Review

Recent Advances and Developments in Bacterial Endophytes Identification and Application: A 20-Year Landscape Review

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Recent Advances and Developments in Bacterial Endophytes Identification and Application: A 20-Year Landscape Review

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Abstract: endophytes are a diverse group of microorganisms that reside within tissues of plants without causing any harm and have gained significant attention due to their role in plant growth promotion, stress tolerance and disease resistance. Interactions between plant tissues and bacterial endophytes are complex and dynamic, in that understanding their colonization mechanisms remains crucial. Advances in genomic and molecular techniques has provided insights into these interactions, but there is still much to explore. The diversity of these microorganisms leads to different functions among strains, making their isolation and characterization challenging. Therefore, researchers utilize a range of culture-independent molecular techniques and high-throughput sequencing technologies to elucidate the diversity of bacterial endophytes. Identifying and understanding these beneficial bacteria is crucial for their applications in agriculture, biotechnology, and environmental sustainability. Researchers can overcome the limitations and improve known techniques, from refining sample collection techniques to developing new computational algorithms. This review provides an overview of recent progress in bacterial endophytes identification, exploring both traditional and emerging techniques. It furthermore discusses broad applications of bacterial endophytes in various fields and highlights directions for future research aimed at enhancing our understanding and utilization of these valuable microorganisms.

Keywords: Bacterial endophytes; plant growth-promoting bacteria (PGPB); Isolation; Identification; Application

1. Introduction

In different ways, diverse microorganisms (bacteria, fungi, archaea and oomycota) are naturally connected to plants. Endophytic microorganisms are widespread in plants and their relationships with host plants has been described as a balanced symbiotic continuum that ranges from mutualism throughout to commensalism but not parasitism [1,2]. They have been reported to be found in nearly all parts of plants from all groups; algae, angiosperms, bryophytes, gymnosperms, lichens, and pteridophytes, including seeds in tall fescue (*Festuca arundinacea*) or other grasses, which are vertically transferred to the next generation [3]. Bacterial endophytes are one class that colonize the internal tissues of the host plant, the stem, leaves, flowers, and fruits together with the seeds, thus developing their lifestyle without causing harm to the host cells and showing no external sign of infection or negative effect on their host [4]. Bacterial endophytes benefit from plants in that plants provide protected living space and constant supply of nutrients [5,6]. In turn, bacterial endohytes form a beneficial relationship with their host plants, providing them with various benefits such as enhanced plant growth [7], improved phytoremediation efficiency [8], confer host plant tolerance to abiotic and biotic stresses [9], nutrient uptake, hormone production, and protection against

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pathogens [10,11]. Mundt and Hinkel documented the first isolation of endophytic bacteria from the ovules and seeds of 27 plants [12]. Bacterial endophytes are commonly classified into two major divisions based on the plant inhabiting life strategies they exhibit viz., facultative and obligate endophytes [13]. Facultative bacterial endophytes are a group that have a stage in their life cycle where they dwell outside the host plant but may enter the host tissue through infection/slightest opportunity and alternate between host plants and the soil; while obligate bacterial endophytes are strictly hooked and live inside their host plant tissues for their growth [14,15]. The novelty of the present review brings to light the bacterial endophytes dynamics and current understanding status pertaining to the recent advances and developments in bacterial endophytes identification. This study also explores traditional, emerging techniques and omic approaches and further highlighting the challenges and limitations in their identification.

2. Diversity and Distribution of Bacterial Endophytes

Bacterial endophytes can be isolated from two different regions known as the part above ground of the plant (phyllosphere) and the part belowground (rhizosphere), characterising each environment to harbour different bacterial communities shaped by traits of each section. Diversity for the belowground can be regulated by different factors associated to the soil microbial communities and the host factors. The microbiome composition in the root interior is significantly less diverse than the microbiomes in the soil [16]. Numerous studies have documented great bacterial endophytes diversity and further demonstrated that microbial communities are still far more diverse in the endosphere than they are in the rhizosphere [17,18]. The diversity of bacterial endophytes demonstrate that they relate to different plant species and have been found in every plant species studied. Thus, pertaining that an endophyte-free plant is a rare exception in the natural environment. In fact, a plant without associated beneficial bacteria would be less fit to deal with phytopathogens and more susceptive to stress conditions [19,20]. Significant number of studies have analysed and reported on the effects of different environmental variables on bacterial endophyte diversity and have shown that different plant hosts may harbour a similar population of endophytic bacteria [12]. Bacterial endophytes population varies within the same species, from plant to plant, from species to species, from region to region and differs with climatic conditions within the same region. Bacterial endophytes are important and their distribution and population structure within host plants is considerably affected by a wide range of factors such as the hosts' genetic backgrounds, ages, interactions between the inhabiting microorganisms as well as the microbe-plant interaction and environmental conditions [21]. Plant compartments, genotypes and geographic locations are vital factors that shape the endophytic microbiome composition, therefore, such environmental conditions including availability of nutrients, pH, temperature and humidity which determine the distribution ranges of hosts, also determine species distribution among the endophytes [22]. Studies done on *Arabidopsis*, *Oryza*, *Populus* and *Zea* validated that bacterial endophyte communities are generally influenced by combination of the host environment and season, host genotype variation and host species identity [23,24].

Additionally, single host plant species may accommodate multiple bacterial endophytic genera and species; and the bacterial endophytic community range depends widely on the colonized tissue, plant type or endophyte isolation season. The technique (type, concentration and even length of the treatment time with sterilizing agent) employed to investigate these bacterial endophytes is a significant determinant utilised to detect the host plant's bacterial endophytic diversity [25]. Depending on the diversity and composition of microbial communities, bacterial endophytes are often associated with a variety of plant organs of which many may harbour similar species. The most described genera in literature of bacterial endophytes and also common inhabitants of the rhizosphere are *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Microbacterium*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas* and *Serratia*; thus suggesting that endophytic microbiome could be a subpopulation of the rhizosphere inhabiting bacteria [26–31].

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3. Biological Roles, Benefits and Prospective Application of Bacterial Endophytes

Bacterial endophytes use direct or indirect techniques to benefit their host plants in multiple ways. The ability of bacterial endophytes to produce bioactive compounds of agricultural, industrial and pharmaceutical importance makes them interesting candidates for research purposes. They play important roles in different fields of life, ranging from their impacts on host plants, their effects on the environment and human life, thus encompassing a range of beneficial applications in agriculture, biotechnology and environmental management as shown in **Figure 2** [9,32]. The cross-talking between key mechanisms used by bacterial endophytes to benefit the host plant largely contributes to and accounts for signal transduction. Mechanisms that bacterial endophytes employ for plant growth and development comprise processes that include biocontrol [33], bioremediation [34], biotic/abiotic stress reduction [35], endosphere colonization [36], enzyme secretion [37], and induction of plant-resistance [38]. The production of antibiotics, cell wall-degrading enzymes, decreased plant ethylene levels, reduced iron availability to pathogens and the synthesis of pathogen-inhibiting volatile compounds are also just a few examples of the numerous common mechanisms used by bacterial endophytes to promote plant growth and development [39]. Furthermore, bacterial endophytes can indirectly enhance plant growth by stimulating plant responses or producing secondary metabolites against phytopathogens [40].

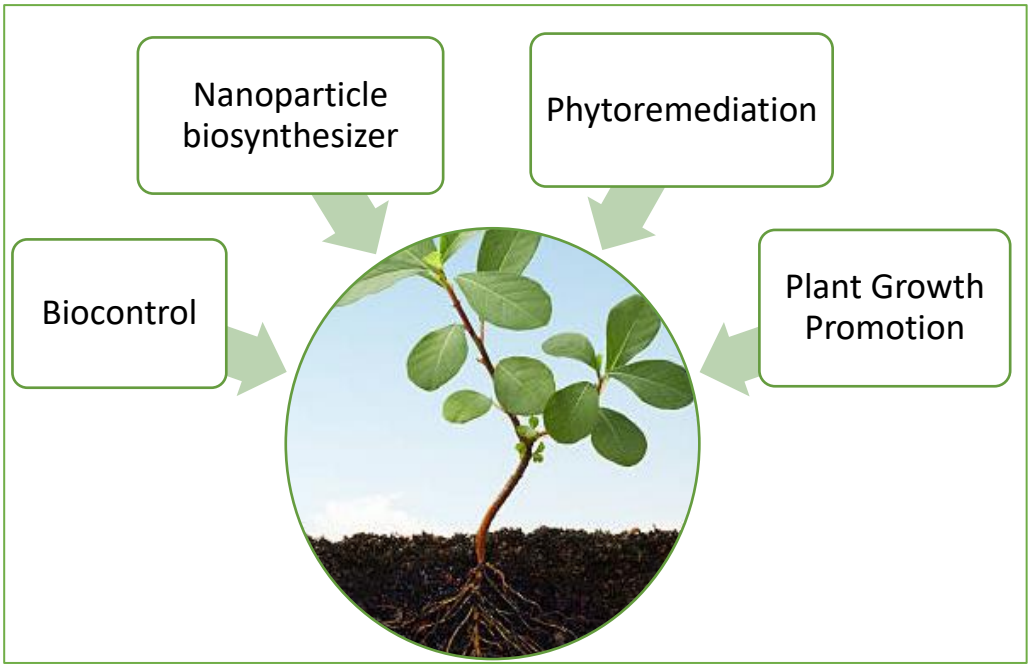

Figure 2. Overview of beneficial roles and application of bacterial endophytes.

3.1. Biocontrol Role

Biocontrol of plant pathogens (phytopathogens) has gained significant attention in the past years due to its potential as a sustainable and environmentally friendly alternative to chemical pesticides and one such approach involves the use of bacterial endophytes, which are beneficial bacteria residing within plant tissues. The design and development of novel antimicrobial agents has attracted considerable attention due to adverse impacts associated with conventional agrochemical pesticides (fungicides, insecticides, and herbicides and nematicides), such as phytotoxicity, pathogen resistance, and chemical residues exposed to the host plants [41]. Agrochemicals execute effective roles to ensure continuous dynamic agricultural structure and control of plant diseases, but extensive use of agrochemicals result in adverse outcome on functioning of the environment, agricultural prosperity and on humans. Bacterial endophytes possess several mechanisms that secrete biocontrol agents to

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inhibit entry and control the growth of phytopathogens among other activities, such as competition for nutrients, production of antimicrobial compounds and induction of plant defence responses [42,43]. Furthermore, other studies have presented evident data on the impact of bioinoculant and endophyte application on development and growth for nutrient absorption in corn, cotton, sorghum and tomato [44,45]. Certain bacterial endophytes such as *B. amyloliquefaciens, B. pumilus, B. subtilis, Pseudomonas fluorescens, P. syringae* and *Serratia marcescens* assist their host plants to elicit antibiotics and induce systemic resistance (ISR), resistance mechanisms which occur when plants successfully activate their defence mechanisms in response to infection by a phytopathogen [46,47]. *Bacillus spp* demonstrated antagonistic activity against *Xylella fastidiosa* that causes olive quick decline syndrome (OQDS) in olive trees [48,49]. Moreover, novel *Streptomyces spp* MBFA-172 in strawberry plant was reported to exhibit biocontrol activity against *Glomerella cingulata* [50]. *Bacillus amyloliquefaciens, B. subtilis, B. velezensis, Lactococcus lactis* and *Leuconostoc mesenteroides* are a good few documented seed associated bacterial endophytes to be used to treat bacterial wilt of tomato and exhibited biocontrol agent properties [51]. Studies by other teams also reported on the ability of *B. velezensis* to inhibit a variety of fungal phytopathogens, *Colletotrichum coccodes, Fusarium avenaceum, Phoma foveat* and *Rhizoctonia solani* on potato in both *in vitro* and field experiments [52,53]. Therefore, isolates of bacterial endophytes can commercially be incorporated to synthesize biopesticides to both protect host plants and maintain a healthy environment [54]. Another study demonstrated that *B. velezensis* IALR619 has potential to inhibit strawberry pathogen growth in greenhouse and possibly increase fruit yield in the field which comfirmed with a study that reported that *B. velezensis* is a typical biocontrol agent used control various soil-borne diseases as well as a plant growth promoting bacteria [55,56]. Furthermore, some biocontrol agents possess plant growth promoting capabilities and their use can help reduce reliance on synthetic chemicals, minimizing environmental damage and promote sustainable agricultural practices. As research in this field continues to progress, exploiting the potential of bacterial endophytes for biocontrol purposes holds great promise for the future of agriculture.

3.2. Nanoparticle Biosynthesizer Role

Biosynthesis and production of nanoparticles (NPs) utilizing bacterial endophytes have emerged as cutting-edge technology, due to the various functions, environmental clean-up, potential bioactivity, non-pathogenic nature and enormous therapeutic applicability of these particles [57]. Biological approach of NPs synthesis over physical and chemical approaches have been reported to have numerous advantages; they are easy, economical, safe and eco-friendly because it requires less energy and produces no harmful waste [58]. Biocompatible and non-toxic NPs are preferred when it comes to drug delivery and medicine. Therefore, their synthesis could easily utilise bacterial endophytes and reports have revealed that numerous researchers have employed prokaryotic and eukaryotic endophytes for the synthesis of gold, silver, copper metal and metal oxide NPs which have been used in all the domains of science [59]. Bacterial endophytes reduce metallic ions during biosynthesise and produce NPs. Furthermore, other researchers have reported NPs synthesis with other elements such as zinc sulfide, copper oxide, cobalt oxide, nickel oxide from endophytes that were isolated from both terrestrial and marine environments. There are numerous potential uses for these biosynthesized NPs in nanomedicine because of their antibacterial, antifungal, antioxidant, antimicrobial, antidiabetic, anticancer and photocatalytic degradation properties [60,61]. AgNPs synthesized using endophytic *Streptomyces spp*. showed antimicrobial, antioxidant and larvicidal activities [62]. Low concentrations of AgNPs synthesized by *Pantoea ananatis* showed antimicrobial activity against *Candida albicans* and *Bacillus cereus* and higher concentrations were active against multidrug-resistant strains of *Enterococcus faecium*, *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus* [63,64]. The endophytic *Streptomyces spp*. isolated from medicinal plants synthesized CuNPs and CuONPs with antibacterial, antifungal, antioxidant and insecticidal activities [65,66]. Fadiji and team demonstrated the function of various NPs synthesized from bacterial endophytes in sustainable agriculture by enhancing plant growth and development, thus

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strengthening resistance to disease [67]. Other studies documented that bacterial endophytes may serve as bio-factories for NPs and those synthesized from them have immense potential in healthcare applications [68]. Silver NPs are widely used specifically in bio-labelling, antimicrobial agents, catalysts and sensors due to their unique optical, electrical and magnetic properties [69].

3.3. Plant Growth Promotion Role

One key area of interest in the study of bacterial endophytes is their role in promoting plant growth through enhancing disease resistance, nutrient uptake, and stress tolerance, which are attributed to several mechanisms employed by endophytes, such as the production of phytohormones (auxin, cytokinin, ethylene, and gibberellin), siderophores, and various enzymes that aid in nutrient acquisition, including nitrogen fixation, phosphate solubilization, and iron and potassium mobilization [70,71]. Additionally, bacterial endophytes can also facilitate plant growth by inducing systemic resistance against phytopathogens and pests, by secondary metabolite synthesis, antibiosis activities ultimately boosting plant responses to environmental stressors leading to healthier host plants and surrounding environment [72,73]. Bacterial endophyte *Pseudomonas spp*. has shown to stimulate pea plant growth and exhibit plant-beneficial traits such as phosphate solubilization, production of indole-3-acetic acid (IAA), siderophores, hydrogen cyanide (HCN), and ammonia (NH_3) [74]. Certain bacterial endophytes genera, including *Acinetobacter*, *Bacillus*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Pantoea*, and *Staphylococcus;* have demonstrated the ability to thaw dormant seeds, enhance seedling growth, protect against phytopathogens, and promote plant development [75,76]. These beneficial properties may explain why such microbes are consistently transmitted to subsequent plant generations through seeds [77]. Bacterial endophytes also play a significant role in protecting plants from pathogens, including bacteria, fungi and nematodes [78]. Notably, the protein BphKLB400 from *Burkholderia xenovorans* LB400 confers herbicide tolerance onto pea plants and facilitates detoxification of multiple herbicides [79]. Under abiotic stress conditions such as drought, salinity, and heavy metal exposure, enzymes like 1-aminocyclopropane-1-carboxylate (ACC) deaminase and IAA, along with phosphate-solubilizing traits, help plants adapt and maintain growth [80]. IAA contributes directly to plant physiological processes like chlorophyll content, increased biomass and root development, while ACC deaminase modulates ethylene levels, mitigating growth inhibition under stress. Endophytes also promote drought tolerance by producing abscisic acid (ABA), volatile compounds, and osmoprotectants, improving plant water retention and osmotic adjustment [81]. Taxonomically, bacterial endophytes are diverse, encompassing Gram-positive and Gram-negative genera such as *Achromobacter*, *Agrobacterium*, *Bacillus*, *Pantoea*, *Xanthomonas*, and others. Many of these microbes produce bioactive compounds with antimicrobial and anticancer activities, positioning them as valuable resources treat a range of illnesses [82,83]. Moreover, their bioactive metabolites have been reported to exhibit antidiabetic, antifungal, immunosuppressive, and anti-inflammatory properties [84–87]. The production of ACC deaminase is one of the key attributes of endophytic bacteria in stimulating plant growth under high concentrations of toxic metals, and lowers ethylene levels during stress, while simultaneously facilitating mineral solubilization [88,89]. Other findings also highlight endophyte-triggered defense responses, *Arthrobacter agilis* produces N,N-dimethyl hexadecylamine, a compound that induces iron uptake by roots against the growth of pathogenic fungi and defense-related gene expression in *Medicago truncatula*, offering protection against *Botrytis cinerea* and *Pseudomonas syringae* without involving the jasmonic acid (JA) pathway [90,91]. Additionally, ACC deaminase and IAA interaction is critical in modulating ethylene levels. Excess ethylene stimulated by IAA-induced ACC synthase activity can inhibit plant growth, which is mitigated by microbial ACC deaminase [92]. The application of the plant growth promoting endophytes producing this enzyme has been linked to enhanced tolerance to various environmental stresses [93]. Specific endophytic strains such as *Pantoea agglomerans* aid in salt stress tolerance through IAA production, whilst *Bacillus licheniformis* promotes overall plant health, inhibits fungal parasites, and synthesizes gibberellins [94,95]. Similarly, *Bacillus stratosphericus* has demonstrated to suppress bacterial and fungal parasites and aid plants in

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phytoremediation of heavy metals and other pollutants, including salt [34,96]. Thus, understanding these diverse mechanisms through which bacterial endophytes promote plant growth lays the foundation for the application of bacterial endophytes in environmental biotechnology and sustainable agriculture.

3.4. Phytoremediation Role

Phytoremediation is a green and sustainable technology that employs plants and their associated microorganisms to remove hazardous organic and inorganic pollutants from the environment [97,98]. Plants have the inherent ability to degrade certain pollutants, and phytoremediation using plants has been recognized as a cost-effective and eco-friendly approach for remediating contaminated sites. Success outcome of phytoremediation largely depends on the combined ability of plants and their bacterial endophytes to tolerate high levels of contamination [99,100]. Microbial degradation of inorganic compounds can result in the production of environmental metabolites such as carbon dioxide, humus, salts, water, and other beneficial bio-products. In addition, bacterial endophytes have been shown to degrade environmental pollutants, including explosives, herbicides, and hydrocarbons [101,102]. In plant-endophyte interactions, plants offer bacterial endophytes nutrients and shelter. In turn, bacterial endophytes with appropriate metabolic activity and degradation pathways enhance the breakdown of organic pollutants while reducing phytotoxicity and evapotranspiration. Moreover, plant growth-promoting endophytes further improve plant development and stress adaptation, especially in contaminated soils and water bodies [103].

Many bacterial endophytes are known for their ability to degrade xenobiotic pollutants including alkanes, hydrocarbons, and pesticides; while also promoting plant growth through mechanisms such as auxin production, nitrogen fixation, and siderophore secretion [104]. Some bacterial endophytes produce IAA and other bioactive compounds that support plant growth and facilitate remediation in heavy metal-contaminated environments [105,106]. Notably, phosphate-solubilizing bacterial genera such as *Pseudomonas*, *Burkholderia*, *Paraburkholderia*, *Novosphingobium*, and *Ochrobactrum* have demonstrated the ability to enhance biomass yield in certain seedlings due to their multifunctional traits. The development of bioinoculants using root-associated endophytes shows great promise in modern agriculture [107]. Given the extensive research on plant growth-promoting bacteria, their integration into organic farming systems has the potential to significantly boost agricultural productivity and contribute to global food security. Experimental bioremediation studies have revealed that the bacterial endophyte *Paenibacillus* spp., isolated from *Tridax procumbens*, achieved significant heavy metal removal eliminating up to 59.4% of copper and 51.4% of zinc, demonstrating its potential for multi-metal remediation [108–110]. Further research is needed to identify novel bacterial endophytes with strong contaminant-degrading abilities. Optimizing their potential can enhance sustainable plant health and contribute to environmentally friendly agricultural practices aimed at improving crop productivity and ecosystem resilience.

Table 1. Beneficial association between bacterial endophytes and host plant.

Role of beneficial bacteria	Bacterial Endophyte	Host plant	References
Enhance plant resistance to phytopathogens	*Bacillus amyloliquefaciens*	*Ginkgo biloba and Panax notoginseng*	[111,112]
	Bacillus sp.	*Curcuma longa*	[113]
	Cohnella sp., Paenibacillus sp. and Pantoea sp.	*Centella asiatica*	[114]
	Phyllobacterium myrsinacearum	*Epimedium brevicornu*	[115]
	Stenotrophomonas maltophilia and Bacillus sp.	*Panax ginseng*	[116]
Improved plant abiotic stress tolerance	*Achromobacter xylosoxidans*	*Catharanthus roseus*	[117]

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	Agrobacterium spp. and Bacillus spp.	*Pteris vittata*	[118]
	Citrobacter putida	*Euphorbia milii*	[119]
	Glutamicibacter halophytocola	*Limonium sinense*	[120]
	Paenibacillus sp.	*Plantago asiatica and Tridax procumbens*	[108,121]
Plant growth promotion	*Bacillus and Paenibacillus spp.*	*Curcuma longa*	[122]
	Bacillus cereus and Bacillus subtilis	*Teucrium polium*	[123]
	Bacillus siamensis	*Coriandrum sativum*	[124]
	Micrococcus luteus and Lysinibacillus fusiformis	*Panax ginseng*	[125]
	Paenibacillus and Bacillus spp.	*Lonicera japonica*	[126]
	Serratia marcescens	*Achyranthes aspera*	[127]
	Variovorax sp.	*Lavandula dentata*	[128]
Promotion of plant metabolites accumulation	*Bacillus subtilis*	*Ligusticum chuanxiong*	[129]
	Burkholderia sp. and Paenibacillus polymyxa	*Panax ginseng*	[130,131]
	Pseudomonas fluorescens	*Atractylodes lancea and Atractylodes macrocephala*	[132,133]
	Pseudonocardia sp.	*Artemisia annua*	[134]

4. Isolation Techniques

Several researchers have extensively studied and reviewed various techniques for isolating bacterial endophytes from various plant tissues, such as seeds, roots, stems, stems, leaves and flowers [135,136]. The most widely accepted isolation technique is whole-surface sterilization, which involves three main steps: (1) surface sterilization of plant tissues, (2) maceration of the sterilized tissues, and (3) culturing of serially diluted macerates [16,137,138]. Surface sterilization is a critical first step of isolation that eliminates all potential pollutants, epiphytic microorganisms, external contaminants, and debris. This is typically achieved by sequential treatment of the plant tissues with sterilizing agents, such as ethanol, hydrogen peroxide, sodium or mercuric chloride, and hypochlorite solutions, for a predetermined period of time to improve the effectiveness of the sterilization procedure; followed by thorough rinsing with sterile distilled water to remove any residual chemicals [139,140]. Effectiveness of sterilization is verified by plating the final rinse water on a culture medium; where the absence of bacterial growth confirms the effectiveness of the sterilization procedure and any growth observed denotes availability of unwanted pollutants [100,141]. A high yield of bacterial colonies after sterilization indicates minimal impact on the internal endophytic population [142,143]. An ideal sterilization protocol must balance the complete removal of surface microbes while preserving the viability of internal endophytes. The sterilizing agents ought to eliminate the epiphytic microbes without causing any damage to the endophytes or host tissue; though this balance is often difficult to achieve because aggressive sterilizing agents can sometimes penetrate plant tissues and harm the endophytes.

Surface sterilization methods may vary depending on the plant species, tissue type, and researcher's preference, however most common used surface-sterilisation protocol followed are those outlined by [144–146]. Mashiane et al. demonstrated surface sterilization of explants using a three-step approach that involved immersion in 70% ethanol for 60sec, followed by rinsing with distilled water and subsequent sterilisation in 3% sodium hypochlorite (NaClO) for 60sec and finally in 70% ethanol for 30sec. Samples were further washed in sterile distilled water three times, for 60sec each. The process involved the inoculation of explants obtained from different parts of the maize plants on nutrient agar, tryptone soy agar, and nutrient broth, and incubated at 27°C for 24 hours to isolate

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endophytic bacteria [147]. In another study, Dasgupta et al. sterilized pooled leaf samples from Eucalyptus clones with 70% ethanol (10 min), 2% NaClO (15 min), and 70% ethanol (10 min), followed by eight sterile water washes. The sterilized leaves were plated on Luria-Bertani (LB) agar to confirm sterility and later used for genomic DNA extraction [148]. Similarly, Abbamondi et al. reported on isolation of 12 bacterial strains from fresh roots using a protocol involving washing with P-buffer containing Tween 40, sterilization in 5% NaClO (5 min), five sterile water rinses, tissue maceration in 10 mM $MgSO_4$, and plating serial dilutions on LB medium. Surface sterility was confirmed by plating the final rinse water [149].

5. Identification and Characterization of Bacterial Endophytes

Accurate identification is essential to understand the diversity, functionality and potential applications of bacterial endophyte populations. Numerous studies have well documented diverse approaches for the assessment of these microorganisms, and a summary of some techniques used to study bacterial endophytes is presented in **Table 2**. Research in this area generally employs both culture-dependent and culture-independent techniques as cornerstone methods for identification and characterization. These typically involve: (i) basic culturing, (ii) DNA extraction, (iii) PCR amplification using specific primers, (iv) sequencing and (v) comparison of the obtained sequences with those available in public databases using BLAST [150–152].

Table 2. Summary of some techniques and characteristics used to study bacterial endophytes.

Techniques	Characteristics
Culture dependent	**Culture- based techniques (by plating)** **Advantages** a. Low cost for easy microbial isolation using nutrient-rich media under specific growth conditions, allowing recovery of pure cultures effectively b. Morphological and biochemical characterization of microbes easily achieved c. Microbial genetic materials (DNA/RNA) easy to extract for further analysis **Disadvantages** a. Laborious and time-consuming processes b. Difficulty in assessing diverse microbial communities due to varied and specific growth requirements c. Undesired microbial proliferation
Culture independent	**Microscope- based techniques (TEM and SEM)** **Advantages** a. Detailed microbial architecture, diversity, structures and colonization patterns are easily visualized **Disadvantages** a. Always bulky and large in size b. Expensive and limited to visualization under light microscope only **Molecular and Omics- based techniques (PCR and sequencing)**

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	Advantages a. Microbial genetic materials (DNA/RNA) are easily extracted b. Enables profiling of the complete microbiome present in the samples c. Determines functional roles of microbes in different biological processes d. Gives comprehensive details on the microbial functions, metabolites production, metabolic pathways, and taxonomy profiling **Disadvantages** a. Plant DNA contamination during endophytic DNA extraction prior to PCR b. Primers designing and sequence analysis requires prior knowledge on sequence information and skilled professionals c. Expensive cost of primers, DNA extraction kits and genomic sequencing d. Low yield of endophytic DNA following 16S rRNA extraction and amplification e. Library preparation may compromise DNA integrity, leading to errors and false results

5.1. Culture-Dependent Techniques

Traditionally, culture-dependent techniques have been utilised to obtain pure bacterial isolates for studying the roles, characteristics, and functional traits of bacterial endophytes. These methods have contributed significantly to the identification and characterization of the diversity of plant-associated bacterial endophytes, however, key limitation is that they capture only a small fraction of the total microbial population, leading to a biased representation of the actual diversity of endophytic populations [153,154]. Culturable bacterial endophytes are identified and characterized through biochemical, morphological, physiological, and molecular approaches [155,156]. These techniques are inherently restricted to microorganisms that can be cultivated under both *in vitro* and *in planta* conditions, excluding the vast majority of unculturable microbes. As a result, culture-dependent approaches do not provide a comprehensive overview of the endophytic bacterial population [157,158]. Some studies analyzed the bacterial endophyte diversity in various tissues of *Dendrobium officinale* and demonstrated that culture-dependent methods yielded a skewed and limited diversity profile. Comparable observations have been reported in other studies, which found that these methods tend to underestimate microbial diversity when compared to culture-independent approaches [153,159].

Bacterial colonies exhibiting distinct morphologies are typically sub-cultured on freshly prepared nutrient media and preserved on nutrient agar slants for downstream analysis. The composition of the nutrient medium plays a critical role in supporting bacterial growth, with carbon- and nitrogen-rich media such as nutrient agar and potato dextrose agar commonly used to encourage the proliferation of bacterial endophytes [160]. In addition to culturing, microscopy and staining techniques can be employed to visualize the colonization patterns, morphology, and cellular structures of bacterial endophytes. Various types of microscopes; including transmission electron microscopes, bright-field microscopes, fluorescence microscopes, and laser scanning microscopes have been instrumental in bacterial endophyte identification [158,161]. These techniques are frequently complemented by biochemical assays to evaluate the metabolic and physiological capabilities of bacterial endophytes. Despite their value, culture-dependent approaches have significant limitations. They only allow the recovery and analysis of culturable microorganisms, leaving most unculturable endophytic populations unexplored. It is estimated that only around 5% of bacterial endophytes have been identified using conventional culturing techniques, as many

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bacterial strains have highly specific metabolic and physiological requirements that are challenging to replicate *in vitro* [162,163].

5.2. Culture-Independent Techniques

Literature has confirmed the occurrence of bacterial endophytes in host plants through various culture-independent assays [164]. Researchers can examine the diversity and functionality of bacterial endophytes using vast of molecular techniques once they have been isolated. Researchers can investigate the diversity and functionality of these bacterial endophytes using an array of molecular techniques once they have been isolated. Culture-independent techniques bypass the need for cultivation, providing a fast and accurate taxonomic landscape of endophytic bacteria. This allows the study of microbial communities in their natural environment [160]. As high-throughput techniques, they offer a more comprehensive understanding of endophyte diversity and community structure. Additionally, culture-independent techniques have elucidated the makeup of bacterial communities in a variety of plants, including bananas, beans and rice and medicinal plants such as aloe vera [87,165]. Molecular and visualization approaches, as examples of culture-independent techniques, enable the identification of unculturable bacterial endophytes within host plants, including those that grow slowly or are present in low abundance and thus overlooked by culture-dependent methods [166–168]. Microscopy is the most direct method for observing bacterial endophytes, and the exact location of these endophytes within plant tissue can be determined using both electron and light microscopy [169,170]. While light microscopy provides a general overview of the structures and morphology, it is limited in resolution compared to electron microscopy, which enables high-resolution ultrastructural analysis. Electron microscopy helps detect bacterial endophytes, reveal their extent of colonization, and visualize bacteria–host interactions and their establishment within the plant environment. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) yield valuable information about the surface and internal structures of bacterial endophytes, respectively [155,171,172].

Morare and colleagues morphologically identified bacterial endophytes from *Crinum macowanii* using gram staining, and high-resolution images were created using SEM micrographs to detail the shapes and confirm identities. Macroscopic identification of bacterial endophyte colonies involved assessing colony color, consistency, elevation, margin, opacity, and surface structure. Isolates were further subjected to a gram stain reaction and viewed under an oil immersion compound bright-field microscope at 100× magnification [173,174]. Biochemical assays such as catalase, oxidase, urease, coagulase, and starch hydrolysis tests are also employed to validate the biochemical activity of bacterial endophytes [3,164]. However, the use of different culture-independent techniques depends on the experimental design and research objectives. The choice of one technique over another is often subjective, based on the researcher's hypotheses and available resources. Having a thorough understanding of the advantages and limitations of each technique increases the possibility of obtaining more comprehensive data and insights. Advancements in meta-omics provide modern tools to better understand the activities of bacterial endophytes in plants [175,176]. Ultimately, the most effective approach might involve combining modern techniques with traditional methods; a strategy that has been shown to reduce error rates in some next-generation sequencing methods and improve data quality.

5.2.1. Meta-Omics Approaches

a. Metagenomics

Metagenomics utilizes modern genomic techniques to analyze genome sequences obtained directly from various environments, providing insights into the diversity, genetics and physiology of unculturable microorganisms. This approach has been a widely used method for studying microbiome populations, as it examines both single marker genes and the complete genomes of all organisms present, providing valuable information on genetic structure, novel genes, functional roles

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and evolutionary relationships within microbial communities [177,178]. The application of metagenomic techniques has demonstrated significant potential in identifying the role of bacterial endophytes in essential plant biological processes, such as biodegradation, nitrification, plant growth promotion, phytoremediation and the suppression of phytopathogens. Compared to traditional culture-dependent methods, metagenomics provides an unbiased perspective, although it comes with limitations such as high sequencing costs, interference from plant DNA, low extraction efficiency and small DNA yields [179–181]. employed a metagenomics approach to analyze bacterial endophyte communities in tomatoes and discovered secondary metabolites capable of suppressing nematode infections in roots. Similarly, functional genes associated with nitrogen metabolism, IAA and tryptophan biosynthesis, siderophore production and phosphate solubilization have been identified in root-associated endophytic microbiomes in maize using a shotgun sequencing. High-throughput sequencing technologies such as Illumina and 454 pyrosequencing have been utilized to explore diverse bacterial endophytes in oak and sorghum plants [182,183]. Furthermore, shotgun metagenomic sequencing, coupled with bioinformatics analysis has revealed taxonomic composition and predicted functional genes involved in plant growth promotion. In the *Panax ginseng* bacterial community, genes related to ACC deaminase activity, phosphate solubilization, 4-phytase, methanol utilization, and nitrogen metabolism have been identified through metagenomic analysis. Additionally, metagenomics has facilitated the study of genes responsible for bacterial attachment, carbohydrate metabolism, responses to temperature fluctuations, osmotic stress, pH regulation and secretion systems [184–186].

b. Metatranscriptomics

Metatranscriptomics focuses on studying the expression of all messenger RNA (mRNA) transcripts in microbial cells associated with various plants. This technique provides insight into transcribed genes, enabling the understanding of genetic profiles and functional similarities or differences among bacterial endophytes in diverse environments [151,187,188]. RNA sequencing (RNAseq) allows comprehensive transcriptome analysis of microbial communities under different conditions, establishing a direct link between microbial genetic composition and the functional roles of expressed genes. These techniques have widely been used in plant-microbe interaction studies, as they characterize active microbial genes with specific functions and identifies those driving symbiotic relationships between microbial communities and plant hosts. In this context, proteins play a key role in promoting plant growth under stress conditions by acting as precursors for various bacterial endophyte-induced mechanisms [189,190]. Moreover, stress-responsive genes from bacterial endophytes can act as catalytic regulators of microRNAs (miRNAs), which modulates the expression of essential genes involved in plant responses to abiotic stress in crops such as wheat, rice, Arabidopsis, and Medicago. miRNA169 and miRNA169c have been reported to enhance stress responses in tomato and rice plants. Additionally, some studies have also explored the role of miRNAs in mediating metal stress in two rice subspecies, *Indica* and *Japonica* using RT-PCR analysis [191–194]. Bacterial endophytes also contribute to plant stress tolerance through the secretion of ACC deaminase, making them promising candidates for bioinoculants that support plant survival under challenging conditions such as drought, salinity and extreme temperatures. The effectiveness of metatranscriptomic studies is enhanced when combined with metagenomic approaches. While metagenomics identifies genes present and absent in culturable and non-culturable bacteria, metatranscriptomics compares transcriptomes of related bacterial species, shedding light on microbial community responses to environmental changes. Together, these techniques provide valuable insights into endophytic bacteria within ecosystems, potentially leading to the discovery of novel genes and their functions [187,195]. One key advantage of metatranscriptomics is that it does not require probes or primers, allowing for an unbiased sequencing of microbial transcripts. Additionally, it provides information on the expression of non-coding genes and small RNAs, further enhancing its utility in microbial profiling. As direct sequencing methods advance, metatranscriptomics will continue expanding microbial community databases, helping to address

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emerging research questions. Despite its advantages, the technique encounters various setbacks, and such includes the recovery of mRNA transcripts as it is unstable metatranscriptomics faces several challenges. Additionally, separating mRNA from other RNA types, including tRNA, rRNA, and miRNA, remains a complex process. The presence of humic compounds that co-exist with nucleic acids can also interfere with accurate sequencing results [196–199].

c. Metaproteomics

Metaproteomics studies gene expression at the protein level within microbial communities, offering insights into their functional activities, including biochemical processes, bioremediation and degradation pathways. This technique allows for the classification and quantification of proteins from bacterial endophytes, aiding in the understanding of gene functions, DNA transcription to mRNA and subsequent protein translation [151,187,188]. Metaproteomics provides cutting-edge capabilities for analyzing microbial functions under biotic and abiotic conditions, such as enzyme activity in metabolic pathways, protein identification and osmotic regulation. In one study, metaproteomic analysis has identified the endophytic bacterium *Gluconacetobacter diazotrophicus* in drought-prone sugarcane soils. Another study compared metaproteomic profiles of ratoon sugarcane in the rhizosphere with those of the plant itself, revealing that 24.77% of the proteins found in soil originated from bacteria, with many upregulated proteins linked to membrane transport and signal transduction [150,200]. Metaproteomics has emerged as an essential tool for linking microbial taxonomic diversity with functional profiles. However, analyzing proteomes in mixed microbial communities remains challenging due to their complexity, with only a small fraction (approximately 1%) of the total metaproteome typically identified. The absence of genetic data further complicates microbial proteome studies, making them incomplete. Additionally, protein extraction and sample preparation are hindered by interfering substances such as alkaloids, organic acids, polysaccharides, polyphenols, lipids, and secondary metabolites. Despite these challenges, metaproteomic studies have yielded valuable findings. Other teams identified diazotrophic methanotrophs in rice roots using metaproteomic analysis, while bacterial proteins linked to amino acid, carbohydrate, and lipid metabolism have been reported in endophytic genera such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* [9,201,202]. Metaproteomics has been applied in environmental research, particularly in soil-microbe and plant-microbe interactions, highlighting its potential in agricultural biotechnology. Analyzing the genomes of endophytes from plants in drought-prone soils could help predict specific functional protein genes associated with bacterial and fungal adaptations [188,200,203]. However, metaproteomics is still in its early stages and literature on the protein expression of bacterial endophytes in major crops remains limited. Future research should focus on expanding metagenomic data from diverse microbial environments to enable a more comprehensive characterization of bacterial endophyte communities. Advancing metaproteomic techniques will be crucial in unveiling the protein functions and metabolic pathways of endophytic bacteria, ultimately enhancing their application in sustainable agriculture [204–206].

d. Metabolomics

Metabolomics focuses on the study of metabolites in living cells and has been widely applied across various biological fields, although less literature is available documenting its use in identifying functional characteristics of microbial endophytes [5,207]. Research has documented numerous bacterial metabolites analyzed using techniques such as capillary electrophoresis-mass spectrometry (CE-MS), liquid and gas chromatography-mass spectrometry (LC-MS and GC-MS) and nuclear magnetic resonance spectroscopy (NMR). In plants, metabolites are chemical compounds produced through metabolic pathways that play essential roles in growth, development and defense mechanisms [186,208,209]. The application of metabolomics in crop production holds great potential for determining metabolite composition at different developmental stages. Bacterial endophytes contribute significantly to plant growth and survival under extreme environmental conditions by producing metabolites that support adaptation and survival. Studies investigating the metabolic

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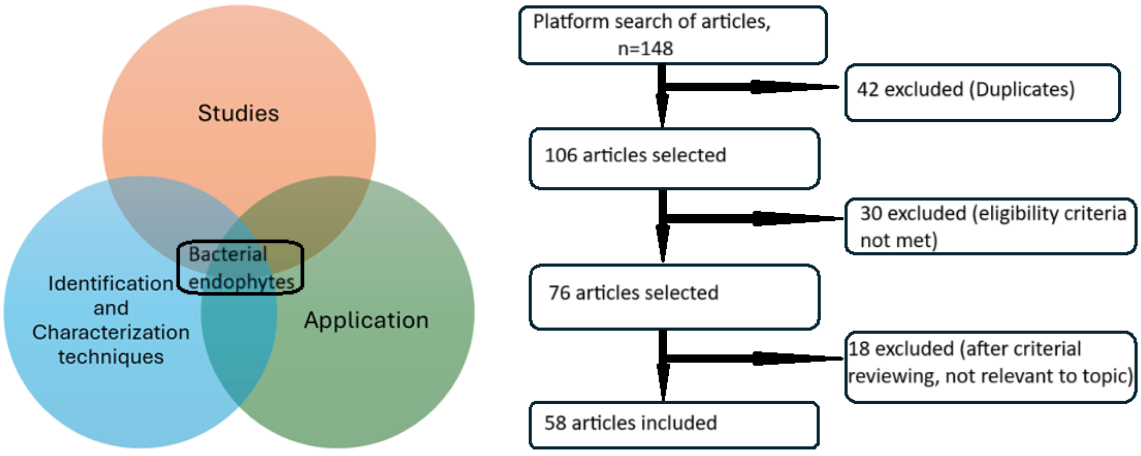
potential of bacterial endophytes have revealed diverse functional secondary metabolites that drive microbial adaptation, interactions and lifestyle shifts in plant-associated environments [178,210,211]. Notable metabolites produced by bacterial endophytes include sespenine (*Streptomyces* spp.), spoxazomicins (*Streptosporangium oxazolinicum*), siderophores (*Pseudomonas aeruginosa*), serobactin A (*Herbaspirillum seropedicae*), valienamine (*Burkholderia kirkii*), pavettamine (*Burkholderia* spp.), and coronatine (*Pseudomonas syringae*). Microbial metabolite profiling plays a crucial role in identifying novel compounds involved in cellular processes and microbial interactions. This approach also offers potential for the discovery of alternative antibiotics derived from bacterial endophytes, contributing to advancements in biotechnology and medicine [202,212–214].

6. Materials and Methods

In order to ensure comprehensive review of the literature and achieve the objective of the review process and analysis, a thorough search of published articles on research terms related to the bacterial endophytes diversity, isolation, identification and application was undertaken. Platforms such as Google Scholar, NCBI, PubMed, ResearchGate, Science Direct, Scopus, Semantic Scholar, Springer Link, Web of Science Master's and Doctoral dissertations etc were used to retrieve studies from 2014 to 2024 and enabled us to access scientific publications more easily. Following the guidelines described by Moher et al of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), all information collected was used to structure and contribute to the build-up of review and the results of this search were collected and grouped using bibliographic management tool (Zotero), enabling relevant articles to be identified and integrated into context [215]. However, thorough scrutiny and assessment of titles, abstracts, and conclusions in the literature was carried out manually to determine their relevance. The search terms and flowchart of the literature review process is shown in Figure 1.



(**a**) (**b**)

Figure 1. Survey methodology used in this review: (**a**) Search terms used; (**b**) flowchart of the literature review process.

7. Conclusions and Future Outlook

As research progresses, the translation of these findings into practical applications will play a crucial role in addressing global challenges related to food security, environmental degradation, and sustainable development. The study of bacterial endophytes is a challenge in the field of research despite the efforts to exploit these bacteria to produce bioactive compounds. However, understanding the mechanisms underlying plant-microbe interactions and developing practical applications for commercial use are important directions for future exploration. In addition, advances in molecular techniques and bioinformatics have enabled researchers to identify and characterize

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bacterial endophytes more efficiently, shedding light on their diverse roles within plant microbiomes. However, further research multi-interdisciplinary scientific approaches including molecular genetics, metabolomics, genome mining, bioinformatics etc. is needed to fully understand the mechanisms underlying the beneficial interactions between endophytes and host plants, their possible exploration in assisting stress tolerance and disease control in plants with the view of combating diverse agricultural problems and ensuring food safety, as well as their potential impact on crop productivity and sustainability in various agroecosystems. Therefore, more studies are necessary to optimize the application of endophytic bacteria in different crops and environmental conditions. This type of knowledge base is crucial for future advancement of more effective biocontrol and plant-specific growth-promoting chemicals. Furthermore, there is a need for more studies focusing on the diversity and function of endophytic communities in different ecosystems to fully grasp their ecological significance. Integrating advanced omics techniques promises to provide a more comprehensive understanding of the complex interactions between plants and bacterial endophytes. Ultimately, these advancements will contribute towards harnessing the full potential of bacterial endophytes for improving crop health and yield in the future.

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Abbreviations

The following abbreviations are used in this manuscript:

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AgNPs	silver nanoparticles
BLAST	basic local alignment search tool
CE-MS	capillary electrophoresis-mass spectrometry
DNA	deoxyribonucleic acid
GC-MS	gas chromatography-mass spectrometry
IAA	indole-3-acetic acid
ISR	induce systemic resistance
JA	jasmonic acid
LB	luria-bertani
LC-MS	liquid chromatography-mass spectrometry
mRNA	messenger rna
miRNAs	micrornas
NCBI	the national center for biotechnology information
NMR	nuclear magnetic resonance spectroscopy
NPs	nanoparticles
OQDS	olive quick decline syndrome
PCR	polymerase chain reaction
PGPB	plant growth-promoting bacteria
PRISMA	preferred reporting items for systematic reviews and meta-analyses
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal rna
tRNA	transfer rna
SEM	scanning electron microscopy
TEM	transmission electron microscopy

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