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RESEARCH PAPER

Enhanced nematicidal potential of $MnCl_2$-fortified *Bacillus* sp. cell-free supernatant against *Meloidogyne incognita*

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ABSTRACT

Meloidogyne incognita causes major yield losses, underscoring the need for sustainable control strategies. This study is the first to demonstrate that micronutrient fortification and pH modulation of *Bacillus* sp. SK07 cultures enhance the nematicidal activity of their cell-free supernatant (CFS) and alter metabolite composition. *Bacillus* sp. SK07 was cultured in Nutrient Broth supplemented with $MnCl_2$ at 0, 50, or 75 ppm, with pH adjusted to 6 or 8. A 10% (v/v) CFS was evaluated in vitro against eggs and second-stage juveniles (J2) over 7 days using a completely randomized design with five treatments and five replicates. The most effective treatment—50 ppm $MnCl_2$ at pH 8—resulted in 96.4% egg-hatch inhibition and 63.6% J2 mortality at 168 hours, significantly higher than the unfortified control (91.8% and 49.4%, respectively). Chemical analysis revealed substantial changes in metabolite composition: unfortified cultures exhibited 9 peaks, whereas the optimized treatment produced 27 peaks, indicating increased chemical diversity. Several bioactive compounds, including fatty acid derivatives and aromatic esters, were identified. These compounds belong to diverse chemical classes such as acids, esters, alcohols, and hydrocarbons, many of which are known for their nematicidal activity. These findings demonstrate that $MnCl_2$ fortification at pH 8 effectively enhanced the bioactivity of *Bacillus* sp. SK07, offering a promising and sustainable approach for managing *M. incognita*.

Keywords: Bioassay, GC–MS, hatch inhibition, root-knot nematode, secondary metabolites

INTRODUCTION

Root-knot nematode (*Meloidogyne incognita*) is a major plant pathogen in Indonesia. Infection causes severe crop losses, reducing yields by 26.5–73.3% (Sikandar et al., 2020; Subedi et al., 2020). In tropical agroecosystems, Meloidogyne spp. are among the dominant plant-parasitic nematodes and frequently associated with significant crop damage (Nabilah et al., 2021). The presence of *M. incognita* also weakens host resistance to biotic and abiotic stresses (Thakur et al., 2024). This nematode has an extensive host range, notably infecting economically important crops such as tomatoes. Second-stage juveniles (J2) represent the critical invasive stage where nematodes penetrate plant roots using their stylets, extract nutrients, and establish reproductive sites within root tissues. The resulting root damage predisposes plants to secondary infections by soil-borne fungal and bacterial pathogens, exacerbating the overall impact on crop productivity (Azlay et al., 2023; Tapia-Vázquez et al., 2022). Therefore, effective control strategies against *M. incognita* are essential to mitigate agricultural losses.

Synthetic nematicides such as carbofuran and dazomet have been commonly used because they are effective and easy to apply (Ebone et al., 2019). However, continuous use of synthetic chemicals poses environmental concerns, including the development of resistant nematode populations, detrimental impacts on beneficial soil organisms, and disruption of soil ecosystems (Desaeger et al., 2020; Tiwari, 2024). Consequently, sustainable and environmentally friendly alternatives, particularly biological control methods, have gained considerable interes. Biological control has been widely explored in Indonesia, with

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various agents showing effectiveness in suppressing root-knot nematodes and reducing plant damage (Nur et al., 2016; Swibawa et al., 2024).

Biological control (biocontrol) refers to the practice of managing pests using living organisms or their metabolic products (Lahlali et al., 2022). Biocontrol agents can directly inhibit pathogen populations or indirectly strengthen host plant defenses (Pandit et al., 2022). Among promising biocontrol candidates, *Bacillus* sp. has demonstrated significant potential against *M. incognita*. Several studies have reported that Bacillus spp. are capable of suppressing Meloidogyne populations while promoting plant growth under tropical conditions (Winarto et al., 2024). Recent studies have also highlighted the nematicidal efficacy of *Bacillus subtilis* when combined with carbon fibers and silica nanoparticles, achieving reductions in *Meloidogyne* spp. populations by up to 90% in vitro (Karačić et al., 2024; Villarreal-Delgado et al., 2018). However, field applications of biological control agents often encounter reduced efficacy due to complex ecological factors, such as predation of antagonistic bacteria by indigenous soil microorganisms and environmental fluctuations influencing microbial survival and metabolic activity (Khan & Mohiddin, 2023).

Secondary metabolites produced by microorganisms are crucial elements in biocontrol strategies, representing diverse bioactive compounds derived from primary metabolites such as amino acids, acetyl coenzyme A, mevalonate, and intermediates from the shikimate pathway (Ahmad et al., 2021; Maulidia et al., 2020). Examples of these compounds include volatile organic compounds, toxins, and hydrolytic enzymes such as proteases, chitinases, and glucanases (Maulidia et al., 2020). Specifically, *Bacillus* sp. produces a variety of antifungal metabolites, notably cyclic lipopeptides such as surfactin, iturin, and fengycin (Liu et al., 2019). These lipopeptides, composed of seven to ten amino acid residues linked to β-amino acids (iturin) or β-hydroxy fatty acids (surfactin and fengycin), have demonstrated the ability to degrade pathogen structures predominantly composed of chitin and proteins, indicating their potential as nematicidal agents (Nadeem et al., 2021). Moreover, *Bacillus* species also secrete hydrolytic enzymes, including proteases and chitinases, further contributing to their nematicidal capacity (Asyiah et al., 2021; Prihatiningsih et al., 2021).

The composition and yield of secondary metabolites vary significantly depending on microbial species, growth media, and cultivation conditions. Recent advancements in microbial cultivation techniques emphasize media fortification strategies to enhance metabolite production. One promising approach involves the addition of manganese chloride ($MnCl_2$) to nutrient-rich growth media, such as Nutrient Broth (NB), to support bacterial growth and metabolism (Adiwena et al., 2023; Khanna et al., 2019). $MnCl_2$ is readily available, economically viable, and plays a role as a cofactor in enzymatic reactions, promoting bacterial growth. Additionally, chloride ions (Cl^-) in $MnCl_2$ may contribute to maintaining osmotic balance and pH stability, which are crucial parameters for bacterial proliferation (Adiwena et al., 2023; Bosma et al., 2021). Optimal pH conditions for bacterial growth typically range between 6.7 and 7.5, significantly influencing bacterial density and secondary metabolite production (Baatout et al., 2007; Ratzke & Gore, 2018).

Thus, fortification of bacterial growth media with $MnCl_2$ is expected to enhance the composition, diversity, and concentration of secondary metabolites produced by *Bacillus* sp. Therefore, $MnCl_2$-enhanced cell-free supernatants from *Bacillus* sp. may offer a novel and environmentally sustainable solution for managing *M. incognita*. In this study, we focus on *Bacillus* sp. SK07, a locally isolated strain with previously unreported nematicidal potential. Unlike commonly studied *B. subtilis* or *B. thuringiensis* strains, SK07 activity is evaluated under modified culture conditions involving $MnCl_2$ fortification and pH modulation, providing a novel framework for enhancing microbial metabolite profiles.

This study aimed to evaluate the effect of $MnCl_2$-fortified culture media on secondary metabolite production of *Bacillus* sp. SK07 and its nematicidal activity against *M. incognita*.

MATERIALS AND METHODS

Research Site. The research was conducted from June to December 2024, followed by data analysis and interpretation completed by March 2025. All experimental procedures and observations were carried out at the Laboratory of Plant Pest Control Technology, Faculty of Agriculture, Universitas Jember, Indonesia.

Source of *Bacillus* sp. Isolate and *M. incognita* Inoculum. The *Bacillus* sp. isolate (SK07) used in this study was an endophytic bacterium previously isolated and characterized by Asyiah et al. (2020). The *M. incognita* inoculum was obtained from the Plant Protection Laboratory collection, maintained on tomato

plants (*Solanum lycopersicum*), and periodically verified for purity.

Propagation of *Bacillus* sp. SK07. The *Bacillus* sp. SK07 isolate was propagated by streaking one loopful of bacterial culture onto Nutrient Agar (NA) plates and incubated at room temperature for 48 hours or until sufficient bacterial growth was visible (Asyiah et al., 2020; Asyiah et al., 2021).

Production of Cell-Free Supernatant from *Bacillus* sp. SK07. A single loopful of propagated bacterial colonies was inoculated into 100 mL of Nutrient Broth (NB) medium contained in Erlenmeyer flasks. The growth media were modified by fortification with manganese chloride ($MnCl_2$) at concentrations of 50 ppm and 75 ppm, with pH adjusted to either 6 or 8. A control medium without $MnCl_2$ supplementation and without pH adjustment was also prepared, resulting in a total of five treatments.

$MnCl_2$ concentrations of 50 and 75 ppm and pH values of 6 and 8 were selected as physiologically relevant variations influencing *Bacillus* metabolic responses without imposing cytotoxic stress (Adiwena et al., 2023). The bacterial cultures were incubated for seven days at room temperature with continuous agitation at 45 rpm using a rotary shaker. After incubation, cultures were centrifuged at 6000 rpm for 10 min. The supernatants were filtered through sterile syringe membrane filters (0.45 µm followed by 0.22 µm), transferred to sterile containers, and immediately used in bioassays (Maulidia et al., 2020; Pradana et al., 2025).

Extraction of Eggs and Juveniles (J2) of *M. incognita*. *M. incognita* eggs were extracted from infected tomato roots. Roots were washed under running water to remove soil and debris, then cut into approximately 1 cm segments. The root pieces were immersed in 1% NaOCl solution and agitated for 15 minutes. The suspension was passed through sieves of mesh No. 212 (0.074 mm) and No. 600 (0.023 mm) to separate eggs from debris. Extracted eggs were collected and used for subsequent assays.

To obtain second-stage juveniles (J2), eggs were incubated in sterile water at room temperature for seven days until hatching occurred, and freshly hatched J2 were collected for immediate use (Wang & Zhang, 2024).

In Vitro Efficacy Test of *Bacillus* sp. SK07 Cell-Free Supernatant. Bioassays were conducted in sterile Petri dishes (6 cm diameter). Each dish contained 100 juveniles (J2) or eggs treated with 10% (v/v) cell-free supernatants. Mortality of juveniles and percentage of non-hatching eggs were recorded daily for seven days at 24-hour intervals.

The experiment employed a Completely Randomized Design (CRD) consisting of five treatments with five replicates each: 1) control (NB without $MnCl_2$ and without pH adjustment); 2) NB + 50 ppm $MnCl_2$ (pH 6); 3) NB + 50 ppm $MnCl_2$ (pH 8); 4) NB + 75 ppm $MnCl_2$ (pH 6); 5) NB + 75 ppm $MnCl_2$ (pH 8).

Analysis of Secondary Metabolite Profile. Secondary metabolites of *Bacillus* sp. SK07 were analyzed using Gas Chromatography–Mass Spectrometry (GC-MS). Samples from the most effective treatment were filtered, and a 1 µL aliquot was injected using a split injection method.

The GC-MS analysis was performed using a SHIMADZU QP2010S equipped with an Rtx-5 MS column (30 m). Helium was used as carrier gas with ionization energy of 70 eV. Operating conditions included: column oven temperature 70 ºC, injection temperature 300 ºC, pressure 13.7 kPa, total flow 28.0 mL/min, column flow 0.50 mL/min, and linear velocity 25.9 cm/s.

The run time ranged from 3.20 min to 70.00 min with 3 min equilibrium time. Relative metabolite percentage were calculated based on peak area normalization. Retention times and mass spectra were compared with the Wiley9.LIB database for compound identification (Asyiah et al., 2025).

Analisis Data. Percentage data were transformed using arcsine square root transformation prior to analysis. Transformed data were subjected to Analysis of Variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test at 95% confidence level using DSTAT version 1.101 software (Adiwena et al., 2023; Maulidia et al., 2020; Pradana et al., 2025).

RESULTS AND DISCUSSION

Ovicidal and Nematicidal Activity of *Bacillus* sp. SK07 Cell-Free Supernatant. Fortification and pH adjustment of the *Bacillus* sp. SK07 culture medium enhanced ovicidal and nematicidal activities against *M. incognita*. The ovicidal assay revealed differences in the efficacy of cell-free supernatants produced under various culture conditions. At 24 hours post-treatment, no egg hatching was observed in the control

treatment, similar to the supernatant produced in NB fortified with 50 ppm $MnCl_2$ at pH 8. In contrast, other treatments showed minimal hatching, with unhatched egg percentages of 99.6% for 75 ppm $MnCl_2$ at pH 6, 98.8% for 75 ppm $MnCl_2$ at pH 8, and 98.0% for 50 ppm $MnCl_2$ at pH 6.

At 96 hours post-treatment, the control exhibited 95.6% unhatched eggs. This value was not significantly different from treatments with 50 ppm $MnCl_2$ at pH 6 (93.2%), 75 ppm $MnCl_2$ at pH 6 (96.8%), and 75 ppm $MnCl_2$ at pH 8 (96.4%). The highest ovicidal effect was obtained from the 50 ppm $MnCl_2$ at pH 8 treatment, which resulted in 98.4% unhatched eggs and differed significantly from the control.

A similar trend was observed at 168 hours post-treatment. The control showed 91.8% unhatched eggs, which was not significantly different from treatments involving 50 ppm $MnCl_2$ at pH 6 (91.8%), 75 ppm $MnCl_2$ at pH 6 (93.8%), and 75 ppm $MnCl_2$ at pH 8 (93.4%). However, the supernatant produced in NB fortified with 50 ppm $MnCl_2$ at pH 8 exhibited the strongest ovicidal activity, with 96.4% unhatched eggs, significantly higher than the control. Detailed results are presented in Figure 1.

Assesment of mortality among second-stage juveniles (J2) of *M. incognita* revealed varying efficacy associated with different media fortifications. At 24 hours post-treatment, the control exhibited a J2 mortality rate of 2%. Statistical analysis indicated no significant difference between the control and

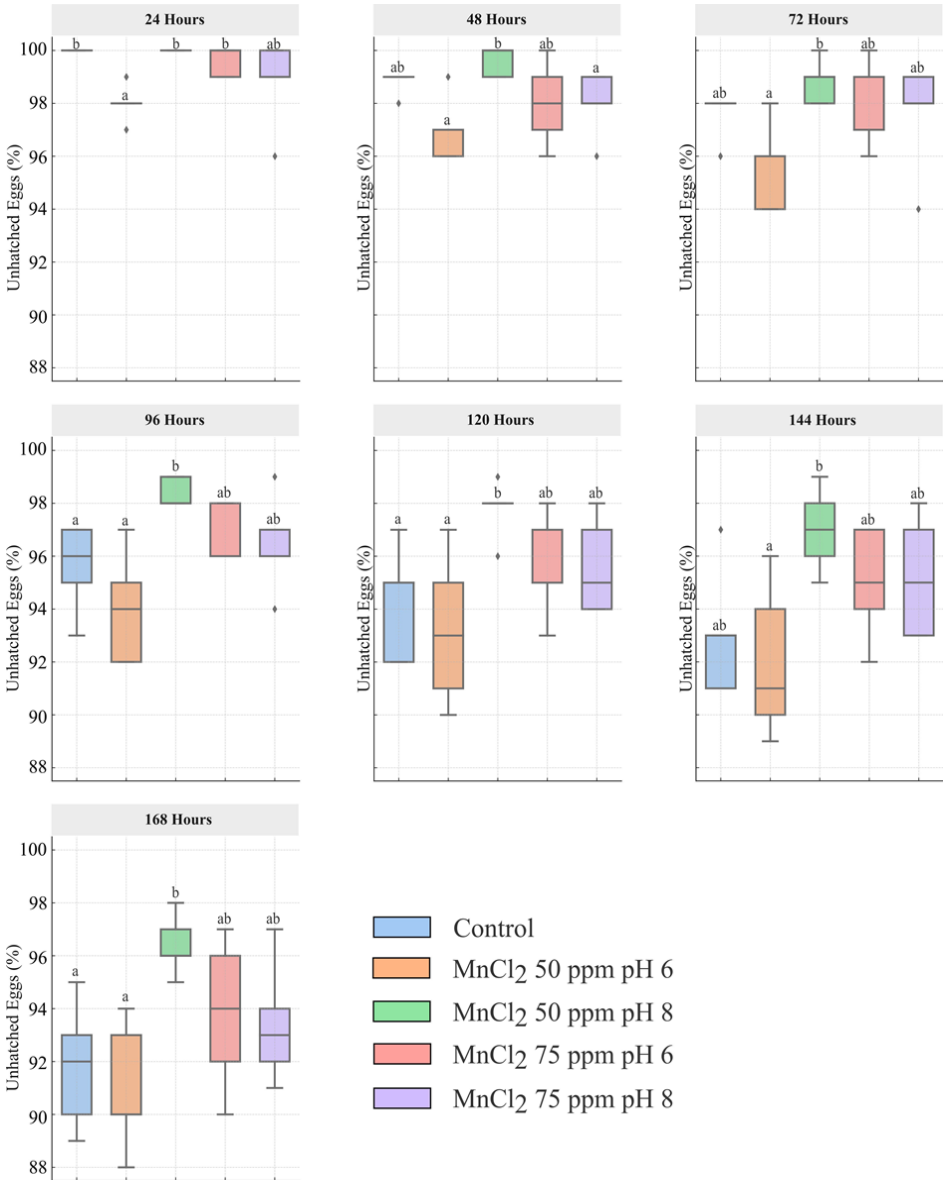


Figure 1. Ovicidal effect of *Bacillus* sp. SK07 cell-free supernatant on *M. incognita* eggs. Identical letters above the boxplots at the same time point indicate no significant difference according to Tukey's HSD post hoc test ($p \leq 0.05$).

treatments involving cell-free supernatants produced in NB fortified with 50 ppm $MnCl_2$ at pH 8 (5.2%), 75 ppm $MnCl_2$ at pH 6 (3.4%), and 75 ppm $MnCl_2$ at pH 8 (2.6%). The highest mortality at this time point (5,2%) was observed in the treatment with 50 ppm $MnCl_2$ at pH 8.

At 96 hours post-treatment, the control treatment showed an average mortality of 36%. This value did not differ significantly from treatments with 50 ppm $MnCl_2$ at pH 6 (29%), 50 ppm $MnCl_2$ at pH 8 (41.2%), 75 ppm $MnCl_2$ at pH 6 (25.2%), and 75 ppm $MnCl_2$ at pH 8 (31.8%).

By 168 hours post-treatment, J2 mortality in the control reached 49.4%. This mortality rate was not significantly different from treatments utilizing cell-free supernatants produced in NB fortified with 50 ppm $MnCl_2$ at pH 6 (60.4%), 75 ppm $MnCl_2$ at pH 6 (44.6%), and 75 ppm $MnCl_2$ at pH 8 (57.8%). However, the treatment involving 50 ppm $MnCl_2$ at pH 8 demonstrated the highest mortality rate of 63.6%, significantly different from the control. Detailed results are illustrated in Figure 2.

Various environmentally friendly approaches, including biological agents and organic amendments, have been reported to suppress *Meloidogyne* populations effectively (Nur et al., 2016). The observed ovicidal and nematicidal activities are consistent with previous reports indicating that *Bacillus* spp. produce

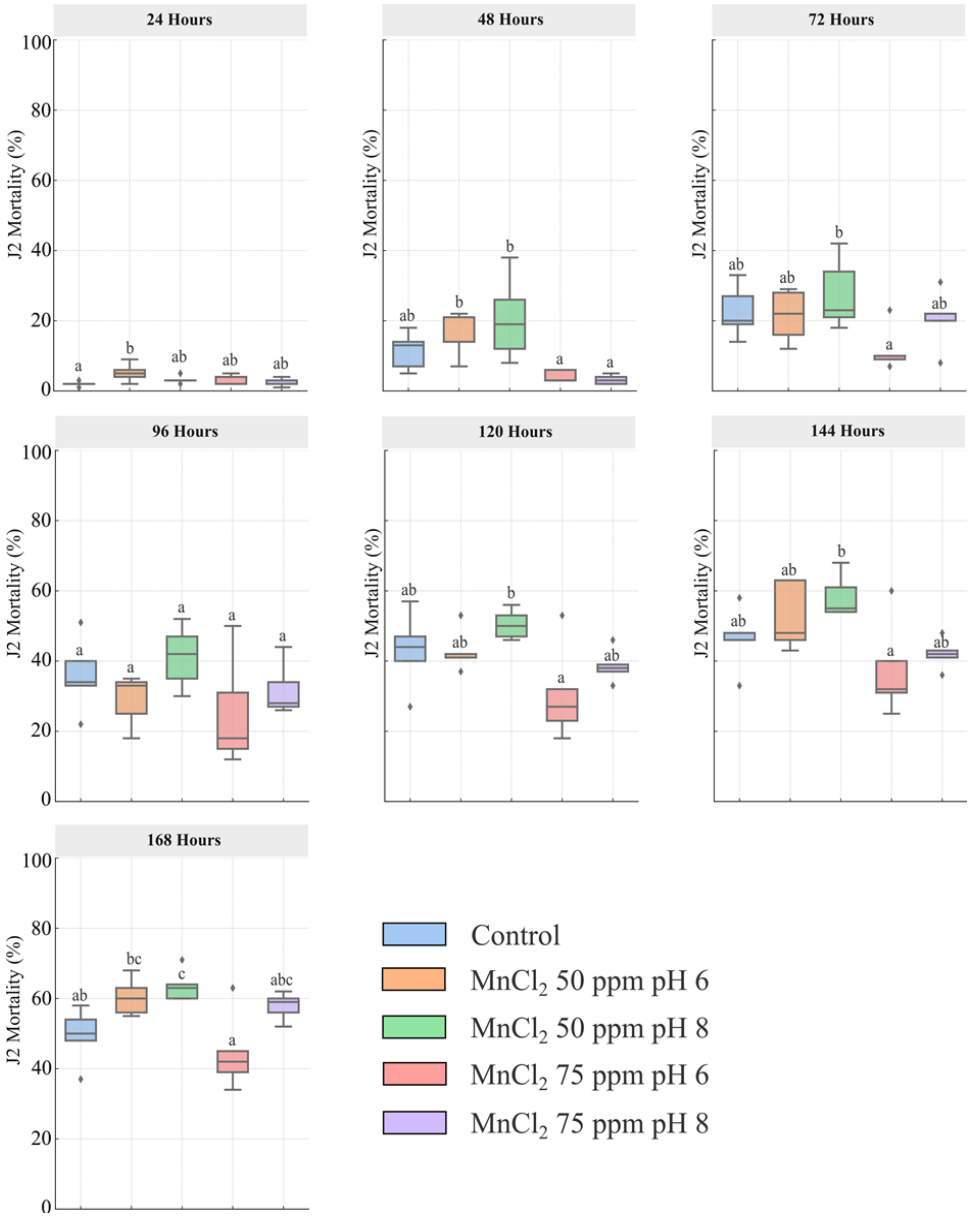


Figure 2. Nematicidal effect of *Bacillus* sp. SK07 cell-free supernatant on J2 of *M. incognita.* Identical letters above the boxplots at the same time point indicate no significant difference according to Tukey's HSD post-hoc test ($p \leq 0.05$).

bioactive metabolites that suppress *Meloidogyne* spp. These findings are also in line with studies showing that biological agents can effectively reduce egg hatching and juvenile populations of root-knot nematodes (Swibawa et al., 2024; Engelbrecht et al., 2018; Vasantha-Srinivasan et al., 2025).

These metabolites disrupt nematode cuticles, inhibit egg hatching, and induce juvenile mortality. *In vitro* studies have reported mortality rates of second-stage juveniles ranging from 80–100% within 24–72 hours following exposure to cell-free supernatants from *B. subtilis*, *B. cereus*, and *B. firmus*, a long with egg hatch inhibition exceeding 90% (Cao et al., 2019; Xiong et al., 2015).

The effectiveness of *Bacillus*-derived cell-free supernatants has also been validated in greenhouse and pot experiments, where applications significantly reduced root galling and nematode populations. Similar results have been reported for *Bacillus* spp. under tropical conditions, demonstrating their potential as biocontrol agents (Winarto et al., 2024; Maulidia et al., 2020; Singh & Siddiqui, 2010). Commercial formulations such as BioNem containing *B. firmus* have demonstrated comparable efficacy to chemical nematicides in field trials (Terefe et al., 2012).

Several classes of *Bacillus*-produced metabolites contribute to nematicidal effects, including cyclic lipopeptides such as surfactins, fengycins, and iturins. These compounds disrupt membrane integrity, damage nematode cuticles, and induce oxidative stress, leading to high juvenile mortality and reduced egg hatching (Saiyam et al., 2024; Théatre et al., 2022; Gowda et al., 2022; Nadeem et al., 2021). Additionally, non-proteinaceous metabolites such as cyclic dipeptides, trans-aconitic acid, sphingosine, and phytosphingosine exhibit strong nematicidal activity through metabolic disruption and oxidative stress mechanisms (Jamal et al., 2019; Zhai et al., 2019; Adiwena et al., 2023; Li & Zhang, 2023).

Secondary Metabolite Profiles of *Bacillus* sp. SK07 Cell-Free Supernatants. Gas Chromatography–Mass Spectrometry (GC–MS) analysis of *Bacillus* sp. SK07 cultured without $MnCl_2$ supplementation revealed nine chromatographic peaks, corresponding to nine secondary metabolites (Figure 3; Table 1). In contrast, the treatment showing the greatest biological activity—NB supplemented with 50 ppm $MnCl_2$ at pH 8—produced 27 chromatographic peaks (Figure 4), indicating a markedly enriched metabolite profile

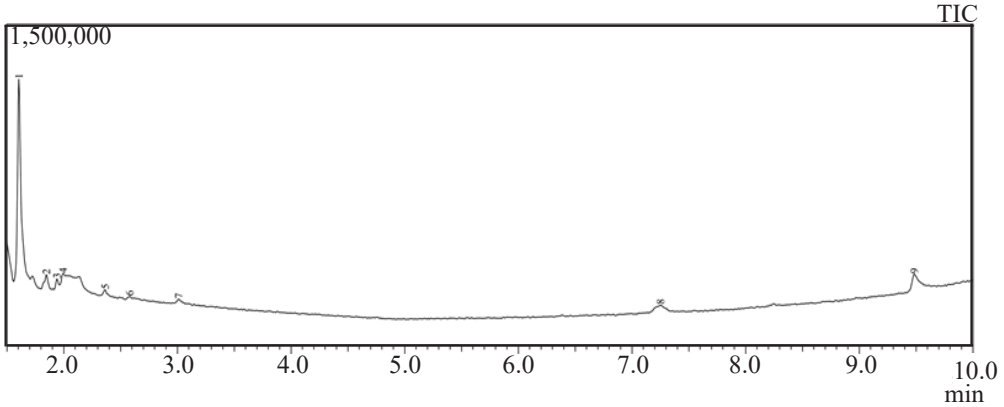


Figure 3. GC–MS chromatogram of secondary metabolites produced by *Bacillus* sp. SK07 cultured in nutrient broth without $MnCl_2$ fortification, showing nine identified compounds.

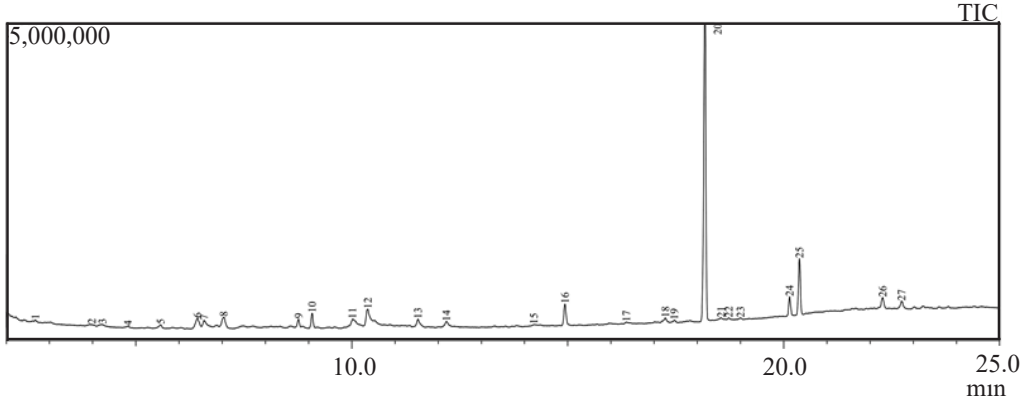


Figure 4. GC–MS chromatogram of secondary metabolites produced by *Bacillus* sp. SK07 cultured in nutrient broth supplemented with 50 ppm $MnCl_2$ (pH 8), showing 27 identified compounds.

(Table 2).

Among the identified compounds, 2-(2-ethylhexoxycarbonyl) benzoic acid was the predominant, accounting for 50.50% of the total percentage area, whereas docosane, 11-decyl-, was detected at the lowest abundance (0.23%). The metabolites belonged to diverse chemical classes, including heterocyclic compounds, carboxylic acids,

Table 1. Secondary metabolites identified by GC–MS from *Bacillus* sp. SK07 cultured in nutrient broth without $MnCl_2$ fortification

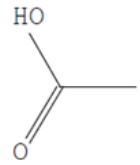
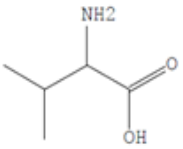
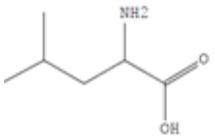
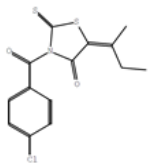
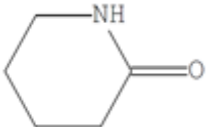
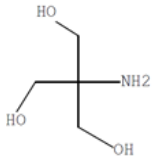
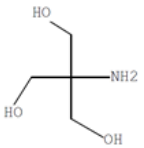
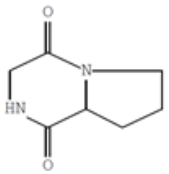
Peak #	Retention time	Area percentage (%)	Compound name	Formula	CAS registry number	Chemical structure
1	1.605	63.99	Acetic acid (CAS)	$C_2H_4O_2$	64-19-7	
2	1.847	4.34	D-Valine	$C_5H_{11}NO_2$	640-68-6	
3	1.940	1.16	2-Amino-4-methylpentanoic acid	$C_6H_{13}NO_2$	0-00-0	
4	1.994	12.58	3-(4′-Chlorobenzen-ecarboxyl)-5-pro-pylidene(1′-methyl) thiazolidine-4-one-2-thione	$C_{14}H_{12}Cl$-NO_2S_2	0-00-0	
5	2.364	1.84	Piperidinone (CAS)	C_5H_9NO	27154-43-4	
6	2.582	0.42	1,3-Propanediol, 2-amino-2-(hydroxymethyl)-(CAS)	$C_4H_{11}NO_3$	77-86-1	
7	3.011	0.58	dl-Ornithine	$C_5H_{12}N_2O_2$	616-07-9	-
8	7.251	4.64	1,3-Propanediol, 2-amino-2-(hydroxymethyl)-(CAS)	$C_4H_{11}NO_3$	77-86-1	
9	9.484	10.45	1,4-Diaza-2,5-dioxobicyclo[4.3.0] nonane	$C_7H_{10}N_2O_2$	19179-12-5	
	Total	100.00				

Table 2. Secondary metabolites identified by GC–MS from *Bacillus* sp. SK07 cultured in nutrient broth supplemented with 50 ppm $MnCl_2$ at pH 8

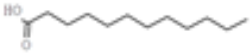
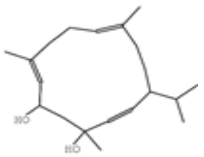
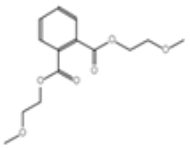
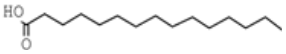
Peak #	Retention time	Area percentage (%)	Compound name	Formula	CAS registry number	Chemical structure
1	2.671	0.32	Dodecanoic acid (CAS)	$C_{12}H_{24}O_2$	143-07-7	
2	3.979	0.63	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)- (CAS)	$C_{20}H_{34}O_2$	7220-78-2	
3	4.226	0.52	Flavone 4′-OH, 5-OH, 7-di-O-glucoside	$C_{27}H_{30}O_{15}$	0-00-0	-
4	4.820	0.35	d-Mannoheptulose	$C_7H_{14}O_7$	3615-44-9	-
5	5.574	0.72	Hexadecanoic acid, methyl ester (CAS)	$C_{17}H_{34}O_2$	CAS:112-39-0	
6	6.424	2.91	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester (CAS)	$C_{14}H_{18}O_6$	117-82-8	
7	6.580	2.48	Pentadecanoic acid (CAS)	$C_{15}H_{30}O_2$	1002-84-2	
8	7.034	3.23	Octadecanoic acid, ethyl ester (CAS)	$C_{20}H_{40}O_2$	111-61-5	
9	8.765	1.64	9-Octadecenoic acid, methyl ester (CAS)	$C_{19}H_{36}O_2$	2462-84-2	
10	9.081	2.28	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS)	$C_{19}H_{34}O_2$	112-63-0	
11	10.015	3.12	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	0-00-0	

Table 2. Countinued. Secondary metabolites identified by GC–MS from *Bacillus* sp. SK07 cultured in nutrient broth supplemented with 50 ppm $MnCl_2$ at pH 8

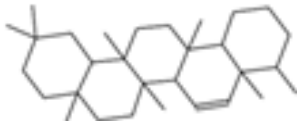
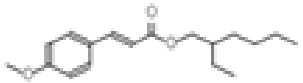
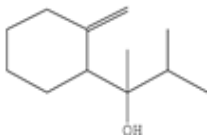
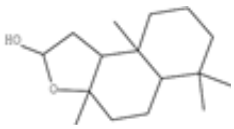
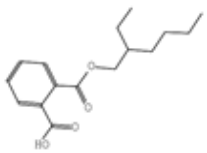
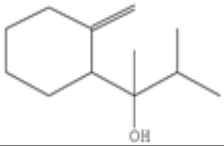
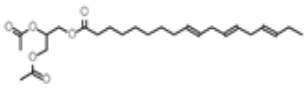
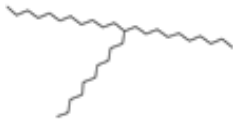
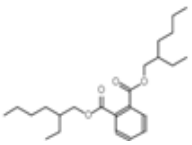
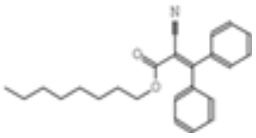
Peak #	Retention time	Area percentage (%)	Compound name	Formula	CAS registry number	Chemical structure
12	10.370	5.08	9,12-Octadecadi-en-1-ol (CAS)	$C_{18}H_{34}O$	1577-52-2	
13	11.533	1.75	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	5466-77-3	
14	12.191	1.84	D:A-Friedoolean-6-ene (CAS)	$C_{30}H_{50}O$	56588-25-1	
15	14.213	0.50	1-Eicosanol (CAS)	$C_{20}H_{42}O$	629-96-9	
16	14.935	3.85	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	5466-77-3	
17	16.359	0.33	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethyl-cyclopropyl)methyl] cyclopropyl]methyl]-, methyl ester (CAS)	$C_{22}H_{38}O_2$	10152-71-3	
18	17.259	0.96	3-Methyl-2-(2-methylene-cyclohexyl) butan-2-ol	$C_{12}H_{22}O$	0-00-0	
19	17.467	0.39	3a,6,6,9a-Tetramethyld odecahydronaphtho[2, 1-b]furan-2-ol	$C_{16}H_{28}O_2$	52811-62-8	
20	18.177	50.50	1,2-Benzenedi-carboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	4376-20-9	
21	18.550	0.84	3-Methyl-2-(2-methylene-cyclohexyl) butan-2-ol	$C_{12}H_{22}O$	0-00-0	

Table 2. Countinued. Secondary metabolites identified by GC–MS from *Bacillus* sp. SK07 cultured in nutrient broth supplemented with 50 ppm $MnCl_2$ at pH 8

Peak #	Retention time	Area percentage (%)	Compound name	Formula	CAS registry number	Chemical structure
22	18.727	0.44	1-Linolensaeure-sn-glyceryl ester-2,3-diacetate	$C_{25}H_{40}O_6$	0-00-0	
23	19.001	0.23	Docosane, 11-decyl- (CAS)	$C_{32}H_{66}$	55401-55-3	
24	20.136	2.85	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS)	$C_{24}H_{38}O_4$	117-81-7	
25	20.365	8.44	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- (CAS)	$C_{30}H5_0$	111-02-4	
26	22.290	2.13	9-Octadecenamide (CAS)	$C_{18}H_{35}NO$	3322-62-1	
27	22.729	1.67	1,1-Diphenyl-2-cyano-2-carbo-octoxy-ethylene	$C_{24}H_{27}NO_2$	0-00-0	
	Total	100.00				

esters, alcohols, amides, hydrocarbons, and complex aromatic molecules. This compositional diversity indicates metabolic plasticity of *Bacillus* sp. SK07 and demonstrates that micronutrient fortification combined with pH modulation enhance secondary metabolite biosynthesis.

In unfortified media, *Bacillus* sp. SK07 primarily produced simple volatile metabolites such as acetic acid, which has reported to exhibit nematicidal activity against *Meloidogyne* spp. Acetic acid disrupts nematode cuticles, damages pseudocoelomic nuclei, and induces cytoplasmic vacuolization, ultimately leading to mortality (Ntalli et al., 2020). Exposure to acetic acid also inhibits egg differentiation and hatching (Ye et al., 2022). Thiazolidine derivatives detected in the control treatment may also contribute to antimicrobial and potential nematicidal activity (Abdel Hafez et al., 2018).

Fortification with 50 ppm $MnCl_2$ and pH adjustment to 8 substantially altered the metabolite composition of *Bacillus* sp. SK07. The number of detected metabolites increased from 9 in the control to 27 in the fortified treatment. This shift may be attributed to physiological stress induced by elevated manganese concentration and alkaline conditions, which modulate *Bacillus* metabolic pathways (Park et al., 2022). Manganese functions as an essential micronutrient hat supports sporulation and enzymatic activity; however, elevated levels can induce stress responses that stimulate secondary metabolite production (Mols & Abee, 2011; Zuber, 2009). The disappearance of acetic acid and the emergence of more complex aromatic esters, such as mono(2-ethylhexyl) phthalate, further illustrate metabolic reprogramming under these

conditions.

Several metabolites identified in the fortified treatment have documented nematicidal activity. Dodecanoic acid suppresses *M. incognita* and inhibits nematode motility (Lu et al., 2020). Methyl hexadecanoate reduces gall formation, egg masses, and egg hatching while acting as a repellent to J2 (Zhang et al., 2012). Methyl (9Z,12Z)-octadeca-9,12-dienoate disrupts enzymatic activity in nematode respiratory chains (Hussain et al., 2023; Themuhi et al., 2025). The dominant compound, 2-(2-ethylhexoxycarbonyl) benzoic acid, belongs to the phthalate ester group and has been reported in *Bacillus*-derived metabolites with pesticidal potential (Balogun et al., 2022).

The altered metabolite profile correlated with the highest in vitro efficacy against *M. incognita*. The fortified treatment (50 ppm $MnCl_2$, pH 8) caused 63.6% J2 mortality at 168 hours and inhibited egg hatch by 96.4%. These values are significant, considering that *M. incognita* juveniles typically survive up to 60 days under normal conditions (Tsai, 2008). Microscopic observations revealed structural damage in treated nematodes, including lysis and disrupted internal tissues, while eggs exhibited shell rupture and cytoplasmic degradation. Such damage is consistent with the action of fatty acids and ester compounds that disrupt membranes and denature proteins (Maulidia et al., 2020; Pradana et al., 2025).

The enhanced production of bioactive metabolites compounds under $MnCl_2$ fortification highlights the potential of nutritional modulation to optimize *Bacillus*-based biocontrol strategies. Future studies should include comparisons with standard nematicides, targeted metabolite quantification, and greenhouse or field validation.

CONCLUSION

Fortifying *Bacillus* sp. SK07 cultures with 50 ppm $MnCl_2$ at pH 8 markedly enhanced the nematicidal performance of the resulting cell-free supernatant against *M. incognita*, achieving 96.4% egg-hatch inhibition and 63.6% juvenile (J2) mortality at 168 hours, compared with 91.8% and 49.4%, respectively, in unfortified control. This enhanced bioactivity was associated with pronounced metabolomic reprogramming, as GC–MS profiles increased from 9 to 27 peaks and shifted from simple volatile compounds to a broader spectrum of bioactive classes. The metabolite profile was dominated by 2-(2-ethylhexoxycarbonyl) benzoic acid (50.50%) and included fatty acid esters such as methyl hexadecanoate and methyl (9Z,12Z)-octadeca-9,12-dienoate. This study demonstrates that targeted micronutrient fortification combined with pH modulation can optimize *Bacillus* metabolite production and enhance nematicidal activity. These findings provide a practical and formulation-oriented framework for modulating microbial metabolite output to improve ovicidal and juvenile control efficacy, supporting the development of environmentally friendly biocontrol strategies against root-knot nematodes. Future research should validate these findings under greenhouse and field conditions, elucidate the modes of action of key metabolites, optimize application rates and exposure times, and assess crop safety as well as soil microbiome interactions.

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AUTHORS' CONTRIBUTIONS

DRA and APP contributed to conceptualization, methodology, and investigation. DAS and MWJ conducted data curation and formal analysis. SFA, SDK, and YH contributed to visualization and manuscript review and editing. KN and APP prepared the original draft. All authors have read and approved the final manuscript.

COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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