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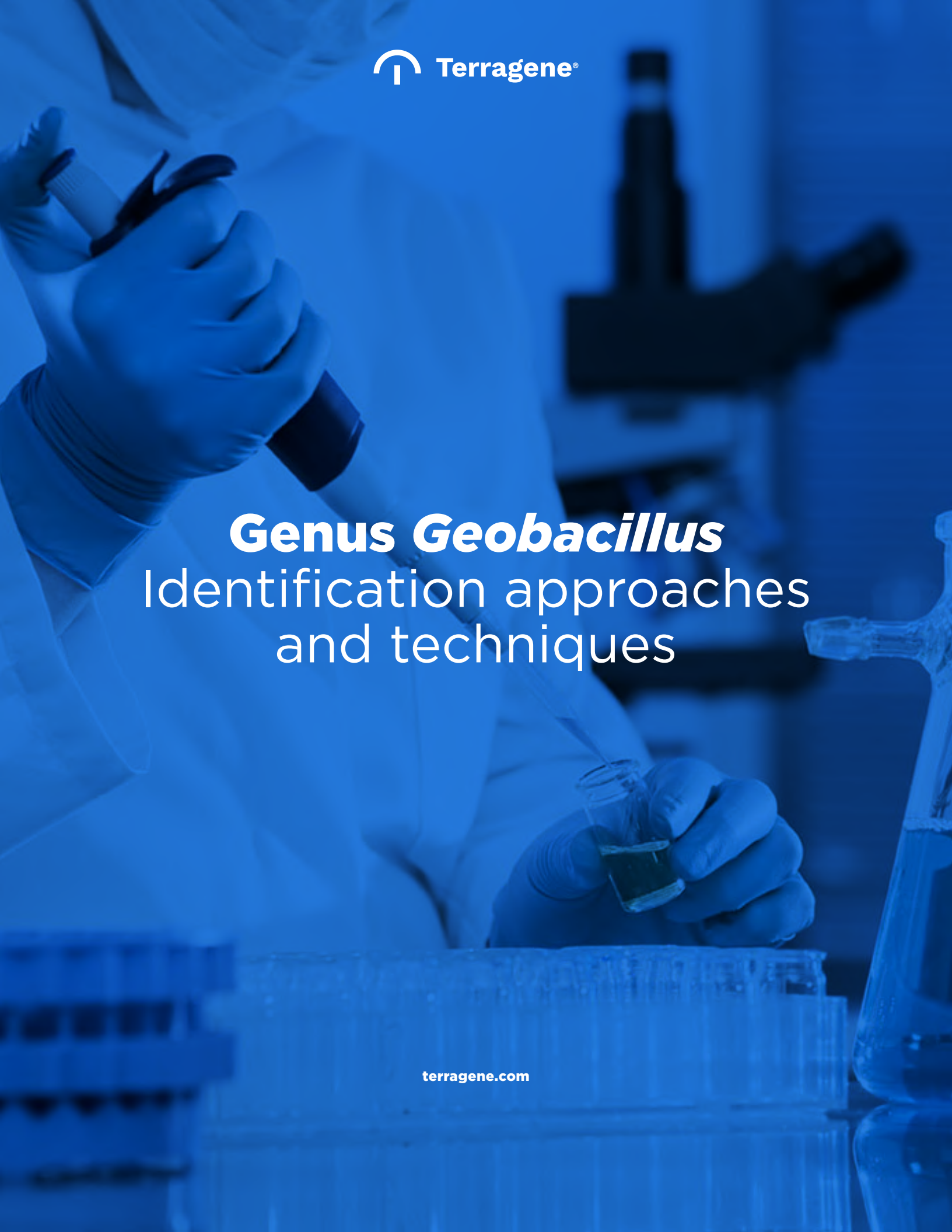

Genus *Geobacillus*
Identification approaches and techniques

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1. Introduction

Geobacillus stearothermophilus is one of the most widely used microorganisms in the production of biological indicators. These thermophilic bacilli are particularly relevant due to their ability to form spores that are highly resistant to various lethal agents, making them ideal for assessing and validating sterilization processes.

G. stearothermophilus belongs to the genus *Geobacillus*. A genus is a biological category that groups together different species sharing certain characteristics. These shared traits exist because they originate from a common ancestor, thereby reflecting their evolutionary relationship. Species within the genus *Geobacillus* (*Geobacillus spp.*) are Gram-positive, spore-forming, rod-shaped bacteria adapted to thermophilic environments. They have been isolated from extreme habitats such as hot springs, oil reservoirs, and marine sediments, as well as from food products including refined sugar, canned goods, dairy products, and dehydrated vegetables.

Originally, this group of bacteria was classified within the genus *Bacillus*. With the advancement of molecular biology techniques, sufficient differences were identified to reassign them to their own genus. Nevertheless, the taxonomic classification of these bacteria is still evolving, not only as a result of improvements in identification methods but also due to changes in phylogenetic criteria. This poses a challenge when establishing reliable methods for their accurate identification.

Since *G. stearothermophilus* strain widely used across a broad range of industries, having a robust identification method is essential as part of routine production control. Many methods are able to correctly identify organisms at the genus level; however, complications often arise when attempting to distinguish at the species level.

Historically, bacterial identification was based on phenotypic tests that assess characteristic traits of bacteria, such as growth patterns in different culture media or specific metabolic properties. Today, more precise alternatives are available, including mass spectrometry (MALDI-TOF) and genetic sequencing. The latter played a key role in the taxonomic redefinition of the genus *Geobacillus*.

The purpose of this material is to describe and compare the three techniques most commonly used today for the identification of *G. stearothermophilus*, highlighting their advantages and limitations for application in industrial contexts. These techniques include biochemical tests, mass spectrometry by MALDI-TOF MS, and 16S rRNA gene sequencing.

2. Identification methods

2.1. Biochemical tests

Biochemical tests aim to identify microorganisms based on their phenotypic characteristics, particularly by observing how they respond to specific compounds. These tests rely on the microorganisms' ability to use different nutrient sources, their resistance or susceptibility to certain drugs, and the expression of specific enzymes that reflect their metabolic activity.
In practice, these techniques involve exposing a pure culture of the microorganism to a series of reagents or substrates and then observing whether a reaction occurs. This reaction, mediated by enzymes inherent to the microorganism, is usually evidenced by a visible change (for example, a color shift or the generation of fluorescent compounds), which allows for the inference of its biochemical profile.

However, the expression of these characteristics can vary considerably depending on the culture conditions and the stimuli required for enzymatic activation, particularly temperature. For this reason, manually performed assays demand significant time, expertise, and strictly controlled conditions to avoid misinterpretation. To overcome this limitation, automated systems were developed, allowing the simultaneous and automated evaluation of multiple biochemical reactions. This system employs specific cards depending on the type of microorganism to be identified. Each card contains compartments with different reagents, which are automatically

inoculated upon loading the sample. Throughout the assay, the instrument detects colorimetric or fluorescent changes, which are then interpreted by internal software that compares the results against its proprietary database. If the database is sufficiently robust and representative, it is possible to achieve identification at the genus level, and in some cases even at the species level.

Nevertheless, even automated systems present certain limitations, since different species within the same genus may exhibit very similar biochemical profiles, which can lead to misidentifications. This is particularly relevant in genera such as *Geobacillus sp.*, where phenotypic differences between closely related species may be subtle or inconsistent.

Moreover, biochemical properties do not always reflect the complexity of a microorganism with accuracy, as they are susceptible to environmental factors and may vary over time or in response to external conditions. Once again, the reliability of the result largely depends on the quality, updating, and comprehensiveness of the database against which the obtained profiles are compared.

This phenomenon is clearly evident in the case of *G. stearothermophilus*, where the expression of certain biochemical traits can vary within the same species depending on the environment from which it is isolated. For instance, some *G. stearothermophilus* strains are capable of using lactose as a carbon source, a feature that was not previously considered in standard biochemical tests. Burgess et al. (2017) demonstrated this phenomenon when they observed phenotypic differences among groups of *G. stearothermophilus* strains, with those isolated from dairy products being the only ones that tested positive in lactose assays. This illustrates how responses and adaptations to different environments can influence biochemical profiles, representing a limitation to the ability of these methods to achieve accurate identification.

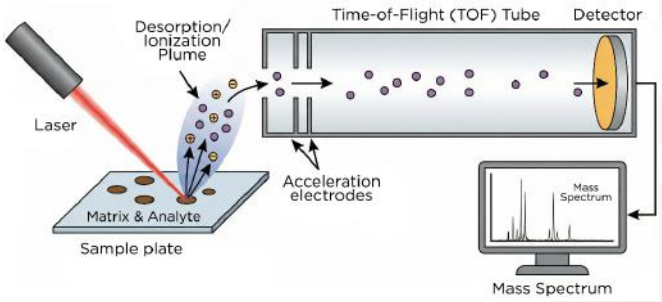
2.2. MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry)

Another widely used technique for microbial identification is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Although the name may sound complex, the underlying principle is relatively straightforward: the technique identifies a sample based on its protein fingerprint, which is obtained as a "spectrum" by analyzing the mass-to-charge ratio of the molecules present.

MALDI-TOF MS can be performed either directly from a cultured colony or from a protein extract. In the case of Gram-positive bacteria, the latter approach is generally preferred, as the thick peptidoglycan cell wall can interfere with the quality of the spectrum. In any case, the protocols for analysis are usually provided by the instrument manufacturers or available in the scientific literature, and they can be adapted to suit the specific laboratory.

The process involves ionizing the proteins from the sample, which are then accelerated in an electric field within the mass spectrometer. The charged particles travel along a path until they reach a detector, and the time they take to arrive—known as the "time of flight"—allows the determination of their mass-to-charge ratio. The result is a characteristic spectrum of peaks that serves as a "protein fingerprint" of the sample, which can then be compared against a reference database for identification.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS)



This identification is expressed as a score that is automatically calculated by the device, indicating the degree of match between the obtained spectrum and those stored in the database. As with other methods, the reliability of this technique largely depends on the quality and coverage of the reference database. In addition, many commercial software packages have limited databases for non-pathogenic microorganisms such as *Geobacillus*, and spore-forming species exhibit different protein expression in their vegetative and spore forms, which increases the likelihood of errors in comparison and precise species determination. For this reason, many laboratories incorporate their own spectra from frequently used strains, although it remains essential to confirm the identification using molecular techniques. When working with phylogenetically closely related species, their protein profiles may be very similar, potentially leading to ambiguous identifications. One strategy to improve identification specificity is to

focus the analysis on ribosomal proteins, as these are expressed consistently by microorganisms regardless of environmental factors.

In summary, MALDI-TOF MS is a rapid and versatile technique with high potential for microbiological diagnostics, as samples can often be analyzed without any pre-treatment. However, its accuracy strongly depends on the reference database used and may require complementary methods to confirm more complex identifications, as in the case of *G. stearothermophilus*.

2.3. 16S rRNA Gene Sequencing

One of the most widely used reference methods for bacterial identification is 16S ribosomal RNA (rRNA) gene sequencing. This approach has proven to be highly effective both for identifying microorganisms and for establishing phylogenetic relationships among them. Since its implementation, it has enabled the accurate classification of a large number of bacterial species, including those that are difficult to culture using conventional methods.

The 16S gene is present in all bacteria and encodes a component of the 30S ribosomal subunit. It is a highly conserved region within the same genus, but it also contains variable regions that allow differentiation between closely related species. Thanks to this combination of conservation and variability, it has become an ideal molecular marker for taxonomic studies.

Moreover, since this region is present in virtually all microorganisms, it is possible to design universal primers that allow amplification of the target fragment across a wide range of species.

The identification process begins with DNA extraction from a pure culture of the microorganism, followed by amplification of the 16S gene using PCR with specific primers. In most cases, the amplified product is analyzed by electrophoresis to verify that a fragment of the

Criteria	Biochemical tests	MALDI-TOF	16S rRNA Sequencing
Identification level	Genus (sometimes species)	Genus/species	Species (sometimes subspecies)
Response time	24–48 h	30 min aprox	24–48 h
Requires pure culture	Yes	Yes	Yes
Cost	Low/Medium	High	High (if outsourced)
Database dependency	High	High	High, with public databases available
Specialized personnel	Low/Medium	Medium	High
Application in industrial quality control	Limited	Good	Excellent

expected size has been obtained. The fragment is then purified and sequenced, most commonly using the Sanger method. Finally, the resulting sequence is compared against public databases such as NCBI or RDP, or against private ones.
The goal of this comparison between the unknown sequence and the reference sequences is to generate an alignment. The higher the base-pair match, the higher the alignment score, and the more reliable the sequence identification.

Unlike biochemical or spectrometric methods, 16S sequencing relies on broad and regularly updated databases, which significantly increases the likelihood of accurate identification, even among phylogenetically closely related species such as those of the genus *Geobacillus*. In addition, many commercial sequencing services offer complete identification from a submitted sample, facilitating its adoption in laboratories that lack molecular biology infrastructure.

Due to its specificity and robustness, 16S gene sequencing is the most recommended method to ensure the traceability and identity of strains used in the production of biological indicators. Although it requires trained personnel and certain technical resources, its ability to resolve complex identifications makes it a fundamental tool for quality assurance.

3. Conclusion

Accurate identification of *G. stearothermophilus* is a critical aspect of quality assurance in industrial processes where this strain is used. Identification can be approached through various techniques, each with specific advantages and limitations that must be considered according to the context. Automated biochemical tests provide an accessible initial approach but may not always yield conclusive results. MALDI-TOF MS, on the other hand, enables rapid and reliable identification, though its accuracy depends on the quality of the reference database used.

16S gene sequencing remains the most precise and traceable method. In this regard, Terragene® performs 16S rRNA gene sequencing on master batches of strains acquired from the American Type Culture Collection (ATCC®), an internationally recognized reference organization for certified strain preservation. This ensures robustness and excellence in Terragene®'s production processes by achieving correct identification of the various strains used in biological indicator manufacturing, in compliance with high international standards such as ISO 13485:2016 / NS-EN ISO 13485:2016 quality management system requirements.

Given the challenges presented by this genus, it is highly recommended to use at least two complementary methods to ensure accurate and well-supported identification. This approach not only enhances process reliability but also strengthens the quality of the final product.

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