex acts synergistically by forming pores in the host cell membrane, leading to cell lysis and disruption of intestinal function (Dietrich et al., 2021).

The distribution of these virulence genes has also been reported in various food samples, including lettuce, frozen foods, and ready-to-eat foods, with varying prevalence rates (Qu et al., 2021; Guo et al., 2021; Yu et al., 2020). This variation is influenced by sample type, isolate source, and environmental conditions (Qu et al., 2021; Guo et al., 2021). Detection of virulence genes is generally performed using conventional PCR with gene-specific primers (Yennie et al., 2022). This method provides advantages in rapidly and specifically detecting the virulence potential of isolates and can be used to evaluate the pathogenic risk of foodborne isolates (Enosi Tuipulotu et al., 2021). Furthermore, PCR also allows simultaneous detection of multiple virulence genes using multiplex PCR, thereby improving analytical efficiency (Yennie et al., 2022).

However, PCR-based detection of virulence genes also has limitations, as it cannot determine whether the detected genes are actively expressed or producing toxins (Enosi Tuipulotu et al., 2021). In addition, the presence of PCR inhibitors, poor DNA quality, and the need for optimized reaction conditions represent major challenges in the application of this method (Yennie et al., 2022). Genetic variation among strains may also affect amplification efficiency and detection accuracy (Ahaotu et al., 2013).

IV. CONCLUSION

Based on the review findings, the combination of phenotypic and genotypic approaches provides more comprehensive identification results in detecting and confirming the presence of *Bacillus cereus* in soybean milk. The phenotypic approach serves as an initial step to observe colony morphology, microscopic characteristics, and biochemical properties, thereby enabling preliminary bacterial identification. However, the phenotypic approach has limitations in differentiating species with similar characteristics. The genotypic approach using conventional PCR provides higher specificity and sensitivity for species confirmation through the detection of specific genes. Nevertheless, this method also has limitations; therefore, the combined use of phenotypic and genotypic approaches is essential to achieve more accurate and comprehensive identification results.

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