

Antibacterial Activity of Stigmatellin Y from Marine *Bacillus amyloliquefaciens* against MDR *Pseudomonas aeruginosa*

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ARTICLE INFO

Original Article

Keywords: Multidrug-resistant *Pseudomonas aeruginosa*, Stigmatellin Y, Marine *Bacillus amyloliquefaciens*, UHPLC-UV-MS

Received: 27 Aug. 2025

Received in revised form: 29 Jun. 2026

Accepted: 10 Aug. 2026

DOI: 10.61882/JoMMID.14.2.130

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ABSTRACT

Introduction: Hospital-acquired infections caused by *Pseudomonas aeruginosa* strains frequently exhibit resistance to both monotherapy and combination therapy with antibiotics because of acquired and intrinsic resistance mechanisms. Therefore, there is an urgent need for novel and potent antibiotics to treat infections caused by multidrug-resistant (MDR) *P. aeruginosa*. Bioactive compounds from marine *Bacillus* species have the potential to inhibit the growth of MDR *P. aeruginosa*. **Methods:** In the present investigation, a *Bacillus* species was isolated from seawater using Zobell Marine Agar. The isolate was identified by morphological, biochemical, and 16S rRNA gene sequencing analyses. Ethyl acetate was used to extract extracellular bioactive secondary metabolites from the cell-free supernatant of the isolated strain. Column chromatography (silica gel, 100–200 mesh) was employed to further fractionate the crude extract, and the agar well diffusion method was used to assess antibacterial activity against MDR *P. aeruginosa*. The bioactive fraction F1 was putatively annotated using ultra-high-performance liquid chromatography–ultraviolet–mass spectrometry (UHPLC-UV-MS). **Results:** The marine bacterial isolate was identified as *Bacillus amyloliquefaciens*. The crude extract and its purified fraction F1 inhibited the growth of MDR *P. aeruginosa*. The column chromatography–purified bioactive fraction F1 showed zones of inhibition ranging from 15.0 to 16.7 mm against MDR *P. aeruginosa*. UHPLC-UV-MS analysis putatively annotated bioactive fraction F1 as stigmatellin Y, exhibiting a retention time of 28.50 min and a mass-to-charge ratio (*m/z*) of 507.2725 ([M+Na]⁺). **Conclusion:** This marine *B. amyloliquefaciens* strain produces the potent compound stigmatellin Y, which exhibits activity against MDR *P. aeruginosa*. This finding highlights the strain's potential as a source of novel therapeutic agents.

INTRODUCTION

P. aeruginosa poses a severe global health threat, causing high morbidity and mortality in burn and cystic fibrosis patients [1]. It is listed as a critical pathogen by the World Health Organization (WHO) and is highly adaptable [2]. *P. aeruginosa* exhibits resistance to multiple antibiotic classes, including aminoglycosides, β -lactams, and quinolones [3]. Combination therapy with two or three antimicrobial agents is recommended to treat infections caused by MDR *P. aeruginosa* to enhance efficacy and prevent additional resistance [4]. Although this approach provides broader coverage and synergistic effects, adverse effects have been reported, including nephrotoxicity, ototoxicity, superinfections, and the emergence of resistant strains [5, 6].

One promising approach to address MDR *P. aeruginosa* infections is the isolation of potent bioactive molecules from

environmental microorganisms, which may offer efficacy comparable to last-line antimicrobial agents [7]. Continuous screening and characterization of new bioactive molecules are essential for developing effective therapeutic agents against infections caused by MDR *P. aeruginosa*. Several microbial genera, including *Natrinema*, *Streptomyces*, *Bacillus*, and *Halobacillus*, have been reported to produce bioactive compounds against MDR *P. aeruginosa*. These include the anti-biofilm agent stigmatellin Y [8], quorum-sensing-inhibiting compounds from *Natrinema* [9], the antibacterial metabolites mollemycin A [10] and picolinamycin [11], and quorum-sensing inhibitors from *Bacillus* species [12].

Stigmatellin Y is a chromone derivative produced via a polyketide biosynthetic pathway and was originally isolated

from myxobacteria such as *Stigmatella aurantiaca* [13]. It is known for its inhibitory effect on the mitochondrial cytochrome bc1 complex [13] and its potential for biofilm inhibition [8, 14]. *Bacillus subtilis* BR4, isolated from seawater in Tamil Nadu, India, was reported to also produce stigmatellin Y [8]. Moreover, marine *Bacillus* species other than *B. subtilis* may offer advantages over myxobacteria in stigmatellin Y production due to faster growth rates, greater genetic diversity, and better suitability for large-scale fermentation [8].

The marine environment provides a unique ecological niche for bacteria that produce bioactive compounds [15], because the high pressures, salinity, and hypoxia characteristic of these environments can trigger secondary metabolite production in resident microorganisms [16, 17]. Marine *Bacillus* species, in particular, have emerged as promising sources of such metabolites [8, 18]. Therefore, the present investigation aimed to isolate a potent bioactive compound from marine *Bacillus* spp. and to assess its activity against MDR *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Isolation and identification of a marine *Bacillus* isolate. A seawater sample was collected from the coastal waters of Goa, Arabian Sea (15.5551° N, 73.7515° E) in 2024. The sample was serially diluted in sterile saline and spread-plated onto Zobell Marine Agar (ZMA). Twenty morphologically distinct bacterial colonies were selected and screened for antimicrobial activity against five MDR *P. aeruginosa* clinical isolates using the agar well diffusion method. Isolate MI03, which exhibited the strongest inhibitory activity against MDR *P. aeruginosa*, was identified at the genus level as *Bacillus* sp. based on cultural and biochemical characteristics, including colony morphology, Gram staining, and catalase and oxidase tests. Species-level identification of this isolate was performed using molecular methods. The 16S rRNA gene was amplified using the universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR amplification was performed using an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Sequence similarity was assessed by comparing the amplified sequence against the NCBI nucleotide (nt) database using the BLASTn tool. Identification was confirmed based on a percentage identity cutoff of $\geq 98.7\%$, E-value $< 10^{-3}$, and query coverage of $\geq 95\%$.

Production and extraction of bioactive compounds from strain MI03. Strain MI03 was cultivated in 250 mL of Zobell Marine Broth using 500-mL Erlenmeyer flasks and incubated for 72 h at 30 °C on a rotary shaker at 180 rpm. The culture broth was centrifuged at $11,000 \times g$ for 15 min in a refrigerated centrifuge (REMI R-247M) to obtain the cell-free supernatant. An equal volume of ethyl acetate (250 mL) was added to the cell-free supernatant in a separating funnel, and the mixture was shaken vigorously for 20 min at

25 ± 2 °C. The phases were allowed to separate by gravity for 30 min, and the organic layer was collected. The organic extract was concentrated to dryness at 40 °C using a rotary evaporator at reduced pressure. The dried extract was fractionated by column chromatography on silica gel (100–200 mesh) using a mobile phase of hexane–ethyl acetate–methanol (7:3:1). The eluent was collected in five distinct fractions (F1–F5) based on thin-layer chromatography (TLC) profiles (silica gel 60 F₂₅₄ plates, visualized under UV light at 254 and 365 nm). The dried fractions were reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 500 µg/mL.

Antibacterial activity of purified fractions against MDR *P. aeruginosa*. Five MDR clinical isolates of *P. aeruginosa* (PA2, PA3, PA5, PA6, and PA7), which exhibited resistance to multiple antibiotic classes including aminoglycosides, β -lactams, and carbapenems, were confirmed as MDR by standard disk diffusion assays according to CLSI M100 guidelines. These strains were isolated from clinical specimens at the Government Hospital and Medical College, Nanded, Maharashtra, India, and identified by 16S rRNA gene sequencing with the universal primers 27F and 1492R (as described above). The agar well diffusion method was used to assess antibacterial activity. Mueller-Hinton Agar (MHA) plates were inoculated by streaking with a sterile cotton swab immersed in each *P. aeruginosa* culture adjusted to a 0.5 McFarland standard. Wells (6-mm diameter) were punched in the agar, and 100 µL of each fraction (500 µg/mL) or DMSO (negative control) or streptomycin (500 µg/mL; 50 µg per well, positive control) was added to each well. The plates were incubated at 37 °C for 24 h, and the diameters of the inhibition zones were measured in millimeters using a digital caliper. All assays were performed in triplicate (n = 3) on three separate days (inter-day reproducibility).

Characterization of bioactive fraction F1 by UHPLC-UV-MS. The potent fraction F1 was characterized by ultra-high-performance liquid chromatography–ultraviolet–mass spectrometry (UHPLC-UV-MS) using an Agilent 6550 Q-TOF mass spectrometer coupled to an Agilent 1260 Infinity II UHPLC system. Chromatographic separation was performed on an Agilent Eclipse XDB-C18 column (3 × 150 mm, 3.5 µm) at a flow rate of 0.3 mL/min. The injection volume was 15 µL. Mobile phase A consisted of 0.1% formic acid in HPLC-grade water, and mobile phase B consisted of 0.1% formic acid in HPLC-grade acetonitrile. The 30-min gradient elution program was as follows: 5% B (0–2.00 min), linear ramp to 95% B (2.00–22.00 min), 95% B hold (22.00–25.00 min), return to 5% B (25.00–26.00 min), and equilibration at 5% B (26.00–30.00 min). Mass spectrometry was performed in positive ion mode using dual Agilent Jet Stream (AJS) electrospray ionization (ESI) with the following source parameters: sheath gas temperature, 350 °C; capillary voltage, 3500 V; nozzle voltage, 1000 V; drying gas flow, 8 L/min; nebulizer gas pressure, 35 psi; and nebulizer gas temperature, 300 °C. The scan range was 100–1700 m/z. The mass spectrometer was calibrated using the Agilent ESI-L reference mass calibration kit. Compound

annotation was supported by spectral matching against the Agilent METLIN Personal Compound Database and Library (PCDL, version B.08.00), with a similarity (DB) score threshold of > 90 considered a confident match.

RESULTS

Isolation and identification of *B. amyloliquefaciens*. Twenty morphologically distinct colonies were observed on ZMA. Initial screening revealed that isolate MI03 exhibited

the largest zone of inhibition against the five MDR *P. aeruginosa* clinical isolates. Isolate MI03 was identified as *B. amyloliquefaciens* based on colony morphology, Gram-staining characteristics, biochemical tests, and 16S rRNA gene sequencing. Phylogenetic analysis showed 99.33% sequence identity between isolate MI03 (GenBank accession PV534022) and *B. amyloliquefaciens* strain ZYB-150 (NCBI RefSeq accession PQ839300.1) (Table 1 and Figure 1).

Table 1. BLASTn sequence alignment results showing similarity of isolate MI03 with entries in the NCBI nucleotide database

Bacterial isolate	Most similar NCBI nt database entry	Max score	Total score	Query coverage (%)	E-value	Per. identity (%)	Accession length (bp)	Accession Number
MI03	<i>B. amyloliquefaciens</i> strain ZYB-150.seq. 16S ribosomal RNA gene, partial sequence	1086	1086	100%	0.0	99.33%	1304	PQ839300.1

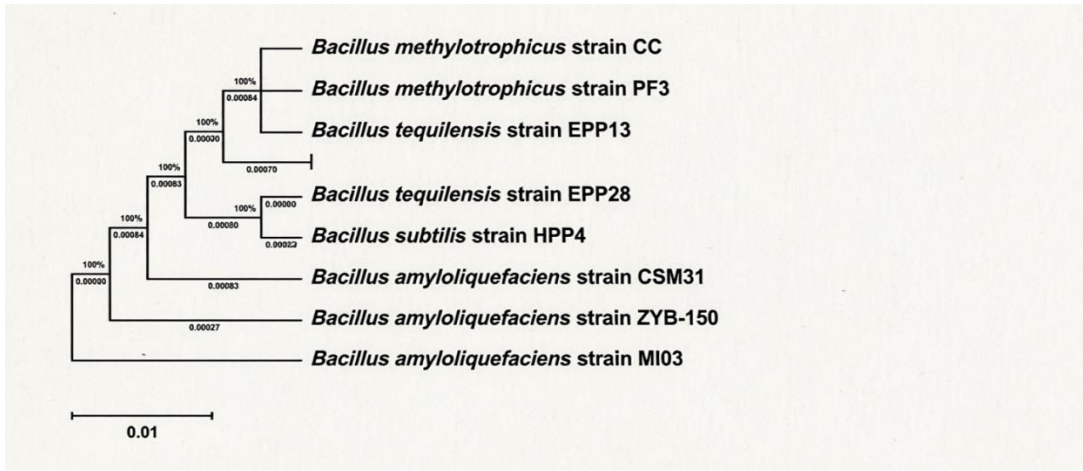


Fig. 1. Phylogenetic tree showing the taxonomic position of the marine isolate *Bacillus amyloliquefaciens* MI03 (GenBank accession PV534022) relative to closely related *Bacillus* reference strains based on 16S rRNA gene sequence analysis. The tree was constructed using the neighbor-joining method in MEGA11 software with 1,000 bootstrap replicates. Bootstrap support values (percentage of 1,000 replicates; all nodes = 100%) are shown above the nodes, and the corresponding branch lengths are shown below the branches. The scale bar represents 0.01 nucleotide substitutions per site. Isolate MI03 clusters within the *B. amyloliquefaciens* lineage together with the reference strains ZYB-150 (NCBI RefSeq PQ839300.1) and CSM31, and is clearly separated from *B. subtilis*, *B. tequilensis*, and *B. methylotrophicus*, supporting its identification as *B. amyloliquefaciens*.

Production and purification of the bioactive compound. The culture of *B. amyloliquefaciens* MI03 in Zobell Marine Broth reached stationary phase after 72 h of incubation at 30°C. The ethyl acetate extract of the cell-free supernatant exhibited inhibitory activity against all five

MDR *P. aeruginosa* strains and was fractionated by column chromatography into five distinct fractions (F1–F5). Only fraction F1 exhibited antibacterial activity against MDR *P. aeruginosa* (Figure 2 and Table 2).

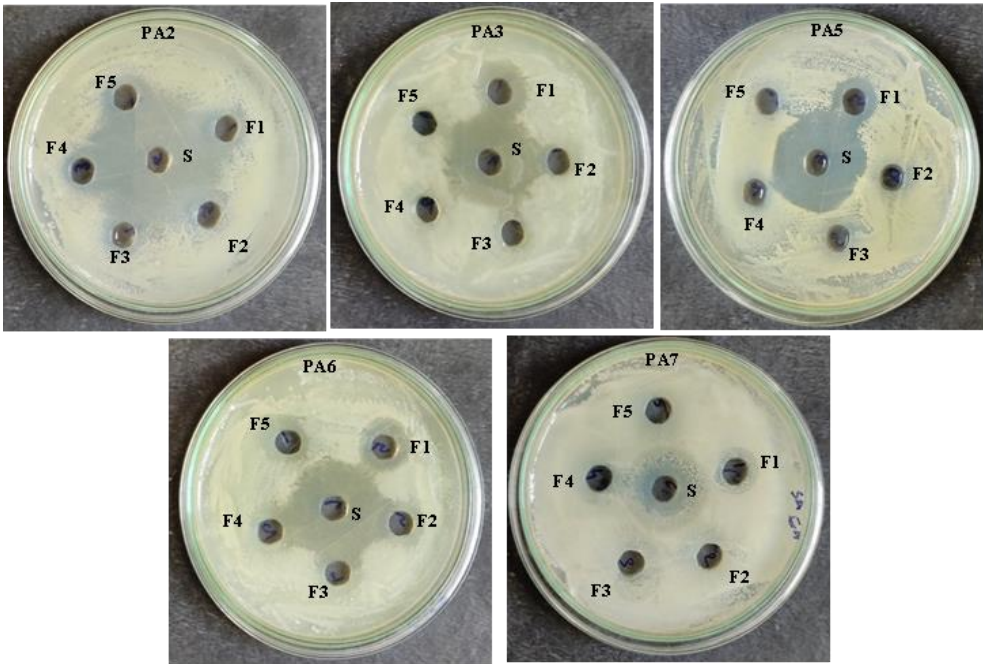


Fig. 2. Antibacterial activity of purified fractions (F1–F5) from *B. amyloliquefaciens* MI03 ethyl acetate extract against MDR *P. aeruginosa* strains (PA2, PA3, PA5, PA6, and PA7) using the agar well diffusion method. S, streptomycin (50 µg, positive control); D, DMSO (negative control). Clear zones indicate growth inhibition. Scale bar, 10 mm.

Table 2. Antibacterial activity of ethyl acetate extract fractions from *B. amyloliquefaciens* MI03 against MDR *P. aeruginosa* strains. Zone diameters are expressed as mean ± SD (n = 3 independent experiments performed on three separate days).

Fraction of ethyl acetate extract from isolate <i>B. amyloliquefaciens</i> MI03	<i>P. aeruginosa</i> PA2	<i>P. aeruginosa</i> PA3	<i>P. aeruginosa</i> PA5	<i>P. aeruginosa</i> PA6	<i>P. aeruginosa</i> PA7
	Zone of inhibition (mm), Mean ± SD, n = 3				
F1	16.3 ± 1.2	16.7 ± 1.5	15.7 ± 1.5	15.0 ± 1.0	16.3 ± 1.2
F2	–	–	–	–	–
F3	–	–	–	–	–
F4	–	–	–	–	–
F5	–	–	–	–	–
DMSO (negative control)	–	–	–	–	–
Streptomycin (50 µg)	28.0 ± 1.0	25.7 ± 1.5	26.0 ± 1.0	27.7 ± 1.5	25.0 ± 1.0

Note: –: no inhibition detected.

Characterization of the bioactive fraction F1. UHPLC-UV-MS analysis of fraction F1 enabled the putative annotation of stigmatellin Y. The compound was detected at a retention time of 28.50 min and showed an intense [M+Na]⁺ adduct ion at *m/z* 507.2725, consistent with the molecular formula C₂₉H₄₀O₆ (neutral monoisotopic mass 484.2831 Da). The observed mass matched the theoretical value with a mass deviation of 0.47 ppm. Compound

annotation was supported by spectral database searching against the Agilent METLIN PCDL (version B.08.00), yielding a similarity score of 90.56 for the top-ranked match. The putative annotation as stigmatellin Y was based on the agreement of retention time, accurate mass, and spectral matching (Level 2 confidence per the Metabolomics Standards Initiative [19]) (Figure 3 and Table 3).

Table 3. Chromatographic and mass spectral data of stigmatellin Y from UHPLC-UV-MS analysis.

Compound	Molecular formula	Retention time (min)	Neutral mass (Da)	Observed adduct	<i>m/z</i> (observed)	DB score ^a
Stigmatellin Y	C ₂₉ H ₄₀ O ₆	28.50	484.2831	[M+Na] ⁺	507.2725	90.56

^a Score based on spectral matching against Agilent METLIN PCDL B.08.00; threshold for confident match: > 90. Identification confidence: Level 2 (putatively annotated compound) per Metabolomics Standards Initiative [19].

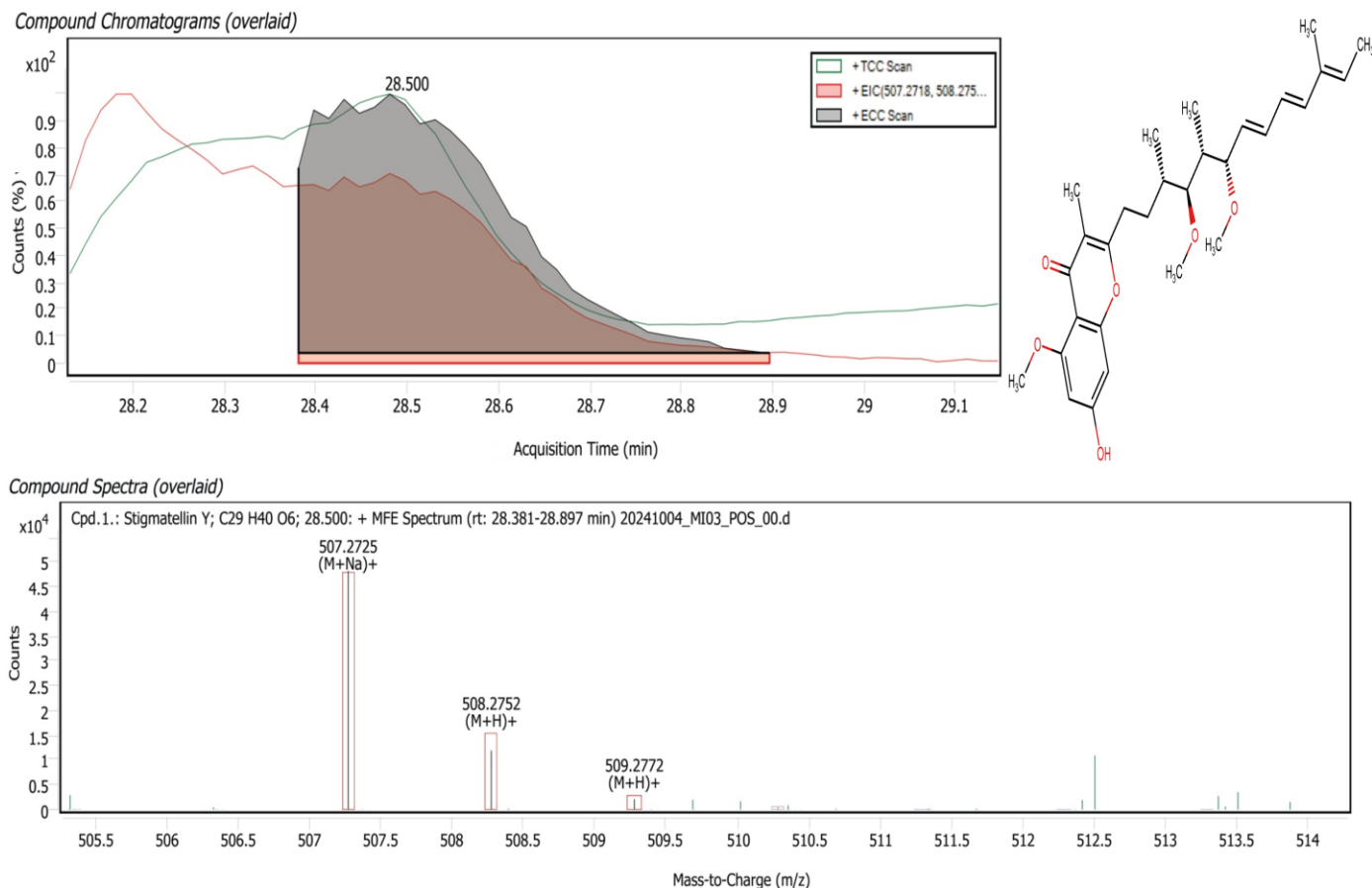


Fig. 3. Structural elucidation of fraction F1 from ethyl acetate extract of *Bacillus amyloliquefaciens* MI03 by UHPLC-UV-MS

DISCUSSION

In the present investigation, we report the isolation of stigmatellin Y from a marine *B. amyloliquefaciens* strain MI03 and its antibacterial activity against MDR *P. aeruginosa*. Stigmatellin is a natural product first isolated from *Stigmatella aurantiaca* [13]; it is a potent inhibitor of the respiratory cytochrome bc1 complex [13] and exhibits toxic effects on yeast and filamentous fungi. Three recently described stigmatellins, stigmatelic acid, iso-methoxystigmatellin A, and stigmatellin C, were isolated from *Vitiosangium cumulatum* MCy10943^T [20]. Myxobacteria are characterized by slow growth rates compared to conventional industrial bacteria such as *Escherichia coli* and *B. subtilis*, leading to longer production times and yielding mixtures of stigmatellin derivatives [21].

Boopathi *et al.* [8] isolated stigmatellin Y from *B. subtilis* BR4 and demonstrated its antibacterial activity against *P. aeruginosa* ATCC 27853. Subsequent work by the same group [14] suggested that stigmatellin Y inhibits biofilm formation by interfering with the PQS–PqsR-mediated quorum-sensing system; however, this mechanism remains to be validated in our strain. *B. amyloliquefaciens* and related species offer advantages

over *B. subtilis* for stigmatellin Y production in terms of growth rate, genomic diversity, and fermentation scalability [22, 23].

Marine environments are valuable sources of bioactive *Bacillus* strains [15, 24]. In this study, strain MI03 was isolated from seawater off the coast of Goa and identified as *B. amyloliquefaciens* by phenotypic and genotypic methods, consistent with previous reports of marine *B. amyloliquefaciens* [25, 26]. Ethyl acetate effectively extracted the bioactive metabolites from strain MI03 [27], and the crude extract inhibited all five MDR *P. aeruginosa* clinical isolates. While polar solvents such as methanol and ethanol can extract a broader range of metabolites [28], we selected ethyl acetate to target the extracellular lipophilic fraction.

UHPLC-UV-MS analysis of fraction F1 yielded the putative annotation of stigmatellin Y based on retention time, accurate mass, and spectral matching. Boopathi *et al.* [8] reported stigmatellin Y from *B. subtilis* BR4 with $[M+H]^+$ at m/z 485.2899 by LC-MS/MS, whereas we detected $[M+Na]^+$ at m/z 507.2725 under our ESI conditions. Both observations correspond to the same neutral mass of 484.2831 Da, supporting the identity of stigmatellin Y. To our knowledge, this is the first report

of stigmatellin Y production by marine *B. amyloliquefaciens*. However, several limitations should be noted: (i) the antibacterial activity was assessed only by agar diffusion at a single concentration without minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) determination; (ii) the hypothesized anti-biofilm mechanism remains untested in our strain; and (iii) cytotoxicity against eukaryotic cells was not evaluated, despite the known mitochondrial toxicity of stigmatellin. Future studies should address these gaps through dose-response assays, mechanistic investigations, and preclinical safety profiling.

In conclusion, *B. amyloliquefaciens* strain MI03 produces stigmatellin Y, which is active against MDR *P. aeruginosa*. This marine strain offers a promising platform for stigmatellin Y production, with potential for media optimization and scale-up to meet industrial requirements.

ACKNOWLEDGEMENT

Bhagvat Lad gratefully acknowledges MAHAJYOTI, Nagpur, Maharashtra, India, for providing the Mahatma Jyotiba Phule Research Fellowship (MJPRF). Sanjay Chavan thanks the University Grants Commission (UGC), New Delhi, India, for his research fellowship.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

DATA AVAILABILITY

All data are included within this article. The 16S rRNA gene sequence of strain MI03 has been deposited in GenBank under accession number PV534022.

AI DISCLOSURE

The authors did not use artificial intelligence (AI) tools in the preparation of this manuscript.

AUTHORS' CONTRIBUTIONS

BL: Methodology, Investigation, Formal analysis, Writing – original draft preparation. TAK: Supervision, Data curation, Validation, Formal analysis, Writing – review and editing. SC: Writing – review and editing.

ETHICS STATEMENT

This study did not involve human participants or animal subjects. Strain MI03 (*B. amyloliquefaciens*) was isolated from marine samples collected for this study. Because the research involved only environmental microbial sampling, formal ethical approval was not

required under Indian institutional and national guidelines.

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Cite this article:

Lad B, Chavan S, Kadam TA. Antibacterial Activity of Stigmatellin Y from Marine *Bacillus amyloliquefaciens* against MDR *Pseudomonas aeruginosa*. *J Med Microbiol Infect Dis*, 2026; 14 (2): 130-136. DOI: 10.61882/JoMMID.14.2.130.