Current Trends in Biotechnology and Pharmacy
Vol. 19(3) 2422-2432, July 2025, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2025.3.27

LC–MS and GC-MS-based bioactive metabolites profiling of endophytic bacterium from *Humulus lupulus* and production of Indole-3-acetic acid

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Abstract

There is a global effort to discover natural compounds that can augment plant growth and replace synthetic chemicals. Phytohormones have been extensively employed in the development and cultivation of diverse types of cash crops. The global population has witnessed sustained yearly growth in the past decades, resulting in an increased demand for food. Consequently, advancements in weed production have become fundamental. Synthetic weed control chemicals are pervasively employed to regulate crops; however, their excessive usage contributes to environmental pollution, the buildup of harmful residues in water and soil, toxicity to mammals, and the emergence of herbicide resistance. Several synthetic phytohormones Various types of synthetic phytohormones have been synthetically created. However, the severe toxicity of these synthetic hormones frequently results in the curtailment of plant growth. Indole-3-acetic acid (IAA), a hormone in the auxin group, is crucial in regulating diverse growth-related processes in plants. The present investigation reveals the endophytic bacterium *Bacillus Cereus* SKAM2 associated with the highly valued plant *Humulus lupulus* and investigates its metabolites in agriculture and medicine. Furthermore, the dried extract was characterized using GC-MS and LC-MS. The identified compounds have been documented to demonstrate notable bioactive compounds. Our results indicated that endophytic bacteria from *Humulus lupulus* have great potential to produce indole-3-acetic acid and bioactive secondary metabolites.

Keywords: Endophytes, Metabolite, Bacteria Sustainability, Indole-3-acetic acid, Medicinal plant

Introduction

The exploration and characterization of microorganisms from diverse environments have always been fundamental for uncovering their genetic variety and metabolic attributes. De Bary (1) first highlighted the term “endophytes” in 1866, emphasizing their importance. Endophytes are well-known non-pathogenic microorganisms living in various parts of the plant without inducing any disease (2). Their major role is to enhance metabolism and plant growth, inhabiting the tissues for a portion or the complete duration of their life cycle(3–7). The rising scientific attention in studying endophytes in plant tissues is fuelled by their contribution to bioactive compound production, plant protection, growth promotion, and metabolic support(8). These products, fabricated through eco-friendly technology, will deliver substantial advantages to both consumers and farmers. A study focusing

on plant endophyte bacteria revealed host-specific traits. Research on plant endophytic bacteria uncovered that environmental variables could affect host-specific traits (9).Extensive research has demonstrated the impact of endophytes on plant growth and defense against pathogens. Owing to their essential relationship with plants, plant endophytes are acquiring significant scientific emphasis as promising sources of novel natural products(10). Plant metabolites have wonderful potential to provide pharmacologically significant compounds that promote drug discovery (11). Furthermore, conventional methods for procuring secondary metabolites from medicinal plants encounter several difficulties, such as seasonal fluctuations, Divergences between market demand and availability, biodiversity loss, the critical status of certain plant species, and rising costs (12). Plants are not only the fabricator of their secondary metabolites. Habitually, the presence of endophytes influences and regulates the biosynthesis of these metabolites (13). Previous studies reported that some common genera of bacterial endophytes are *Pseudomonas, Bacillus, Pantoea,* and *Streptomyces* (14).The global population has been rising consistently each year in the last decades, contributing to a greater need for food. As a result, improvements in crop production have become essential (15). To control weeds, synthetic herbicides are mainly employed. However, Excessive consumption of these herbicides resulted in ecological disruption, promoting harmful substances in soil and water, and toxicity to vertebrates (16,17). Due to health concerns, human and animal toxic herbicides have been restricted in various countries. To reduce reliance on these toxic herbicides, bioherbicides increasingly manage weed control (18). Among all plant phytohormones, Indole-3-acetic acid (IAA) is one of the most important phytohormones. As a key metabolite, IAA regulates growth processes such as callus formation, cell elongation, proliferation, and differentiation in response to environmental cues (19). Furthermore, previous studies explore IAA, also engaging an anti-inflammatory (20), hepatoprotective effect (21), anticancer (22), and boosting the efficacy of chemotherapy in pancreatic cancer (23). IAA is synthesized chemically; however, most chemicals used in the process cause environmental pollution. Therefore, the synthesis of IAA through artificial cultivation using the PGPRs isolated from nature would be more eco-friendly (24). This study focuses on endophytic bacteria present in the leaves of *Humulus lupulus,* a well-known climbing, perennial plant from the Cannabaceae family that exhibits a dioecious growth habit (12). The plant is widely employed for beer production and is rich in phytochemicals. Various metabolites demonstrated their beneficial potential for agricultural, pharmacological, and medicinal applications. Compounds produced from hops are widely used for various applications, including insecticides, antimicrobial agents, cancer treatments, and preservatives (25). The current study focuses on revealing the metabolite profiling through LC-MS and GC-MS of the isolated endophytic bacterium *Bacillus cereus* SKAM2 from fresh Leaves of the Hops (*Humulus lupulus*) plant. The isolated strain reveals many metabolites that are used in various fields, such as medicinal applications and agricultural metabolites, such as IAA was quantified using Ultra-performance liquid chromatography (UPLC). Furthermore, IAA was also produced

Materials and Methods

Source of endophytic bacterium

Healthy and fresh leaves from *Humulus lupulus* plants were used and preserved within the campus premises. The isolation of endophyte bacteria following previously documented methods (26). Tap water was used to remove the surface debris; furthermore, it was rinsed with distilled water. The leaves were surface sterilized by submerging them in 70% ethanol for 1 minute, followed by exposure to a 4% sodium hypochlorite solution for 5 minutes. The leave was rinsed three times with sterile distilled water under aseptic conditions in a laminar flow hood. The leaves were snipped and placed

on Luria agar plates, which were incubated at 37°C for 24 hours. Microorganism growth was observed and transferred to a fresh Luria agar plate and stored at 4°C.

Morphological identification

Gram staining was employed for the morphological identification of bacteria. HiBacillus identification kit (KB013) from Himedia Laboratories Pvt. Ltd, Mumbai, India was used to biochemically characterize the isolated endophytic bacterium. Biochemical tests including glucose, trehalose, ONPG, Voges-Proskauer, catalase, arginine, arabinose, citrate, mannitol, sucrose, nitrate reduction, and malonate (27).

Molecular identification of an endophytic bacterium

The isolated endophytic bacterium was identified using the molecular technique of 16S rRNA gene sequencing, following the method described by (28), with some modifications. The endophytic bacterium identified using molecular techniques of 16S rRNA gene sequencing. The isolated strain was cultured overnight on Luria broth media to acquire pure cultures. A cream-colored colony was selected as the DNA template for PCR. The colony was dissolved in autoclaved nuclease-free water. The 16S rRNA gene sequencing was amplified using the forward (27F) 5-AGAGTTTGATCMTGGCTCAG-3 and reverse (1492R) 5-GGTTACCTTGTTACGACTT-3. Sanger sequencer (Seq Studio 8 Flex Genetic Analyzer Thermo Fisher Scientific, Waltham, MA, USA) was employed. The obtained DNA sequence was implemented using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed using a bootstrap value of 1000.

Culture conditions of the endophytic bacterium

An isolated endophytic strain was cultured on both Luria broth media (HiMedia, Mumbai, India) and M9 Minimal Medium Salts (HiMedia, Mumbai, India). Bacterium was cultured on both media until they reached the stationary phase. The bacterial cells were collected from the broth by centrifugation. For extracellular IAA production supernatant was dried at room temperature. For intracellular IAA production, the bacterial cell pellet was dissolved in HPLC-grade methanol (Sigma). The solution was then sonicated at 80 A° for 120 seconds, employing 10-second on/off cycles. Following sonication, the mixture was centrifuged at 10,000 RPM for 10 minutes at 4°C (29). The resulting supernatant was further used for IAA evaluation.

Analysis of volatile compounds using gas chromatography-mass spectrometry (GC-MS)

The GC-MS technique was employed to characterize the organic compounds present in the dried extract (30) using Agilent 5977B GC/MSD (Santa Clara, California, United States). The GC conditions were employed, a fused silica column (60 meters long, 0.25 mm internal diameter, and coated with 0.25 µm of DB-5ms ultra-inert material). 1 a sample volume of the extract was injected, and helium was used as the carrier gas. The oven temperature program began at 30°C for 1 minute, then increased at 10°C per minute to 40°C, where it was held for 5 minutes. The temperature was then raised at 40°C per minute to 250°C and held for 5 minutes, followed by a final increase at 40°C per minute to 275°C, which was held for 10 minutes. The helium flow rate was set to 1 ml/min, and the MSD transfer line temperature was maintained at 275°C. The electron energy was set at 70 eV. The volatile constituents in the extract were identified by comparing the GC–MS spectra to those in the National Institute of Standards and Technology (NIST) MS data library.

Analysis of bioactive compounds using Liquid chromatography-mass spectrometry (LC-MS) of dried extract

The dried extract was analyzed by LC-MS, and was carried out as described by (31) Kuźniar et al. 2021 with modifications. The Dionex Ultimate 3000, Thermo Scientific. For the separation of bioactive compounds, the column

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Hypersil Gold C18 (2.1mm x 100mm, 3.0µm) was used with a temperature of 25 °C. The flow amount was employed at 0.320 ml/min (320µl/min). The method duration was 08 minutes. The mobile phase carried buffer A: 0.1% Formic acid in water and Buffer B: 0.1% Formic acid in methanol. Scientific was used for mass spectrometry (MS) detection, and ESI mode was used for Ionization with Probe Heater Temp: 320 °C, capillary temperature: 270 °C, capillary voltage: (+) 4.0 kV, aux gas flow rate (arbitrary unit): 5, S-Lens RF Level: 50, Sheath Gas flow rate (arbitrary unit): 30, sweep gas flow rate: 0, scan range: 100-1500 m/z, resolution: 70,000. Thermo Fisher Scientific Compound Discoverer 3.3 was used for the identification and investigation of the bioactive compounds.

Thin layer chromatography (TLC)

TLC was employed for the qualitative assessment of IAA. TLC Silica gel (60GF254, 20 × 20 cm, Merck) was used with the mobile phase of ethyl acetate:chloroform: formic acid (55:35:10, v/v/v). Spots displaying Rf values matching those of standard IAA were visualized under UV illumination at 254 nm (32).

Quantification of IAA by UPLC.

Quantification of IAA was conducted as mentioned by(33)Szkop & Bielawski, 2013. UPLC analysis of the IAA from intracellular and extracellular IAA production, Waters Acquity UPLC (United States) was used with BEH C18(100mm×2.1mm). The extract was solubilized in 1 mL of HPLC-grade methanol and subjected to chromatographic separation at 37°C. The mobile phase consists of solvent A: B:- 40: 60 (0.01% Glacial acetic acid in water: Acetonitrile), Wavelength: 265nm, Column Temp.: 35, particle size 1.7 µm, Detector Used: PDA eλ Detector and run time: 5 mins was employed. The flow rate of the mobile phase was kept at 0.3 mL/min, with an injection volume of 1 1 of the respective sample. The generated peak was compared with the retention time of authentic IAA. Indole-3-acetic acid (Central Drug House (P) Ltd), solubilized in HPLC-grade methanol, is used as a standard.

Results and Discussion

Endophytic bacterium isolation and characterization

In the current investigation, endophyte-isolated bacteria from the medicinal plant *Humulus lupulus*. Gram staining and the biochemical test reveal bacteria characterized as *Bacillus sp*. Molecular identification was carried out by amplifying the 16S rDNA gene from genomic DNA using Polymerase Chain Reaction (PCR), followed by sequencing, which confirmed the bacterial identity as *Bacillus cereus* SKAM2 (Accession No PQ305264). Homology analysis employing the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed a sequence similarity of approximately 100% with *Bacillus Cereus*. To strengthen the evidence for the species-level identification, a phylogenetic tree was constructed employing the neighbor-joining method based on the retrieved sequence data. Figure 1 illustrates the phylogenetic tree of isolated bacteria.

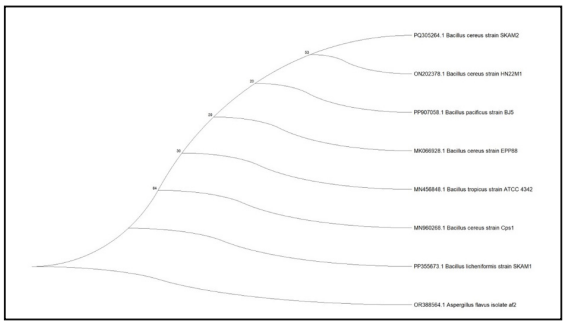

Figure.1: Phylogenetic tree of an endophytic bacterium

Analysis of volatile compounds using GC-MS.

Gas Chromatography-Mass Spectrometry (GC–MS) is an effective analytical technique for characterizing volatile compounds by evaluating retention times, resolution peak profiles, peak areas, mass fragmentation patterns, and molecular weights. Furthermore, a dried

extract from the isolated strain *Bacillus Cereus* SKAM2 was subjected to GC-MS analysis for detailed profiling of volatile compounds. Figure 2 demonstrates the chromatogram obtained from the GC-MS analysis, and Table 1 describes the GC-MS analysis of the dried extract and highlights a summary of the detected compounds, including their retention times, associated biological activities, and molecular formulas.

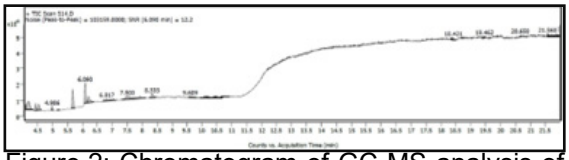

Figure.2: Chromatogram of GC-MS analysis of dried extract

LC-MS analysis.

Examining bioactive compounds of the isolated strain *Bacillus Cereus* SKAM2 using LC-MS. It revealed catalogs of metabolites. Table 2 describes the complete details of metabolite profiling, including the molecular formula and bioactivity of the compounds using LC-MS. Figure 3 shows the chromatogram of the LC-MS of the dried extract.

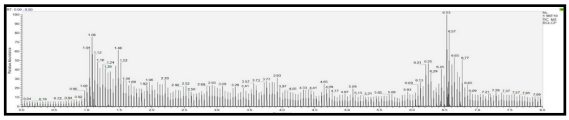

Figure 3: LC-MS analysis of dried extract

Table 1: GC-MS analysis of dried extract.

S. No	PubChem ID	Name of Compounds	Retention time (Minutes)	Area %	Molecular Formula	Activity (based on cited literature)	References
2	546203	2-Azido-2, 4,4,6,6-pentame thylheptane	6.090	92.01	C12H25N3	Anti-inflammatory	(34)
3	6329174	Silane, [(methylsilyl)methyl](silylmethyl	7.503	53.36	C3H14Si3	-	
4	6329012	1,3,5-Trisilahexane, 5-methyl	8.333	31.22	C4H16Si3	-	
5	85983070	Dimethyl-mercapto-arsine	4.986	16.36	C2H7AsS	-	

Table 2: Compounds and their properties detected in LC-MS analysis

S. No	Identified compound	PubChem ID	RT	Area Max	Molecular Formula	Activity	References
1	Norepinephrine	439260	1.078	71919501063	C8 H11 N O3	Neurotransmitter	(35)
2	Propranolol	4946	6.541	66249262491	C16 H21 N O2	Anticancer	(36)
3	L-phenylalanine	6140	1.496	20121367044	C9 H11 N O2	Antiproliferative Activity	(37)
4	L-Isoleucine	6306	1.201	16476360370	C6 H13 N O2	Plant Resistance	(38)
5	Indole-3-acetic acid	802	4.745	319184960.3	C10 H9 N O2	Plant growth-promoting	(39)
6	Carbofuran	2566	4.647	5065031086	C12 H15 N O3	pesticides	(40)
7	Apaziquone	5813717	3.922	14329072752	C15 H16 N2 O4	Treatment of bladder cancer Anticancer of Oral Cancer	(41,42)

8	Paracetamol	1983	1.08	11194814554	C8 H9 N O2	fever and acute pain	(43)
9	3-Isobutylhexahydro-pyrrolo[1,2-a]pyra-zine-1,4-dione	102892	3.597	7100421543	C11 H18 N2 O2	Antimicrobial	(44)

TLC

TLC confirmed the qualitative assessment IAA produced by the endophytic bacterium *Bacillus cereus* SKAM2. On the dried extract, a band corresponding to the IAA standard was observed, with the Rf value measured at 0.99.

Quantification of IAA via UPLC

UPLC was employed for the quantification of IAA from the dried extract. The dried extract of *Bacillus* Cereus SKAM2 cultured in Luria Broth yielded IAA concentrations of 1.37 mg/ml in extracellular and 1.11 mg/ml in intracellular. When cultured in a minimal medium, the IAA levels were measured at 0.05 mg/ml extracellular and 0.01 mg/ml intracellular. Figures 4A and 4C demonstrate the UPLC chromatograms for extracellular IAA secretion in Luria Broth and Minimal Medium, respectively. Figures 4B and 4D illustrate the UPLC chromatograms for intracellular IAA secretion in Luria Broth and Minimal Medium from the *Bacillus* Cereus SKAM2.

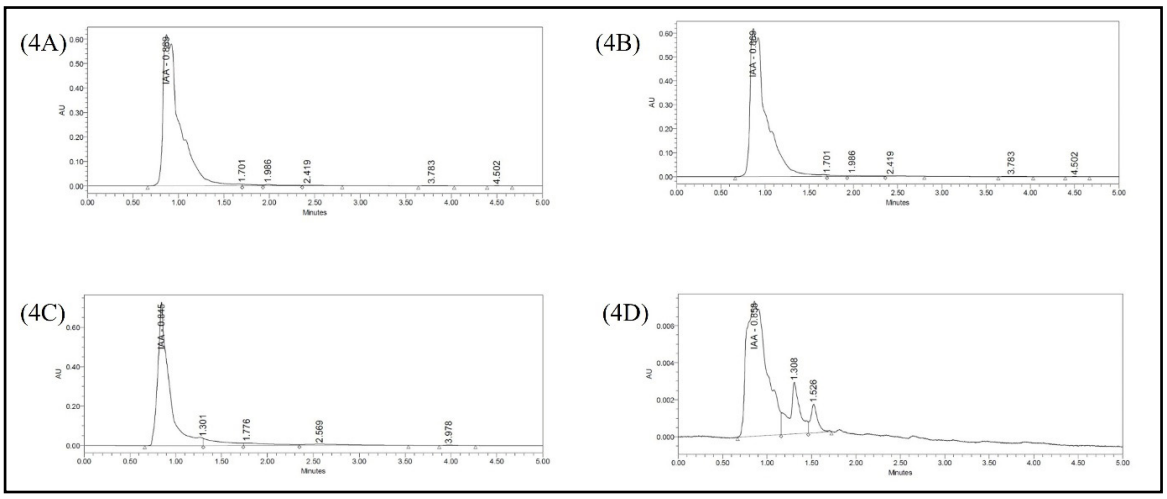

Figure 4 describes the IAA production in different media from *Bacillus* Cereus SKAM2.

Discussion

The advantages granted by metabolites from endophytes, primarily bacterial endophytes, have unlocked new avenues for exploring inadequately studied or obscure habitats and assessing how the microenvironment impacts the tuning of metabolite profiles. IAA is an auxin that governs various components of plant growth. IAA is considered hydrophilic; it can calmly diffuse across cell membranes in its protonated form, without the need for a dedicated transporter (45,46). The capacity to generate IAA is a crucial factor for screening beneficial bacteria. Furthermore, plants rely on the fabrication of phytohormones, such as IAA, to stimulate their growth and serve to alleviate both biotic and abiotic stresses (47,48). Bacteria utilize them to utilize to synthesize IAA to interact with plants. Bacteria-synthesized IAA plays a major role in promoting root development of plants and enhancing their ability to absorb nutrients from the soil and water (49,50). To generate safe ag-

ricultural products and minimize dependence on chemical herbicides, IAA-producing bacteria have the potential to cornubite as an alternative for IAA production. The rapid advancement of biological resources for eco-friendly farming is essential to mitigate ecosystem pollution caused by the excessive use of toxic agricultural compounds (51). The global requirement for the IAA is projected to reach USD 36 million by 2028, reflecting a compound annual growth rate of 5.5% (39). Expanding upon previous research emphasizing the crucial role of IAA in promoting plant-microbe interactions and supporting plant growth, our study focuses on evaluating endophytic bacteria linked with *Humulus lupulus* for their ability to fabricate IAA. Key insights from our literature review: *Bacillus Cereus* 1st isolated from the hops plant. *Bacillus Cereus* is to gram-positive bacterium and has to wide range of applications, including plant growth promotion and producing therapeutics (52). Bacteria associated with plants are known as endophytic bacteria and play a significant role in enhancing plant growth. Endophytic bacteria are acknowledged for their ability to fabricate several plant growth hormones, resulting in notable improvements in the growth and development of the host plant (7). An example of such a bacterium in our study is Bacillus *Cereus* SKAM2. Previous studies have shown that various bacilli group bacteria are capable of producing IAA (47). In our study, IAA production by the isolated strain was significantly higher in Luria broth (1.37 mg/ml extracellular, 1.11 mg/ml intracellular) compared to minimal medium (0.05 mg/ml extracellular, 0.01 mg/ml intracellular), indicating the influence of nutrient-rich conditions on IAA biosynthesis. Minimal media are regarded as economical substitutes and are recognized as appealing substrates for industrial fermentation processes (53). Various endophyte bacteria were reported for IAA production. (14) isolated various bacilli groups of endophyte bacteria and their yield of IAA, *Bacillus cereus* HRT1 7.8±0.2 µg/ml, *Bacillus megaterium* HST16 8.4±0.3 µg/ml, and *Bacillus aryabhattai* HSN1 8.9±0.3 µg/ml. Furthermore, Endophyte bacteria are a rich source of diverse metabolites. Dried extract of *Bacillus Cereus* SKAM2 was introduced to GC-MS analysis to profile the metabolites. Profile suggested compounds 2-Azido-2,4,4,6,6-pentamethylheptane reported for such anti-inflammatory activity (34). Moreover, metabolite profiling of *Bacillus Cereus* SKAM2 was done using the LC-MS. Profiling uncovers diverse compounds that have potential roles in agriculture and therapeutics. Compounds crucial to agriculture, including L-Isoleucine, play a major role in plant resistance (38)Li et al. 2021, and IAA also plays a role in plant growth promotion (54). Furthermore, therapeutic compounds reveal various biological activities, including antimicrobial, anticancer, antiproliferative activity, and neurotransmitter effects. Infectious conditions can result in elevated global fatality levels due to antimicrobial resistance (55,56) Among the identified compounds, 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione has been reported as antibacterial (44)Mangamuri et al. 2016. Identified anticancer compounds such as propranolol (36) and Apaziquone have been reported for their efficacy in treating cancer (41) and bladder cancer (42). Other compounds identified, named L-phenylalanine, reported for antiproliferative activity (37). Moreover, paracetamol, also identified by LC-MS, has been reported for treating fever and acute pain (43). The detection of notable metabolites via GC-MS and LC-MS analysis draws attention to the importance of their characterization and purification. Broadening this present study to embed the isolation of bioactive compounds and the evaluation of their biological activities could add significant value. Progressing with research on the purification and in-depth characterization of these compounds will be vital to endorsing their potential impact.

Conclusion

Our investigation highlighted that bacteria associated with the medicinal plant *Humulus lupulus* can synthesize plant growth-promoting hormones, including indole-3-acetic acid. These observations propose that isolat-

ed strains of *Bacillus cereus* SKAM2 have the potential to be applied across various sectors of medicine and agriculture. Addressing global challenges and enhancing food production to support the growing global population. Furthermore, sustaining the scarce and endangered *Humulus lupulus* species has gradually become crucial in facilitating the investigation of their endophytic microbes, potentially leading to the discovery of rich metabolites with diverse applications. The investigation also contributed to the optimization of abiotic factors to enhance IAA yield.

Acknowledgments

The authors acknowledge the Jaypee Institute of Information Technology, Noida, for providing all necessary support for the successful execution of the study.

Conflict of Interest

The authors report that there are no competing interests to declare.

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