GC–MS-BASED METABOLOMIC PROFILING AND FUNCTIONAL CHARACTERIZATION OF *Bacillus albus* REVEALING PLANT GROWTH-PROMOTING AND BIOCONTROL TRAITS

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Abstract:

In this experiment, rhizobacteria were separated from tomato rhizosphere soil and their capacity to generate rhizobacteria that promote plant growth was examined. These include the ability to produce IAA, ammonia, and phosphorus. Morphological analyses were used to identify the bacterial isolates, and 16S rDNA sequencing was used to validate their identity. Ethyl acetate solvent was used to extract metabolites from isolates. The isolates' volatiles were found using GC-MS. The discovered chemicals' peak area and retention duration were revealed by the GC MS data. The results included a large number of main and secondary metabolites. These included alcohols, phenolics, organic acids, fatty acids, and organic volatiles. Among the metabolites discovered were acetoin, hexadecanoic acid, octadecanoic acid, and 2,3-butanediol. Among these metabolites' functions include the synthesis of phytohormones and antibacterial activity. These metabolic components suggest that *Bacillus albus* contributes to plant growth via various processes, including disease suppression, mineral solubilization, and modification of plant signaling pathways. Its flexibility is enhanced by both primary metabolites needed for nutrition cycling and secondary metabolites associated with biocontrol. In conclusion, the application of GC-MS based metabolite analysis is highly beneficial for comprehending the biochemical mechanism of PGPR functioning. These findings suggest that *Bacillus albus* is a great bacteria to use for biofertilization and biocontrol. It is recommended to conduct additional study through molecular characterisation and field experiments.

Key words: Rhizobacteria, PGPR activity, Auxin, Metabolites, *Bacillus albus*

Introduction:

In order to preserve the ecosystem and promote sustainable agriculture, it has been necessary to use fewer fertilizers and fungicides in the agricultural sector. These days, the utilization of beneficial microorganisms as inoculants in biotic processes like biofertilization and biocontrol is growing [1]. To improve plant growth and lessen the impact on the environment, biofertilizers and biocontrol techniques are frequently applied to seeds and seedlings [1]. In addition to increasing soil fertility and plant productivity while lowering the need for artificial fertilizers, soil microbes play important roles in the natural cycle of elements. The soil layer connected to plant roots, known as the rhizosphere, is home to a variety of microbial communities, including plant growth-promoting rhizobacteria (PGPRs). PGPs have the most

advantageous symbiotic connection between plant roots and related microorganisms [2]. Iron chelation, phosphate solubilization, nitrogen fixation, and phytohormone synthesis are a few of these direct processes. However, among the indirect ways that PGPR promotes plant growth are the synthesis of anti-phytopathogen chemicals and the reduction of abiotic stresses like salinity and drought [3]. Plant growth-promoting rhizobacteria (PGPR) are known to be able to increase drought-expression genes, such as dehydrin, malondialdehyde, and aquaporins, along with hormonal homeostasis, antioxidants, and exopolysaccharides, as well as bacterial synthesis of phytohormones (indole acetic acid, cytokinin, and abscisic acid), antioxidants, and 1-aminocyclopropane-1-carboxylate (ACC). Furthermore, it is known that metabolites and volatile organic compounds (mVOCs) produced by PGPR bacteria are important factors influencing how microorganisms and plants interact. These substances may have a direct or indirect impact on plant development, disease prevention, and resistance to abiotic stress [4]. The secondary metabolites of organisms include a variety of chemical classes, including terpenes, alcohols, hydrocarbons, ketones, esters, carboxylic acids, and several volatile sulfur-containing chemicals [5]. Plants use a range of metabolic pathways that activate a number of phytohormones, growth regulators, and signaling molecules in order to defend themselves. Plant defense is aided by the interaction or cross-talk between these processes and events [6]. In addition to being economical, studies have shown that PGPR increases the antioxidant activity of plants. Through a variety of methods, including biological nitrogen fixation, phosphate solubilization, phytohormone synthesis, and biocontrol processes, PGPR produced as bio-inoculants is an essential component of the soil microbiota to increase crop productivity [7].

The metabolomics approach uses the chemical composition of natural products to find bioactive chemicals. Big data that will result in bioactive molecules from these natural materials is produced by combining chemical profiles with bioactivity data. For the measurement of metabolites found in various biological samples, GC-MS has continuously been the most used analytical method. The excellent chromatographic resolution of gas chromatography and mass spectrophotometry (GC-MS) is attributed to the high sensitivity and specificity of mass spectrophotometry [8]. The metabolic products, such as carbohydrates, fatty acids, organic acids, and amino acids, can be analyzed using GC-MS's comparatively high repeatability, high resolution, high quality sensitivity, and good throughput analysis when compared to other methods [9]. The long-term existence of the producer organism depends on secondary metabolites. Increased production of secondary metabolites occurs during the shift

from the active to the stationary phase [10]. Secondary metabolite extraction heavily relies on organic solvents. The intracellular and extracellular secondary metabolites will be extracted using solvents such as ethanol and chloroform. Secondary metabolite activity is increased when organic solvent is added to the growth medium. The solvent's dissolving capacity indicates the compound's activity [11]. The best method for determining the quantity and proportion of components in a complicated mixture of volatile substances is gas chromatography (GC). Additional details regarding each separated compound's molecular mass, elemental composition when using high resolution mass spectrometry, functional groups, and, in some situations, molecular geometry and spatial isomerism are revealed when it is combined with mass spectrometry (GC-MS) [12].

Gram-positive, aerobic, motile, endospore-forming, catalase-producing *Bacillus* species may colonize a variety of environments, even in harsh environments. Numerous secondary metabolites, including enzymes, antibacterial chemicals, anticancer, antialgal, and biosurfactant products, are known to be produced there [13]. The identification of metabolites from the genus *Bacillus* and their pharmacology, including how they affect cancer cells, have been the focus of contemporary research. Because of their high rate of proliferation and treatment resistance, glioblastomas are thought to be the most deadly forms of brain cancer [14]. Numerous metabolites with more research into their biological activity can be found when *Bacillus* spp. extracts are subjected to GC-MS technology [15]. This analytical method lays the groundwork for comprehending the possible pharmacological characteristics of the metabolites produced by *Bacillus* species by providing priceless information on their composition [16]. *Bacillus* species. have gained attention because to their broad-spectrum antifungal properties, long shelf life in bio-formulations, and efficient colonization of plant tissues [17]. The synthesis of structurally varied antimicrobial compounds that have shown different antagonistic activity against bacterial and fungal phytopathogens accounts for around 4-5% of the genome of *Bacillus* species. The most important of these antimicrobials are cyclic-lipopeptides (CLPs), which include iturins, fengycins, and surfactins and are essential for *Bacillus* species to colonize roots [18].

The potential of *Bacillus* species as biofertilizers and biocontrol agents has been the subject of numerous studies; nevertheless, little is known about the full metabolomic profile, account of the features that promote plant development, secondary metabolites, and their variations produced by a single strain[19]. *Bacillus sp.* has been shown in numerous studies to boost plant development and eliminate a range of environmental stressors and plant illnesses. For instance,

Vessey and Buss [20] found that co-inoculation of *Bradyrhizobium japonicum* and *Bacillus cereus* accelerated root formation and increased root biomass, which in turn encouraged soybean plant growth. The aerobic, spore-forming genus *Bacillus* gram-positive members are presently being investigated as possible striga control agents [21]. The main cause of this shift in focus is that, unlike gram-negative bacteria, bacilli produce a wide range of antibiotics that offer protection against root infections, develop endospores, and are prevalent in many soils [22]. Among the antibiotics made are lipopeptide surfactins, iturin, and fengycin, which are highly effective at inhibiting the growth of a wide range of plant diseases and the activity of fungi [23]. Adoption of *Bacillus* species in agriculture should be promoted due to their advantages as potential PGPR in terms of biofertilization, biological control, and bioremediation, all of which have a positive effect on crop productivity and ecosystem functioning. The use of PGPR will surely become a reality as technology advances in the formation of efficient research and development. It will be useful in crucial processes that guarantee the stability and productivity of agro-ecosystems, leading us to an ideal agricultural system. It has been shown that certain *Bacillus* species stimulate plant development by generating phytohormones such IAA, cytokinin, and gibberellic acid. The PGPR bacteria manufacture IAA, a crucial auxin, using two main pathways: the IAM pathway, which uses indole-3-acetamide to form IAA, and the tryptophan-dependent method, which uses tryptophan from the soil with the aid of the tryptophanase enzyme. Dimethylallyl diphosphate and ATP are converted into cytokinin by the IPT enzyme, which is responsible for cytokinin biosynthesis [24].

In order to evaluate the function of these metabolites in plant growth promotion activities, the current study plans to do a thorough GC-MS analysis of metabolites produced by *Bacillus albus* collected from rhizospheric soil. Significant insight into the metabolic activity linked to *B. albus*'s PGPR activities will be gained by identifying key bioactive chemicals through metabolite analysis in comparison to control samples. The investigation will aid in determining the chosen bacteria's functional role in the rhizosphere.

Methods and materials:

A sample is taken from the tomato plant's rhizosphere (rhizoplane) in a tomato farm close to Vengalayapalem in the Guntur district.To check for the existence of any bacterial strains on bacterial media, the collected sample is serially diluted.Using the spread plate method, the soil sample is serially diluted and inoculated on nutrient agar plates.In order to obtain pure cultures,

colonies from the mother plate are now treated to the streak plate technique. Each colony is then evaluated for qualitative analysis of plant hormone production (auxin) and nutrient production (ammonia, phospate) activities.The powerful strain exhibiting all of these traits is now isolated and kept in glycerol stock for additional examination.After the isolated strain was submitted to the NCBI for rRNA sequencing, it was discovered that it was *Bacillus* albus (PX690623).

Culture conditions:

The isolated *Bacillus albus* strain was cultivated in sterile nutrient broth medium and shaken at 120–150 rpm for 48 hours at 30 °C.

Preparation of media for auxin analysis:

Two concentrations of L-tryptophan, 0 and 1000µg/ml, were utilized in duplicate for auxin quantitative analysis. Four flasks containing fifteen milliliters of L-broth were produced and autoclaved for a single strain. 0.2 g of L-tryptophan was added to 20 ml of autoclaved distilled water to create a stock solution. 1.5 milliliters of stock L-tryptophan were added to L-broth at a concentration of 1000µg/ml. The appropriate Bacillus strains were added to each flask. Culture flasks were kept in a shaker incubator at 37°C for 72 hours at 130 rpm. Bacterial cultures were harvested following incubation. To get the supernatant, the culture was centrifuged for ten minutes at 5000 rpm. Two milliliters of Salkowski reagent were applied to a test tube containing one milliliter of bacterial supernatant. For thirty minutes, test tubes were incubated in the absence of sunlight. Following incubation, optical densities were measured at 535 nm and the pink color development was noted [25].

Preparation of media for ammonia analysis:

The isolates' ammonia production was calculated using the method outlined by Marques et al. (2010) [26]. For 48 hours, bacteria were cultivated in 10 mL of peptone broth at 30°C. Following incubation, 0.5 mL of Nessler's reagent is added to the isolated bacteria. Ammonia would be present if the hue changed from yellow to dark brown [27].

Methods for sample preparation for GCMS analysis:

The solvent extraction approach was employed to isolate metabolites from a *Bacillus* albus cell-free culture (100 mL).After adding 100 mL of ethyl acetate (EtOAc) to the cell-free broth, the mixture was periodically vortexed for five minutes to allow pressure to build up.Because

of their varying densities, this was allowed for five to fifteen minutes to allow the phases to separate. The top organic layer was ethyl acetate due to the lower density of EtOAc (ρ = 0.90 g/mL).After adding a few drops of saturated NaCl or methanol to help with phase separation, the emulsion was centrifuged for five minutes at 3,000 xg. The ethyl acetate fraction's top layer was gathered. Fresh ethyl acetate was used for two or three further extractions (3-4 extractions).

GCMS analysis:

According to Gheda et al., *Bacillus albus* extract was analyzed using a Gas Chromatography-Mass Spectrometer (Shimadzu GC-MS QP2010SE, Autosampler: AOC 20i) to identify its various secondary phytochemical compounds. The GC-MS analysis was performed by inserting 1 µ L of the methanolic extract onto a column (SH-5Sil-MS 30 m, 0.25 mm ID, 0.25 µ m), using helium as the carrier gas. The temperature was set to vary from 50 to 250 degrees Celsius for 2.50 minutes, ramp up to 8 degrees Celsius, hold for five minutes, then rise by 5 degrees Celsius per minute to 280 degrees Celsius per minute, and hold for two minutes.Turbo-Mass ver-5.2 software was utilized to handle mass spectra and chromatograms with a delay time of 4.00 minutes, a split ratio of 20:1, a scan range of 50 to 600 Da, and an injector temperature of 280 2 ◦ C using a solvent. The GC-MS computer system's integration of the National Institute of Standards and Technology (NIST) database with the compounds' mass spectra served as the basis for compound identification.

Results:

Control

The *Bacillus albus* extract GCMS chromatogram displayed several peaks. The control sample's GC–MS analysis showed a complicated chromatographic profile with several peaks visible over the retention time range of roughly 4.6 to 50.2 minutes. A wide range of primary and secondary metabolites were present, according to the total ion chromatogram (TIC). Retention period and comparison with the NIST library database led to the identification of 115 chemicals. Erucic acid (26.65%) was the most prevalent metabolite found in the control sample, followed by 1,3,2-Dioxaborinane, 2,4-diethyl-5-methyl-6-propyl (5.96%), 1-Pentacosanol (4.86%), and 2,5-Piperazinedione derivatives (4.39%). Significant levels of chemicals like 1-Nonadecene (3.39%) and Pyrrolo[1,2-a]pyrazine derivatives (3.74%) were also found (Fig. 1)

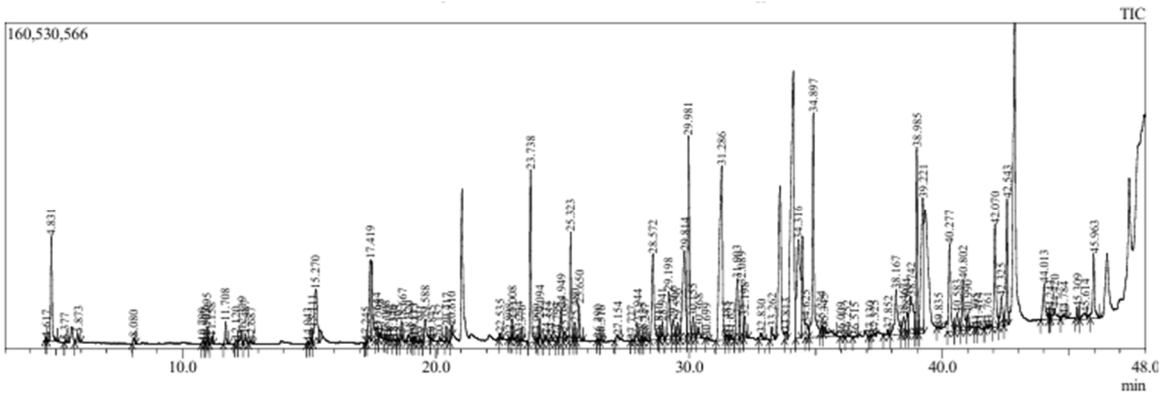
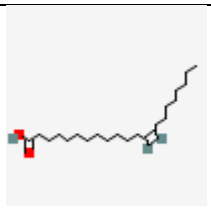
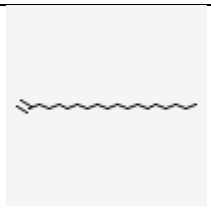
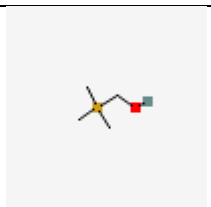
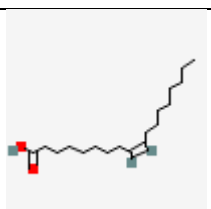
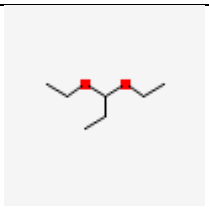
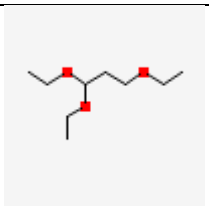
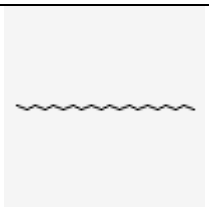
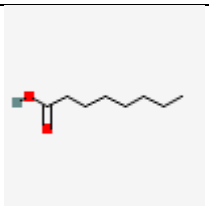
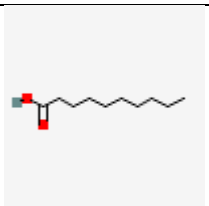
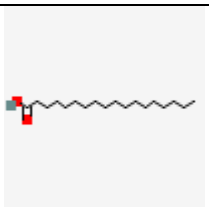


Fig 1: Chromatogram of control

Table 1: List of GCMS compunds present in the control sample

Peak#	R. Time	Area %	Compound Name	Structure
114	49.615	26.65	Erucic acid	
66	29.981	3.39	1-Nonadecene	
2	4.831	1.69	Trimethylsilyl methanol	
90	38.167	1.50	Oleic acid	

1	4.617	0.03	Propane, 1,1-diethoxy-	
10	11.708	0.29	Propane, 1,1,3-triethoxy-	
43	24.094	0.36	Eicosane	
16	15.111	0.24	Octanoic acid	
33	20.152	0.05	Decanoic acid	
92	38.604	0.45	Octadecanoic acid	

Erucic acid, a monounsaturated fatty acid with 22 carbon atoms and one double bond (22:1 n-9), found commonly in mustard and rape seed oil, may be metabolized by *Bacillus* genus bacteria. The erucic acid, commonly studied for its role in reducing the toxicity of vegetable oil during fermentation, may be metabolized by the genera *Bacillus*, *Pseudomonas*, and *Rhodococcus* [28]. Trimethylsilylmethanol from the control substance is not a result of microbe metabolism but a side effect of derivatization during GC-MS analysis [29].

During sample preparation, aldehydes react with alcohols to generate propane, 1,1-diethoxy- (propionaldehyde diethyl acetal), which was found in the control sample. Acetalization is a

well-documented chemical system process that can take place under extraction and GC-MS settings. This compound's existence in the control indicates that it is not a microbiological metabolite but rather a solvent-derived artifact [30].

The presence of propane and 1,1-diethoxy-in the control chromatogram indicates the formation of acetal derivatives during sample preparation. During extraction and GC-MS analysis, a well-known chemical reaction between alcohols and aldehydes might result in the formation of acetals. The presence of this chemical in the control suggests that it is a contaminant produced from the solvent rather than a bacterial metabolite [31]

Aldehydes react with alcohol solvents during sample preparation and GC–MS analysis to produce the acetal chemical propane, 1,1,3-triethoxy-, which is visible in the control chromatogram. These triethoxy chemicals are well-known byproducts of chemical acetalization and etherification reactions, not microbial metabolism. The presence of this molecule in the control proves that it was a solvent-derived artifact and rules out its role in bacterial metabolic activity [32].

The long-chain n-alkane eicosane, which is present in the control chromatogram, is thought to be a non-biological hydrocarbon that often arises from column bleed, solvent impurities, or environmental pollution. These alkanes are frequently observed in GC-MS background profiles and are unrelated to microbial metabolic activity under normal growth circumstances. Although certain specialized microorganisms can degrade long-chain hydrocarbons, their presence in control samples suggests a non-biological origin in the current study.1 Nonadecene, a long-chain terminal alkene seen in the control chromatogram, is thought to be a non-biological hydrocarbon that usually arises from column bleed, solvent impurities, or environmental pollution. Although some microbes have the capacity to synthesize long chain alkenes in specific conditions, the presence of such molecules in the control chromatograms in this study indicates that there might be either an analytical reason or environmental source rather than biological synthesis by any organisms [33, 34]. Octanoic acid (caprylic acid), a medium chain fatty acid detected in the control, is generally known to exist as a contaminant in the medium, solvents, and lipids degraded in the process of sample preparation.Similar results have been reported in GC-MS analyses, where fatty acids are frequently detected in blanks and culture medium due to non-biological sources [35].

Media components, solvent contaminants, or lipid breakdown during sample preparation often yield the medium-chain fatty acid decanoic acid (capric acid), which was detected in the control

chromatogram. Although decanoic acid is known to have antibacterial effects and participate in microbial metabolism, its presence in the control sample suggests a non-specific origin unrelated to bacterial production. The same findings have been made in GC-MS tests, where fatty acids obtained from non-biological materials are usually present in the samples of media and blanks [36].

For example, oleic acid, which belongs to a group of monounsaturated fatty acids and is found in the chromatogram of the control sample, may originate from media constituents, lipids, and even solvent contaminants. Even though oleic acid takes part in the membrane formation of bacteria and has antibacterial activity, the detection of this compound in the control sample shows that this fatty acid does not originate from the process of microbial biosynthesis [37].

The long chain fatty acid octadecanoic acid (stearic acid) identified by the control sample has often been seen to be an impurity originating from the solvent used, contamination of the culture media with lipids, or breakdown of complex biological molecules during sample extraction. While stearic acid can be considered as a constituent part of membranes and involved in lipid metabolism, its identification in the control sample does not reflect its origin from microbial biogenesis. Studies using GC-MS technique have revealed long chain fatty acid in media blanks [36].

Primary metabolites:

The sample's GC–MS chromatogram showed a complicated metabolic profile with many well-resolved peaks spread between 4.6 and ~46 min retention time, suggesting the presence of a variety of volatile and semi-volatile chemicals.

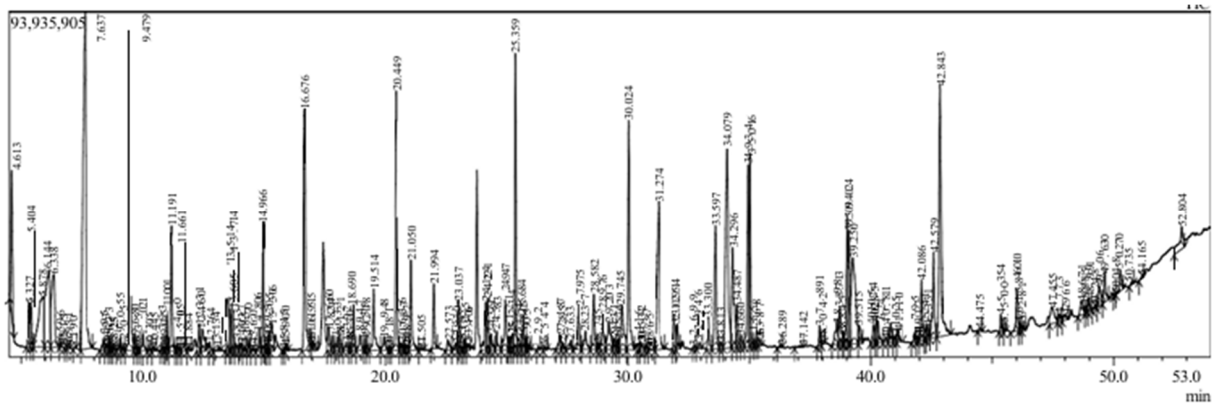
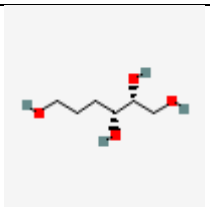
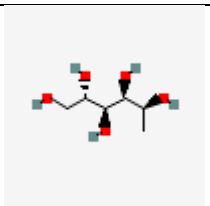
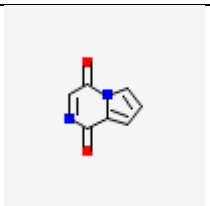
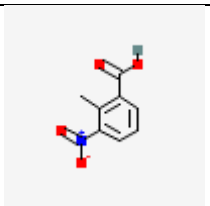
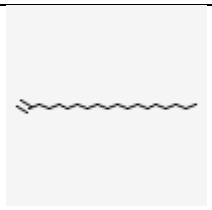
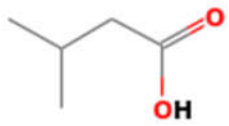
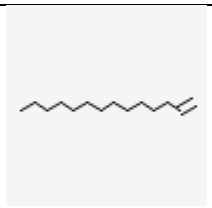
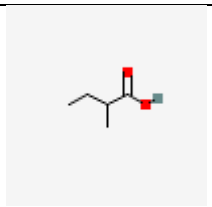
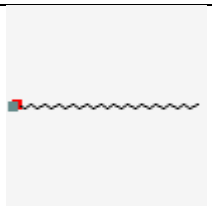
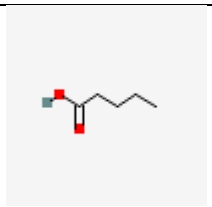
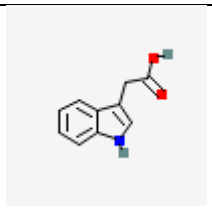


Fig 2: Chromotogram of primary metabolites of *Bacillus albus*

Table 2: List of primary metabolite compounds in the

Peak#	R. Time	Area %	Compound Name	Structure
11	7.637	7.80	1,6-Dideoxygalactitol	
19	9.479	5.06	2,5-Monomethylene-L-rhamnitol	
120	34.079	4.38	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-	
52	16.555	3.28	Benzoic acid, 2-methyl-3-nitro-	

88	25.359	2.94	1-Nonadecene	
5	6.144	2.72	Butanoic acid, 3-methyl-	
68	20.449	2.66	1-Tetradecene	
6	6.338	2.02	Butanoic acid	
136	39.024	1.52	1-Hexacosanol	
28	11.191	1.47	Pentanoic acid	
116	32.946	0.08	IAA ethyl ester (Indole-3-acetic acid ethyl ester)	

Bacillus organisms do not frequently generate L-fucitol, which is also known as 1,6-dideoxy galactitol, as their primary end product. However, the role of L-fucitol is vital as a structure in studies related to sugar metabolizing enzymes for the Firmicutes family of bacteria.

Various *Bacillus* species, such as *Bacillus subtilis* and *Bacillus licheniformis*, synthesize methyl butanoic acid, which may also be called isovaleric acid. It is commonly produced during food product fermentation and frequently results from leucine breakdown [38].

2,5-Monomethylene-l-rhamnitol is a volatile substance that can be found in a variety of bacterial settings.It is often a metabolite.Although it seems 2,5-Monomethylene-1-rhamnitol may play a role in the analysis of micro-organisms where the GC-MS technique is used to analyze volatile substances, it cannot be classified as the major metabolite of *Bacillus* because amino acids, organic acids, and nucleic acids are classified as major metabolites of *Bacillus* bacteria. [39]

The following is a description of this nitroaromatic compound known as Benzoic acid, 2-methyl-3-nitro- which is also popularly called 3-nitro-o-toluic acid. It has been found as an intermediate product during the breakdown of the pesticide substance, namely pendimethalin by the *Bacillus* bacteria such as *B. subtilis*.

Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro is among the group of compounds that make up the diketopiperazines which have antibacterial properties. Diketopiperazines have been observed to be produced by the *Bacillus* albus and the antibiotics generated from *Bacillus tequilensis* inhibit multidrug resistant Staphylococcus aureaus highly effectively.Furthermore, its broad-spectrum effectiveness against bacterial and fungal infections has been reported in recent research. Together, these results imply that this metabolite plays a major role in *Bacillus albus's* ability to biocontrol and promote plant growth [40].

Some *Bacillus* strains create volatile organic compounds (VOCs) that contain tetradecene, a long-chain ß-olefin with antifungal properties [41].

Butanoic acid, a short-chain fatty acid linked to primary metabolism and strong antibacterial activity, was found in the GC–MS profile of *Bacillus albus*. Through cytoplasmic acidification and membrane integrity disturbance, short-chain fatty acids have been shown to suppress pathogenic bacteria. Butanoic acid and other microbial metabolites in the rhizosphere help to maintain microbial balance and reduce soil-borne diseases [42].

The identification by GC-MS of *Bacillus albus* yielded hexacosanol, a fatty alcohol containing long chain of fatty acids. The presence of hexacosanol in a biological organism is known to be associated with bioactivity and lipid metabolism. Fatty alcohols, as reported, are capable of exhibiting antibacterial properties by disrupting the membrane structure of microorganisms. These lipids play a role in microbial stabilization and act against soil-borne diseases. These results imply that 1-hexacosanol supports *Bacillus albus*'s ecological fitness and biocontrol effectiveness [43].

Pentanoic acid, which is among the short-chain fatty acids with functions in metabolism and bacteria, was identified through GC-MS testing on the bacterium *Bacillus albus*. The function of short-chain fatty acids is to help maintain microbial balance in the environment by reducing soil pathogens.Additionally, they may function as signalling molecules that cause plants to develop systemic resistance. These results imply that pentanoic acid is crucial to *Bacillus albus*'s PGPR activity and biocontrol activity [44].

Higher production of growth-associated primary metabolites, such as precursors for auxin biosynthesis and nitrogenous substances involved in ammonia formation, is supported by the observed rise in OD, which is directly correlated with increased metabolic flux. In accordance with the theory that the process of metabolite biosynthesis is directly proportional to increased biomass formation efficiency, and that the formation of primary metabolites depends on growth.

The optimization approach proved its value in relation to the functions performed by PGPRs, as it not only enhanced bacterial growth but also facilitated physiological conditions favoring synthesis of primary metabolites.

Secondary metabolites

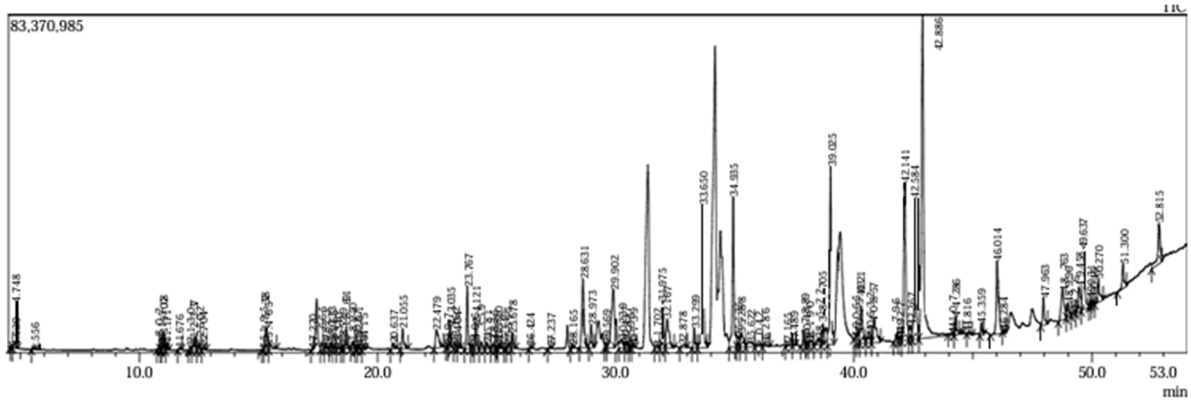
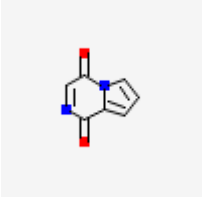
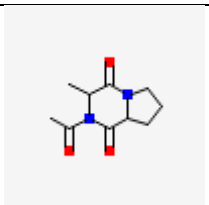
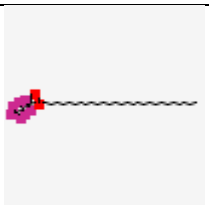
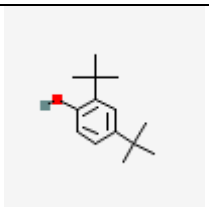
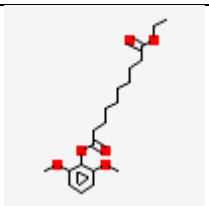
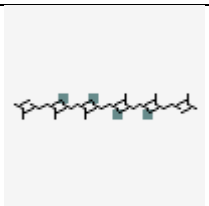
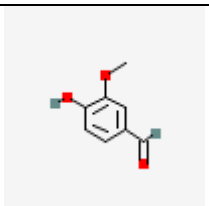
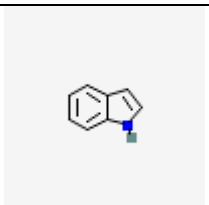


Fig 3: chromatogram of secondary metabolites of *Bacillus albus*

Table 3: List of Secondary metabolite compounds in the GCMS analysis

Peak#	R. Time	Area %	Compound Name	Structure
87	42.886	16.31	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	

49	28.631	3.07	N-acetyl-3-methyl-1,4-diazabicyclononan-2,5-dione	
104	52.815	2.17	Heptacosyl heptafluorobutyrate	
35	23.767	1.86	2,4-Di-tert-butylphenol	
95	48.763	1.90	Sebacic acid, 2,6-dimethoxyphenyl ethyl ester	
102	50.270	0.96	Squalene	
29	21.055	0.73	Vanillin	
21	18.340	0.08	Indole	

The diketopiperazine class of bioactive metabolites generated from amino acids includes pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, which was found in the GC-MS profile of *Bacillus albus*. The antibacterial action of these substances against a variety of bacterial and fungal diseases is well established. Diketopiperazines also reduce pathogen virulence and biofilm development by acting as quorum sensing inhibitors. Additionally, they function as

signaling molecules that strengthen plant defense mechanisms against phytopathogens by causing systemic resistance. These combined characteristics imply that this molecule is essential to *Bacillus albus*'s ability to biocontrol and promote plant growth [45].

Heptacosyl heptafluorobutyrate is one of the bioactive substances that can be found in the chemical profiling of extracts from certain *Bacillus* strains. It is recognized as one of these bacteria's secondary metabolites. [46].

N-acetyl-3-methyl-1,4-diazabicyclononan-2,5-dione is a significant secondary metabolite produced by *Bacillus* species, especially *Bacillus amyloliquefaciens*. It is frequently described in writing as a derivative of 3-methyl-1,4-diazabicyclononan. It is another well-known bioactive secondary metabolite made by a number of organisms, such as *Bacillus subtilis* and other *Bacillus* species. It is a phenolic chemical with potent antibacterial, antifungal, and antioxidant properties that frequently helps the producing organism regulate endocide.

Some of the secondary metabolites produced by the genus *Bacillus* are sebacic acid and 2,6-dimethoxyphenyl esters derivatives, including sebacic acid 2,6-dimethoxyphenyl tridecyl ester and its derivatives such as di(2,6-dimethoxyphenyl) ester. While extracting *Bacillus* samples that have antimicrobial activities, there were frequent instances where phenolic compounds, organic acids, and phthalates accompanied these bacteria.

Squalene, which is a biologically active hydrocarbon belonging to the triterpenoid class and serves as an important molecule in isoprenoid synthesis, was identified through the *Bacillus albus* GC-MS analysis. As per the literature, squalene has antioxidant and antibacterial abilities that aid in the protection of cells as well as inhibiting pathogenic microorganisms. The antioxidant ability of this compound enhances rhizosphere efficiency under oxidative stress conditions. Furthermore, it is known that terpenoid molecules take role in signaling mechanisms and plant–microbe interactions that affect plant defensive responses [47].

Vanillin, a phenolic secondary metabolite with important plant growth-promoting and biocontrol capabilities, was found in the GC–MS profile of *Bacillus* spp. By interfering with the integrity of membranes and metabolic processes, phenolic substances like vanillin demonstrate potent antibacterial activity against a variety of bacterial and fungal diseases.

A crucial product from tryptophan metabolism, indole is found in the GC-MS profile of *Bacillus* species. IAA, a plant growth hormone that is crucial for plant development, is derived

from indole. It is essential for plant growth because it encourages the formation of roots and the uptake of nutrients from the soil. Also, it induces systemic resistance by activating plant defense mechanisms. Additionally, indole has anti-biofilm and antibacterial properties that lessen the pathogenicity of pathogens [48].

The growth optimization data unequivocally shows that environmental factors, metabolite synthesis in *Bacillus albus*, and nutrition availability are strongly positively correlated.

AUXIN

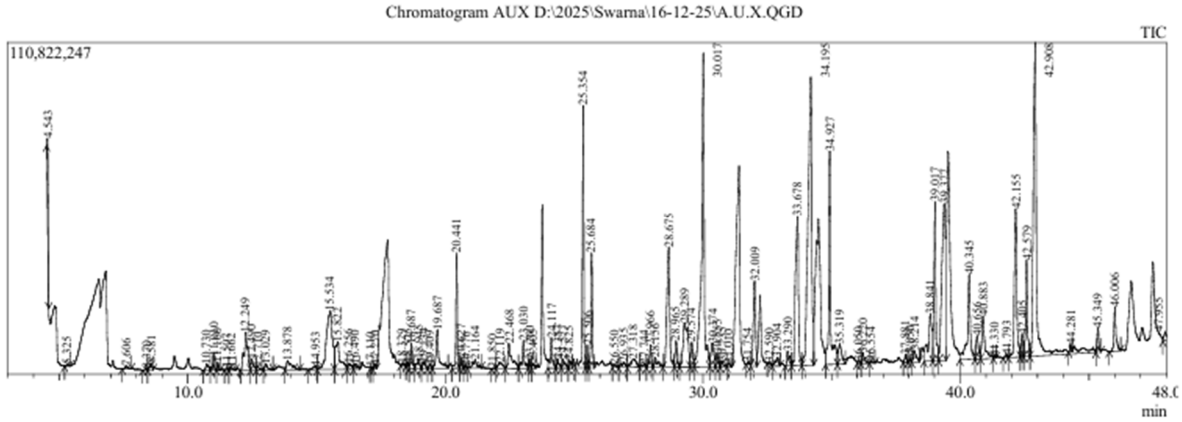


Fig 4 : chromatogram of auxin production

Peak#	R. Time	Area %	Compound Name
88	45.270	10.57	Indole-3-acetic acid (IAA)
69	34.927	4.22	Indole
68	34.195	10.79	Boroxine, diethyl methyl-

Table 4: List of GCMS compounds in auxin production

The indole-3 acetic acid (IAA), which acts as the plant growth hormone responsible for root development and growth in plants, is manufactured by *Bacillus* bacteria. In order to reinforce the interaction between microorganisms and plants, PGPR or plant growth hormones have been formulated.

From the results obtained through searches in various online sources, it is evident that boroxine, diethyl methyl- (CAS No. 727708-54-5) is a boron-based chemical compound, which has many

applications and is expected to have great potential when fighting antibiotic-resistant organisms like the *Bacillus*. The boroxine derivative mentioned above was not probably produced naturally, but it is rather the outcome of the reaction itself.

It is clear that the IPyA pathway is the best choice for the production of indole-3-acetic acid (IAA) from B. albus, whereby biosynthesis involves L-tryptophan converted to IPyA, IPyA decarboxylated to indole-3-acetaldehyde and finally IAA oxidation.

IPyA biosynthesis pathway is considered reliable for the reasons discussed below: IPyA is a labile intermediate that gets oxidized to indole-3-acetaldehyde and other intermediates. As a result, it becomes difficult to detect IPyA in the metabolome of bacteria owing to its fast breakdown. The occurrence of IPyA in GC-MS studies is not an indication of the IPyA biosynthetic pathway; however, the absence of IPyA cannot disprove its biosynthesis due to the labile nature of the intermediate.

The other pathway through which IAA can be synthesized may be IAM. In the IAM pathway, tryptophan converts to IAM, which further converts through hydrolysis to give IAA [49]. IAM is more stable than IPyA. Therefore, in instances where this pathway is utilized to produce auxins, IAM will be present. Their nonexistence in the metabolic profiling of the bacteria makes them very unlikely. Another option that the bacteria could have chosen to produce auxin may be the IAM pathway, though it is not the likely option. In the IAM pathway, tryptophan converts to IAM, which then undergoes hydrolysis to produce IAA. IAM is relatively stable; hence, it is usually present when the pathway is active. Since no IAM metabolite was detected in the metabolic profiling of B. albus, there is no evidence to prove its presence.

The pathway of IAA synthesis that would be considered the least likely pathway is the tryptamine (TAM) pathway. The TAM pathway is associated with the series of chemical reactions involved in the formation of IAA through oxidation of tryptamine formed by the conversion of tryptophan into indole-3-acetaldehyde. Common products found within this pathway include tryptamine and serotonin-like amines, among others [49]. It is evident that there was no presence of any of these intermediates in the GC-MS data set of the present study. It is, therefore, reasonable to conclude that *Bacillus* albus primarily utilizes the IPyA pathway for IAA synthesis in the plant, but the IAM and TAM pathways cannot be ruled out.

The results of the GC–MS analysis of both primary and auxin-enriched metabolite extracts from *Bacillus albus* indicated that a considerable number of indolic metabolites were present in the extracts. Namely, these metabolites include indole and IAA. In other words, IAA

comprised close to 10% relative peak areas in the metabolite extracts. The determination of indole is vital, as indole is considered a crucial component in the synthesis of IAA.

FOR AMMONIA

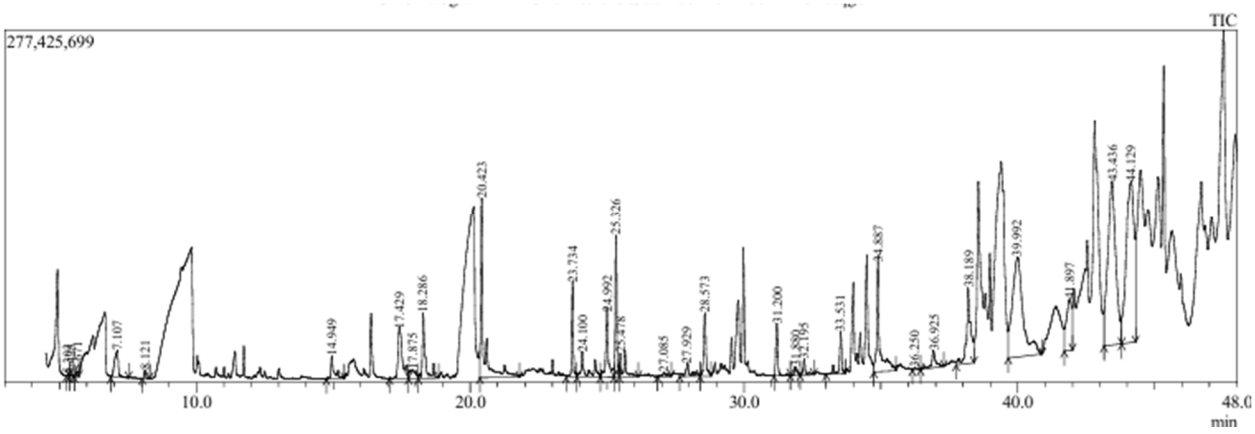


Fig 5: Chromotogram of ammonia production

Table 5: List of compounds in the production of ammonia

Peak#	R. Time	Area %	Compound Name
18	28.573	2.10	3-Methyl-1,4-di azabicyclo[4.3.0] nonan-2,5-dione, N-acetyl-
19	18.329	1.53	Pyrrolo[1,2-a] pyrazine-1,2-dione, hexahydro-
25	36.925	1.02	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-
31	52.932	1.12	Bis(cis-13-docosenamido) methane

9	18.286	2.68	Benzonitrile, 2-methyl-

It can be seen from the metabolite profile that the nitrogen secondary metabolism of B. albus was rather common for a plant growth-promoting bacterium, especially with respect to the production of diketopiperazines, piperazinediones, fatty amides, and cyclic peptides – all known characteristic nitrogenous metabolites of *Bacillus* PGPR strains. Compared with the most well-studied members of the genus, namely, *B. subtilis*, *B. velezensis*, *B. amyloliquefaciens*, and *B. pumilus*, the profile of *B. albus* revealed an abundance of pyrrolo-pyrazines and diazabicyclic compounds – a feature pointing at equally effective peptide turnover and amino acid degradation/deamination and ammonia release/production.

In this regard, B. albus would have good abilities to suppress pathogens. However, the presence of benzonitrile (2.68%) and diazabicyclo diketone (2.10%), which are not found abundantly in other *Bacillus* strains, suggests the development of a unique nitrogen secondary metabolism in this species or strain, which might enhance its competitiveness in rhizospheric conditions compared with a number of traditional bioinoculants based on *Bacillus* bacteria. At the same time, the detection of diketopiperazines clearly shows that *B. albus* belongs to the group of *Bacillus* PGPR species.

The ammonia production optimization results (OD_{420} nm) have confirmed the nitrogenous metabolites produced by *Bacillus* albus, including diketopiperazines, piperazine diones, and nitrogen-based cyclic substances, as detected in GC-MS analysis. Of all the physical parameters analyzed, maximum production of ammonia has been seen at pH 8.0 (OD_{420} = 2.284), followed by constant maximum production at pH ranging from 7.0 to 9.0, which means that slightly basic pH is favorable for the metabolism of nitrogen. Temperature optimization has shown maximum ammonia production at 35-40°C (OD_{420} = 3.554 – 3.478).

From the various parameters, peptone (5g/L) produced the highest amount of ammonia with OD value of 4.075. This implies that moderate amount of nitrogen favors effective ammonification while increased amounts of nitrogen reduce the efficiency of ammonification as a result of metabolic imbalances. Likewise, lower concentration of NaCl (1g/L; OD = 2.473) favored increased production of ammonia compared to the higher concentrations which had

slight effect on metabolic efficiency. This is supported by the GC–MS analysis of nitrogenous compounds in form of pyrrolo-pyrazine diketopiperazines and diazabicyclic compounds formed during amino acid metabolism and peptide breakdown.

Discussion:

Based on the GC-MS analysis, it has been established that the current experiment has offered knowledge about the biochemistry related to various phases in the life of microbes by comparing the metabolic composition in the control, primary, and secondary metabolites. In view of the results obtained in the previous validation process through metabolomics experiments, it is plausible to say that the absence of any active biochemical in the control signifies that the metabolites discovered in the test samples are naturally generated. Some of the major metabolites in the primary metabolome include fatty acids, alcohols, and hydrocarbons, signifying the presence of biological activity in producing membranes and energy.

Some other studies have also made similar observations when investigating the metabolomics of microbes where the major metabolites are involved in biological processes that promote cell division. Culturing the *Bacillus* albus in the traditional manner led to the growth of bacteria where the log phase takes place after 6 and 12 hours of incubation. Further analysis was conducted using metabolites taken from the mid-log phase that occurred between 8 and 10 hours of incubation.

The stationary phase of *Bacillus* albus was observed after 12 to 24 hours of incubation under standard culture conditions, as well as the appearance of a certain modification in the secondary metabolites' profile. This period was suitable for extraction and GC-MS analysis due to the presence of a stationary growth phase and intensified secondary metabolite formation. It had been characterized by chemical diversity and the presence of compounds of complex structure. It should be noted that many chemical compounds investigated in this paper were pyrrolo[1,2-a]pyrazines. It is known that these chemicals are quite active secondary metabolites produced by bacteria. Thus, according to modern publications, various *Bacillus* species produce pyrrolo[1,2-a]pyrazine-1,4-diones having potent antibacterial activity (Vikram et al., 2024). The high presence of heterocycles with nitrogen atoms can be attributed to some physiological conditions that stimulate the development of secondary metabolic processes. Secondary metabolite biosynthesis is vital for inter-microorganism signalling and interaction, which takes place during the stationary growth stage. Pyrrolo-derivatives are important compounds in drug

discovery due to their wide-spectrum antibiotic, antifungal, and medicinal characteristics, as noted in recent review studies [51]. Further evidence on the antibacterial and antioxidant properties of the extract can be derived from the inclusion of phenolic substances like 2,4-di-tert-butylphenol in its makeup. Extensive research has highlighted the significance of phenolic compounds in microbial biocontrol mechanism. Just as lipid-derived metabolites have been established to take part in adaptation and stress resistance processes in microbes, long chain fatty alcohols (hexacosanol) and hydrocarbon derivatives identified in this study can play a significant role in membrane maintenance and bioactivity. Importantly, the predominance of nitrogen-containing compounds in the secondary metabolite fractions highlights their importance in bioactivity.

The reason behind the antibacterial and cytotoxic behaviour of nitrogen heterocycles such as derivatives of pyrrolo and pyrazine is that they have the potential to react with biological targets of microorganisms and enzymes [52]. Considering all the factors discussed above, it can be seen that there is a noticeable metabolic shift from the primary metabolism to secondary metabolism. This is due to the reason that primary metabolism takes place during growth, whereas secondary metabolism occurs specifically for the purpose of producing various biologically active compounds.

The prevalence of pyrrolo[1,2-a] pyrazine derivatives in the current study highlights the importance of these compounds in PGPR-based biological control applications. They have been shown to control a broad spectrum of diseases affecting plants and are often referred to as antimicrobial secondary metabolites synthesized by rhizobacteria, particularly *Bacillus* bacteria [53]. Recent studies have shown that PGPRs actively inhibit phytopathogens in the rhizosphere by secreting various types of antimicrobial agents, including peptides, phenazines, and heterocyclic metabolites [53]. Furthermore, the ecological role of pyrrolo-pyrazine derivatives in protecting plants from pathogens has been validated based on their characterization as potent antifungal agents effective against soil-born diseases [54]. In addition to being recognized as a good supplier of antimicrobial compounds, PGPR was also found to produce phenols like 2,4-di-tert-butylphenol. Phenols are important for plant resistance against biotic and abiotic stresses due to their properties of antioxidants and bactericides. Moreover, they induce systemic resistance (ISR) in which PGPR enhances the plant's ability to withstand biotic stress [55]. The GC-MS profile's indole derivatives, especially indole-3-acetic acid (IAA), indicate the possibility of phytohormone synthesis. IAA, which is known to be produced by PGPR, has a major impact on root architecture by encouraging

nutrient uptake efficiency, lateral root development, and root elongation. According to a recent study, PGPR like *Bacillus* aryabhattai directly link microbial metabolites to plant physiological responses by improving nutrient absorption and metabolite production [56]. Furthermore, the discovery of organic acids and fatty acid derivatives suggests a function in the mobilization of nutrients, specifically phosphate solubilization and mineral availability. One important way that these microorganisms enhance plant development and production is by PGPR-mediated nutrient absorption. As per recent studies, the benefits of PGPRs include enhanced nutrient absorption, increased chlorophyll concentration, and increased plant metabolism that contribute towards increasing plant yield [57]. Hydrocarbons and fatty alcohols like hexacosanol have been identified, signifying more roles played by them in root colonisation and adapting to the environment. Lipid substances not only increase microbial survival in the rhizosphere but also enable microbes to interact with plants. Furthermore, it has been demonstrated that PGPR-produced secondary metabolites greatly boost plant biomass and encourage the build-up of advantageous plant metabolites, suggesting their wider significance in plant growth and productivity [57].

One can observe that there is the presence of high molecular weight biologically active substances that are commonly linked to the ecological functions like competition, communication, and antibiosis because the secondary metabolites have a higher percentage of high retention time compounds. It cannot be denied that the theory on the late synthesis of secondary metabolites as a means to improve the fitness of the microorganism and its association with host plants has much validity. It must be emphasized that the presence of nitrogen heterocycles in the secondary metabolites is one more reason for their significance. There are many heterocycles that perform various biological roles like communication and anti-microbial functions. These compounds are formed from metabolic products of amino acids.

From the results of GC-MS, different aromatic compounds and indoles are observed. These may include intermediates of the reaction of precursors of amino acids in tryptophan biosynthesis reactions and their benzene acetic acid derivatives. Despite not being detected as a separate peak in the analysis of GC-MS, detection of these precursors gives a hint that the pathway of auxin synthesis using tryptophan precursors, particularly indole-3-pyruvic pathway, is activated. For instance, in the bacteria species of genus *Bacillus*, tryptophan undergoes a transformation into indoles; the latter undergoes further transformations into IAA to produce auxins. Detection of different nitrogen-containing heterocycles and aromatic acids

in the present research gives an idea of activation of this pathway. This is because it is very clear that IAA exerts its effects, even at minimal concentrations because of its high bioactivity [58, 59]. Bacterial synthesis of auxins plays an important role in regulation of plant roots architecture, including root elongation and branching to ensure nutrient uptake and proper growth. Similar results have been demonstrated in other *Bacillus* PGPRs by linking the presence of indole compounds in the GC-MS analysis with auxin production and plant growth [60, 61].

A variety of nitrogen compounds were found to have been detected in the results of the GC-MS test. The following derivatives of benzonitriles (approximately 2.68%), piperazinediones and pyrrolopyrazines (0.3-1.5%), and amino acid compounds like S-methyl-L-cysteine were revealed. In addition to that, ammonium hydroxide is regarded as the final product of the process which certainly shows that ammonia has been synthesized in the course of metabolic reactions. The above mentioned compounds belong to active nitrogen metabolism which implies their presence as a result of amino acid deamination reaction followed by NH_3 formation. The hypothesis about the role of the corresponding enzymatic reactions including oxidative deamination and urease activity in ammonia formation can be made. Diketopiperazine derivatives play the same role in promoting active nitrogen turnover due to amino acid synthesis and breakdown[61,59]. In particular, ammonia formation leads to increased nitrogen availability in rhizospheres contributing to further plant growth and development. In support of the hypothesis, the positive effect of ammonia-forming strains on plant development was proved before.

The finding of both ammonia and auxin molecules at the same time implies the use of multiple approaches by *Bacillus albus* for the promotion of plant growth. Auxin production is mainly involved in root growth, whereas ammonia acts as an additional source of nitrogen for the plant. Plant growth and nutrient uptake are improved when these mechanisms cooperate. This dual activity is shown in effective PGPR strains, where phytohormone production and food cycle metabolic pathways work together [60]. Such integration improves the bacterium's ecological fitness in the rhizosphere and strengthens plant-microbe interactions. *Priestia megaterium* is widely known for its strong ability to solubilize phosphate, mostly by the formation of organic acids such as gluconic acid [62]. The primary PGPR mechanism is nutrient mobilization, albeit it also produces auxin. Conversely, *Bacillus* albus has a more complete metabolic profile, characterized by a variety of metabolites that include auxin precursor and nitrogen-verified

ammonia production. This suggests that *B. albus* possesses a more comprehensive PGPR system that integrates nitrogen metabolism and phytohormone synthesis.

Examples of *Bacillus* species used for PGPR that synthesize large amounts of IAA include *Bacillus subtilis* and *Bacillus licheniformis*. Unlike these two types of bacteria, *Bacillus albus* possesses dual functions that involve ammonia leakage and synthesis of auxin precursors simultaneously. Diketopiperazines play an important role in facilitating communication and anti-microbial properties. This suggests that the system is closely connected with metabolic processes and displays several PGPR properties concurrently. The saltwater-adapted *Rossellomorea aquimaris* [63]. However, its importance in fostering plant growth is extremely limited due to its reduced role in auxin synthesis and nitrogen metabolism. In contrast, *Bacillus albus* demonstrates strong rhizosphere flexibility and actively engages in pathways linked to auxin synthesis of ammonia cycle of nitrogen. It can be seen from this that B. albus is better adapted for niches that involve the use of plants while R. aquimaris is ecologically specialized to thrive in marine habitats. In *B. albus*, there is a combination of primary metabolism (organic and amino acid production), nitrogen metabolism (ammonia production), and auxin production. This combination makes *B. albus* more efficient as a PGPR organism as it facilitates the growth of roots as well as nutrition of plants. The results obtained correlate well with recent developments in the field of rhizosphere microbiology [64,65].

Conclusion:

The current work establishes Bacillus albus's potential as a Plant Growth-Promoting Rhizobacterium (PGPR) and offers a thorough assessment of its metabolite profile using GC-MS analysis. The strain's metabolic adaptability and functional significance in the rhizosphere are demonstrated by the discovery of a wide range of primary and secondary metabolites. Organic acids and related molecules are examples of primary metabolites that may play a major role in the mobilization of nutrients, especially in the solubilization of phosphates and the improvement of soil fertility. Concurrently, the identification of bioactive secondary metabolites, such as fatty acids, phenolic compounds, and volatile organic compounds like acetoin and 2,3-butanediol, suggests a high potential for promoting plant growth via phytohormone signalling, antimicrobial activity, and systemic resistance induction.

These metabolite classes' cohabitation shows that Bacillus albus uses a variety of complementary strategies to promote plant growth and fend off phytopathogens. Its potential as a bioinoculant for sustainable agriculture systems is highlighted by this multifunctional

characteristic. All things considered, the results of this investigation help close the current knowledge gap about the metabolomic basis of PGPR activity in Bacillus albus. A strong framework for comprehending its function in plant–microbe interactions is provided by the combination of GC–MS-based metabolite profiling and functional interpretation. In vivo validation through greenhouse and field trials, along with molecular and genomic investigations to clarify the regulatory pathways controlling metabolite production, should be the main focus of future study.

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