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Identification and Antimicrobial Susceptibility Profile of Bacterial Contaminants Isolated From Biofertilizer Bioreactors

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Abstract

Microbial contamination in biofertilizer bioreactors represents a major challenge for industrial production, yet studies integrating molecular, phenotypic and antimicrobial characterization of bacterial contaminants remain limited. Bacterial isolates recovered from biofertilizer bioreactors with process deviations were identified by 16S rRNA gene sequencing (primers 27F/1492R) and further characterized by phenotypic and biochemical assays. Antimicrobial susceptibility was determined by the Kirby–Bauer disk diffusion method, while the antibacterial activity of a hydroethanolic extract of *Copaifera malmei* was evaluated by broth microdilution and membrane permeability assays. The isolates were identified as *Micrococcus luteus* and *Bacillus cereus* sensu lato, showing > 99% 16S rRNA gene sequence similarity. The *B. cereus* isolate exhibited multidrug resistance to ampicillin, penicillin, cefoxitin and ceftazidime, remaining susceptible only to tetracycline and erythromycin. Phenotypic and biochemical analyses corroborated the molecular identification of the *B. cereus* isolate. In contrast, *M. luteus* was susceptible to all antimicrobials tested. The hydroethanolic extract of *C. malmei* showed limited antibacterial activity, with minimum inhibitory concentrations of 5000 µg/mL against *B. cereus* and 312 µg/mL against *M. luteus*, although membrane permeability assays indicated increased leakage of intracellular material in the latter. The integration of molecular, phenotypic and antimicrobial susceptibility analyses provides a robust approach for the characterization of bacterial contaminants in biofertilizer bioreactors. The multidrug-resistant profile of the *B. cereus* isolate reinforces the importance of continuous microbiological surveillance and stricter sanitation strategies in industrial biofertilizer production.

1. Introduction

The biofertilizer industry has experienced accelerated global expansion, driven by the demand for sustainable agriculture. However, maintaining microbiological purity in large-scale bioreactors remains a critical challenge. Microbial contamination not only compromises the quality of the final product but also results in substantial economic losses due to batch disposal and production flow interruptions. In this context, the identification of these microorganisms is fundamental for contaminant traceability and the implementation of effective corrective measures [1, 2].

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Bacteria from the genera *Bacillus* and *Micrococcus* are reported as contaminants in industrial environments due to their ubiquity and, in the case of *Bacillus* spp., their ability to form endospores and persistent biofilms [3, 4]. In addition to taxonomic identification, characterizing the antimicrobial susceptibility profile of these isolates is vital for understanding the risks of resistance dissemination and for guiding sanitation protocols [5]. Concurrently, the search for new antimicrobial agents derived from biodiversity, such as *Copaifera* species from the Brazilian Cerrado, emerges as an exploratory alternative for the control of industrial pathogens [6, 7].

Strengthening collaboration between industry and research institutions is fundamental to promote technological innovation, enhance quality control, and ensure the sustainability of industrial processes.

This interaction favors the integration of scientific knowledge with the demands of the productive sector, enabling the identification of microbiological challenges and the development of more efficient strategies for their prevention and control. This study aimed to identify bacterial contaminants isolated from biofertilizer bioreactors with process deviations, characterize their susceptibility profile to conventional antibiotics, and exploratorily evaluate the antimicrobial activity of the *Copaifera malmei* extract.

2. Material and Methods

2.1 Sample collection and bacterial isolation

Samples were aseptically collected from a bioreactor that presented process deviations in an industrial biofertilizer unit, located in the city of Lucas do Rio Verde, Mato Grosso, Brazil. Although the samples were collected from the same bioreactor, the isolates originated from different batches. Isolation was performed on nutrient agar (NA) under incubation at 35 ± 2°C for 24 h. Morphologically distinct colonies were purified by successive streaking [8, 9].

2.2 Molecular identification

Genomic DNA was extracted using a commercial Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), strictly following the manufacturer's instructions. Amplification of the 16S rRNA gene was performed by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), widely used for molecular identification of bacteria [10, 11]. PCR products were purified and subjected to Sanger sequencing. The obtained sequences were edited, evaluated for quality, and compared with those deposited in the GenBank database (NCBI) using the BLASTn tool, with taxonomic identification based on the highest sequence identity percentage [12, 13]. The sequences were processed and deposited in GenBank under accession numbers PZ535511 and PZ535512.

2.3 Phenotypic and biochemical characterization

The *B. cereus* group isolate was characterized using a phenotypic and biochemical approach. Motility was evaluated in SIM (Sulfide-Indole-Motility) medium by the stab method, while rhizoid growth was determined by observing colonial morphology on nutrient agar after incubation at 35 ± 2°C for 24 h [14, 15]. Hemolytic activity was determined on blood agar containing 5% defibrinated blood, with isolates classified according to the hemolysis pattern observed [16]. The presence of parasporal crystals was investigated by Smirnoff staining in sporulated cultures, according to the methodology described for differentiation of *B. cereus* group species [17]. Growth at 6°C was evaluated after incubation in appropriate culture medium, following recommendations of ISO 7932 for characterization of *B. cereus* group isolates. Biochemical characterization was performed on Mannitol Egg Yolk Polymyxin (MYP) medium, simultaneously evaluating mannitol fermentation and lecithinase production, as described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual [18]. Together, these assays

provided phenotypic and biochemical characteristics compatible with isolates belonging to the *B. cereus* group. Tests were performed in triplicate.

2.4 Antimicrobial susceptibility testing

Susceptibility profile was determined by the disk diffusion method (Kirby-Bauer) following CLSI M100 guidelines. The following antimicrobials were tested: ampicillin (10 µg), penicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), tetracycline (30 µg), and erythromycin (15 µg). Results were interpreted based on zone diameter formation. Tests were performed in triplicate with three repetitions. This assay was conducted with the isolates and with the *B. cereus* ATCC 10876 strain.

2.5 Plant extract and MIC determination

Stem bark of *C. malmei* was collected in the Cerrado (Voucher deposited at the Herbarium of the Federal University of Mato Grosso under No. 46321). The ethanolic extract was obtained by maceration (hydroethanolic, 72h) and concentrated in a rotary evaporator. The Minimum Inhibitory Concentration (MIC) was determined by broth microdilution (CLSI M07) at concentrations ranging from 3.12 to 10000 µg/mL, with an inoculum of 0.5 McFarland. Tests were performed in triplicate with three repetitions.

2.6 Membrane permeability assay

Intracellular material leakage (absorbance at 260 nm) was measured after exposure of cells to the extract at the MIC for 2h. 0.1% SDS was used as a positive control and untreated cells as a negative control [19]. Tests were performed in triplicate with three repetitions.

3 Results and Discussion

3.1 Bacterial identification

Two bacteria were isolated, and analysis of the 16S rRNA gene sequences allowed identification of the isolates as *B. cereus* sensu lato and *M. luteus* (> 99% similarity and E-value 0) (Fig. 1).

In the *B. cereus* group, phenotypic and biochemical methods are widely employed as initial screening tools, allowing preliminary differentiation based on morphological and metabolic characteristics. In the present study, these methods were used as an initial identification step, subsequently complemented by 16S rRNA gene sequencing. Although 16S rRNA analysis is widely applied in bacterial identification, its discriminatory capacity within the *B. cereus* group is limited due to the high genetic similarity among phylogenetically close species. Thus, the combined approach adopted in this work provides greater robustness in the identification of isolates [20, 13, 21].

3.2 Table 1. Polyphasic identification of the isolate belonging to the *Bacillus cereus* group based on molecular and phenotypic characteristics.

Approach	Factor analyzed	Observed result	Taxonomic interpretation
Molecular	Sanger sequencing of 16S rRNA gene	99.65% identity with *Bacillus cereus* strains	Compatible with *Bacillus cereus* group (*sensu lato*)
Phenotypic	Motility test (SIM)	Motility positive	Phenotypically excludes *B. anthracis*
Phenotypic	Rhizoid growth	Absent	Phenotypically excludes *B. mycoides*
Phenotypic	Hemolysis profile	β-hemolysis	Phenotypically excludes *B. anthracis*
Phenotypic	Parasporal crystals	Absence	Makes *B. thuringiensis* unlikely
Phenotypic	Growth at 6°C	Absent	Disfavors psychrotrophic species of the group
Biochemical	MYP (mannitol/lecithinase)	Mannitol negative and lecithinase positive	Corroborates identification as *B. cereus*. Excludes *B. megaterium*
Conclusion	Data integration	Convergent molecular, phenotypic and biochemical profile	Identification compatible with *B. cereus*

3.3 Phenotypic and Biochemical tests

The phenotypic and biochemical tests corroborate the molecular identification of the isolate as *B. cereus*. Positive motility and β-hemolysis allowed differentiation from *B. anthracis*, a species typically non-motile and non-hemolytic [20, 22]. The absence of rhizoid growth phenotypically excluded *B. mycoides*, while the absence of parasporal crystals made identification as *B. thuringiensis* unlikely, since this species is characterized by the production of crystalline inclusions during sporulation, considered one of its main phenotypic markers [23]. Furthermore, the absence of growth at 6°C was compatible with *B. cereus* and disfavors psychrotrophic species of the group, such as *B. weihenstephanensis* [24]. On MYP medium, the isolate showed negative mannitol fermentation and positive lecithinase activity, a profile characteristic of *B. cereus* and consistent with the criteria described in the FDA's Bacteriological Analytical Manual, which uses acid production from mannitol as a differential characteristic from *B. megaterium* [25]. Together, the phenotypic, biochemical, and molecular results were concordant, supporting the identification of the isolate as *B. cereus*. Regarding *M. luteus*, the intense yellow pigmentation constitutes a typical phenotypic characteristic of the species, resulting from carotenoid production and widely used as an auxiliary criterion for its microbiological identification [26].

3.4 Antimicrobial susceptibility

The antibiogram results revealed marked differences between the isolates (Table 2). While *M. luteus* showed susceptibility to all antimicrobials tested, the industrial *B. cereus* isolate exhibited a multidrug

resistance profile, remaining susceptible only to tetracycline and erythromycin. The susceptibility profile was consistent with that observed for the reference strain *B. cereus* ATCC 10876, reinforcing the reproducibility of the disk diffusion assay. This pattern is consistent with studies demonstrating that members of the *B. cereus* group exhibit intrinsic resistance to β-lactam antibiotics, mainly due to the production of β-lactamases, in addition to variable resistance to aminoglycosides and fluoroquinolones, depending on the origin and selective pressure of the isolates [27, 28]. In industrial environments, the occurrence of multidrug-resistant strains represents an additional challenge for microbiological control, reinforcing the need for continuous monitoring and phenotypic and molecular characterization of contaminants to support effective control strategies [29].

Table 2
Antimicrobial susceptibility profile of the isolated contaminants.

Antimicrobial	**Pharmacological class**	**Concentration (µg)**	***B. cereus* (Industrial isolate)**	***B. cereus* ATCC 10876**	***M. luteus* (Industrial isolate)**
Ampicillin	β-Lactam (Aminopenicillin)	10	R	R	S
Penicillin	β-Lactam (Natural penicillin)	10	R	R	S
Cefoxitin	β-Lactam (Cephamycin)	30	R	R	S
Ceftazidime	β-Lactam (Third-generation cephalosporin)	30	R	R	S
Tetracycline	Tetracycline	30	S	S	S
Erythromycin	Macrolide	15	S	S	S

3.5 Activity of Copaifera malmei extract

As a complementary evaluation, the antimicrobial potential of the hydroethanolic extract of *C. malmei* against the bacterial isolates was investigated. The stem bark extract showed MIC of 5000 µg/mL for *B. cereus* and 312 µg/mL for *M. luteus*. The nucleotide leakage assay, performed with *M. luteus*, measuring absorbance at 260 nm, demonstrated increased absorbance, indicating that the extract promoted the release of intracellular material compatible with increased cytoplasmic membrane permeability. Although the hydroethanolic extract of *C. malmei* did not show high antimicrobial activity, this study constitutes a preliminary evaluation of its potential against industrial contaminants. The reduced activity may be related to the nature of the crude extract, suggesting that strategies such as bio-guided fractionation and isolation of active compounds may result in fractions and compounds with greater antimicrobial efficacy [6, 30].

3.6 Industrial implications

The detection of multidrug-resistant *B. cereus* underscores the need for more rigorous sterilization and sanitation protocols. The resilience of this microorganism in industrial environments is often potentiated by biofilm formation, which requires continuous microbiological monitoring to avoid persistent process failures.

4. Conclusion

This study demonstrated that the integration of molecular identification, phenotypic characterization, and antimicrobial susceptibility evaluation constitutes an efficient approach for the characterization of bacterial contaminants in biofertilizer bioreactors. This strategy enabled the identification of a *B. cereus* isolate with a multidrug resistance profile and an *M. luteus* isolate, providing relevant information for microbiological monitoring and the improvement of quality control programs in industrial processes. Furthermore, the results obtained represent a preliminary evaluation of the antimicrobial potential of the hydroethanolic extract of *C. malmei*, indicating that future studies involving bio-guided fractionation, chemical characterization, and evaluation of isolated compounds may contribute to obtaining fractions with greater activity against bacterial contaminants of industrial interest. Thus, the results reinforce the importance of integration between research institutions and the industrial sector to address microbiological challenges associated with biotechnological processes. This collaboration can contribute to the development of evidence-based quality control strategies, as well as to technological advancement and sustainability of the biofertilizer industry in Brazil and on the international stage.

Declarations

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Author contributions

Tarcísio Renan Pereira Sousa Resende: Bibliographic research, antimicrobial tests with plant extracts, molecular identification of isolates, antibiotic testing, and manuscript writing.

Geiziquele de Lima: Phenotypic and biochemical tests for *B. cereus*.

Vanessa Mendes Rêgo: DNA extraction, PCR performance, and molecular identification of isolates.

Sikiru Olaitan Balogun: Experimental design, manuscript writing, manuscript supervision.

Marcondes Alves Barbosa da Silva: Manuscript writing, manuscript supervision.

Reginaldo Vicente Ribeiro: Experimental design and manuscript supervision.

Conflict of interest. The authors declare no conflict of interest.

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Figures

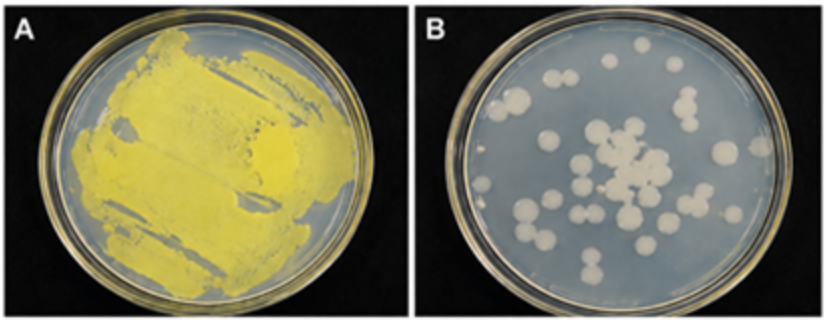


Figure 1

Colonial morphology of bacterial isolates cultured on nutrient agar. **(A)** *Micrococcus luteus*, showing colonies with intense yellow pigmentation, characteristic of the species. **(B)** *B. cereus*, showing opaque, white-cream colored colonies.