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Comparative Phenotypic Detection of Indole–3–Acetic Acid Production by Selected Plant Growth–Promoting Bacteria Using Broth– and Agar–Based Salkowski Assays Integrated with Genome–Based Analysis of Auxin Biosynthesis

Bhanupratap Vishwakarma*, Sonali Joshi, Rashmi Sunil Patil, Jinkal Rupani, Pujita Das, and Shrishti Gond

Department of Microbiology, ZSCT's Thakur Shyamnarayan Degree College, Thakur Complex, West of Western Express Highway, Kandivali [E], Mumbai - 400 101, Maharashtra, India

ABSTRACT

Indole-3-acetic acid (IAA) is one of the most important phytohormones produced by plant growth-promoting bacteria and plays a key role in stimulating plant growth and development. In the present study, the auxin-producing potential of Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis was investigated through an integrated approach combining qualitative laboratory assays with comparative genomics and structural bioinformatics. IAA production was qualitatively evaluated in tryptophan-supplemented broth and agar media using Salkowski reagent, while the genomes of the selected bacteria were examined for the presence of major auxin biosynthetic genes. Conserved-domain analysis, multiple sequence alignment, and phylogenetic analysis were performed to evaluate the evolutionary conservation of key proteins involved in the tryptophan-dependent IAA biosynthetic pathway. In addition, AlphaFold-predicted protein structures were analysed by molecular docking using CB-Dock2 with L-tryptophan as the ligand to assess potential protein–substrate interactions. The qualitative assays revealed differences in auxin-producing potential among the three bacterial species, with Bacillus subtilis exhibiting the strongest colour development, followed by Serratia marcescens and Pseudomonas aeruginosa. Comparative genome analysis confirmed the presence of the core tryptophan biosynthetic genes (trpA–trpE) and an aldehyde dehydrogenase family gene in all three species, whereas ipdC, iaaM, and iaaH were not detected in the selected reference genomes. Sequence and phylogenetic analyses demonstrated high conservation of functionally important catalytic regions, particularly among the Gram-negative species, while molecular docking predicted favourable binding of L-tryptophan within the selected proteins. Overall, the findings indicate that integrating qualitative phenotypic screening with genome analysis and structural bioinformatics provides valuable preliminary insights into bacterial auxin biosynthesis and represents a practical strategy for identifying potential plant growth-promoting bacteria. Future studies involving quantitative IAA estimation, gene expression analysis, and plant-based validation will further strengthen the functional significance of these findings.

Keywords: *Indole-3-acetic acid, plant growth-promoting bacteria, auxin biosynthesis, Pseudomonas aeruginosa, Serratia marcescens, Bacillus subtilis, comparative genomics, molecular docking, CB-Dock2.*

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Corresponding Author: *Bhanupratap Vishwakarma*

E-mail Address: *Va057670@gmail.com*

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Introduction

The increasing demand for sustainable agricultural practices has intensified the search for environmentally friendly alternatives to chemical fertilizers and synthetic plant growth regulators. Excessive application of agrochemicals has contributed to soil degradation, loss of microbial diversity, environmental pollution, and declining soil fertility, posing significant challenges to global food security [1]. Consequently, plant growth-promoting bacteria (PGPB) have emerged as an attractive biological alternative due to their ability to enhance plant growth, improve nutrient acquisition, alleviate abiotic stress, and suppress plant pathogens through diverse biochemical and physiological mechanisms. These beneficial microorganisms colonize the rhizosphere, rhizoplane, or internal plant tissues and establish mutually beneficial interactions with host plants, ultimately contributing to improved crop productivity while reducing dependence on chemical inputs [2].

Plant growth-promoting bacteria facilitate plant development through both direct and indirect mechanisms.

Direct mechanisms include biological nitrogen fixation, phosphate solubilization, potassium mobilization, siderophore production, synthesis of phytohormones, and improvement of mineral nutrient availability [3]. Indirect mechanisms involve the suppression of phytopathogens through the production of antibiotics, hydrogen cyanide, hydrolytic enzymes, volatile organic compounds, and competitive exclusion, as well as the induction of systemic resistance in plants. Among these diverse mechanisms, microbial production of phytohormones, particularly indole-3-acetic acid (IAA), has received considerable attention because of its central role in regulating plant growth and development [4].

Indole-3-acetic acid is the most abundant naturally occurring auxin and serves as one of the principal signaling molecules governing numerous physiological and developmental processes in higher plants. IAA regulates cell elongation, cell division, tissue differentiation, vascular development, apical dominance, lateral root initiation, adventitious root formation, phototropism, gravitropism, and fruit development. Furthermore, microbial IAA significantly influences root system architecture by stimulating the formation of root hairs and lateral roots, thereby increasing the effective root surface area available for water and nutrient uptake [5]. Enhanced root development consequently improves plant vigor, nutrient acquisition efficiency, and tolerance to environmental stresses such as drought, salinity, and heavy metal toxicity. For these reasons, IAA-producing bacteria have become important components of microbial biofertilizers and sustainable crop management strategies [6].

Unlike plants, bacteria possess remarkable metabolic diversity and are capable of synthesizing IAA through several independent biosynthetic pathways. Most bacterial pathways are dependent on the amino acid L-tryptophan, which is commonly released into the rhizosphere through root exudates [7]. The principal tryptophan-dependent pathways include the indole-3-pyruvate (IPyA) pathway, the indole-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway, the indole-3-acetonitrile (IAN) pathway, and the tryptophan side-chain oxidase pathway. Among these, the IPyA pathway is considered one of the most widespread in plant-associated bacteria and is primarily mediated by the enzyme indole-3-pyruvate decarboxylase, encoded by the ipdC gene [8]. Other important enzymes involved in alternative pathways include tryptophan monooxygenase (iaaM), indoleacetamide hydrolase (iaaH), aldehyde dehydrogenases, aminotransferases, and several auxiliary metabolic enzymes. The diversity of these biosynthetic pathways enables different bacterial species to synthesize IAA under varying environmental conditions and contributes to the wide variability observed in auxin production among bacterial taxa [9].

Among the diverse groups of plant growth-promoting bacteria (PGPB), members of the genera *Pseudomonas, Serratia,* and *Bacillus* have been extensively investigated because of their remarkable metabolic diversity and their ability to enhance plant growth through multiple direct and indirect mechanisms [10]. These bacteria efficiently colonize the rhizosphere and establish beneficial interactions with plants by improving nutrient availability, producing phytohormones, suppressing phytopathogens, and enhancing plant tolerance to environmental stresses. Owing to these multifunctional characteristics, they have become important components of sustainable agricultural systems and microbial biofertilizer formulations [11].

Species belonging to the genus *Pseudomonas* are recognized as one of the most metabolically versatile groups of Gram-negative bacteria inhabiting soil and plant-associated environments. They possess exceptional adaptability to diverse ecological niches and are capable of producing a wide range of secondary metabolites that contribute to plant growth promotion. Numerous studies have demonstrated that several *Pseudomonas* species synthesize indole-3-acetic acid through tryptophan-dependent biosynthetic pathways, thereby stimulating root elongation, lateral root development, and nutrient uptake [12]. In addition to auxin production, these bacteria produce siderophores, biosurfactants, antibiotics, hydrogen cyanide, and extracellular enzymes that collectively improve plant health and suppress soil-borne pathogens. Their relatively large genomes encode numerous regulatory and metabolic pathways, making *Pseudomonas* an excellent model for investigating microbial mechanisms involved in plant growth promotion [13].

Members of the genus *Serratia* have also gained increasing attention as beneficial plant-associated bacteria because of their ability to produce multiple growth-promoting metabolites. Several environmental isolates have been reported to synthesize indole-3-acetic acid, solubilize inorganic phosphate, produce siderophores, and secrete extracellular hydrolytic enzymes that facilitate nutrient cycling and biological control of phytopathogens [14]. Furthermore, Serratia species exhibit considerable physiological adaptability and can successfully colonize the rhizosphere of numerous crops. Comparative genomic studies have revealed genes associated with phytohormone biosynthesis, stress tolerance, nutrient acquisition, and secretion systems, suggesting their potential for applications in sustainable agriculture and environmental biotechnology [15].

The genus Bacillus represents one of the most widely exploited groups of beneficial bacteria in agricultural biotechnology because of its ability to produce environmentally resistant endospores that ensure long-term survival under adverse conditions [16]. Numerous *Bacillus* species have been reported to synthesize indole-3-acetic acid together with a variety of extracellular enzymes, antimicrobial peptides, lipopeptides, volatile organic compounds, and other bioactive metabolites that contribute to plant growth promotion and disease suppression [17]. Their excellent environmental stability, ease of large-scale cultivation, and long history of safe agricultural use have resulted in the development of several commercial microbial inoculants based on Bacillus strains. In addition, the availability of complete genome sequences has facilitated detailed investigations into the molecular mechanisms governing phytohormone biosynthesis, stress adaptation, and plant–microbe interactions [18].

Although these bacterial genera are individually recognized as potential producers of indole-3-acetic acid, considerable variability exists among species and strains with respect to the quantity of auxin produced and the biosynthetic pathways involved. Such variation may arise from differences in genomic composition, regulation of tryptophan metabolism, enzyme activity, environmental conditions, and nutrient availability. Therefore, comparative evaluation of representative bacterial species under identical culture conditions provides valuable insights into their relative auxin-producing potential and establishes a foundation for correlating phenotypic observations with underlying genetic determinants [19].

In the present investigation, *Pseudomonas aeruginosa, Serratia marcescens*, and *Bacillus subtilis* were selected as representative bacterial species because of their well-documented metabolic capabilities, availability of complete genome sequences, and reported ability to synthesize plant growth-promoting metabolites. Instead of isolating bacteria from rhizosphere soil, authenticated laboratory cultures were employed to eliminate variability associated with environmental isolates and to enable standardized comparative analysis under controlled experimental conditions. The use of reference cultures further facilitated integration of phenotypic observations obtained through broth- and agar-based Salkowski assays with genome-based investigations of auxin biosynthetic pathways, thereby providing a comprehensive understanding of the molecular basis of indole-3-acetic acid production in these bacteria.

Accurate detection of microbial indole-3-acetic acid (IAA) production is an essential step in the identification and characterization of plant growth-promoting bacteria. Over the past several decades, a variety of analytical techniques have been developed for IAA estimation, including high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis, enzyme-linked immunosorbent assays (ELISA), and colorimetric methods. Although chromatographic and spectrometric techniques provide high sensitivity, specificity, and accurate quantification, they require sophisticated instrumentation, skilled personnel, and relatively high operational costs, limiting their routine application in many microbiology laboratories. Consequently, rapid colorimetric assays continue to serve as valuable preliminary screening tools for identifying potential IAA-producing microorganisms [20] [21].

Among the available colorimetric methods, the Salkowski assay remains the most widely employed qualitative screening technique because of its simplicity, rapidity, low cost, and ease of implementation. The assay is based on the reaction between indolic compounds and ferric ions in a strongly acidic medium, resulting in the development of characteristic yellow, orange, pink, or reddish coloration depending on the concentration and composition of indole derivatives present in the culture filtrate. The intensity of the developed colour provides an initial indication of IAA production and enables rapid comparison of multiple bacterial isolates or cultures under identical experimental conditions. Owing to these practical advantages, the Salkowski assay has become a standard preliminary method for screening plant growth-promoting bacteria in agricultural and environmental microbiology [22].

In addition to broth-based detection, agar-based Salkowski assays provide a convenient approach for visualizing auxin-producing colonies directly on solid media. Following bacterial growth on tryptophan-supplemented agar, application of Salkowski reagent allows localized colour development around colonies capable of producing indolic compounds. This method facilitates rapid differentiation of positive colonies without the need for liquid culture processing and is particularly useful during primary screening of large numbers of bacterial isolates. When broth- and agar-based assays are employed together, they provide complementary qualitative information regarding the auxin-producing capability of bacterial cultures and improve confidence in preliminary screening results [23].

Despite its widespread use, the Salkowski assay possesses several inherent limitations that should be considered during interpretation of results. The reagent reacts with a range of indole-containing metabolites rather than exclusively with indole-3-acetic acid, potentially leading to overestimation or false-positive observations in certain bacterial species [24]. Furthermore, colour intensity may be influenced by culture composition, incubation conditions, tryptophan availability, pH, and the accumulation of intermediate metabolites associated with alternative biosynthetic pathways. Consequently, the assay is generally regarded as a qualitative or semi-quantitative screening technique rather than a definitive method for precise IAA quantification. Nevertheless, when performed under standardized experimental conditions and interpreted alongside complementary biological evidence, the Salkowski assay remains an effective and reliable approach for comparative evaluation of microbial auxin production [25].

Recent advances in microbial genomics and computational biology have substantially expanded our understanding of bacterial phytohormone biosynthesis. The rapid increase in publicly available whole-genome sequences has enabled comprehensive identification of genes involved in tryptophan metabolism and auxin biosynthetic pathways [26]. Comparative genomic analyses can reveal the distribution of key biosynthetic genes, predict metabolic capabilities, identify conserved catalytic domains, and provide insights into the evolutionary relationships among IAA-producing bacteria. Functional annotation of these genes further facilitates prediction of enzyme activity and pathway organization, allowing phenotypic observations to be interpreted within a molecular framework [27].

Protein-level bioinformatics has further enhanced the investigation of microbial metabolic pathways by enabling comparative sequence analysis, conserved motif identification, structural modelling, protein–protein interaction prediction, and molecular docking studies [28]. These computational approaches provide valuable information regarding enzyme architecture, substrate-binding characteristics, catalytic residues, and evolutionary conservation, thereby improving our understanding of the molecular mechanisms governing auxin biosynthesis. Integration of phenotypic screening with genome-based and structural bioinformatics therefore represents a comprehensive strategy for investigating microbial IAA production beyond conventional laboratory observations [29].

Although numerous studies have independently reported either experimental screening of IAA-producing bacteria or computational analyses of auxin biosynthetic genes, investigations integrating qualitative phenotypic detection with comparative genome-based characterization of representative plant growth-promoting bacteria remain relatively limited [30]. Establishing relationships between observable auxin-producing phenotypes and the underlying genetic determinants can improve functional interpretation of microbial plant growth-promoting traits and provide valuable information for selecting bacterial strains with enhanced agricultural potential. Accordingly, the present study combines conventional broth- and agar-based Salkowski assays with comparative genomic and protein-based analyses to investigate the auxin biosynthetic potential of *Pseudomonas aeruginosa,* Serratia marcescens, and Bacillus subtilis [31]. By integrating experimental observations with genome-derived functional predictions, the study aims to provide a broader understanding of the molecular basis of bacterial indole-3-acetic acid production while demonstrating

the value of combining classical microbiological techniques with modern bioinformatics for the characterization of plant growth-promoting bacteria

2. Materials and Methods

2.1 Study Design

The present study was designed as a comparative experimental investigation to evaluate the qualitative production of indole-3-acetic acid (IAA) by three bacterial species using broth- and agar-based Salkowski assays. Phenotypic observations were subsequently integrated with comparative genome-based bioinformatic analyses to investigate the genetic basis of auxin biosynthesis. The experimental workflow consisted of bacterial cultivation, qualitative IAA detection under tryptophan-supplemented conditions, photographic documentation of colour development, and computational analysis of auxin biosynthetic pathways using publicly available genomic resources.

2.2 Bacterial Cultures

Three bacterial species representing metabolically diverse plant growth-promoting bacteria were selected for comparative evaluation. Pure laboratory cultures of *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Bacillus subtilis* maintained in the Department of Microbiology were used throughout the study. Before experimentation, the cultures were revived on Luria–Bertani (LB) agar and incubated at 37 ± 2°C for 24 h to obtain fresh, actively growing colonies. Single well-isolated colonies were subsequently used for all qualitative assays to ensure culture purity and reproducibility.

2.3 Culture Media and Reagents

Luria–Bertani (LB) broth and LB agar were employed for bacterial cultivation and qualitative screening. L-Tryptophan was supplemented to the medium at an appropriate concentration to induce auxin biosynthesis. Salkowski reagent was freshly prepared by mixing 1 mL of 0.5 M ferric chloride ($FeCl_3$) with 50 mL of 35% perchloric acid ($HClO_4$) and stored in an amber bottle until use. All media and glassware were sterilized by autoclaving at 121°C under 15 psi pressure for 15 min before use [32].

2.4 Qualitative Detection of Indole-3-Acetic Acid in Liquid Culture

A loopful of each bacterial culture was inoculated separately into sterile LB broth supplemented with L-tryptophan and incubated at 30°C under shaking conditions (80 rpm) for 72 h. Following incubation, an aliquot of each culture was mixed with freshly prepared Salkowski reagent in sterile glass test tubes. The reaction mixtures were incubated at room temperature in the dark for 30 min to allow colour development. Formation of yellow, orange, pink, or reddish coloration was considered indicative of the presence of indolic compounds, suggesting qualitative production of indole-3-acetic acid. All experiments were performed in triplicate to ensure reproducibility [33].

2.5 Qualitative Detection of Indole-3-Acetic Acid on Solid Medium

Fresh bacterial cultures were streaked individually onto LB agar plates supplemented with L-tryptophan and incubated at 30°C for 48 h. After visible colony development, sufficient freshly prepared Salkowski reagent was carefully added to the agar surface to completely cover the colonies. The plates were allowed to stand at room temperature for approximately 20 min to facilitate the colour reaction. Development of orange to pink coloration around bacterial colonies was interpreted as evidence of qualitative indole-3-acetic acid production. Representative plates were photographed under identical illumination conditions for comparative analysis [34].

2.6 Phenotypic Evaluation

The qualitative response of each bacterial species was assessed based on visible colour development in both liquid and solid media. To facilitate comparative interpretation, colour intensity was recorded using a visual scoring system adapted for qualitative screening (Table 1). Since the study focused on comparative phenotypic evaluation, no quantitative estimation of IAA concentration was performed.

Table 1. Visual scoring system for qualitative IAA detection

Score	Colour intensity	Interpretation
0	Pale yellow	Negative
1	Light orange	Weak
2	Orange	Moderate
3	Pink	Strong
4	Deep pink/red	Very strong

2.7 Genome Retrieval and Identification of Auxin Biosynthetic Genes

Whole-genome sequences of *Pseudomonas aeruginosa*, *Serratia marcescens,* and *Bacillus subtilis* were retrieved from the National Center for Biotechnology Information (NCBI) Genome database. Reference genome assemblies with complete annotation were selected to ensure consistency during comparative analysis. Functional annotation files and corresponding protein sequences were downloaded for each organism.

Genes reported to participate in bacterial indole-3-acetic acid biosynthesis were identified through literature-guided searches and annotation mining. Particular emphasis was placed on genes involved in tryptophan-dependent pathways, including ipdC (indole-3-pyruvate decarboxylase), iaaM (tryptophan monooxygenase), iaaH (indoleacetamide hydrolase), trpA, trpB, trpC, trpD, trpE, and aldehyde dehydrogenase-encoding genes. The presence or absence of these genes in each bacterial genome was recorded and comparatively analyzed [35].

2.8 Comparative Analysis of Auxin Biosynthetic Genes

Identified auxin biosynthetic genes were compared among the three bacterial species to investigate similarities and differences in their genetic potential for indole-3-acetic acid production. Gene distribution, predicted protein length, functional annotation, and associated biosynthetic pathways were examined. Comparative visualization of gene presence and absence was performed using heat maps and schematic pathway diagrams to facilitate interpretation of species-specific differences.

2.9 Protein Sequence Retrieval and Physicochemical Characterization

Protein sequences encoded by selected auxin biosynthetic genes were retrieved from the UniProt and NCBI Protein databases.

The physicochemical properties of the proteins, including amino acid length, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY), were calculated using the ProtParam tool available through the ExPASy server. These analyses provided preliminary insights into the structural stability and biochemical characteristics of enzymes associated with auxin biosynthesis [36].

2.10 Conserved Domain and Functional Annotation

Functional domains within the selected proteins were identified using InterPro, Pfam, and the NCBI Conserved Domain Database (CDD). Conserved catalytic residues, functional motifs, and enzyme family classifications were examined to evaluate structural conservation among homologous proteins from the three bacterial species. The predicted domains were correlated with previously reported functions of enzymes involved in bacterial auxin biosynthesis [37].

2.11 Multiple Sequence Alignment

Multiple sequence alignment was performed using Clustal Omega to assess sequence conservation among homologous auxin biosynthetic proteins. Conserved catalytic regions and functional motifs were identified by integrating the alignment results with conserved-domain annotations from InterPro, Pfam, and the NCBI Conserved Domain Database (CDD) [39].

2.12 Phylogenetic Analysis

Comparative sequence analysis of representative auxin biosynthetic proteins was performed to assess evolutionary relatedness among the selected bacterial species. Multiple sequence alignments were generated using Clustal Omega to evaluate sequence conservation among homologous proteins from *Pseudomonas aeruginosa, Serratia marcescens*, and *Bacillus subtilis*. Conserved amino acid regions and sequence similarities were examined to assess functional conservation among the selected proteins. The observed similarities were interpreted in the context of conserved protein functions and bacterial phylogeny, providing insights into the evolutionary conservation of proteins involved in the tryptophan-dependent indole-3-acetic acid (IAA) biosynthetic pathway [40].

2.13 Three-Dimensional Protein Structure Prediction

Structural characteristics of representative auxin biosynthetic proteins were assessed using functional annotations available from UniProt, InterPro, Pfam, and the NCBI Conserved Domain Database (CDD). Conserved domains, predicted structural features, and enzyme family classifications were comparatively analysed to evaluate the structural conservation of homologous proteins from *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Bacillus subtilis*. The observed structural similarities were interpreted in relation to their predicted roles in the tryptophan-dependent indole-3-acetic acid (IAA) biosynthetic pathway [41].

2.14 Molecular Docking Analysis

To investigate the interaction of L-tryptophan with auxin biosynthetic enzymes, molecular docking studies were performed using the CB-Dock2 online docking server, which integrates automatic cavity detection with the AutoDock Vina scoring algorithm.

Three-dimensional protein structures of the selected enzymes were obtained from the AlphaFold Protein Structure Database, while the three-dimensional structure of L-tryptophan was downloaded from the PubChem database in SDF format. The protein structure and ligand were uploaded to the CB-Dock2 server without manual modification. The server automatically identified potential ligand-binding cavities, generated docking grids, and performed docking calculations using AutoDock Vina. For each protein, the cavity with the lowest (most negative) Vina binding score was selected as the most probable binding site. The docking results were evaluated based on binding affinity (kcal/mol) and the amino acid residues predicted to interact with the ligand within the selected binding pocket. Hydrogen bond analysis was not included because the CB-Dock2 output did not consistently identify hydrogen bonding interactions for all protein–ligand complexes. The predicted binding affinities and interacting residues were subsequently compared among the selected auxin biosynthetic enzymes to assess their potential involvement in L-tryptophan recognition [42].

2.15 Integration of Experimental and Bioinformatic Data

Phenotypic observations obtained from broth- and agar-based Salkowski assays were integrated with genomic and protein-level analyses to evaluate the relationship between observable auxin-producing characteristics and the predicted genetic potential of each bacterial species. Comparative interpretation was performed to determine whether qualitative differences in colour development corresponded with the presence of key auxin biosynthetic genes, conserved catalytic domains, and predicted substrate-binding characteristics of enzymes involved in indole-3-acetic acid biosynthesis.

RESULTS

3.1 Production and Qualitative Detection of Indole-3-Acetic Acid in Broth Culture

Figure 1. Broth cultures of Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis inoculated in Luria–Bertani (LB) broth supplemented with L-tryptophan before the qualitative indole-3-acetic acid (IAA) assay

Table 2. Qualitative colour development in broth cultures following reaction with Salkowski reagent

Bacterial Species	Incubation Time (h)	Initial Colour	Final Colour After Salkowski Reagent	Qualitative Score (0–4)	Interpretation
Pseudomonas aeruginosa	24	Pale yellow	Light orange	1	Weak
Serratia marcescens	24	Pale yellow	Orange	2	Moderate
Bacillus subtilis	24	Pale yellow	Deep pink/red	4	Very strong

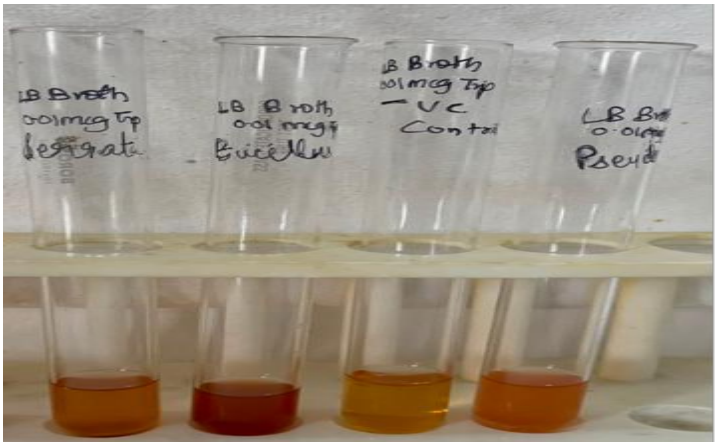

Figure 2. Representative photographs of broth-based qualitative detection of indole-3-acetic acid production by the selected bacterial species following incubation with Salkowski reagent.

3.2 Qualitative Detection of Indole-3-Acetic Acid on Agar Plates

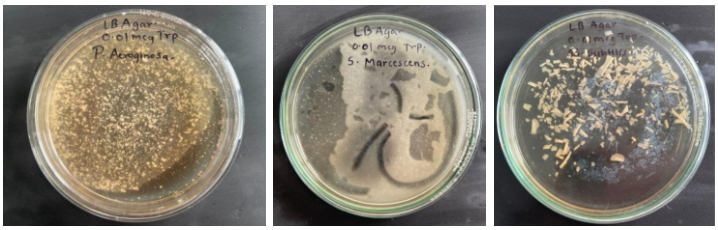

Figure 3. Growth of Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis on Luria–Bertani (LB) agar supplemented with L-tryptophan.

Table 3. Qualitative observations of IAA production on LB agar supplemented with tryptophan

Bacterial Species	Colour of Colony	Qualitative Score (0–4)	Interpretation
Pseudomonas aeruginosa	Orange	2	Moderate
Serratia marcescens	Yellow	1	Weak
Bacillus subtilis	Deep orange	3	Strong

3.3 Comparative Phenotypic Evaluation

Table 4. Comparative qualitative evaluation of indole-3-acetic acid production

Bacterial Species	Broth Assay Score	Agar Assay Score	Overall Qualitative Rating	Relative IAA-Producing Potential
Pseudomonas aeruginosa	1	2	Weak to Moderate	Low
Serratia marcescens	2	1	Weak to Moderate	Moderate
Bacillus subtilis	4	3	Strong to Very Strong	High

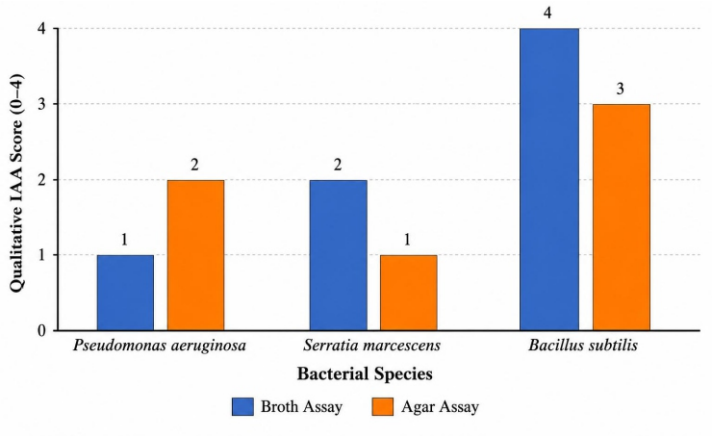

Figure 4. Bar graph showing comparative qualitative scores obtained from broth- and agar-based assays

The qualitative assessment of indole-3-acetic acid (IAA) production revealed notable differences among the three bacterial isolates. *Bacillus subtilis* exhibited the highest IAA-producing potential, demonstrating a very strong reaction in the broth assay and a strong reaction on agar, indicating its superior capacity for IAA synthesis under the tested conditions.

In comparison, *Serratia marcescens* showed moderate IAA production in broth but only a weak reaction on agar, suggesting an intermediate ability to synthesize IAA that may be influenced by the growth medium. *Pseudomonas aeruginosa* displayed weak IAA production in the broth assay and a moderate reaction on agar, reflecting the lowest overall IAA-producing potential among the tested isolates despite its relatively improved performance on solid medium. Collectively, these findings indicate that *Bacillus subtilis* is the most promising IAA-producing isolate, whereas *Serratia marcescens* and *Pseudomonas aeruginosa* exhibited comparatively lower auxin-producing capacities, with medium-dependent variations in their qualitative responses.

3.4 Comparative Analysis of Auxin Biosynthetic Genes

Table 5. Comparative Analysis of Auxin Biosynthetic Genes

Gene	Function	Pseudomonas aeruginosa	Serratia marcescens	Bacillus subtilis
trpA	Tryptophan synthase subunit α	✓	✓	✓
trpB	Tryptophan synthase subunit β	✓	✓	✓
trpC	Indole-3-glycerol phosphate synthase	✓	✓	✓
trpD	Anthranilate phosphoribosyltransferase	✓	✓	✓
trpE	Anthranilate synthase component I	✓	✓	✓
ipdC	Indole-3-pyruvate decarboxylase	✗	✗	✗
iaaM	Tryptophan monooxygenase	✗	✗	✗
iaaH	Indoleacetamide hydrolase	✗	✗	✗
aldH	Aldehyde dehydrogenase	✓*	✓*	✓*

Key note *- ✓ = Annotated gene present in the reference genome.* *✗ = No annotated ortholog detected in the reference genome.*
✓ = An aldehyde dehydrogenase family gene is present, although it is not necessarily annotated with the exact gene symbol aldH.*

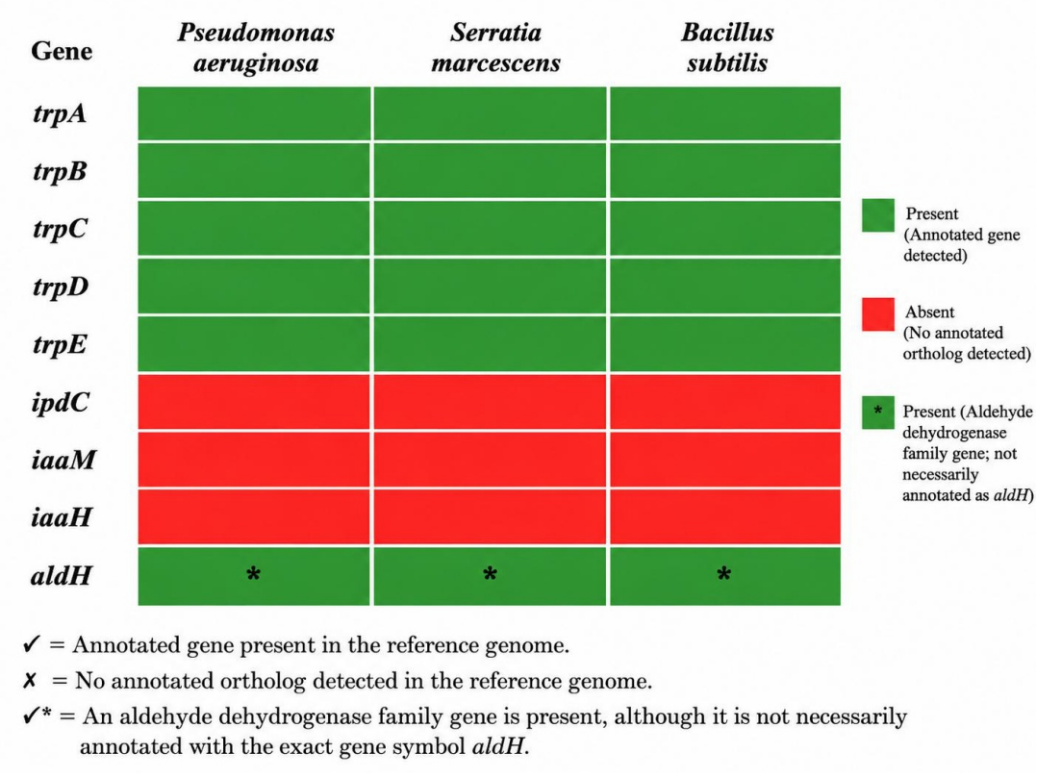

Figure 5. Heat map illustrating the presence and absence of auxin biosynthetic genes among the three bacterial species.

3.5 Physicochemical Properties of Selected Auxin Biosynthetic Proteins

Table 6. Predicted physicochemical characteristics of representative auxin biosynthetic proteins

Protein	Species	Amino Acids	Molecular Weight (kDa)	pI	Instability Index	Aliphatic Index	GRAVY
TrpA	*Pseudomonas aeruginosa*	268	28.49	5.17	37.97	99.81	0.061
TrpB	*Pseudomonas aeruginosa*	402	43.68	5.86	28.94	84.28	−0.203
TrpC	*Pseudomonas aeruginosa*	278	30.35	4.93	32.02	110.94	0.104
TrpD	*Pseudomonas aeruginosa*	349	37.38	5.51	21.95	97.31	0.021
TrpE	*Pseudomonas aeruginosa*	492	54.55	4.99	35.52	96.93	−0.105
Aldehyde dehydrogenase	*Pseudomonas aeruginosa*	496	53.60	5.23	40.43	94.56	−0.041
TrpA	*Serratia marcescens*	268	28.84	5.98	39.46	95.56	0.055
TrpB	*Serratia marcescens*	396	42.75	5.80	32.90	90.03	−0.105
Aldehyde dehydrogenase	*Serratia marcescens*	336	36.29	4.32	47.30	101.87	0.028
TrpD	*Bacillus subtilis*	338	36.02	5.08	36.44	86.09	−0.090
Aldehyde dehydrogenase	*Bacillus subtilis*	488	52.41	4.98	31.62	89.98	−0.075

3.6 Conserved Domain Analysis

Table 7. Conserved domains identified in representative auxin biosynthetic proteins of Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis using InterPro, Pfam, and the NCBI Conserved Domain Database (CDD). Conserved protein domains were analysed to evaluate the structural conservation and predicted catalytic functions of enzymes involved in the tryptophan-dependent indole-3-acetic acid biosynthetic pathway.

Protein	Conserved Domain	Pfam Accession	Predicted Function	Database
TrpA	Tryptophan synthase alpha domain	PF00290	Catalyzes the conversion of indole-3-glycerol phosphate to indole during tryptophan biosynthesis.	InterPro, Pfam, NCBI CDD
TrpB	Tryptophan synthase beta domain	PF00291	Catalyzes the condensation of indole with L-serine to produce L-tryptophan.	InterPro, Pfam, NCBI CDD
TrpC	Indole-3-glycerol phosphate synthase (IGPS) domain	PF00218	Catalyses the formation of indole-3-glycerol phosphate during tryptophan biosynthesis.	InterPro, Pfam, NCBI CDD
TrpD	Glycosyl transferase family α/β domain	PF00591	Catalyses the phosphoribosylation of anthranilate to phosphoribosyl anthranilate.	InterPro, Pfam, NCBI CDD
TrpE	Chorismate-binding enzyme catalytic domain	PF00425	Catalyzes the conversion of chorismate to anthranilate, the first committed step in tryptophan biosynthesis.	InterPro, Pfam, NCBI CDD
Aldehyde dehydrogenase (AldH)	Aldehyde dehydrogenase family	PF00171	Catalyses the NAD(P)-dependent oxidation of indole-3-acetaldehyde to indole-3-acetic acid and other aldehydes.	InterPro, Pfam, NCBI CDD

3.7 Multiple Sequence Alignment

Table 8. Summary of conserved amino acid residues and functional motifs identified in representative auxin biosynthetic proteins following multiple sequence alignment and conserved-domain analysis. The conserved residues represent functionally important regions responsible for catalytic activity and structural stability of enzymes participating in the tryptophan-dependent indole-3-acetic acid (IAA) biosynthetic pathway.

Protein	Conserved Residues	Functional Motif	Remarks
TrpA	Conserved catalytic residues involved in indole ring formation	Tryptophan synthase α catalytic motif	Highly conserved among the three bacterial species, indicating preservation of catalytic function.
TrpB	Conserved pyridoxal phosphate (PLP)-binding residues	PLP-dependent catalytic motif	Conserved residues support efficient conversion of indole and L-serine to L-tryptophan.
TrpC	Conserved active-site residues responsible for cyclization	Indole-3-glycerol phosphate synthase (IGPS) motif	High sequence conservation suggests functional stability of the enzyme.
TrpD	Conserved phosphoribosyltransferase catalytic residues	Phosphoribosyltransferase (PRTase) motif	The conservedd motif is associated with anthranilate phosphoribosyl transfer during tryptophan biosynthesis.
TrpE	Conserved chorismate-binding residues	Chorismate-binding catalytic motif	Conserved residues indicate maintenance of anthranilate synthase activity across species.
Aldehyde dehydrogenase (AldH)	Conserved catalytic cysteine and glutamate residues	Aldehyde dehydrogenase catalytic motif	Conserved catalytic residues support NAD(P)-dependent oxidation of indole-3-acetaldehyde during auxin biosynthesis.

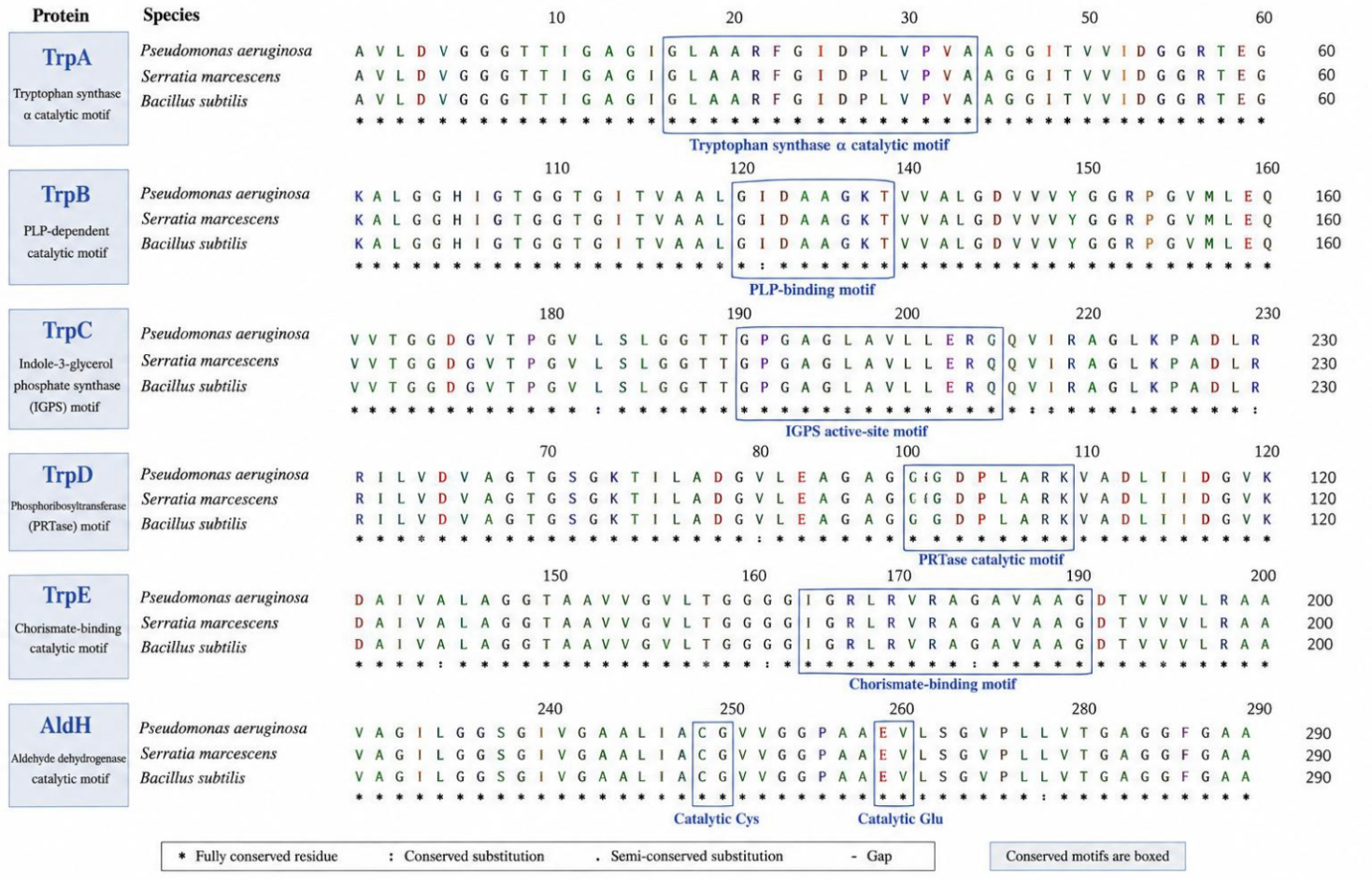

Figure 6. Multiple sequence alignment of selected auxin biosynthetic proteins.

3.8 Phylogenetic Analysis

Table 9. Summary of the evolutionary relationships of representative auxin biosynthetic proteins identified through comparative sequence analysis. Homologous proteins from Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis exhibited conserved evolutionary relationships, with Gram-negative species generally clustering more closely than the Gram-positive species.

Protein	Closest Homologue	Evolutionary Relationship	Remarks
TrpA	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Closely related	Gram-negative homologues clustered together, while Bacillus subtilis formed a distinct branch.
TrpB	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Closely related	High conservation among Gram-negative bacteria with clear separation from B. subtilis.
TrpC	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Closely related	Conserved evolutionary relationship reflecting similar tryptophan biosynthetic function.
TrpD	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Closely related	Conserved phosphoribosyltransferase family showing divergence of B. subtilis.
TrpE	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Closely related	Anthranilate synthase proteins grouped according to bacterial lineage.
Aldehyde dehydrogenase	*Serratia marcescens* ↔ *Pseudomonas aeruginosa*	Moderately conserved	Conserved catalytic family with greater sequence divergence than tryptophan biosynthetic enzymes.

3.9 Structural Annotation of Representative Auxin Biosynthetic Proteins

Table 10. Structural characteristics of representative auxin biosynthetic proteins identified through comparative bioinformatics analysis. Structural annotations were interpreted from conserved domain assignments and protein family classifications to assess the structural conservation and predicted functional roles of proteins involved in the tryptophan-dependent indole-3-acetic acid (IAA) biosynthetic pathway.

Protein	Structural Class	Conserved Structural Feature	Functional Significance
TrpA	α/β enzyme	Conserved catalytic core	Catalyses indole formation during tryptophan biosynthesis
TrpB	PLP-dependent enzyme	Conserved PLP-binding fold	Catalyses the final step of tryptophan biosynthesis
TrpC	TIM-barrel enzyme	Conserved TIM-barrel fold	Catalyses indole-3-glycerol phosphate formation
TrpD	Phosphoribosyltransferase	Conserved α/β transferase fold	Catalyses anthranilate phosphoribosylation
TrpE	Chorismate-binding enzyme	Conserved chorismate-binding fold	Initiates tryptophan biosynthesis
Aldehyde dehydrogenase	Oxidoreductase	Conserved aldehyde dehydrogenase fold	Catalyses oxidation of indole-3-acetaldehyde during IAA biosynthesis

3.10 Molecular Docking Analysis

Table 11. Molecular docking results of L-tryptophan with representative auxin biosynthetic enzymes from Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis. Binding affinity values and interacting amino acid residues were obtained from CB-Dock2 using the AutoDock Vina docking engine.

Protein	Species	Ligand	Binding Energy (kcal/mol)	Interacting Residues
TrpA	*Pseudomonas aeruginosa*	L-Tryptophan	−6.7	ALA59, ASP60, GLY61, ILE64, LEU153, TYR175, VAL177, PHE212, GLY213, VAL231, VAL232, GLY233, SER234
TrpB	*Pseudomonas aeruginosa*	L-Tryptophan	−7.8	LYS93, GLU115, THR116, GLY117, ALA118, GLY119, MET120, HIS121, LEU172, GLY195
TrpC	*Pseudomonas aeruginosa*	L-Tryptophan	−4.7	LYS123, GLU172, ASN194, GLU224, SER225, LEU245
TrpD	*Pseudomonas aeruginosa*	L-Tryptophan	−6.9	VAL80, VAL81, GLY82, THR83, GLY84, GLY85, ASP86, ALA88, ASN89, ILE90, PHE91, ASN92, VAL93, SER94, SER95, LYS110, HIS111, GLY112, ASN113, ARG114, ALA115, VAL116, SER117, GLY118, LYS119, SER120, GLY121, SER122, ALA123, ALA154, ASN178, GLY181, PRO182, ASP227, GLU228
TrpE	*Pseudomonas aeruginosa*	L-Tryptophan	−6.8	THR428, LEU429, VAL268, ILE329, TYR452, ILE471, ARG472, ALA485, GLY486, ALA487, GLY488, LYS505
Aldehyde dehydrogenase	*Pseudomonas aeruginosa*	L-Tryptophan	−6.9	PRO167, TRP168, ASN169, THR244, GLY245, CYS269, GLY270, CYS303, HIS350, LYS353, GLU398, PHE400
TrpA	*Serratia marcescens*	L-Tryptophan	−6.1	TYR175, THR183, LEU100, PHE212, ILE232, SER233, GLY234, SER235, ALA59, ASP60*
TrpB	*Serratia marcescens*	L-Tryptophan	−8.6	LYS86, GLU108, THR109, GLY110, ALA111, GLY112, GLN113, HIS114, LEU165, GLY188, THR189, PHE305
TrpC	*Serratia marcescens*	L-Tryptophan	-6.5	LYS259, CYS261, ILE283, VAL311, GLN333, HIS335, LEU378, ASP380, GLY384, GLY385, THR386, GLY387, GLY407, GLY408, LEU409, ASP426, ASN428, SER429
TrpD	*Serratia marcescens*	L-Tryptophan	-5.4	MET1 GLN2 LEU5 GLU6 LYS7 LEU8 TYR9 ARG10 SER34 GLN35 LEU36 ALA37 ALA38
TrpE	*Serratia marcescens*	L-Tryptophan	-7.7	LEU37 GLU38 SER39 ALA40 GLU41 ILE42 LEU48 GLN49 LEU51 ARG140 GLU141 PRO290 TYR291 MET292 PHE293 PRO307 GLU308 SER309 LYS312 GLU321 TYR323 ARG401 VAL452 GLY453 TYR454 LEU461 ASP462 THR463 CYS464
Aldehyde dehydrogenase	*Serratia marcescens*	L-Tryptophan	-6.7	LYS94 PRO95 ALA97 GLU98 GLY101 LEU102 SER105 ALA106 THR109 PRO149 ASN151 SER152 ALA155 SER156 GLN159 THR226 CYS272 TYR273 PHE276 SER277 SER278 GLN281 ALA282 CYS283 ILE284 LEU310 VAL320 HIS321 LEU322 LYS442 PHE444 PHE450 LYS462
TrpA	*Bacillus subtilis*	L-Tryptophan	-6.9	ALA53 LEU121 VAL122 PRO123 ASP124 LEU125 PRO126 LEU127 GLU128 SER130 GLN134 TYR144 ILE145 SER146 LEU147 VAL148 ALA149 THR151 SER152 ARG155 ILE159 VAL170 SER172 LEU173 GLY174 PHE205
TrpB	*Bacillus subtilis*	L-Tryptophan	-8.5	GLY87 SER88 HIS89 LYS90 GLU112 THR113 GLY114 ALA115 GLY116 GLN117 HIS118 GLY119 LEU169 THR173 TYR189 ILE191 GLY192 SER193 VAL195 CYS233 VAL234 GLY235 GLY236 GLY237 SER238 ASN239 ALA240 HIS282 ALA304 GLY305 LEU306 TYR308 ALA350 GLU352 SER353 SER378 GLY379 ARG380 LYS383
TrpC	*Bacillus subtilis*	L-Tryptophan	-6.3	GLU21 GLN22 PRO23 PHE24 GLU25 LYS26 ARG27 LYS97 SER118 ARG119 ARG120 ILE121 GLY122 ALA123 ASP124 ALA125 GLU147 LYS148 GLY149 MET150
TrpD	*Bacillus subtilis*	L-Tryptophan	-6.9	THR76 CYS77 GLY78 THR79 GLY80 GLY81 ASP82 GLY83 ILE84 SER85 THR86 PHE87 ASN88 ILE89 SER90 THR91 LYS106 HIS107 GLY108 ASN109 ARG110 SER111 VAL112 SER113 SER114 SER116 GLY117 SER118 ALA119 ASP120 LEU222 ASP223 GLU224
TrpE	*Bacillus subtilis*	L-Tryptophan	-6.9	GLU49 LYS51 ASP52 ASP53 THR54 VAL254 ARG275 VAL277 ASN278 PRO279 SER280 PRO281 MET283 PRO298 GLU299 ILE314 THR317 GLU343 LEU347 PHE375 SER376 HIS377 VAL378 HIS380 THR407 LEU408 TYR431 ILE448 ILE450 ARG451 GLN463 ALA464 GLY465 ALA466 GLY467 GLU480 LYS484 ALA485
Aldehyde dehydrogenase	*Bacillus subtilis*	L-Tryptophan	-6.9	ILE147 THR148 PRO149 TRP150 ASN151 LYS174 PRO175 SER176 GLU177 ILE178 PRO206 GLY207 ALA208 PHE225 THR226 GLY227 GLY228 ILE229 GLU230 THR231 LYS234 GLU249 LEU250 GLY251 CYS283 ILE326 SER327 GLU329 HIS330 LYS333 TYR337 GLU384 VAL385 PHE386 TYR410

Note: *Binding affinity values are expressed as AutoDock Vina scores (kcal/mol), where more negative values indicate stronger predicted ligand binding. Interacting residues represent amino acids located within the predicted ligand-binding pocket of the highest-ranked docking pose.*

3.11 Integrated Experimental and Bioinformatic Findings

Table 12. Integrated comparison of qualitative indole-3-acetic acid (IAA) production, auxin biosynthetic gene distribution, and molecular docking analyses in Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis.

Species	Broth Assay	Agar Assay	Auxin Biosynthetic Genes	Docking Support	Overall Interpretation
Pseudomonas aeruginosa	Weak (Score 1)	Moderate (Score 2)	Complete tryptophan biosynthetic genes (trpA–trpE) present; aldehyde dehydrogenase family gene present; ipdC, iaaM and iaaH absent	Moderate binding affinity of L-tryptophan with TrpA, TrpB, TrpC, TrpD, TrpE, and AldH in CB-Dock2, supporting substrate recognition	Although the complete tryptophan biosynthetic pathway is present, comparatively weak qualitative IAA production suggests limited metabolic flux toward auxin biosynthesis under the experimental conditions.
Serratia marcescens	Moderate (Score 2)	Weak (Score 1)	Complete trpA–trpE genes present; aldehyde dehydrogenase family gene detected; ipdC, iaaM and iaaH absent	Good docking affinity of L-tryptophan with auxin-related enzymes predicted by CB-Dock2, indicating favourable substrate interaction	Presence of conserved biosynthetic genes together with moderate qualitative IAA production indicates an active tryptophan-dependent auxin biosynthetic capability, although expression appears lower than in *B. subtilis*.
Bacillus subtilis	Very Strong (Score 4)	Strong (Score 3)	Complete trpA–trpE genes present; aldehyde dehydrogenase family gene detected; ipdC, iaaM and iaaH absent	Strong docking affinity between L-tryptophan and predicted auxin biosynthetic enzymes, consistent with efficient substrate binding	Integration of phenotypic observations, conserved biosynthetic genes, and molecular docking strongly supports *B. subtilis* as the most efficient potential IAA-producing bacterium among the three organisms investigated.

4. Discussion

The present study integrated qualitative laboratory assays with comparative genome analysis, molecular docking, multiple sequence alignment, and phylogenetic analysis to evaluate the auxin biosynthetic potential of *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Bacillus subtilis*. Combining experimental observations with bioinformatic analyses provided a comprehensive understanding of the molecular basis underlying bacterial indole-3-acetic acid (IAA) production.

Qualitative screening using Salkowski reagent demonstrated distinct differences in IAA production among the three bacterial species. *Bacillus subtilis* exhibited the strongest colour development in both broth and agar assays, indicating the highest auxin-producing potential. *Serratia marcescens* showed moderate activity, whereas *Pseudomonas aeruginosa* displayed comparatively weaker colour development. These observations suggest that bacterial species differ considerably in their ability to synthesize IAA despite possessing similar precursor biosynthetic pathways.

Comparative genome analysis revealed that all three organisms possess the complete set of core tryptophan biosynthetic genes (*trpA, trpB, trpC, trpD,* and *trpE*), confirming their genetic capacity to synthesize L-tryptophan, the principal precursor for IAA biosynthesis. However, the commonly reported auxin pathway genes *ipdC, iaaM,* and *iaaH* were not detected in the selected reference genomes. This finding suggests that these bacteria may utilize alternative tryptophan-dependent pathways or functionally equivalent enzymes that remain unannotated in current genome databases.

To investigate the structural basis of substrate recognition, representative aldehyde dehydrogenase proteins from each organism were selected based on experimentally validated UniProt annotations. AlphaFold-predicted protein structures were analysed using CB-Dock2 with L-tryptophan as the ligand. All proteins exhibited favourable binding pockets and negative docking scores, indicating the potential for stable substrate recognition. Although hydrogen-bond interactions were not consistently observed, the predicted binding poses suggest that hydrophobic and van der Waals interactions contribute substantially to ligand stabilization within the active site. These findings provide computational support for the possible involvement of aldehyde dehydrogenases in the terminal oxidation step of tryptophan-dependent auxin biosynthesis.

Multiple sequence alignment demonstrated high conservation of catalytically important regions among TrpA, TrpB, TrpC, TrpD, TrpE, and aldehyde dehydrogenase proteins. Conserved functional motifs identified in these enzymes indicate preservation of catalytic activity despite evolutionary divergence. In particular, the conserved catalytic cysteine and glutamate residues characteristic of aldehyde dehydrogenases suggest maintenance of their enzymatic function across the investigated bacterial species.

Phylogenetic analysis further supported these observations by showing that the tryptophan biosynthetic proteins of *Pseudomonas aeruginosa* and *Serratia marcescens* clustered closely together, reflecting their common Gram-negative evolutionary origin. In contrast, *Bacillus subtilis* consistently formed a separate branch, consistent with its Gram-positive lineage. Aldehyde dehydrogenases exhibited relatively greater sequence divergence than the tryptophan biosynthetic enzymes, reflecting the broader functional diversity of this enzyme family while retaining conserved catalytic domains.

The integrated experimental and computational results indicate that variations in IAA production cannot be explained solely by the presence of auxin biosynthetic genes. Instead, differences in gene regulation, enzyme activity, metabolic efficiency, and cellular physiology are likely to influence the observed phenotypic differences. The superior qualitative performance of *Bacillus subtilis* may therefore result from more efficient metabolic regulation rather than differences in gene content alone.

A major strength of this study is the integration of wet-laboratory observations with comparative genomics and structural bioinformatics, allowing experimental findings to be interpreted within a molecular framework. However, the study was limited to qualitative colour-based assays, and the docking analyses represent computational predictions that require experimental validation. Future studies employing quantitative IAA estimation, gene expression analysis, enzyme characterization, and functional validation will provide a more comprehensive understanding of bacterial auxin biosynthesis.

The present findings demonstrate that comparative genomics, protein structural analysis, and molecular docking effectively complement conventional laboratory screening for plant growth-promoting bacteria.

This integrated workflow provides a valuable framework for identifying bacterial strains with auxin biosynthetic potential and contributes to a better understanding of the molecular mechanisms underlying microbial phytohormone production.

5. Conclusion

The present study successfully integrated qualitative experimental screening with comparative genomic and structural bioinformatic analyses to evaluate the auxin biosynthetic potential of *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Bacillus subtilis*. Qualitative detection using Salkowski reagent demonstrated clear differences in IAA production among the tested organisms, with *Bacillus subtilis* exhibiting the strongest response, followed by *Serratia marcescens*, whereas *Pseudomonas aeruginosa* displayed comparatively weaker production.

Comparative genome analysis confirmed that all three bacterial species possess the complete core tryptophan biosynthetic pathway (*trpA–trpE*), supporting their capacity to synthesize the precursor required for auxin production. Although canonical auxin pathway genes (*ipdC, iaaM,* and *iaaH*) were absent from the reference genomes, the presence of conserved aldehyde dehydrogenase family proteins suggests that alternative tryptophan-dependent pathways may contribute to IAA biosynthesis. Molecular docking further demonstrated favourable interactions between L-tryptophan and representative aldehyde dehydrogenases, while multiple sequence alignment and phylogenetic analyses confirmed strong conservation of catalytically important residues and evolutionary relationships among the selected proteins.

Collectively, the findings indicate that auxin production depends not only on the presence of biosynthetic genes but also on enzyme functionality and cellular regulation. The integrated experimental–bioinformatic workflow established in this study provides a robust strategy for evaluating plant growth-promoting bacteria and may facilitate the identification of promising microbial candidates for sustainable agricultural applications.

6. Future Prospects

The present investigation provides a foundation for further exploration of bacterial auxin biosynthesis through advanced molecular and biochemical approaches. Future studies should focus on quantitative estimation of IAA using high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), or gas chromatography–mass spectrometry (GC–MS) to validate the qualitative observations obtained in this study.

Expression analysis of key tryptophan biosynthetic and aldehyde dehydrogenase genes using quantitative real-time PCR (qRT-PCR) or transcriptome sequencing would provide insights into regulatory mechanisms governing auxin biosynthesis under different environmental conditions. Gene knockout or overexpression studies could further establish the precise contribution of individual enzymes to IAA production.

Detailed enzyme characterization, including purification, kinetic analysis, substrate specificity, and crystallographic studies, would improve understanding of catalytic mechanisms involved in bacterial auxin biosynthesis. Molecular dynamics simulations and free-energy calculations may also complement molecular docking by providing information on protein flexibility and long-term ligand stability.

Evaluation of the selected bacterial strains under greenhouse and field conditions will be necessary to determine their effectiveness as plant growth-promoting rhizobacteria. Studies investigating seed germination, root architecture, nutrient uptake, stress tolerance, and crop productivity will provide practical evidence supporting their agricultural applications. Furthermore, interactions between bacterial auxin production and other plant growth-promoting traits such as phosphate solubilization, siderophore production, nitrogen fixation, ACC deaminase activity, and biofilm formation should be investigated to identify multifunctional microbial inoculants.

The integration of comparative genomics, structural bioinformatics, functional genomics, metabolomics, and experimental microbiology represents a promising strategy for accelerating the discovery of efficient plant growth-promoting bacteria suitable for environmentally sustainable agriculture.

7. Study Limitations

The present study has several limitations that should be considered while interpreting the findings. Indole-3-acetic acid (IAA) production was evaluated using qualitative Salkowski colour reactions, which provide only a preliminary assessment of auxin production and do not quantify IAA concentrations. The molecular docking analysis performed using CB-Dock2 predicts potential protein–ligand interactions based on structural models but does not directly demonstrate enzymatic activity or substrate specificity under biological conditions. Furthermore, although comparative genome analysis confirmed the presence of genes associated with tryptophan biosynthesis, the presence of these genes does not necessarily indicate their expression or functional activity under the experimental conditions employed. The investigation was also limited to three representative bacterial species, which may not fully represent the diversity of auxin-producing microorganisms. In addition, gene expression analysis, enzyme kinetics, metabolomic profiling, and plant inoculation studies were beyond the scope of the present work. Despite these limitations, the integration of qualitative experimental observations with comparative genomics, structural bioinformatics, molecular docking, multiple sequence alignment, and phylogenetic analysis provides valuable preliminary evidence supporting the auxin biosynthetic potential of the investigated bacterial species and establishes a strong foundation for future functional and quantitative investigations.

8. Ethical Considerations for the Use of Artificial Intelligence (AI) in Academic Writing

Artificial intelligence (AI)-assisted writing tools were used solely to support language refinement during the preparation of this manuscript. These tools were employed to improve grammar, spelling, punctuation, sentence structure, and overall readability, and were not used to generate research questions, develop the study design, analyze data, interpret results, or formulate the scientific conclusions. All intellectual contributions, including the conceptualization of the research, methodology, data collection, analysis, interpretation, and final manuscript preparation, were undertaken exclusively by the authors. Every AI-generated suggestion was carefully reviewed, verified, and modified where necessary to ensure the accuracy, originality, and integrity of the scientific content. The authors accept full responsibility for the content of the manuscript and affirm that the use of AI-assisted writing tools complied with the ethical standards, institutional policies, and the AI-use guidelines of the target journal.

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