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Research Article

Molecular identification of rice bran isolate bacteria based on 16s rRNA gene: A comparative study of dna extraction methods

Trifan Budi[1,2], Muhammad Shobihul Khoir[3*]

[1]School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand

[2]Animal Genomic and Bioresource Research Unit (AGB Research Unit), Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

[3*]Chemistry Department, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim Malang

*Correspondence: muhammadshobihulkhoir@gmail.com

Abstract

DNA extraction serves as a critical preliminary step for molecular identification, and the application of such molecular techniques has become indispensable for analyzing bacterial diversity in rice bran isolates. This study aimed to confirm bacterial species based on 16S rRNA gene analysis. Two DNA isolation methods were compared: a chemical method utilizing *Cetyl Trimethyl Ammonium Bromide* (CTAB/NaCl) and an enzymatic method employing a commercial kit. The CTAB/NaCl method yielded DNA with a purity of 1.83 for isolate BR, while the enzymatic method produced higher purity values (2.04 for isolate TR). Amplification of the 16S rRNA gene was performed using universal primers 27F and 1492R. Sequence data were analyzed using BLAST-N and phylogenetic tree reconstruction software. BLAST-N analysis revealed that both BR and TR isolates belong to the genus *Bacillus*, with 99% identity values. Phylogenetic analysis further demonstrated that both isolates are closely related to *Bacillus methylotrophicus* strain CBMB205. The novelty of this research lies in the comparative evaluation of DNA extraction methods specifically optimized for rice bran isolates, a substrate with complex composition that poses unique challenges for DNA isolation, as well as the first molecular confirmation of *Bacillus methylotrophicus* from Indonesian rice bran.

Keywords: *Bacillus methylotrophicus*, CTAB method, enzymatic method, 16S rRNA gene, rice bran bacteria

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1. Introduction

Molecular identification has become an essential approach in modern microbiology, offering precision that traditional phenotypic methods cannot achieve. Phenotypic methods relying on morphological and biochemical characterization often fail to provide clear species-level discrimination due to similarities among distinct bacterial taxa [1]. This limitation is particularly problematic when analyzing complex substrates such as rice bran, where multiple species with similar phenotypes may coexist.

Indonesia generates approximately 13 million tons of rice bran annually, an abundant agricultural byproduct rich in carbohydrates, proteins, and lipids that harbors significant but underexplored bacterial diversity [2,3]. Previous research has documented the isolation of cellulolytic bacteria such as *Bacillus subtilis* and *Bacillus cereus* from rice bran; however, these studies relied predominantly on phenotypic methods that cannot definitively resolve species identities within the closely related *Bacillus* species complex [3,4]. The estimation of bacterial biodiversity has been further constrained by the inherent difficulties associated with cultivating bacteria from natural environments, with approximately 99% of bacteria remaining unculturable using standard techniques [5].

The 16S rRNA gene has emerged as the gold standard molecular marker for bacterial taxonomy, containing conserved regions for universal amplification and variable regions for species discrimination [1,6]. This gene enables the construction of phylogenetic trees that illustrate evolutionary relationships among bacteria, providing insights into their ecological and biotechnological potential [7]. However, successful molecular identification is highly dependent on the quality of extracted DNA. Rice bran presents unique technical challenges for DNA extraction due to high levels of polysaccharides, lipids, and phenolic compounds that co-purify with DNA and inhibit downstream enzymatic reactions such as PCR [8].

Enzymatic methods using commercial kits have gained popularity due to their simplicity and ability to yield high-purity DNA. However, these kits are relatively expensive and may not be accessible to all research laboratories, particularly in developing countries [9]. Conversely, chemical methods utilizing surfactants such as CTAB (Cetyl Trimethyl Ammonium Bromide) offer a cost-effective alternative. Modified CTAB protocols have been successfully applied to various bacterial species, including *Staphylococcus aureus* and *Shigella dysentriae* [9]. Nevertheless, systematic comparative studies of DNA extraction methods specifically optimized for rice bran isolates remain absent from the literature.

Bacillus methylotrophicus strain CBMB205, originally isolated from rice rhizosphere soil in Korea, has been characterized as a Gram-positive, aerobic, rod-shaped bacterium with plant-growth-promoting properties, including phosphate solubilization and phytohormone production [10,11]. Complete genome sequencing revealed 19 phosphatase genes and 14 thiamine synthesis-related genes, highlighting its biotechnological potential for agricultural applications [11]. Despite the growing interest in rice bran-associated bacteria and the recognized potential of *Bacillus methylotrophicus*, its presence in Indonesian rice bran has not been previously documented through molecular methods.

The novelty of this research lies in four interconnected aspects: (1) methodological innovation through the first systematic comparison of CTAB/NaCl versus enzymatic DNA extraction methods specifically evaluated for rice bran bacterial isolates; (2) geographical novelty as the first molecular investigation of *Bacillus methylotrophicus* from Indonesian rice bran; (3) phylogenetic advancement through near-full-length 16S rRNA gene sequencing for high-resolution species identification within the *Bacillus subtilis* complex; and (4) practical application by establishing cost-effective molecular identification protocols suitable for resource-limited laboratory settings in developing countries.

Based on the background above, this study aimed to: (1) evaluate the effectiveness of CTAB/NaCl and enzymatic DNA extraction methods for bacterial isolates from rice bran; (2) identify bacterial species from rice bran isolates based on 16S rRNA gene sequence analysis; and (3) construct phylogenetic trees to determine the genetic relationship of the isolates with related bacterial species.

2. Materials and Methods

2.1 Materials

Two bacterial isolates, designated BR and TR, were obtained from the Biotechnology Laboratory, Department of Chemistry, Universitas Islam Negeri Maulana Malik Ibrahim Malang. These isolates were originally cultured from local rice bran samples collected from traditional rice mills in East Java, Indonesia. Bacteria were maintained on Nutrient Agar (NA) medium (Merck, Germany) and propagated in Nutrient Broth (NB) medium (Merck). Reagents for DNA isolation included CTAB 2% (Merck), NH_4COOH 5 M (Merck), NaCl 5 M (Merck), SDS 10% (MP Biomedicals, USA), chloroform (Merck), isopropanol (Merck), TE buffer (10 mM Tris from Vivantis, 10 mM EDTA from Merck) pH 8, isoamyl alcohol (AppliChem, Germany), phenol (Merck), and 70% ethanol. Enzymatic DNA extraction was performed using a commercial kit (GeneAll, Korea). DNA qualification utilized agarose gel electrophoresis (SciencePreneur, India), TBE buffer (tris, boric acid, EDTA), TAE buffer (acetic acid, EDTA), EtBr (ethidium bromide), and loading dye (Vivantis, Malaysia). PCR amplification employed the Maxime PCR Premix Kit (i-Taq, Korea) with universal primers 27F (5'-AGAGTTTGATCATGGCTGA-3') and 1492R (5'-TACGGCTACCTTGTTACGA-3') (Integrated DNA Technologies, USA).

2.2 Methods

2.2.1 Bacterial Cultivation

BR and TR isolates were cultured on NA medium at 25°C for 24 hours to obtain pure colonies. Subsequently, single colonies were transferred to NB medium and incubated at 30°C with shaking at 150 rpm for 18 hours to reach exponential growth phase. Microscopic examination (BioRad, USA) was performed at 1000× magnification following Gram staining to assess bacterial morphology.

2.2.2 Enzymatic DNA Isolation

A 3 mL aliquot of bacterial culture was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 6000 × g for 2 minutes (BioRad, USA). After removing the supernatant, the pellet was resuspended in 100 µL R1 buffer and 20 µL lysozyme (20 mg/mL), followed by vortex mixing for 15 seconds. The sample was incubated at 37°C for 20 minutes to facilitate cell lysis, then centrifuged at 10000 × g for 3 minutes. The supernatant was discarded, and the pellet was resuspended in 180 µL R2 buffer with 20 µL proteinase-K (20 mg/mL). Following incubation at 65°C for 20 minutes, 20 µL RNase A (10 mg/mL) was added to remove RNA contaminants, and the sample was incubated at 37°C for 5 minutes. Subsequently, 440 µL BG buffer (binding buffer) was added, and the sample was incubated at 65°C for 10 minutes, followed by addition of 200 µL absolute ethanol. The entire mixture was transferred to a spin column and centrifuged at 10000 × g for 1 minute. The column was washed with 650 µL washing buffer and centrifuged at 10000 × g for 1 minute, followed by an additional centrifugation step at full speed for 2 minutes to remove residual ethanol. The DNA pellet was eluted by adding 50-100 µL elution buffer to the column membrane, incubating at room temperature for 2 minutes, and centrifuging at 10000 × g for 1 minute. Isolated DNA was stored at -20°C for long-term preservation.

2.2.3 Chemical DNA Isolation (CTAB/NaCl Method)

A 3 mL bacterial culture was centrifuged at 10000 rpm for 2 minutes. The pellet was resuspended in 567 µL TE buffer by gentle pipetting, then 30 µL of 10% SDS and 3 µL of 5 M NH_4COOH were added to facilitate cell lysis and protein precipitation. The solution was incubated at

37°C for 1 hour with occasional mixing. Following lysis, 100 µL of 5 M NaCl was added to increase ionic strength, followed by 80 µL of 2% CTAB prepared in 0.7 M NaCl. The mixture was incubated at 65°C for 10 minutes to form CTAB-nucleic acid complexes. An equal volume of chloroform:isoamyl alcohol (24:1) was added, and the mixture was gently inverted for 5 minutes, then centrifuged at 12000 rpm for 4 minutes to separate phases. The aqueous phase (upper layer) was carefully transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to further purify the DNA, followed by centrifugation at 12000 rpm for 5 minutes. The aqueous phase was transferred to a clean tube, and DNA was precipitated by adding 0.6× volume of cold isopropanol. The sample was incubated at -20°C for 1 hour to enhance precipitation, then centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol, centrifuged at 12000 rpm for 5 minutes, and air-dried at room temperature for 15-20 minutes. The DNA pellet was dissolved in 30 µL TE buffer with gentle heating at 55°C for 5 minutes if necessary, and stored at -20°C.

2.2.4 DNA Qualification and Quantification

DNA quality was assessed by agarose gel electrophoresis (0.8% gel in 1× TAE buffer) at 80 V for 45 minutes. Gels were stained with ethidium bromide (0.5 µg/mL) and visualized under UV light using a gel documentation system (BioRad, USA). DNA concentration and purity were determined using a Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, USA) by measuring absorbance at 260 nm (DNA), 280 nm (protein), and 230 nm (polysaccharides/phenols). The A260/A280 ratio was used to evaluate protein contamination (optimal range: 1.8-2.0), while the A260/A230 ratio indicated polysaccharide/phenol contamination (optimal range: 2.0-2.2).

2.2.5 16S rRNA Gene Amplification

PCR amplification of the 16S rRNA gene was performed using a thermocycler (BioRad, USA) with a 25 µL reaction mixture containing 12.5 µL Master Mix PCR (2×), 1 µL each of forward primer (27F, 10 µM) and reverse primer (1492R, 10 µM), 2 µL DNA template (approximately 50 ng), and 8.5 µL nuclease-free water. The thermal cycling protocol consisted of initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 20 seconds, and extension at 72°C for 2 minutes. A final extension step was performed at 72°C for 3 minutes to ensure complete amplification. PCR products (5 µL) were visualized by electrophoresis on 1% agarose gel at 80 V for 40 minutes. Positive amplicons (approximately 1500 bp) were purified using a gel extraction kit (GeneAll, Korea) following the manufacturer's protocol and sent to Gemini Singapore Science Park via PT. Genetics Science Indonesia (Jakarta) for Sanger sequencing using the same primers.

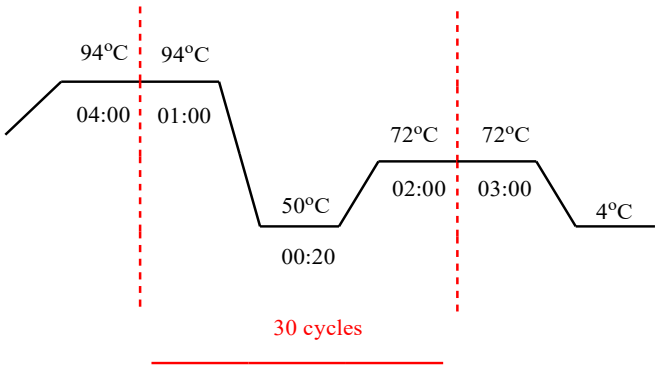


Figure 1. Cycles of PCR (*Maxime* PCR Premix Kit- *i*-Taq)

2.2.6 Sequence Analysis and Phylogenetic Reconstruction

Sequence chromatograms were manually inspected for quality using ChromasPro version 1.7 (Technelysium, Australia). Low-quality bases at the ends were trimmed, and contiguous sequences were assembled using BioEdit version 7.2 . The resulting 16S rRNA gene sequences (1124 bp for BR, 1320 bp for TR) were compared against the GenBank database using BLAST-N (Basic Local Alignment Search Tool for Nucleotides) with default parameters (NCBI, https://blast.ncbi.nlm.nih.gov). The top 20

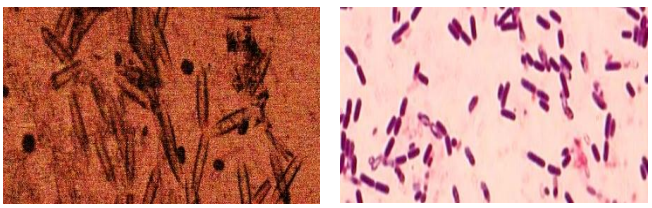
sequences with highest similarity were retrieved for phylogenetic analysis.

Multiple sequence alignment was performed using ClustalW implemented in MEGA6 with default parameters (gap opening penalty: 15, gap extension penalty: 6.66) . Phylogenetic trees were constructed using the neighbor-joining (NJ) method based on the Kimura 2-parameter model, and the maximum-likelihood (ML) method based on the Tamura 3-parameter model with gamma distribution (T92+G), as determined by the best-fit substitution model selection in MEGA6. Nodal support was assessed using bootstrap analysis with 1000 replications. The resulting trees were visualized and annotated using FigTree version 1.4.2.

Result and Discussion

3.1. Morphological Characteristics of Bacterial Isolates

Microscopic examination revealed that both BR and TR isolates exhibited rod-shaped morphology and were classified as Gram-positive bacteria. These isolates demonstrated optimal growth at temperatures ranging from 25-35°C, categorizing them as mesophilic bacteria. The presence of subterminal endospores was also observed, a hallmark characteristic of the genus *Bacillus*.



(a) (b)

Figure 2. Gram staining of BR and TR, rice bran isolate

Morphological identification serves as an initial screening step before molecular confirmation. The rod-shaped, Gram-positive, endospore-forming characteristics observed in this study are consistent with members of the *Bacillus* genus, as previously described by Madhaiyan et al. [10]. Endospore formation is a distinctive feature of *Bacillus* species that enables survival under adverse environmental conditions, including heat, desiccation, and chemical exposure [12]. This ability contributes to the widespread distribution of *Bacillus* species in various environments, including agricultural byproducts such as rice bran.

Previous studies on rice bran isolates have also reported similar morphological characteristics. Lokapirnasari et al. [3] described cellulolytic bacteria isolated from fermented rice bran as rod-shaped and Gram-positive, consistent with *Bacillus* species. Jannah [4] also reported that bacterial isolates from rice bran exhibited morphological features typical of the genus *Bacillus*. However, morphological identification alone cannot definitively determine species identity, particularly within the *Bacillus subtilis* complex, where multiple species share nearly identical morphological features [13]. This limitation underscores the necessity of molecular identification using 16S rRNA gene sequencing.

The mesophilic nature of these isolates (optimal growth at 25-35°C) aligns with the ambient temperature conditions of rice storage and processing in tropical Indonesia. This temperature adaptation suggests that these bacteria are indigenous to the rice bran environment rather than contaminants from other sources. Furthermore, mesophilic *Bacillus* species are known to produce enzymes with optimal activity at moderate temperatures, making them suitable for various industrial applications [14].

3.2. Comparative Evaluation of DNA Extraction Methods

DNA extraction is a critical determinant of success in molecular identification studies. The quality and purity of extracted DNA directly influence the efficiency of PCR amplification, sequencing accuracy, and reliability of downstream analyses [8]. In this study, three DNA extraction methods were compared: standard 2% CTAB, modified CTAB/NaCl, and enzymatic commercial kit.

3.2.1. Qualitative Analysis

Agarose gel electrophoresis demonstrated that all three methods successfully extracted DNA from both BR and TR isolates, as evidenced by distinct high molecular weight bands without smearing. The absence of smearing indicated minimal DNA shearing and degradation. Notably, the CTAB/NaCl method produced brighter bands compared to the enzymatic method, suggesting higher DNA yields. This observation is consistent with Fitriya et al. [9], who reported that CTAB-based methods produced higher DNA yields from Gram-positive bacteria due to more effective cell lysis.

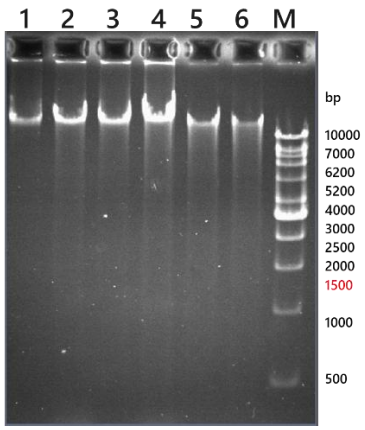


Figure 3. Electropherogram of DNA isolation

The thick peptidoglycan layer of Gram-positive bacteria poses a challenge for DNA extraction, requiring more rigorous lysis conditions compared to Gram-negative bacteria [15]. CTAB-based methods, particularly when combined with mechanical disruption or enzymatic pre-treatment (lysozyme), effectively penetrate the peptidoglycan layer and release genomic DNA. The enzymatic kit method also includes lysozyme treatment but uses spin column purification, which may result in some DNA loss during the washing steps, explaining the lower yields observed [16].

3.2.2. Quantitative Analysis

Spectrophotometric analysis revealed marked differences in DNA purity and yield among the three methods (Table 1). The modified CTAB/NaCl method produced DNA with acceptable to good purity (A260/A280: 1.83-2.05; A260/A230: 1.91-1.94), while the standard 2% CTAB method showed significant polysaccharide contamination (A260/A230: 1.48-1.52). The enzymatic method produced the highest purity but lowest yields.

Table 1. Quantitative analysis of extracted DNA comparing CTAB and enzymatic methods

Sample	Concentration (ng/µL)	A260/280	A260/230	Purity Assessment
BR 2% CTAB	164.36 ± 12.3	1.76	1.52	Protein & polysaccharide contamination
TR 2% CTAB	1139.16 ± 89.2	2.19	1.48	RNA & polysaccharide contamination
BR CTAB/NaCl	175.98 ± 15.6	1.83	1.91	Acceptable
TR CTAB/NaCl	540.91 ± 42.7	2.05	1.94	Good
BR enzymatic	116.45 ± 9.8	2.00	1.89	Good
TR enzymatic	72.78 ± 6.5	2.04	1.92	Good

Note: Values represent mean ± standard deviation of three independent extractions.

The A260/A280 ratio indicates protein contamination (values <1.8) or RNA contamination (values >2.0) [17]. The A260/A230 ratio is particularly important for polysaccharide-rich samples, with values

below 1.8 indicating significant polysaccharide or phenolic compound contamination [18]. Rice bran contains approximately 25% carbohydrates, predominantly as cellulose, hemicellulose, and starch [3], which readily co-precipitate with DNA during alcohol precipitation.

The superior performance of the modified CTAB/NaCl method can be explained by the mechanism of CTAB-based DNA extraction. CTAB (cetyltrimethylammonium bromide) is a cationic detergent that forms insoluble complexes with polysaccharides when the sodium chloride concentration is maintained at or above 0.7 M [19]. In the standard 2% CTAB method, the NaCl concentration may be insufficient to prevent CTAB-DNA complex formation, leading to lower yields and co-precipitation of polysaccharides. The modified CTAB/NaCl method incorporates additional NaCl (final concentration approximately 1.4 M), which maintains DNA in solution while CTAB-polysaccharide complexes are removed during organic extraction [20].

The low A260/A230 ratios observed with standard CTAB (1.48-1.52) indicate significant polysaccharide contamination. Similar findings have been reported for other polysaccharide-rich samples. For example, Liu et al. [21] found that standard CTAB protocols produced DNA with A260/A230 ratios below 1.6 from polysaccharide-rich fungi, while modified high-salt CTAB protocols achieved ratios above 1.9. The present study confirms that the same principle applies to bacterial isolates from rice bran, where residual polysaccharides from the growth substrate may adhere to bacterial cells and co-extract with DNA.

The commercial kit method produced the highest purity DNA (A260/A280: 2.00-2.04; A260/A230: 1.89-1.92), reflecting the effectiveness of spin column purification in removing contaminants. However, the lower yields (72.78-116.45 ng/µL) may be insufficient for certain downstream applications requiring high DNA quantities, such as library preparation for next-generation sequencing [16]. For routine PCR and Sanger sequencing applications, the CTAB/NaCl method provides sufficient yield and acceptable purity at substantially lower cost.

3.3. 16S rRNA Gene Amplification

PCR amplification of the 16S rRNA gene using universal primers 27F and 1492R successfully produced amplicons of approximately 1500 bp for all DNA samples, regardless of the extraction method used (**Fig. 4**). This amplicon size corresponds to the near-full-length 16S rRNA gene, which is the standard target for bacterial taxonomic studies [1].

The universal primers 27F and 1492R are widely used for bacterial 16S rRNA gene amplification because they target highly conserved regions at the 5' and 3' ends of the gene, enabling amplification from virtually all bacterial phyla [6]. The 1500 bp amplicon includes nine variable regions (V1-V9) interspersed among conserved regions, providing sufficient phylogenetic information for genus-level and often species-level identification [22].

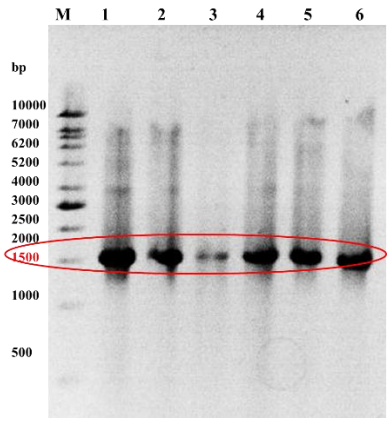


Figure 4. 16S rRNA gene amplification on electropherogram

Successful amplification from all DNA samples, including those with suboptimal purity (e.g., TR 2% CTAB with A260/A230 = 1.48), indicates that PCR can tolerate some degree of polysaccharide contamination. However, the band intensity was notably lower for samples with higher contamination levels, consistent with reports that polysaccharides can inhibit DNA polymerase activity in a concentration-dependent manner [8]. For optimal PCR efficiency, DNA with A260/A230 ratios above 1.8 is recommended [18].

3.4. Molecular Identification via 16S rRNA Gene Sequencing

3.4.1. BLAST-N Analysis

Sequencing of the PCR products yielded high-quality sequences of 1124 bp (BR isolate) and 1320 bp (TR isolate). BLAST-N analysis against the NCBI GenBank database revealed that both isolates showed highest similarity (99% identity) to *Bacillus methylotrophicus* strain CBMB205 (accession number NR_116240) (Table 2).

Table 2. BLAST-N analysis results for BR and TR isolates

Isolate	Closest relative	Accession	Identity (%)	Query cover (%)	E-value
BR	*Bacillus methylotrophicus* CBMB205	NR_116240	99.02	100	0.0
BR	*Bacillus velezensis* FZB42	NR_075005	98.95	100	0.0
BR	*Bacillus amyloliquefaciens* subsp. *plantarum*	NR_117946	98.88	99	0.0
TR	*Bacillus methylotrophicus* CBMB205	NR_116240	99.15	99	0.0
TR	*Bacillus velezensis* CBMB205	CP014838	99.08	99	0.0
TR	*Bacillus siamensis* PD-A10	NR_117274	98.92	99	0.0

Sequence identity of ≥99% is generally considered sufficient for species-level identification in bacteria [23]. However, within the *Bacillus subtilis* complex, which includes *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, and *B. methylotrophicus*, 16S rRNA gene sequences share 98-99% similarity, making definitive species assignment based solely on this gene challenging [13].

Bacillus methylotrophicus CBMB205 was originally isolated from rice rhizosphere soil in Korea and described as a novel species by Madhaiyan et al. [10]. The species name "methylotrophicus" refers to its ability to utilize methanol as a sole carbon and energy source, a relatively uncommon trait among *Bacillus* species. This methylotrophic capability may provide a competitive advantage in the rice bran environment, where methanol can be produced from the demethylation of pectin during microbial metabolism [24].

This study provides the first molecular documentation of *Bacillus methylotrophicus* (syn. *Bacillus velezensis*) from Indonesian rice bran. Previous reports of this species from rice-associated environments include Korea [10,11], China [29], Brazil [30], and India [31]. The identification of closely related strains (99% identity) from Indonesian rice bran suggests that this species may be widely distributed across rice-growing regions of Asia and South America, potentially playing important ecological roles in rice cultivation systems.

3.5. Phylogenetic Analysis

Phylogenetic analysis was performed using the neighbor-joining (NJ) method with the Tamura 3-parameter (T92+G) model in MEGA6 software [32]. The phylogenetic tree (Fig. 4) demonstrated that both BR and TR isolates cluster within the *Bacillus* genus, forming a clade with *Bacillus methylotrophicus* CBMB205, *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, and *Bacillus subtilis* 168. The bootstrap support values for the clade containing BR and TR with *B. methylotrophicus* CBMB205 were 43% and 33%, respectively. While these bootstrap values are below the conventional 70% threshold for strong support [33], they reflect the very high sequence similarity (>99%) among these taxa.

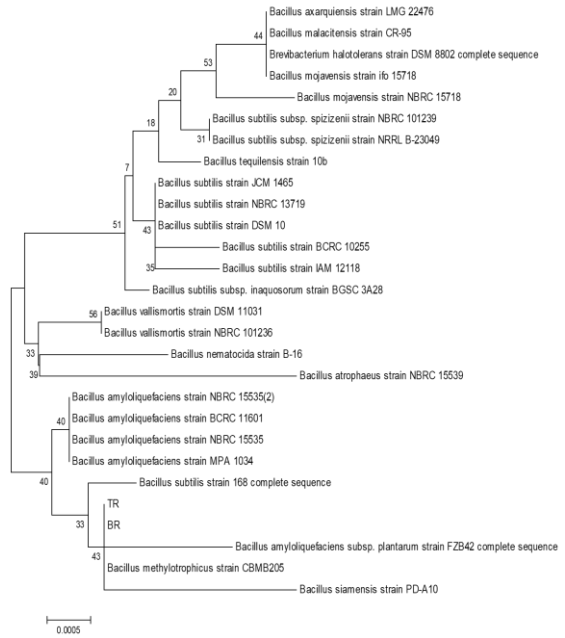


Figure 5. Phylogenetic Tree of BR and TR

The tree topology shows that *Bacillus methylotrophicus* CBMB205 forms a well-supported clade (bootstrap 87%) with *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, consistent with their close genetic relationship and taxonomic synonymy. This clade is sister to *Bacillus subtilis* and other related species, as previously reported by Rooney et al. [13]. The phylogenetic placement of BR and TR within this clade confirms their identity as members of the *Bacillus subtilis* complex and specifically as strains closely related to *B. methylotrophicus* CBMB205. The shorter branch lengths observed for BR and TR compared to reference strains indicate their close genetic relationship, while the distinct branching pattern suggests they may represent a locally adapted Indonesian strain. Similarly, Liu et al. [29] identified *Bacillus methylotrophicus* CH1 from soil samples in China and characterized its chitosanase activity, demonstrating the species' enzymatic versatility. The enzyme showed optimal activity at 55°C and pH 6.0, with potential applications in the production of chitooligosaccharides for agricultural and pharmaceutical uses.

Conclusion

The modified CTAB/NaCl method proved superior to standard CTAB for DNA extraction from rice bran bacterial isolates, achieving acceptable purity (A260/A280: 1.83-2.05; A260/A230: 1.91-1.94) at substantially lower cost than commercial kits. Molecular identification based on 16S rRNA gene sequencing confirmed that both BR and TR isolates are *Bacillus methylotrophicus* strain CBMB205 (syn. *Bacillus velezensis*), with 99% sequence identity and phylogenetic clustering within the *Bacillus subtilis* complex. This study provides the first molecular documentation of this species from Indonesian rice bran, expanding knowledge of its geographical distribution. The optimized CTAB/NaCl protocol offers a practical, cost-effective approach for molecular identification suitable for resource-limited laboratory settings in developing countries

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