分类号：Q81 单位代码：10363
密 级：公开 学号：5220510102

枣内生菌抗菌拮抗菌株的筛选与挖掘

Screening and Mining of Jujube Endophytes as Antagonistic Strains for Antimicrobial Application

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研 究 方 向： 生物技术与工程
论文答辩日期： 2025 年 5 月 15 日

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SCREENING AND MINING OF JUJUBE ENDOPHYTES AS ANTAGONISTIC STRAINS FOR ANTIMICROBIAL APPLICATION

ABSTRACT

One of the most serious global challenges in the present day is the large loss of crop productivity experienced every year due to plant disease or pathogen invasion during pre- or post-harvest storage conditions. Postharvest rot is an urgent problem affecting the storage of winter jujube. An endophyte is an endosymbiont, often a bacterium or fungus, which lives within a plant for at least part of its life cycle without causing apparent disease. As concerns about chemical pesticide resistance, environmental degradation, and food security increase, endophytes provide a promising alternative.

The aim of this study is to isolate endophytes with high antimicrobial activity from jujube fruit. Endophytic strains were isolated from healthy "Dongzao" jujube (*Ziziphus jujuba* Mill. 'Dongzao') fruit. The strains were screened using both pathogenic bacteria, such as *Escherichia coli and Staphylococcus aureus, and* importantly, fungi *Alternaria tenuissima, as* the target pathogen. One bacterium isolate showed high antifungal activity. Using molecular techniques, the bacterium X27 was identified as *Bacillus* sp. The antimicrobial activity was examined using the dual culture method. The results of response surface methodology experiments showed that the optimal values of the fermentation parameters were as follows: glucose concentration of 18 g /L, pH 7.4, agitation speed of 149 rpm, and inoculum size of 9.4% (v/v) with an inhibition rate of 61.7% ,which exhibited an increase from the inhibition rate observed in the one-factor-at-a-time experiments. Using the optimal fermentation conditions, in vivo experiments were carried out and demonstrated a 50% disease inhibition in jujube fruits. Biochemical indices indicated that X27 offers a degree of protection against pathogen-induced sugar degradation yielding 0.28% soluble sugar content. X27 inoculated fruits showed a promising decreased rotting rate in jujube fruit of 40%, revealing a significant 60% difference compared to the control. GC-MS analysis revealed approximately 20 distinct compounds in the fermentation broth of X27. The most abundant compound appears to be Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), (15.26%), which was reported to have significant antifungal activity.The results show a promising area in the field of investigation.

Keywords: Endophytes, jujube fruit, bioactive secondary metabolites, biological control, antagonism

Contents

1 Introduction

In recent years, there has been an increasing interest in the study of novel resources, especially endophytes, which are harvested from plants and could produce chemicals unique to that plant. There has been a noticeable increase in the number of published scientific articles focusing on endophytes and their bioactive products, indicating a growing interest in this field of research. The term "endophytes" refers to a group of microorganisms that grow intra-and/or intercellularly in higher plants' tissues without compromising the plants they reside in (De Bary, 1866) .The term endophyte (endo-within, phyto-plant) to distinguish from epiphytic organisms that reside on the surface of the plant. The term encompasses not only neutral, commensal, and/or beneficial microorganisms but also dormant saprobes and pathogens during their latent phase of life cycle. These microbes establish intimate associations within their host plants, establishing a community in endosphere, and are present in different plant parts. The natural microbiome such as the presence of endophytes is indicative of the healthy plant system(Barman & Dkhar, 2019) .There are several niches and origins of endophytes notably roots (rhizosphere), stems (caulosphere), the soil zone surrounding belowground plant stems (laimosphere), leaves (phyllosphere), flowers (anthosphere), fruits (carposphere), seeds (spermosphere), and more (Compant et al., 2016). Endophytes, ubiquitously present in plants, have a wide range of activities within their hosts. The population of endophytes in a plant species varies widely and is influenced by factors, including the host species, the developmental stage, the density of the inoculum, and the environment (Surjit & Rupa, 2014). In general, endophytes can be divided into two categories: systemic endophytes and non-systemic or transient endophytes based on their biology, mode of spread, evolution, and taxonomy. Systemic endophytes are characterized as those that reside within the internal tissues of plants, forming symbiotic relationships with their host while remaining asymptomatic. Conversely, transient endophytes are those that occupy the host plant tissues for a portion of their life cycle, typically exhibiting no signs of infection under normal circumstances; however, they can act as pathogens when the host experiences stress (Ogbe et al., 2020).There has been a growing scientific interest in plant microbiomes due to their potential to reduce plant stress, improve nutrient efficiency, facilitate nutrient uptake, and promote the growth of plants. The increasing interest in endophytes stems from their potential applications in agriculture, medicine, and environmental sustainability, particularly in the face of global challenges such as climate change and soil degradation.

Understanding the mechanisms through which endophytes operate will be crucial for harnessing their full potential and integrating them into effective agricultural and biotechnological strategies. Recent studies have highlighted the significant impact of endophytes on plant growth and stress tolerance. For instance, the endophytic fungus *Cladosporium halotolerant* and its ability to remediate saline soils, enhancing the growth of common beans (*Phaseolus vulgaris*) under salt stress has been reported. Their findings revealed that *C. halotolerans* improved soil properties, increased chlorophyll levels, and reduced stress indicators in plants, demonstrating the potential of endophytic fungi as bio stimulants in agriculture. This aligns with the broader understanding that endophytes can produce phytohormones and other bioactive compounds that promote plant resilience against abiotic stresses (Muhammad et al., 2024).

The functional diversity of endophytes extends beyond stress tolerance; they also contribute to nutrient acquisition and overall plant health, as demonstrated by the relationship between endophytes and their host plants, which is particularly evident in the *Orchidaceae* family. These microorganisms contribute to mineral solubilization, nitrogen fixation, and overall plant vigor (Choudhary et al., 2023). This mutualistic interaction not only supports the growth of orchids but also underscores the potential of endophytes as a reservoir of bioactive compounds that can be harnessed for sustainable agricultural practices. A great deal of previous research explains that endophytes produce diverse types of bioactive compounds such as alkaloids, saponins, flavonoids, tannins, terpenoids, quinones, chinones, phenolic acids and more. The diversity of these compounds is influenced by factors such as the host plant species and the cultivation medium, indicating that further exploration of endophytic diversity is necessary. Renowned fungal genera such as *Penicillium, Fusarium, Aspergillus, Sclerotium, Myxormia, Alternaria, Colletotrichum, Cladosporium, Diaporthe, and Curvularia* are recognized for their remarkable ability to produce bioactive compounds.

1.1 Exploring endophytic microorganisms

1.1.1 The role of endophytes

Endophytes play an integral role in the health of their host species. This is done through various mechanisms, including antibiosis, the release of lytic enzymes, the activation of secondary metabolites, and the stimulation of hormonal activity reported that the plant microbiome is crucial for plant development, well-being and stress tolerance by supplying nutrients, defending against diseases, and regulating plant physiology. Studies have shown that a varied

and enduring microbial population can boost plant development, improve absorption of nutrients, and lower vulnerability to diseases and abiotic stresses.

Naturally occurring endophytes use a variety of strategies to encourage plant growth. The symbiotic association of plants and endophytes allows plants to more effectively access and absorb essential nutrients, thereby enhancing growth and improving overall plant health. Bacterial endophytes including *Bacillus, Pseudomonas,Microccus* and *Methylobacterium*, can promote nutrient acquisition and plant growth through mechanisms such as N fixation, P,K solubilization, and siderophore production (Fe chelation) (Gopalakrishnan et al., 2015). Phosphate solubilization activity by endophytes has been reported by (Adhikari & Pandey, 2019. Kumar et al., 2016), endophytes can either solubilize insoluble phosphates or release organic phosphates by generating acids like citric, gluconic, and malic acids. *Pseudomonas spp., Bacillus megaterium, Azobacter, Paenibacillus, Thiobacillus* and *Serratia* has been reported to facilitate phosphorus mobilization through solubilization and mineralization processes (Jahan et al., 2013; Kang et al., 2014). Kang et al. (2014) noted that *Bacillus megaterium* regulates the content of amino acids and carbohydrates to promote the growth of mustard plants.

Another strategy employed by endophytes is the production of phytohormones, also known as plant growth regulators. It has been determined that multiple endophytes generate plant growth hormones. Brassinosteroids, strigolactones, auxins, cytokinins, abscisic acid, ethylene, gibberellins, and jasmonates are the primary phytohormones produced by endophytic bacteria (Santoyo et al., 2016; Shahzad et al., 2016). Indole acetic acid (IAA), for example, stimulates cell division, differentiation, and extension; enhances seed and tuber germination; and increases root and xylem development. Fungal endophytes, such as *Penicillium sp., Aspergillus sp., Cladosporium sp., and Fusarium sp.*, produce gibberellins that enhance plant growth and stress resilience (Verma et al., 2022). Other fungal species, including *Aspergillus, Cladosporium, Fusarium, Verticillium,* and various *Ascomycetes,* release triterpenoid saponins and ginsenosides when they colonize Panax ginseng. These compounds play a significant role in promoting root growth and enhancing the plant's tolerance to stress. Similarly, bacterial endophytes, including *Azoarcus, Burkholderia, Gluconobacter, Herbaspirillum, Klebsiella, Pantoea,* and *Rahnella*, synthesize phytohormones like indole-3-acetic acid (IAA) to promote plant growth (Kandel et al., 2017).

One of the most significant and promising roles of endophytes is their ability to act as natural biocontrol agents. Endophytes exhibit their biocontrol effects through several mechanisms, including production of antimicrobial compounds, competition for nutrients and space, and

induction of plant resistance mechanisms. Endophytes are recognized for their ability to synthesize a diverse array of secondary metabolites that exhibit antifungal and antibacterial activities, effectively inhibiting the growth of pathogens. Endophytes produce diverse secondary metabolites, such as alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, and xanthones (Adeleke et al., 2022; Choudhary et al., 2023; Gouda et al., 2016; Singh & Kumar, 2023). Lipopeptides, iturin and surfactin are the primary metabolites synthesized by the *Bacillus* genera. Likewise, *Pseudommonas* produce 2,4-diacetylphloroglucinol(DAPG), pyrrolnitrin, and phenazine metabolites. Endophytes exhibit antagonism towards pathogens through various mechanisms, including antibiosis, competition, and induced resistance (De Silva et al., 2019; Mon et al., 2021) .In a study by Xia et al. (2023) reported the antagonistic activity of the endophytic bacterial strain *Bacillus subtilis* against the pathogen *Colletotrichum fructicola,* and their possible mechanism of action was the secretion of bioactive metabolites, defense enzymes, and siderophores production. Mycoparasitism is another mechanism where endophytes directly attack pathogens. Endophytic fungi penetrate the hyphae of pathogenic fungi, destroying their cell walls by lysing cells. For instance, *Trichoderma flagellatum* exhibits mycoparasitic activity against *Fusarium sp.,* a tracheomycosis-inducing pathogen in *Coffea arabic* (Segaran et al., 2022).

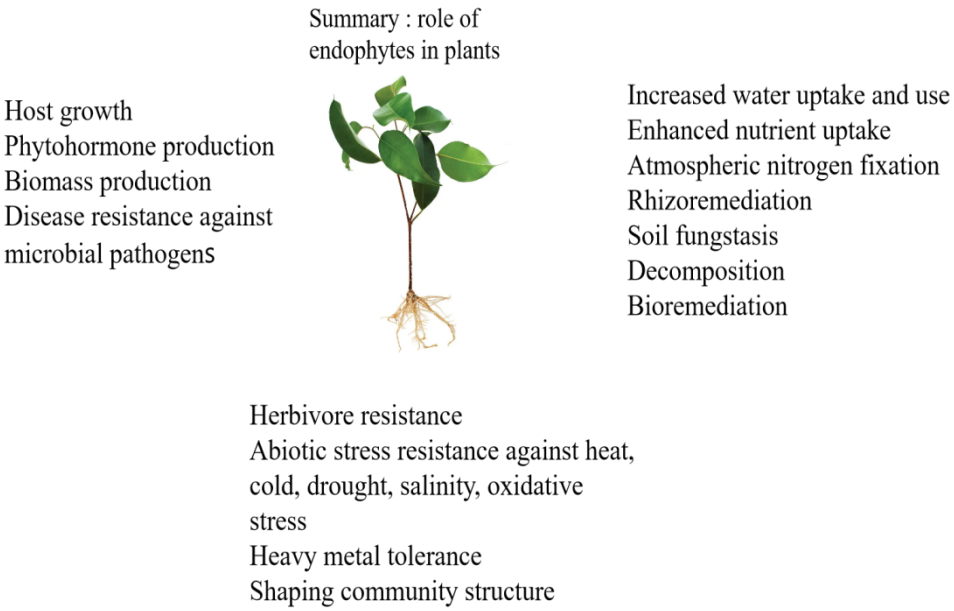

Figure 1.1 General overveiw of the role of endophytes in plant host

1.1.2 Endophytic bacteria

Endophytes have been described in numerous ways, and these definitions have evolved as research has progressed. Bacterial endophytes as defined by Hardoim et al. (2015) a diverse

group of microorganisms that colonize the internal tissues of plants without causing any apparent harm to their hosts. They are found across many phyla, including the *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes* (Wemheuer et al., 2017). There are many more, such as *Bacteroidetes, Planctomycetes, Verrucomicrobia*, and *Acidobacteria,* which have not been documented as much as endophytes. The prevalence and diversity of bacterial endophytes among various plant species are documented extensively. Various plant hosts, environments, and different plant components, including root tissues, stems, leaves, seeds, fruits, tubers, ovules, and nodules, have been cited as the sources from which bacterial endophytes exist. The roots have been notably reported as a greater source of bacterial endophytes (Ali et al., 2023). Bacterial endophytes initially attach to the root surface, also called rhizoplane, and explore the potential entry sites to access the internal plant tissues. Openings in the roots where root hairs or lateral roots emerge, as well as stomata, wounds and hydathodes in the shoots are considered the main entry points that endophytes use to enter the host plant (Hardoim et al., 2015). Bacterial endophytes are predominantly located in the root tissues rather than in the above-ground plant tissues (Ma et al., 2011). Endophytic bacteria tend to occupy the intracellular space more frequently, possibly because of the abundance of inorganic nutrients such carbohydrates, and amino acids during the initial phase of colonization. The presence of endophytes is first observed in the root hairs and later in the root cortex (Hnamte et al., 2024). Lifestyle depends on environmental factors, host and microbial genotypes, and an imbalance in the nutrient exchange between the microbe and the host (Hazarika et al., 2019). Endophytic bacteria have been noted to form mutualistic associations with their host plants, aiding in growth, boosting resilience to abiotic and biotic stressors, and facilitating the buildup of secondary metabolites that confer immunity to the plant. This mutualistic interaction involves the host plant offering nutrients and shelter to the bacteria, which in turn promote the plant's growth and development by enhancing nutrient uptake, fixing nitrogen from the atmosphere, and solubilizing minerals and phosphates. Some of these microbes can also act as antimicrobial agents by producing compounds that protect the plants against pathogens. Few of them improve the capacity of the plants to tolerate environmental stresses such as cold, oxidative envelope, salinity, drought, etc. (Hnamte et al., 2024). Secondary metabolites, such as surfactin, iturin, fengycin, bacillaene, subtilosin A, fusaricidin, polymyxin, 2,4-diacetylephloroglucinol (DAPG), phenazine-1-carboxylic acid, 2-hydroxyphenazine, pyrrolnitrine, viscosinamide, and Orfamide are recognized for their antimicrobial activity. The mechanism through which bacteria inhibit the growth of other microorganisms by synthesizing antimicrobial substances is known as antibiosis

Endophytic bacteria, *Bacillus* species, exhibit antagonistic ability toward fungal pathogens. *Bacillus subtilis* SCB-1 has demonstrated antifungal properties against a variety of fungal pathogens, such as *Alternaria* and *Fusarium* (Hazarika et al., 2019). An endophytic bacterium, sterile *Bacillus subtilis XZ18-3*, indicated good inhibition effects when investigated against *Rhizoctonia cerealis* . The endophytic bacterium seemed to employ different mechanisms including DNA fragmentation, modifications to the susceptibility of cell membrane and accumulation of ROS (Yi et al., 2022). Carbazomycin B metabolite produced by endophytic *Streptomyces roseoverticillatus 63 (Sr-63)* from rice inhibited the growth of *Xanthomonas oryzae pv. oryzae (Xoo)*. It inhibits the pathogen metabolic activity by decreasing the malate dehydrogenase activity and suppressing the pathogen protein expression (Shi et al., 2021). *Bacillus* species are often highlighted in literature for their ability to promote plant growth. *B. subtilis 2S*, isolated from maize stem, and *B. subtilis* 135, isolated from honey suckle root, both promote growth in maize and wheat through the production of IAA (Indole-3-acetic acid) and siderophores. *Bacillus cereus AR156* is a plant growth–promoting rhizobacterium that induces resistance against a broad spectrum of pathogens including *Pseudomonas syringae pv. tomato DC3000*. demonstrated that inoculation of tomato plants with 2,4-diacetylphloroglucinol (DAPG)- and hydrogen cyanide (HCN)-producing *Pseudomonas brassicacearum LBUM300* could significantly reduce bacterial canker symptoms caused by *Clavibacter michiganensis sub sp. Michiganensis.*

1.1.3 Endophytic fungi

In literature, the term fungal endophytes (EF) tend to be used to refer to fungi that live in plant tissues throughout the entire or partial life cycle by establishing a mutually beneficial symbiotic relationship with its host plant without causing any adverse effect or disease. The definition has significantly evolved over time and will inevitably face further redefinition as research in endophyte biology yields new data. For instance, it has been proven that some fungal strains pose some pathogenicity towards certain plant species while having some beneficial characteristics towards other plant hosts (Hardoim et al., 2015). The existence of endophytic fungal interactions dates back approximately 400 million years (Krings et al., 2007). Endophytic fungi primarily consist of *Ascomycetes* and sporadic appearance of *Basidiomycetes* and *Zygomycetes*. Fungal endophytes can be broadly classified into ecological categories or evaluated according to their diversity and functional roles. Some researchers further refine this classification into sub-classes based on various criteria, such as the range of hosts, reproductive modes, the specific plant parts colonized, transmission methods, nutritional sources, and the

ability to manifest symptoms in the host plant (Bamisile et al., 2018; Rodriguez et al., 2009). A large and growing body of literature has reported endophytic fungi inhabiting a diverse group of plant species. Fungal endophyte diversity is significantly influenced by geographical location and is a result of environmental factors including the genotype and physiology of host plants, seasonal growth conditions, and anthropogenic impacts, among other elements (Chang-Kyun et al., 2013).

The diverse biological activities associated with metabolites from endophytic fungi highlight their role as an invaluable source of important compounds. Fungal endophytes prove a beneficial symbiotic relationship with host plants and have been proven to have a high impact on host plants. The association of fungal communities with their host plants improves growth, immunity, and overall development of plants. Via many strategies, such as pathogen suppression through competition, antibiosis, and mycoparasitism, as well as indirectly by generating resistance and triggering the plant's defense system, endophytic microbes improve plant adaptation. Endophytic fungi provide several benefits to crops, primarily by promoting plant growth, which leads to increased agricultural productivity. These fungi enhance plant growth through mechanisms such as improving nutrient uptake, producing plant hormones, boosting water absorption, and adjusting hormone levels in the plant (Fite et al., 2023; Poveda et al., 2020). Research findings consistently prove fungal endophytes can enhance plant growth indirectly by aiding the plant in nutrient uptake including nitrogen, phosphorus, potassium, zinc, and iron (Bilal et al., 2018; Yan et al., 2019). The species *P. liquidambari* establishes a symbiotic relationship with rice, facilitating increased nitrogen accumulation and uptake, which in turn significantly promotes the growth and yield of the rice crop (Yang et al., 2014). Additionally, endophytic fungi can alter plant development by directly affecting their hormone pathways. They can influence plant development either actively or passively by stimulating the synthesis of plant hormones, including auxin, gibberellic acid (GA), abscisic acid, ethylene, and indole-3-acetic acid (IAA)(Baron & Rigobelo, 2022). Bader et al. (2020) that the main auxin produced by fungi is indole-3-acetic acid (IAA).

Trichoderma strains from Argentine Pampas soil, selected for high IAA production and phosphate solubilization ability, were inoculated into tomato seeds, resulting in increased plant height, fresh and dry matter, chlorophyll content, and surface area. Endophytic fungus phytohormones play a significant role in plant protection, increasing defensive responses and tolerance to pathogens through intricate interactions with the plant. Fungal endophytes play a key role in plant–pathogen interactions. It has been documented that endophytic fungi use various mechanisms to suppress pathogen activity (Gao et al., 2010). It has been observed that

endophytic fungi utilize various mechanisms to suppress pathogen activity. Some endophytes trigger the plant's defense system to fend off pathogen invasion, while others produce antimicrobial compounds that directly inhibit pathogen growth. Additionally, certain endophytes compete for ecological niches and nutrients, further limiting pathogen development. Fungal endophytes act as biological control agents, with unique properties that enhance production and stress resistance in the host plant. Isolates of *Epicoccum nigrum, Trichoderma viride, Sclerotinia sclerotiorum, Fusarium tricinctum, Cytospora spp.*, and *Alternaria alternata* were found to significantly control *Diplodia corticola*, the pathogen responsible for wilting, vascular necrosis, and mortality in various oak species (Campanile et al., 2007). Endophytic fungal communities, including *Lasiodiplodia, Acremonium, Cladosporium, Blastomyces, Botryosphaeria, Colletotrichum, Geotrichum, Cordyceps, Diaporthe, Fusarium, Gibberella, Gliocladium, Nectria, Monilochoetes, Pestalotiopsis, Phomopsis, Pseudo fusarium, Pleurotus, Rhizopycnis, Syncephalastrum, Verticillium, Xylaria,* and *Trichoderma,* have been identified as potential antagonists to *Clostridium perenne*(Strobel & Daisy, 2003).Terhonen et al. (2016) reported *Phialocephala sphareoides,* isolated from Norway spruce trees, produces bioactive compounds that inhibit the growth of other fungi. These protective metabolites help the spruce trees resist root infections caused by pathogenic fungi such as *Heterobasidion parviporum* and *Phytophthora pini*.

1.1.4 Endophytic actinomycetes

Endophytic actinobacteria, which exist in the inner tissues of living plants, have attracted increasing attention among taxonomists, ecologists, agronomists, chemists, and evolutionary biologists (Qin et al., 2009, 2011). Identification and classification of actinobacteria are based on a polyphasic approach, comprising morphological, physiological, and molecular studies. The genus Streptomyces is the most prevalent among endophytic actinomycetes, but other genera like *Micromonospora, Actinopolyspora, Nocardia, Saccharoplyspora, Streptoaporangium, Promicronospora, omicronospora and Rhodococcus* follow. According to Passariet al. (2017), there were also reports of *Dietzia, Microtetraspora, Actinocorallia, Verrucocsispora, Isoptericola,* and *Kytococcus*. Golinska et al. (2015) note endophytic actinomycetes have been isolated from diverse parts of different medicinal plants. In most cases, the maximum endophytic actinobacteria have been recovered from roots, followed by stems and leaves. A detailed study conducted on medicinal plants in China led to the successful of isolation of 560 endophytic actinomycetes. These isolates displayed a broad spectrum of antimicrobial activity, underscoring the significant role of endophytic actinomycetes as a crucial reservoir for bioactive compounds. Some of the secondary metabolites produced by

actinomycetes include antibiotics, biopesticide agents, plant growth hormones, antitumor compounds, antiviral agents, pharmacological compounds, pigments, enzymes, enzyme inhibitors, anti-inflammatory compounds, single-cell protein feed, and biosurfactant (Passari et al., 2017; Selim et al., 2021). Numerous bioactive secondary metabolites have been discovered in the field of endophyte research, demonstrating their promise as a rich source of antibacterial agents against a range of pathogenic microbes. In an investigation by Vu et al. (2020) based on phenotypic characteristics, 111 actinomycetes were isolated from roots, stems and leaves *Cinnamomum cassia Presl*, 38 isolates (34.2%) showed antimicrobial activity against at least one of nine tested microbes. 26 isolates (68.4%) showed capability to produce anthracycline-like metabolites. The study confirmed that the 38 antibiotic-producing actinomycetes isolated from *Cinnamomum cassia Presl* were classified into three distinct genera *Streptomyces* was the most dominant genus, followed by *Microbacterium* and *Nocardia*. In a study in Fujian province, China by Shan et al. (2018) 46 endophytic actinobacteria were isolated from 20 tissue samples of nine cultivars based on colony morphology and were further confirmed by 16S rRNA gene sequencing. *Streptomyces* isolates dominate in tea plants, with some likely representing new species. Some rare actinomycetes have also been discovered, among which *Mobilicoccus* and *Piscicoccus sp*. have not been previously reported as plant endophytes. Inhibitory activity against at least one bacterial or fungal pathogen was demonstrated by 43.2% of the tested isolates. These isolates demonstrated a high metabolic potential for producing secondary metabolites with varying roles.

1.1.5 Antimicrobial properties of endophytic microrganisms

One of the primary contributions of endophytes is their ability to produce a diverse array of secondary metabolites with antimicrobial properties. Endophytes are known to produce various secondary metabolites, i.e., terpenoids, diterpenoids, polyketides, alkaloids, steroids, and anthraquinone (Zheng et al., 2021). In most cases, the majority of the compounds have antimicrobial properties, and it is estimated that these properties include protection of the host plant from various pathogens. The antimicrobial compounds produced by the endophytes are considered advantageous over the conventional ones as they are environment-friendly, specifically toxic to certain harmful pathogens, whereas non-toxic to humans (Singh et al., 2017) purely endorses the use of secondary metabolites of endophytic origin as promising sources of antimicrobial compounds. Digra & Nonzom (2023) reported that several factors can influence the antimicrobial activity of endophytic microorganisms, including the host plant, the specific microbial species, and the culture conditions. A study comparing different culture media found that an endophytic fungus cultured on Potato Dextrose Broth (PDB) grew faster,

produced more extracts, and exhibited stronger antimicrobial activity (Hardiningtyas et al., 2023). Additionally, research has shown that the extraction method can impact the recovery of antimicrobial compounds, with extracellular compounds often having higher activity than intracellular ones (Abdul Rahman & Ibrahim, 2021; Hardiningtyas et al., 2023).

A diverse array of mechanisms is employed by endophytes to achieve their antimicrobial effects (Eshboev et al., 2024; Fadiji & Babalola, 2020; Joo et al., 2021). (Fadiji & Babalola, 2020) these mechanisms encompass the production of antimicrobial agents that compromise the integrity of the microbial cell walls, disrupt protein synthesis, or interfere with essential metabolic pathways. Bacterial endophytes synthesize a variety of antimicrobial substances, such as antibiotics, enzymes, and volatile organic compounds (VOCs), which inhibit the growth of pathogens(Lal & Neelam, 2024).Bacterial endophytes utilize several mechanisms to suppress plant pathogens, including the production of antimicrobial compounds, competition for nutrients and space, and induction of systemic resistance (ISR)(Akinsanya et al., 2015; Moreno-Valencia et al., 2024). *Bacillus amyloliquefaciens strain EA19*, an endophyte isolated from *Erigeron annuus*, produces antifungal compounds effective against *Blumeria graminis f. sp., tritici, Magnaporthe oryzae, and Fusarium graminearum.* Bacterial endophytes compete with pathogens for essential resources, limiting pathogen proliferation. For example, given *Bacillus species c*olonize plant roots, effectively blocking access for fungal pathogens seeking to establish themselves (Doty et al., 2022; Pérez-Equihua & Santoyo, 2021; Song et al., 2024). Certain endophytic bacteria can initiate ISR in plants, thereby enhancing the plants' responses against subsequent pathogen invasions. Plant growth-promoting bacteria (PGPB) are capable of synthesizing siderophores, solubilizing inorganic phosphate, and performing nitrogen fixation, indirectly enhancing plant immunity (Moreno-Valencia et al., 2024). Similarly, the fungal endophyte *Penicillium olsonii ML37* reduces *Fusarium* head blight in wheat by inducing local resistance in wheat spikes (Rojas et al., 2022). Certain fungal endophytes exhibit mycoparasitic activity, directly attacking and feeding on other fungi. *Chaetomium globosum* L18 parasitizes pathogens by parallel intergrowth, intertwining, and penetrating their mycelia (Wang et al., 2012). Fungal endophytes often employ various mechanisms to protect host plants from pathogens, including the production of antimicrobial metabolites, mycoparasitism, and the induction of local or systemic resistance.

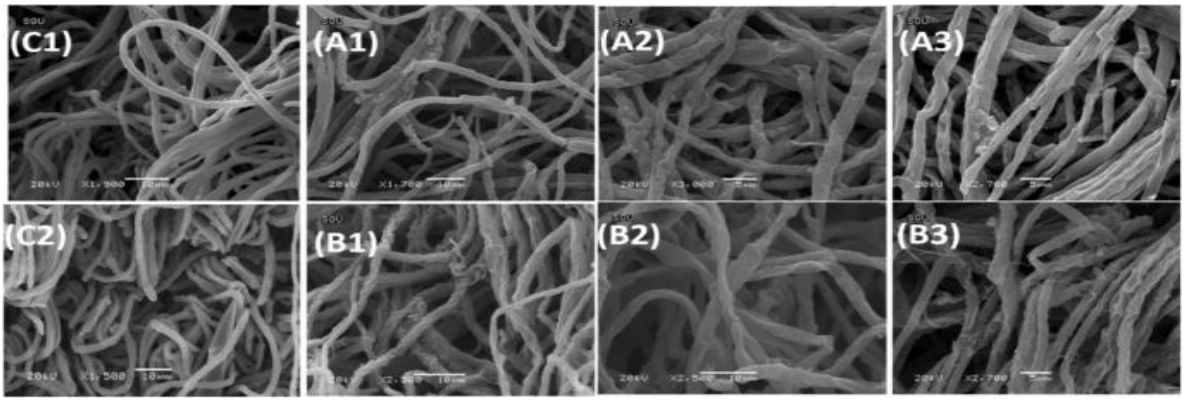

Figure 1.2 Scanning electron micrographs showing morphological abnormalities in the hyphae of *F. solani* and *Alternaria* sp. due to the antagonistic effect of endophytic bacterial strains from date palm (cv. Nighal)

An investigation by Al-Nadabi *et al.* (2021) isolated endophytic bacteria associated with date palm leaves and their in vitro antagonistic potential against fungal pathogens causing leaf spots in date palm. The Scanning Electron Microscopy (SEM) analysis of dual culture assays revealed significant morphological damage to fungal hyphae when co-cultivated with bacterial endophytes. Specifically, *F. solani* hyphae displayed shrinkage, distortion, loss of turgidity, and fragmentation upon exposure to *P. septica* strains B1, B7, B8, and B9. Similarly,

Alternaria sp. hyphae also exhibited shrinkage and disintegration when treated with these bacterial endophytes, contrasting sharply with the healthy, normal, and smooth hyphae observed in control samples

1.1.6 Endophytes: a valuable source of bioactive compounds

There are nearly 300,000 plant species that exist on Earth, and each individual plant hosts one or more endophytes. The endophytic biology of only a handful of these plants has been subject to extensive research (Aleynova & Kiselev, 2023). The internal microbiome of plants plays a crucial role in the production of bioactive secondary metabolites. Endophytes are ubiquitous microorganisms residing in the inner tissues of different plant parts, forming a mutualistic relationship with the host without causing any disease-like symptoms. Endophytes can synthesize compounds that the host cannot synthesize or can only synthesize small amounts, changing the method of metabolite synthesis in the host and affecting metabolite levels (Li et al., 2023). According to Choudhary et al. (2023) endophytes present in plants are considered a potent source of a wide range of various bioactive compounds such as tannins, alkaloids, terpenoids, benzopyranones, quinones, polyketides, chinones, saponins, flavonoids, phenolic acids, steroids, xanthones. Metabolites bearing antibiotic activity can be defined as low-molecular-weight organic natural substances made by microorganisms that are active at low concentrations against other microorganisms. The metabolites produced by endophytes have been found to display a wide range of pharmacological properties, primarily encompassing antimicrobial, antineoplastic, anticancer, antioxidant, anti-inflammatory, antidiabetic, and antidepressant activities (Digra & Nonzom, 2023; Fanai et al., 2024; Pasrija et al., 2022; Strobel, 2018). The production of bioactive compounds/secondary metabolites by endophytes is believed to be directly linked to the evolution of the host plant (Gouda et al., 2016). The potential for applicability for these secondary metabolites across various fields, including agriculture, medicine, and food production, has greatly increased interest in research.

Bioactive compounds play a crucial role in the relationship between endophytes and their host plants, influencing plant growth, defense mechanisms, and overall ecological fitness of the plant. The functions of metabolites produced by endophytic microorganisms vary and depend on the structure of a given compound (Gouda et al., 2016) These molecules often regulate various interactions between plants and microbes, host plants and neighboring plants, and plants and pathogens or grazing animals (Bisht et al., 2025). Consequently, natural bioactive compounds influence the processes in an ecosystem. They have a good potential as biocontrol agents in plant growth promotion and suppression of unwanted plants and insects. Thus, they can play a promising role in sustainable agriculture. Besides, many of these compounds also

possess the properties of antibiotics and anticancer agents and can cure several other diseases of humans (Fanai et al., 2024).

Endophytes enhance the synthesis of SM's, and this is achieved through several mechanisms including but not limited to promoting photosynthesis, regulating gene expression, compound genetic material transfer and other unique metabolite synthesis (Kumari et al., 2023). Laraia et al. (2018) state that the multifaceted functionality of individual bioactive compounds and their complex interactions underscore the importance of understanding these compounds in a broader biological context. These interactions prevent the simplification of their roles into singular outcomes, emphasizing the necessity for integrated research approaches that account for the myriad ways bioactive compounds affect health and ecology.

1.2 Jujube fruit and antagonistic potentional

1.2.1 Jujube the superfruit

China is the largest producer of jujube, out of the 170 species in the genus Ziziphus Mill., Chinese jujube (*Ziziphus jujuba Mill.*) "Dongzao" is the most significant species. It ranks first in the genus with an annual production of over 8 million t, an annual total output value of over 15 billion US dollars, and a cultivation area of about 1.5 million ha (Liu et al., 2020) . China is the primary source of jujube, which has been used for thousands of years in traditional Chinese medicine (TCM) and as a popular dietary supplement. Six provinces, Xinjiang, Hebei, Shandong, Shanxi, Shaanxi, and Henan, account for more than 90% of jujube production output (Liu et al., 2020) .The jujube plant is now extensively available outside of China, in nations including China, Europe, Japan, Korea, India, and the United States (Chen & Tsim, 2020) .The fruit includes many bioactive compounds, including polysaccharides, polyphenols, amino acids, nucleotides, fatty acids, dietary fiber, alkaloids, and other essential nutrients. These components' physiological roles have been thoroughly investigated, and a variety of roles have been discovered. These include antiviral, immunoregulatory, neuroprotective, anticancer, antioxidant, anti-inflammatory, anti-hyperlipidemic, and anti-hyperglycemic reactions (Lu et al., 2021).

Fresh jujube is a perishable commodity, and possesses a limited postharvest lifespan, particularly in the case of fully mature jujube fruit. The preservation duration is less than one week when kept at room temperature due to metabolic activities that increase ripening and senescence, mechanical damage, chilling damage, and the impacts of microbial decay (Deng et al., 2022; Dou et al., 2023).The fruit is particularly sensitive to diseases caused by multiple pathogenic fungus, such as *Alternaria alternata (Fr.) Keissler, Phoma destructiva Plowr.,*

Dothiorella gregaria Sacc. and *Fusicoccum* spp. increasing yield loss (Zhang et al., 2015). On that account the development of strategies to postpone jujube fruit senescence during storage to lengthen the storage duration of fresh jujube is imperative. Black rot is a serious disease caused by *Alternaria tenuissima* (*A. tenuissima*) that affects winter jujubes.

To minimize postharvest loss caused by phytopathogens or illnesses, many chemical pesticides or fungicides have been widely utilized on a large industrial scale or even in laboratories. One major drawback of this approach is that chemical pesticides severely influence the nutritional components, texture, flavor, and quality of fruits and consumer health. The growing number of pathogens with resistant variants against conventional chemicals is also of grave concern (Noumeur et al., 2016). The development of a sustainable approach for substituting destructive agrochemicals with suitable microbial antagonists is necessary due to the adverse impacts of chemical pesticides on fruit quality, human health, ecological balance, and drug-resistant pathogens.

1.2.2 Antagonistic potential of jujube (ziziphus jujuba) fruit endophyte

Endophytes isolated from jujube (*Ziziphus jujuba*) plants have demonstrated significant antagonistic activities against various pathogens, particularly those causing fruit disease (Lang et al., 2018). These beneficial microorganisms inhabit different tissues of healthy jujube plants and have been extensively studied for their biocontrol potential against economically important diseases. Lang et al. (2018) successfully isolated endophytic strains from various parts of healthy jujube plants, including stems, leaves, flowers, and fruits. The researchers isolated a total of 81 endophytic strains from different tissues of winter jujube. Among the isolated endophytes, certain strains have shown remarkable inhibitory effects against *Alternaria alternata*, the causative agent of jujube shrunken-fruit disease. The antagonistic activities of jujube endophytes operate through several mechanisms, including antibiosis, competition, and lysis. These diverse modes of action make endophytes particularly effective biocontrol agents against a range of pathogens. Endophytic bacteria residing in plant roots have demonstrated antagonistic properties that can be harnessed for disease control. The expression of these antagonistic properties varies among different endophytic strains, with some exhibiting broad-spectrum activity against multiple pathogens (Liu et al., 2022). Jujube endophytic bacteria, rhizosphere soil bacteria (Wanget al., 2021), and jujube leaves (Hawar, 2022) have been the primary subjects of previous research on the selection of related antagonistic strains.

Bacillus subtilis strain *St-zn-34*, isolated from winter jujube, exhibits strong inhibitory activity against *Alternaria alternata*, the primary infection pathogen causing jujube fruit shrunken disease ((Lang et al., 2018). In a study by Zou et al. (2024), two endophytic antagonistic

bacteria, identified as *B. velezensis* ZJJDZY and ZJJDYB, have exhibited significant antagonistic effects against various pathogenic fungi responsible for black spot disease. *Brevibacterium halotolera*ns JZ7, an endophytic bacterium, demonstrates strong antagonistic activity against *Fusarium oxysporum*, a primary pathogen causing root rot in jujube (Wang et al., 2021). Gas-chromatography mass-spectrometry analysis showed that the predominantly produced volatile organic compounds produced by strain JZ7 are acetoin, 2,3-butanediol and fenretinide with fenretinide strongly suppressing spore germination of *F. oxysporum* in vitro. *Debaryomyces* nepalensis is an antagonistic yeast that effectively controls postharvest anthracnose of mango fruit and black spot rot of jujube. In this study, a yeast *D. nepalensis* isolated from jujube leaves was utilized on jujube fruit to control postharvest rot decay (Lei et al., 2022). Several *Streptomyces* strains isolated from jujube plants have demonstrated antagonistic activity against various fungal pathogens (Padilla-Gálvez et al., 2021; Passari, Mishra, Gupta, et al., 2015; Passari, Mishra, Saikia, et al., 2015). These examples highlight the diversity of endophytic microorganisms with antagonistic properties in jujube plants, showcasing their potential for sustainable disease management. Antagonistic microorganisms are a green, environmentally friendly, and safe method to replace chemical synthetic fungicides to control postharvest diseases of jujube (Lei, Yi, et al., 2022). The most promising applications of jujube endophytes are their potential as biocontrol agents against various fruit diseases.

1.3 Application of microbial endophytes in plant disease management

Endophytes, including endophytic fungi, endophytic bacteria, and endophytic actinomycetes, exist in various organs, tissues, and intercellular spaces of plants without causing immediate signs of diseases (Card et al., 2016). In current plant disease control, the divergence from chemical pesticide use has been fueled by the public concern about toxicity and environmental impact of these products, the development of pesticide-resistant pathogens, and a rising ban on existing pesticides. An excessive use of these chemicals has resulted in the development of resistance to pests and diseases in traditional plant varieties and in transgenic plants.

Endophytic bacteria play a crucial role in plant defense and stress management through various direct and indirect mechanisms. The use of antagonistic endophytes as biocontrol agents against several plant pathogens is considered an attractive option for the management of some plant diseases due to their nontoxic nature. *Acremonium alternatum* has demonstrated relief from damage from *Plutella xylostella* in beans and it induces resistance against mildew pathogen *Plutella xylostella. Bacillus and Psedomonas* have been extensively studied as effective biocontrol agents. For example, *Bacillus pumilus* and *Pseudomonas chloraphis* can

act as antagonistic strains against pathogens such as *Fusarium oxysporum.* Endophytic bacteria like *Enterobacter spp.* also promote plant growth and enhance resistance to pathogens through the induction of systemic plant defense responses. The focus of recent research has been the commercialization of these biocontrol agents. There is an increasing interest in the formulation of commercially available products to fully utilize these microbial endophytes. De Silva et al. (2019) provides a list of companies and agents that have commercialized this microbial endophyte. For example, *Ampelomyces quisqualis* (AQ10) provided by Ecogen, Inc for effectively managing powdery mildew across various crops.Continuous initiatives are directed toward merging field trials with laboratory research of endophytic agents under varying environmental conditions. Ultimately, successful commercialization of microbial endophytes could significantly contribute to sustainable agriculture, reducing dependence on chemical pesticides and enhancing crop resilience.

1.4 Research purpose, significance and content

1.4.1 Research purpose

In the ever-evolving realm of agriculture, the convoluted interaction between plants and microorganisms has assumed paramount significance. Exploring their unique ability to coexist with their plant hosts, endophytes have unlocked a treasure trove of biological weaponry to fend off pathogens, enhance plant resilience, and all in all, improve plant health. From the synthesis of bioactive secondary metabolites to intricate signaling pathways, these silent allies are masters of biological warfare. In the recent past, endophytic microorganisms have gained popularity as BCAs due to their excellent colonization efficacy and better acclimatizing potential. The excessive use of chemical pesticides in jujube cultivation poses significant risks to the environment, human health, and the sustainability of agricultural systems. Chemical pesticides can disrupt ecological balance, leave harmful residues on fruits, and lead to the development of pesticide-resistant pathogen strains.

This research offers a pathway to combat major jujube diseases, reduce reliance on harmful chemical pesticides, and promote ecological balance within jujube agricultural systems. Endophytes represent a largely untapped microbial resource with potential to provide novel compounds with antimicrobial, antifungal, or antibacterial properties. The full potential of jujube endophytes remains largely unexplored. This research presents a gateway to the vast microbial diversity within other plant species and how tapping into this diversity could lead to the discovery of important new bioactive compounds. This research may highlight the critical role of endophytes in safeguarding plants, especially in mitigating crop losses caused by

diseases and pests, thereby enhancing or improving food security and promoting agricultural sustainability. This research holds significant value as it aims to broaden our comprehension of natural antimicrobial agents, specifically by studying the endophytes present in jujube fruit. Jujube fruit has received little attention as a potential natural antimicrobial source for use in other medical or agricultural applications, so the study identifies the fruit as potentially useful for further exploration of plants that can be used in these areas.

The aim of this project is to isolate endophytes with high antimicrobial activity from jujube fruit. Additionally, the study assesses the potential use of endophytes as natural biocontrol agents in agriculture and the biotechnological potential to develop antimicrobial agents for medicine and in industry. By pursuing these aims, future researchers can unlock the potential of jujube endophytes to enhance crop protection, promote sustainable agriculture, and address the global challenge of antimicrobial resistance. This research can unlock the potential of Jujube endophytes as a valuable resource for crop protection and the discovery of novel antimicrobial agents.

1.4.2 Research significance

Food security and sustainable agriculture are global concerns, and effective pathogen management plays a crucial role in ensuring consistent crop production. Jujube is a valuable fruit with high nutritional and medicinal benefits and its protection against fungal infection is of paramount importance to ensure both market supply and economic sustainability. The increase of antimicrobial resistance and the overuse of harmful chemicals pose a major public health risk. The study sought to mitigate the overuse of chemical fungicides that contribute to water contamination and exposure to harmful agrochemicals. The low cost and ease of maintenance of endophytes make them favorable for large scale production; this combined with controlled fermentation facilitates reproducibility and scalability in commercial settings. However, these microorganisms have not been explored to their full extent in terms of their antibiotic potential, especially in relation to traditional methods of food processing. By characterizing strains with antimicrobial activity properties, this research expands the knowledge base regarding microbial antagonism and the benefit of bacterial suppression of fungal pathogen. Furthermore, it provides a glimpse into how environmental factors such as glucose concentration, fermentation speed, inoculation size pH affects antimicrobial activity, thereby improving our understanding of microbial metabolic optimization for pathogen suppression. By horning in into optimizing such factors can contribute to the field of biotechnology where microbial fermentation is essentially utilized for industrial, agricultural and pharmaceutical application. The understanding and formulation of optimized conditions

for microbial growth and antagonistic activity can also aid in large scale bio formulation production and making feasible commercial use. The study enhances understanding of pathogen-host interaction and the potential for application. The study paves way for exploring Jujube fruit as a plant tissue with potent antimicrobial activity. This research paves the way for future studies that ought to investigate mechanisms of microbial antagonism, extensive biocontrol uses, and genetic alteration to improve microbial effectiveness. Field experiments can also confirm the antagonistic strains, whereas research on microbial synergy might enhance pathogen control. In summary, the significance of the study lies in its multifaceted contribution to agricultural science, environmental sustainability, and food security by identifying and optimizing antagonistic strains in jujube fruit. This research advances theoretical knowledge in plant-microbe interaction and offers a practical solution for disease management. Ultimately the findings support the shift towards sustainable biological based strategies for product for plant protection.

1.4.3 Research content

Isolation of endophytic bacteria from jujube fruit and evaluation of its antimicrobial activity. The isolation of endophytic bacteria with notable antimicrobial properties from jujube fruit was achieved through conventional microbiological methods, marking a successful endeavor in harnessing the potential of these microorganisms.

Comprehensive assessment of the endophytic communities inhabiting jujube fruit tissue. By examining the diversity and potential applications of these microbial communities, the research can inform sustainable agricultural practices and facilitate the discovery of novel antimicrobial agents.

Evaluation of the efficacy of selected bacterial endophyte in suppressing disease development in jujube plants using both in vitro and in vivo assays. This investigation illuminates the potential of beneficial microbial interactions to bolster plant health, encourages sustainable agricultural practices, and opens avenues for further exploration into the mechanisms of endophyte-mediated disease suppression. This research enables the development of more resilient agricultural practices capable of withstanding biotic and abiotic stresses, ultimately aiding in food security efforts in the face of ongoing environmental challenges.

1.4.4 Technical route

This research employed a structured, multi-stage methodology to identify, evaluate, and characterize endophytic microorganisms. The primary objective was to find endophytes demonstrating strong antagonistic properties against plant-pathogenic fungi, ultimately assessing their utility for managing postharvest diseases.

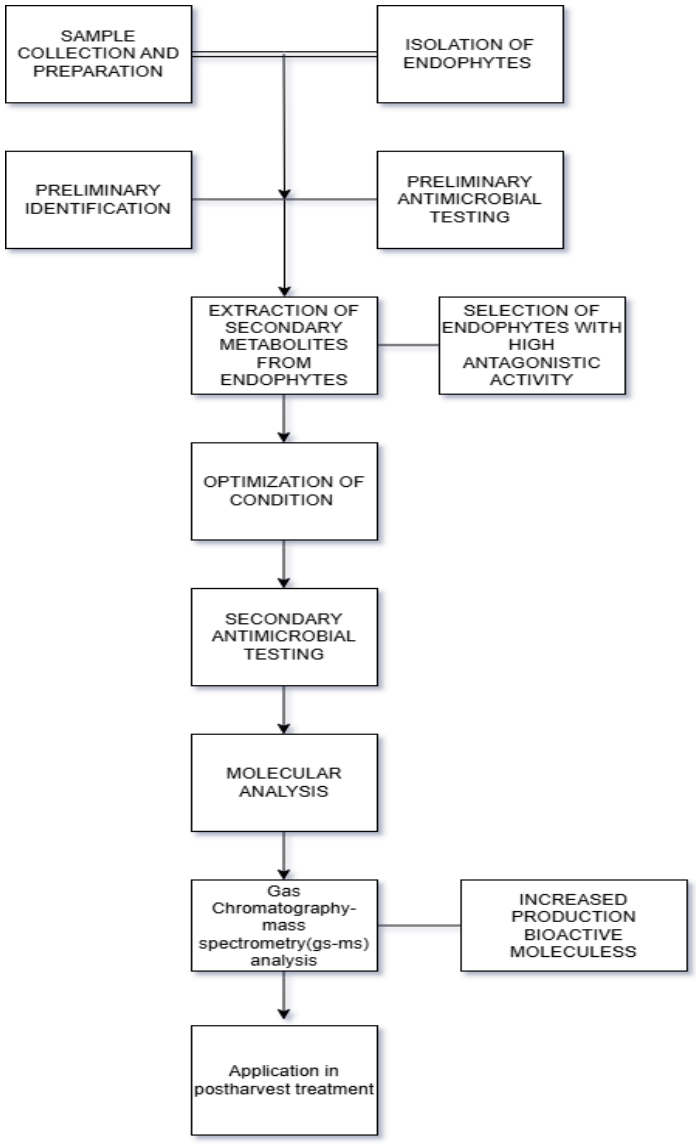

Figure 1.3 Systematic workflow for Endophyte Characterization and Biocontrol Efficacy Assessment

This study will systematically identify and evaluate endophytic microorganisms for their antimicrobial potential. The workflow involves isolating endophytes from plant tissues, screening them for antagonistic activity against phytopathogenic fungi, and then identifying promising isolates. Their secondary metabolites will be extracted, their antimicrobial activity confirmed, and fermentation conditions optimized. Chemical profiling of these metabolites will be done via GC-MS, will be analyzed. Finally, potent strains and their metabolites will be

selected for postharvest application to control fungal pathogens, aiming to develop eco-friendly biocontrol agents.

2 Isolation of endophytic bacteria from jujube and its antimicrobial activities

2.1 Introduction

In recent years, there has been a growing interest in the interactions between seed endophytes and their hosts. Huang et al. (2021) define endophytic bacteria as microorganisms that reside within plant tissues without causing any diseases in those plants. They asymptotically colonize in the intercellular space or cells of plant tissues and organs and establish a harmonious relationship with the host plant. A vast multitude of endophytes are non-pathogenic and evidently commensal with the host plant. This leads to various interactions, including symbiotic, mutualistic, commensalistic, and trophobiotic (Brader et al., 2017). The presence of endophytes in a plant species is considered a symbol of a healthy plant system. Bacterial endophytes are found to be the best producers of various therapeutic compounds. Several studies have demonstrated that endophytes can regulate the development of plant pathogens by competing for space and nutrient resources while maintaining the health of plants by protecting them from abiotic and biotic stresses (Lata et al., 2018). Many different secondary metabolites that have important biological actions are produced by endophytic bacteria. Alkaloids, terpenoids, polyketides, lipopeptides, non-ribosomal peptides, and phenolic compounds are some of these metabolites (Gouda et al., 2016). While some of these substances are specific to the metabolism of bacteria, many of them imitate or improve the bioactive chemicals made by their host plants. Numerous endophytic bacterial species, including *Burkholderia, Bacillus, Streptomyces,* and *Pseudomonas,* are recognized for their ability to generate powerful antibiotic chemicals that effectively combat many human infections. Pimentel et al. (2011) explain that well-known for producing antibiotics, *Streptomyces* species are also a rich source of glycopeptides and polyketides with potent antibacterial activities.

This chapter investigated endophytic microorganism from winter jujube fruit, isolating and identifying them through morphological and molecular methods. The study further explored the antimicrobial potential of these bacterial isolate, specifically focusing on their antifungal activity against *Alternaria tenuissima.* The experimental process involved preparing various culture media, isolating and purifying endophytes, preparing pathogenic bacterial suspensions, fermenting the isolates, and extracting compounds for antimicrobial testing. The results section detailed the isolation process, identification outcomes, and preliminary antimicrobial activity,

providing a foundation for understanding the potential of winter jujube fruit endophytes as a source of antimicrobial compounds.

2.2 Experimental materials and methods

2.2.1 Sample source and test microorganisms

Fresh jujube fruits of *Ziziphus jujuba Mill*., free of any injury, were purchased from the local market around Anhui Polytechnic University in Wuhu.

Pathogenic bacteria *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. Coli);*Pathogenic fungi; A*lternaria tenuissima* were preserved in the microbiology laboratory of Anhui Polytechnic University.

2.2.2 Culture media

Potato dextrose agar (PDA) (in g/L: Potato 200 (peeled, cut up and boiled), glucose 20, Agar 15 and H_20 1L, distilled water) Sterilization at 121℃ for 20 min. YPD media: (in g/L: yeast extract 10, peptone 20, glucose 20, agar powder 15, H_2O 1L distilled water, Sterilization at 110℃ for 30 min. PDA with the winter jujube fruit extract medium (in g/L: Potato 200 (peeled, cut up and boiled), glucose 20; winter jujube fruit extract medium 10 mL, agar powder 15 g, H_2O 1L)Sterilization 121℃ for 20 min; winter jujube fruit extract medium: 10 g winter jujube (including the peel) is cut up, denucleated, grinded and boiled in dH_2O 200 ml for 20 minutes, agar powder 15 g, and H_2O to 1000 ml sterilization at 110 ℃ for 30 min. LB medium (peptone l0 g, yeast extract 5 g, NaCl 10 g). H_2O 1000 ml, pH 7.5, sterilization at 121℃ for 20 min. The pathogen cultivation medium: peptone 10 g, yeast extract 5 g, Na Cl 10 g, agar 15 g, H_20 1000 ml, pH 7.5, sterilization at 121 ℃ for 20 min.

Fermentation medium for optimization. Seed medium: beef extract 5 g，peptone 10 g，NaCl 5 g，agar powder 15 g (no addition in liquid medium) H_2O 1000 mL，pH7.5，sterilization at 121℃ for 20~30 min. Fermentation medium: glucose 10 g (determined by condition design) peptone 10 g，NaCl 1 g，K_2HPO_4 0.1 g，$MgSO_4 \cdot 7H_2O$ 0.05 g，H_2O 1000 ml，pH7.5，sterilization at 121 ℃ for 20 min.

2.2.3 Isolation of endophytes and purification of isolates from winter jujube fruit

The washed and dried winter jujube fruit was disinfected on a super clean bench. The fruits were immersed in 75% alcohol for 3-5 minutes before being rinsed three to four times with sterile distilled water. The fruits were then surface sterilized with 3% sodium hypochlorite solution for 1.5 min before being rinsed three times with sterile distilled water. The whole fruit was then dried using sterile filter paper. The sterile water from the last washing is collected and absorbe100 μL and spread on PDA solid medium to test whether the surface was thoroughly

disinfected. Following disinfection, the jujube fruit was cut into 3~5 mm×3~5 mm on the laminar flow cabinet using sterile blades. Inoculated the pieces on the surface of solid medium with cutting plane down using a sterile tweezers, each dish evenly placed 20~25 pieces. Finally, the dishes are incubated at 28~30℃ for 48~72 h.After the colonies grow out, single colonies are selected and inoculated on the medium by the streak method for inverted culture until pure strains are obtained and further strain preservation.

2.2.4 Morphological identification

The preliminary identification is made by observing colony morphology and cell micro-morphology. The purified single colonies are inoculated on the surface of solid medium by the point-inoculating method. After the colonies grow out, the morphology of each colony, including growth rate, color, shape, and texture, is observed and recorded. The single colonies of each strain are selected and stained to observe the microscopic morphological characteristics of each strain.

2.2.5 Molecular biological identification

The strain with the strongest inhibitory activity was molecularly identified. Bacterial DNA was isolated using the EzupColumn Bacterial Genome Kit. The 16S rDNA ITS regions of the bacterial isolates were amplified with the primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ TACGGCTACCTTGTTACG ACTT 3′). The PCR-amplified products were sequenced by Sangon Bioengineering (Shanghai) Co. The sequenced sequences were searched and compared in the NCBI database, and the sequences with high similarity were downloaded and analyzed by using MEGA 7.0 software and the neighbor-joining (NJ) method for cluster analysis and construction of a phylogenetic tree for species identification.

2.2.6 Preparation of pathogenic bacterial suspension

The pathogenic bacteria *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. Coli)* were activated on the surface of solid LB medium; single colonies were inoculated into 50 mL corresponding liquid medium after activation and shaking and cultured at 37°C at 180 r/min. After incubation to the logarithmic phase (about 12–14 h), it was then centrifuged at 4000 r/min for 20 min; thus, the bacterial precipitate was collected. The bacterial precipitate was resuspended in sterile normal saline and adjusted to an OD_{600} of approximately 1 to prepare the pathogenic bacterial suspension.

2.2.7 Fermentation step and the fermentation extract preparation

A single colony of the activated endophyte is picked up from a solid plate medium of LB and inoculated in 50 mL seed liquid medium (LB), shaking cultured at 37 °C and 180 r/min for about 12 h. After incubation, it is inoculated in fermentation liquid medium at 10% (v/v)

inoculation size, shaking cultured at 37°C and 150 r/min for about 24~36 h. Finally, the fermentation broth was centrifuged at 4000 r/min, and the supernatant was collected. An equal volume of n-butanol solution is added to the supernatant (shaking sufficiently for 3-5 min) for extraction three times. The extraction solution is then mixed, concentrated, and dried at 60 °C under reduced pressure and dissolved with 2 ml of DMSO to obtain the fermentation extract. The sample was filtered through a 0.22 μm membrane to remove any insoluble impurities, residual particulates, or microbial debris that may remain after concentration and drying.

2.2.8 Preliminary antimicrobial activities

Disk method: Agar plates of LB medium were inoculated with a standardized inoculum of the pathogenic bacteria (*Staphylococcus aureus, S. aureus,* and *Escherichia coli, E. coli).* Sterile filter paper discs (about 6 mm in diameter), containing the DMSO-dissolved fermentation extract, are placed on the agar surface using sterile forceps. The plates are inverted and incubated at 37°C for 24 hours to assess the inhibition zone. Sterile filter paper discs containing the DMSO are set as a control for each experiment. Post-incubation, the diameter of the zone of inhibition is measured quantitatively to determine the antimicrobial activity of the isolated strains. The magnitude of the inhibition zone correlates with the degree of antimicrobial efficacy, with measurements meticulously documented for comparative analyses.

The Oxford cup method: 10 mL of sterilized LB agar was poured into a sterile petri dish, evenly spread to cover the entire bottom surface, and left to solidify at room temperature, forming the bottom plate for further experimentation. 1 ml of pathogenic bacteria suspension was added to 100 ml of semi-solid medium held at 50 °C and mixed thoroughly. 10 ml was quickly poured into the bottom plate and mixed well immediately to prepare the upper plate. Sterile Oxford cups were evenly placed on the prepared double-layer medium, with 3 cups per plate, and 200 μL DMSO-dissolved fermentation extract is added into each cup, with three replicates per group. Meanwhile, DMSO was set as a blank control group. After incubating at 37 °C for 24~48 h, the antimicrobial effects will be tested (examination of the inhibition zone).

2.2.9 Antifungal Screening of Bacterial Extracts Against *Alternaria tenuissima*

A mycelial plug of *Alternaria tenuissima* (5 mm diameter, 7 days old) was placed 2 cm away from the edge of a PDA plate. An Oxford cup is placed 2 cm opposite the mycelial plug of *Alternaria tenuissima*. 100 μL of sterile endophytic bacterial extract is applied in an Oxford cup. Control Oxford cup holds sterile DSMO. Inoculated plates are incubated at 25–30 °C for 5–7 days. Antifungal activity is evaluated through the reduction of fungal growth.

$$\text{Inhibition}(\%) = \frac{(\text{control growth} - \text{treatment growth})}{\text{control growth}} \times 100$$

2.3 Results and discussion

2.3.1 Isolation of endophytic microorganism

The choice of growth medium is crucial as it directly affects the number, and the type of endophytic microorganism isolated that can be isolated from plant tissues. The culture media that are used during endophyte isolation will strongly influence the number and diversity of cultivable microorganisms. In the present study YPD and PDA were used to isolate endophytic microorganisms. Although YPD is essentially a yeast/fungus medium, it has plenty of nutrients for bacteria as well. Potato dextrose agar (PDA) is often used for fungal isolation, as it is rich in carbohydrates that are also used for the growth of some bacterial species. However, such media is non-selective, allowing for successful isolation of filamentous fungi and bacterial endophytes from jujube fruit. Surface sterilization was a critical step for removing epiphytic microbes from sample explants. This step proved satisfactory in our study due to the absence of any growth on the control plate. In total, 14 endophytic strains were isolated from the fresh jujube fruit, with 6 with 6 bacteria and the rest fungi. Most of the fungi were isolated from the YPD, whereas the bacterial strains were isolated from PDA media.

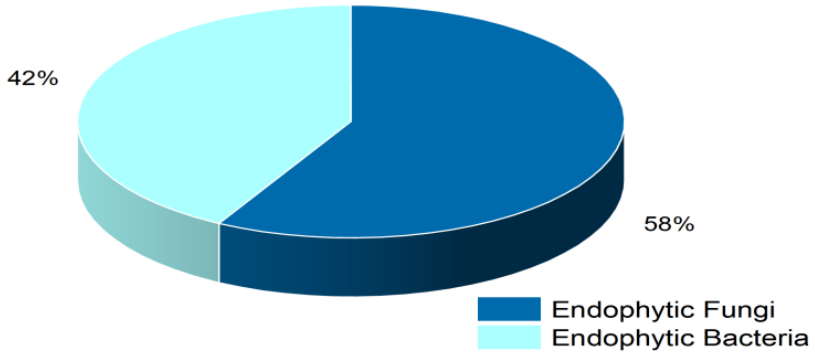

Figure. 2.1 Isolation frequency of endophytic microorganism isolated from jujube fruit.

2.3.2 Preliminary identification

Microscopy and Gram staining were performed to aid in the preliminary identification of microbial endophytes isolated from the jujube fruit. Microscopic inspection allows for visualizing bacteria morphology and of each colony, growth rate, color, shape, and texture, whereas each isolate was subjected to Gram-staining analysis using standard staining procedures. Gram staining allows for differentiating gram-positive from gram-negative bacteria by the characteristics of their cell wall. The preliminary results were important in identifying general taxonomic categories of the isolates before further biochemical and molecular analysis.

2.3.3 Morphological identification

The single colonies of each bacterial strain were isolated and stained to observe the microscope morphological characteristics of each strain. The colonies appeared circular, smooth, and creamy/white, larger, opaque, and easy to pick out; the edge was not neatly round in the different periods of growth, and the longer the culture time, the drier the colony surface became. The microscopic is a short, rod-shaped, and Gram-positive bacterium under a microscope at 400× total magnification.

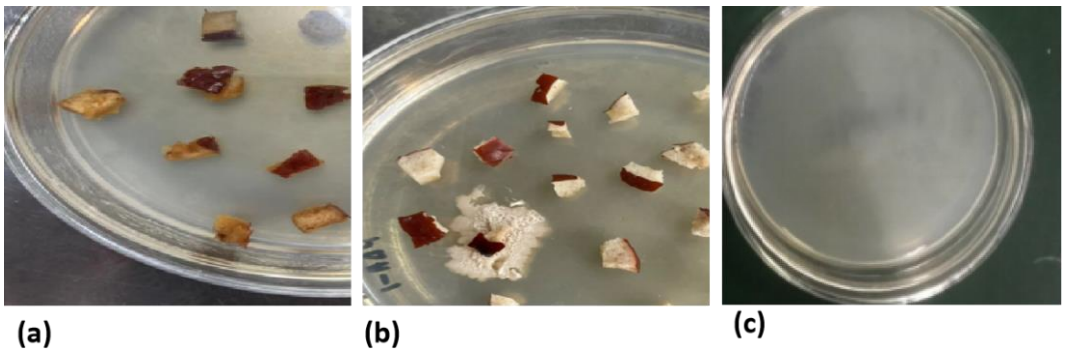

Figure 2.2 Development of bacterial endophyte from jujube fruit. (a) Freshly cut fruits on the PDA medium. Pieces placed on PDA media. (b) Growth of bacterial endophytes after three days on PDA medium in 30°C incubation. (c) Sterility control plates showing no growth of microbes after seven days of incubation at 30 °C.

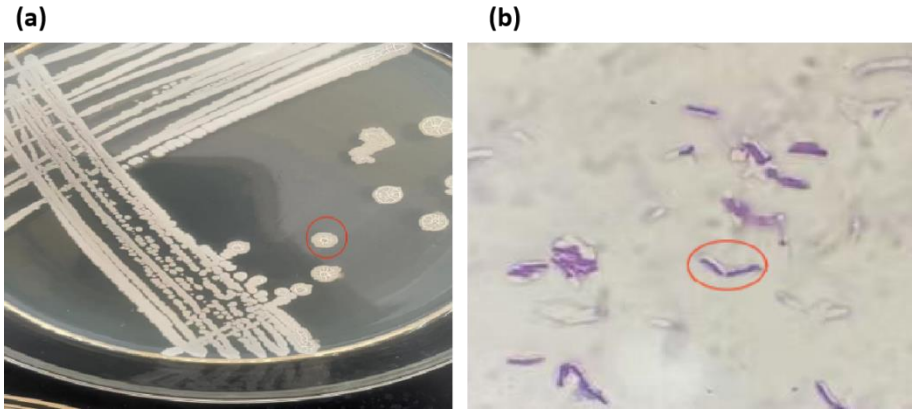

Figure 2.3 Growth of bacterial isolate of jujube fruit on PDA media. (a) The colony morphology on an agar plate (highlighted colony showing typical colony morphology). (b) Microscopic view of Gram-stained bacterial isolates

2.3.4 Molecular identification of the isolate

The amplification of 16S rDNA with the primers 27F and 1492R yielded a band approximately 1500 base pairs in length as shown in Figure 2.4. X27 isolate forms a well-supported cluster with *Calidifontibacillus erzurumensis strain* P2 (NR_180225.1), supported by a bootstrap value of 72, indicating a moderately strong phylogenetic association. This suggests that X27 may belong to the genus *Calidifontibacillus*, a relatively new genus within the *Bacillaceae* family.Further, this cluster forms a monophyletic clade (bootstrap = 100) with *Bacillus vallismortis* DSM 11031, a well-characterized soil bacterium known for plant growth-

promoting and antimicrobial properties. *B. vallismortis* strains have been recorded to exhibit strong inhibition against numerous phytopathogenic fungi such as *Monilinia fructicola* (causing brown rot in peaches), *Alternaria alternata*, *Rhizoctonia oryzae*, *Fusarium oxysporum*, *Colletotrichum species*, and *Magnaporthe grisea* (Chen et al. 2019).The close evolutionary proximity of X27 to *B. vallismortis* suggests that it may possess similar functional traits, particularly secondary metabolite production and environmental resilience.

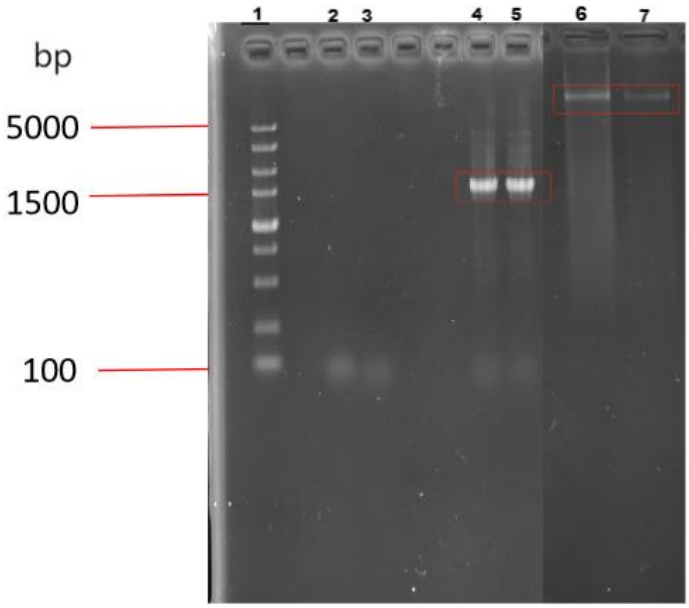

Figure 2.4 Electrophoretic diagram of PCR product for 16S rDNA of X27 strain.

To determine the phylogeny of strain X27, phylogenetic analysis was carried out based on 16S rDNA. The tree was constructed based on the neighbor-joining approach in software MEGA 11.

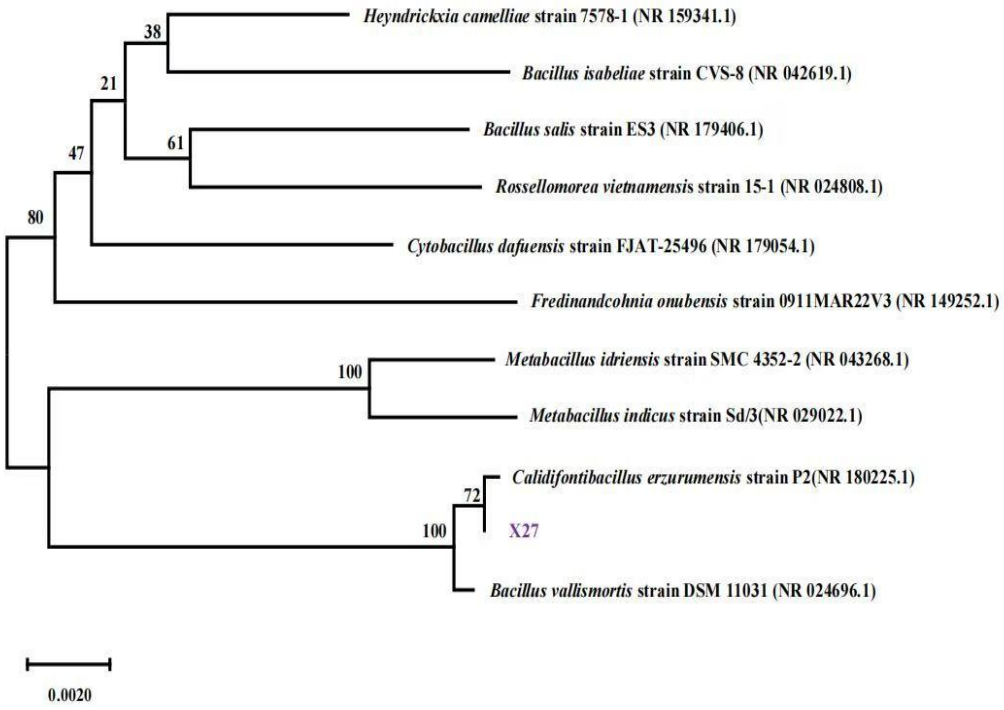

Figure 2.5 Phylogenetic tree constructed based on the 16S rDNA gene sequence of the X27 isolate.

2.3.4 Preliminary antimicrobial activity detection

Bacterial endophytes were primarily screened for their antimicrobial activity following agar diffusion methods against the test bacterial strains *Staphylococcus aureus* and *Escherichia coli.* Among these isolates, only 1 demonstrated antimicrobial activity against the test microorganisms, as shown in Figure 2.6

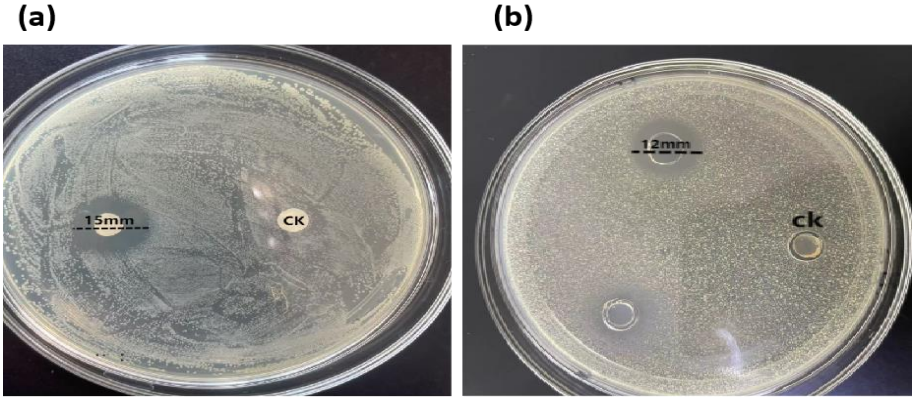

Figure 2.6 Preliminary screening of antimicrobial activity of isolated bacteria. (a) Preliminary screening of antimicrobial activity against E. coli using disk diffusion method. (b) Preliminary screening of antimicrobial activity against S. aureas using oxford cup.

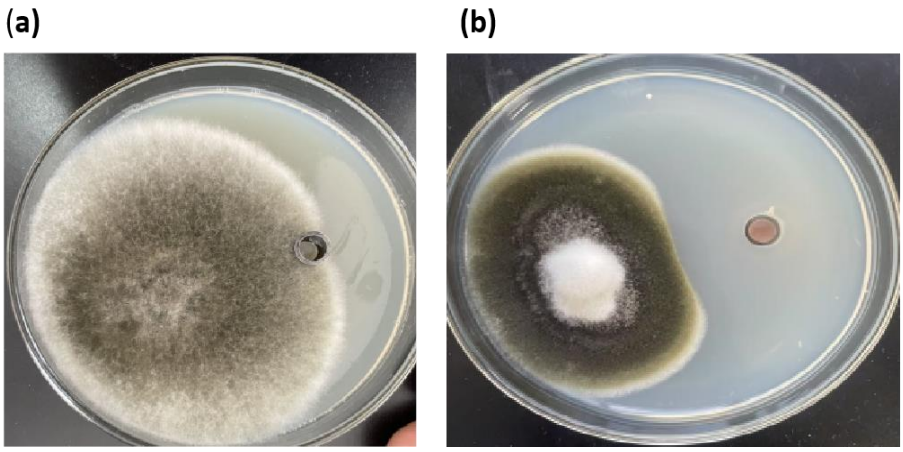

Figure 2.7 Screening of antimicrobial activity of isolated bacteria against Alternaria tenuissima using the dual culture method. (a) control plate demonstrating the growth of Alternaria tenuissima using DMSO. (b)growth of Alternaria tenuissima with the antagonistic isolated bacteria in the oxford cup.

2.4 Summary

A quantitative analysis, depicted in the initial pie chart, revealed a differential isolation frequency between two distinct endophytic populations, designated Endophytic Fungi and Endophytic Bacteria, with the former exhibiting a significantly higher prevalence. Subsequent phylogenetic analysis, utilizing 16S rDNA gene sequencing and comparative genomics, placed the isolate designated X27 close to *Calidifontibacillus erzurumensis strain* P2 (NR_180225.1),inferring a close evolutionary relationship and potential functional homology with previously characterized members of this species. Molecular confirmation of the X27 isolate was achieved through successful polymerase chain reaction (PCR) amplification of the 16S rDNA gene, visualized via agarose gel electrophoresis.The close phylogenetic connection of isolate X27 with *Calidifontibacillus erzurumensis* and *Bacillus vallismortis* indicates its potential for the production of antimicrobial secondary metabolites. Research indicates that *Calidifontibacillus erzurumensis,* a thermotolerant member of the *Bacillaceae* family found in hot springs, demonstrates significant metabolic adaptability and resilience to environmental stressors. *Bacillus vallismortis* is recognized for its ability to produce a variety of antimicrobial substances, including lipopeptides such as surfactins and iturins, which are known for their extensive antibacterial and antifungal properties. This strong evolutionary relationship implies that X27 may possess similar characteristics, positioning it as a promising source for bioactive metabolites with antimicrobial capabilities that could be beneficial in both medical and agricultural fields. Antimicrobial susceptibility assays, employing zone of inhibition methodologies, demonstrated the bioactive potential of the X27 strain or its metabolites against a test microorganism, evidenced by clear zones of growth inhibition. Furthermore, a dual

culture assay, utilizing *Alternaria tenuissima* as a target phytopathogen that causes black rot in jujube, revealed a potent antagonistic interaction mediated by the X27 isolate, characterized by a distinct zone of inhibition surrounding the bacterial sample. Collectively, these findings suggest that the endophytic X27 possesses significant antimicrobial activity, including antifungal activity against *Alternaria tenuissima*, highlighting its potential as a bio control agent for agricultural application.

3 Optimization of medium composition and culture conditions for antifungal activity of a jujube endophytic bacterium x27

3.1 Introduction

Fermentation technology is widely used for the production of various economically important compounds which have applications in the energy various industries. Optimizing the growth medium is a key step before large-scale production.The optimization of the culture medium itself is a multifaceted endeavor, demanding a strategic balance between empirical observation and statistical rigor. Initial medium selection, often involving standard growth media, serves as a starting point. Understanding the optimal temperature, pH, and aeration conditions, as well as the nature of the anti fungal compounds produced, provides crucial insights for tailoring the culture environment (Singh et al., 2017).

Optimizing medium composition and culture conditions is critical for maximizing the antifungal potential of jujube endophytic bacteria, as these factors directly influence microbial growth, metabolite synthesis, and bio control efficacy. By methodically varying factors such as glucose concentrations, pH, inoculation size, etc., The aim is to identify optimal conditions that maximize production of antifungal metabolites. Singh et al., (2017) explains that a clear comprehension of the interaction between the endophytic bacterium and its environment is significant for harnessing antimicrobial potential.The use of unsuitable culture media, the number of viable bacteria in the fermentation process may be low, and few antimicrobial substances are secreted, which diminishes the bio control potential for these microorganisms against diseases. The choice and proportional composition of microbial media components are therefore crucial.

While a one-factor-at-a-time (OFAT) approach offers simplicity, it may overlook crucial interactions between different medium constituents. A one-factor-at-a-time (OFAT) method involves changing one component while keeping others constant, but this approach fails to account for potential interactions among different components. For a more comprehensive optimization, statistical experimental designs, such as response surface methodology (RSM), are employed. RSM uses designs like central composite design (CCD) or Box-Behnken to simultaneously optimize multiple factors, which helps reveal synergistic or antagonistic effects .By employing techniques such as factorial designs, central composite designs, and Box-Behnken designs, RSM enables researchers to efficiently explore the factor space and identify the optimal conditions for maximizing or minimizing the response variable.

3.2 Experimental materials and methods

3.2.1 Optimization of fermentation condition of endophytic bacteria

One-factor at-time is a method used to improve the extraction of bioactive compounds. In this method, one parameter is varied while all the other parameters are kept constant. The effect of a single factor or variable on the antimicrobial activity of the extracted bioactive compound is then measured.

Determination of fermentation time: 24，36，48，60，72 h, respectively.

Determination of glucose concentration: 5，10，15，20，25 g/L

Determination of Initial pH: 6.0, 7.0, 8.0, 9.0, and 10.0

Determination of Shaking Speed: 90, 120, 150, 180, 210 rpm

Inoculation size: 1%, 5%, 10%, 15%, and 20% (using seed liquid as inoculant with the OD_{600} of about 1.0, the inoculation size means the ratio of seed liquid volume to fermentation medium volume).

3.2.2 Response surface optimization tests

To define selective factors that affect antimicrobial activity, OFAT preliminary experiments were performed. The concentration of glucose, the size of the inoculation, and pH, and the speed significantly influenced the results, with optimal results found for glucose concentration to be 20 g/L, inoculation size of 10%, speed of 150 rpm, and pH of 8. They were then chosen for optimization by RSM to study their interactions and optimize the conditions for the highest antimicrobial activity using Design-Expert 12 software (Version 23.1 Stat-Ease Inc; USA), Box-Behnken design, and 29 experiments were performed. Each of the parameters was coded at three levels: -1, 0 and +1.

Table 2.1 Response surface methodology factor level design

Factors	Unit	Factor level		
		-1	0	1
A. Glucose concentration	g/L	5	15	25
B. Initial pH		6	8	10
C. Speed rpm	rpm	90	150	210
D. Inoculation size	% (v/v)	1	10	20

3.2.3 Verification test

The optimal fermentation conditions obtained by the response surface test were used for antifungal test verification, which was repeated 3 times. The optimal antifungal rate obtained

by the test was compared with the predicted value and compared with the original culture conditions before optimization.

3.2.4 Data processing

Each experiment was replicated three times, and the experimental data were organized using Excel 2024, Origin 2022 to produce graphs, and IBM. SPSS 27 was used to analyze the trial data for statistics and significance of differences.

3.3 Results and discussion

3.3.1 One-factor-at-a-time

The OFAT method was used to determine the effect of the main factors, including fermentation time, glucose concentration, initial pH, inoculum sizes, and rotary speeds, on the growth and antifungal activity of the isolated endophytic bacteria X27.

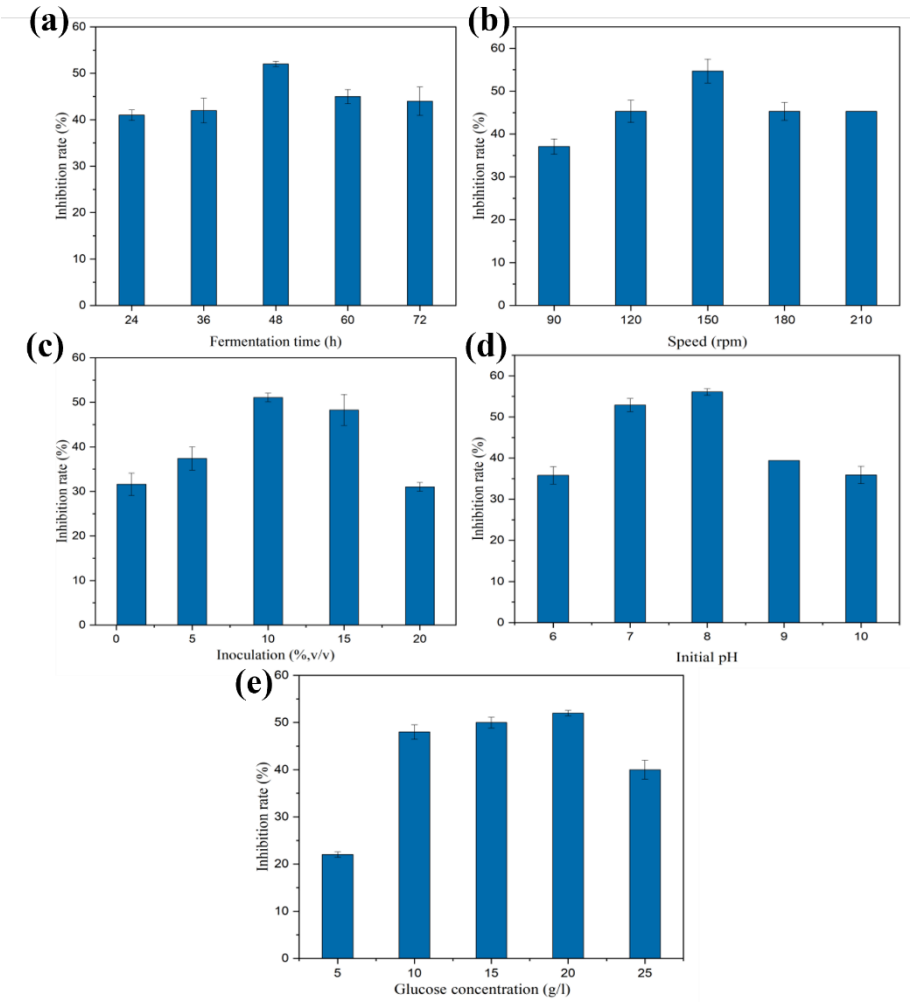

Figure 2.8 O-F-A-T-based analysis of fermentation conditions effect on endophytic bacteria X27 antagonism on Alternaria tenuissima. (a) Fermentation time (h) (b) Speed (rpm) (c) Inoculation size (%, v/v) (d) Initial pH (e) Glucose concentration (g /l).

One-factor-at-a-time experiments were conducted to investigate the effect of different initial pH values, fermentation times, inoculum sizes, rotary speeds, and glucose concentration on the X27 bacteria. The dependence on the length of the fermentation process, which is known to affect both endophytic bacteria's efficacy against different pathogens and the generation of beneficial metabolism. In Figure 2.8(a) the inhibition rate was at its highest with a 48-hour fermentation duration. Therefore, a 48-hour fermentation period has been identified as optimal for endophytic bacteria X27. The rate of inhibition for isolated endophytic bacteria was shown to be a time-dependent trend. At 24 hours, the inhibition rate was 41%, and it increased slightly to 42% at 36 hours. The antifungal activity was most salient after 48 h, with a 52% inhibition rate. This was followed by a decline, with the inhibition rate falling to 45% after 60 hours and 44% after 72 hours. The results denote that 48 hour is the highest fermentation time with increased antifungal activity. The results also indicate a longer incubation period results in a decreased inhibition rate. The timely decrease of inhibitory activity after a certain period of fermentation is connected to the variations in the concentration of bioactive compounds, pH, and depletion of nutrients (Cortez et al., 2023). These factors could be contributing factors to the decreased stability of the bioactive compounds responsible for the inhibition activity thus shown in the present study. The antifungal activity of the isolated endophytic bacteria showed an obvious difference when rotary speed was changed.

With 90 rpm, the inhibition was 37.1%, showing relatively weak antifungal activity in low agitation conditions. On the other hand, increasing the speed to 120 rpm, the inhibition rate was significantly enhanced to 49.3%, indicating the metabolic improvement of the bacteria as well as improvement of secondary metabolites. 150 rpm had the highest inhibition rate (54.7%), suggesting that this velocity was the optimal condition for antifungal metabolite production Figure 2.8(b). With an inoculum size of 1%, the inhibition rate was 31.6%, suggesting low antifungal activity at the lowest inoculation of bacteria X27. The inhibition rate was 37.4% at the inoculum size of 5% which was further increased up to 48.1% at 10% of the inoculum size, indicating that the concentration was optimum for growth and secondary metabolite production. After that point, the inhibition rate decreased to 33.3% at 15% and 31% at 20%, showing that high inoculum size hindered antifungal activity Figure 2.8(c). Numerous studies highlight that the antimicrobial effectiveness of endophytic bacteria varies greatly with changes in pH. The antimicrobial activity of the endophyte remained slightly unchanged between a pH of 7 and 8 (52% and 56%, respectively); however, in highly acidic or highly alkaline conditions, there's a notable decrease in the antimicrobial activity of the X27 against *Alternaria tenuissima.* The highest antimicrobial activity is observed at pH 8, as shown in the above Figure 2.8(d). At these

pH levels, the bacteria's metabolic activity is often enhanced, which is related to the efficient production of antibiotic compounds. On the flip, low and/or high pH acidic or basic will harm the growth of bacteria and harm their metabolism, thus reducing their suppressive effects on pathogens. Figure 2.8(e) shows the effect of glucose concentration on inhibition rate, with 20g/l exhibiting the highest antifungal activity. Figure 2.8(e) shows that 5g/l glucose concentration exhibited the lowest inhibition rate. Glucose concentration is a critical optimization factor in the cultivation of endophytic bacteria for biocontrol application.

3.3.2 Response surface test results

A three-factor and three-level Box-Behnken design (BBD) was used for constructing the models with Design Expert (Version 23.1 Stat-Ease Inc; USA). The main effects, interaction effects, and formulation components were investigated and optimized for the antimicrobial performance of the isolated endophyte X27. The box-Behnken design was used to screen the variables; Table 3.2 displays the experimental design outcomes. After screening, the components were fitted to a model to determine the quadratic regression equation:

$$Y=62.65+11.95A-1.41B-0.2583C-0.5833D-1.49AB+0.3575AC-0.295AD+0.1725BC-1.01BD+0.845CD-17.76A^2-3.74B2-7.17C^2-3.69D^2$$

The predicted R^2 of 0.9345 is in reasonable agreement with the adjusted R^2 of 0.9717; i.e., the difference is less than 0.2. This is an indication that the model has good correlation and a high degree of fitting to the response value. The current model with an F-value of 69.63 indicates the model was statistically significant. The probability of such a large F-value occurring by chance is only 0.01%. P-values below 0.0500 show that model terms are significant. The Lack of Fit F-value of 1.08 indicates that the Lack of Fit is not notably different from the pure error. A Lack of Fit F-value of this magnitude has a 51.34% probability of being caused by random noise. Insignificant lack of fit is desirable.

Table 2.2 Box-Behnken design matrix for optimization of antimicrobial activity of endophytic bacteria

Std	Factor level				Inhibition rate (%)
	A	B	C	D	
1	-1	-1	0	0	27.17
2	1	-1	0	0	52.33
3	-1	1	0	0	32.07
4	1	1	0	0	49.31
5	0	0	-1	-1	54.23
6	0	0	-1	1	50.24
7	0	0	1	-1	46.83
8	0	0	1	1	48.07
9	-1	0	-1	0	25.79
10	1	0	-1	0	55.83
11	-1	0	1	0	24.43
12	1	0	1	0	51.36
13	0	-1	0	-1	54.84
14	0	1	0	-1	53.06
15	0	-1	0	1	54.4
16	0	1	0	1	49.35
17	-1	0	0	-1	23.96
18	1	0	0	-1	48.2
19	-1	0	0	1	23.16
20	1	0	0	1	51.81
21	0	-1	-1	0	53.4
22	0	1	-1	0	57.44
23	0	-1	1	0	56.65
24	0	1	1	0	57.58
25	0	0	0	0	64.34
26	0	0	0	0	59.16
27	0	0	0	0	59.56
28	0	0	0	0	64.76
29	0	0	0	0	63.29

The response surface methodology (RSM) was applied for the evaluation of factors A, B, C, and D on the inhibition rate (%). Table 2.2 presents the results of the experiments, and the inhibition rates are different based on the factor levels.

Table 2.3 Analysis of variance (ANOVA) for the response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	significant
Model	3904.819	14	278.916	69.626	0.000	**
A	1714.586	1	1714.586	428.015	0.000	**
B	23.914	1	23.914	5.970	0.028	*
C	0.801	1	0.801	0.200	0.662	
D	4.083	1	4.083	1.019	0.330	
AB	8.910	1	8.910	2.224	0.158	
AC	0.511	1	0.511	0.128	0.726	
AD	0.348	1	0.348	0.087	0.772	
BC	0.119	1	0.119	0.030	0.866	
BD	4.080	1	4.080	1.019	0.330	
CD	2.856	1	2.856	0.713	0.413	
A^2	2045.933	1	2045.933	510.729	0.000	**
B^2	90.605	1	90.605	22.618	0.000	**
C^2	333.921	1	333.921	83.357	0.000	**
D^2	88.556	1	88.556	22.106	0.000	**
Residual	56.083	14	4.006			
Lack of Fit	40.925	10	4.093	1.080	0.513	
Pure Error	15.157	4	3.789			
Cor Total	3960.902	28				

Significant at the $P<0.05$ level, ** significant at the $P<0.01$ level.

(a) is the representation of the interaction between inoculation size and carbon source. (b) is a representation of the interaction of initial pH and Inoculation size. (c) is the representation of the interaction of speed and carbon source or glucose concentration. (d) is a representation of the interaction of speed and initial pH. (e) is a representation of the interaction of initial pH and carbon source or concentration of glucose. (f) is a representation of the interaction between inoculation size and speed. The F values, as seen in Table 2.3 offer a statistical comprehension of each factor's antifungal effect with its increase or decrease. The incline degree of the surface diagram is directly proportional to the influence degree of factors on the response value. The contour plot illustrates that smaller circles indicate less pronounced interactions between the two factors, while an ellipse signifies a significant interaction. By comparing the F values of each factor, glucose concentration (g/l) and initial pH have the highest influence on the antifungal effect of the isolated endophyte.

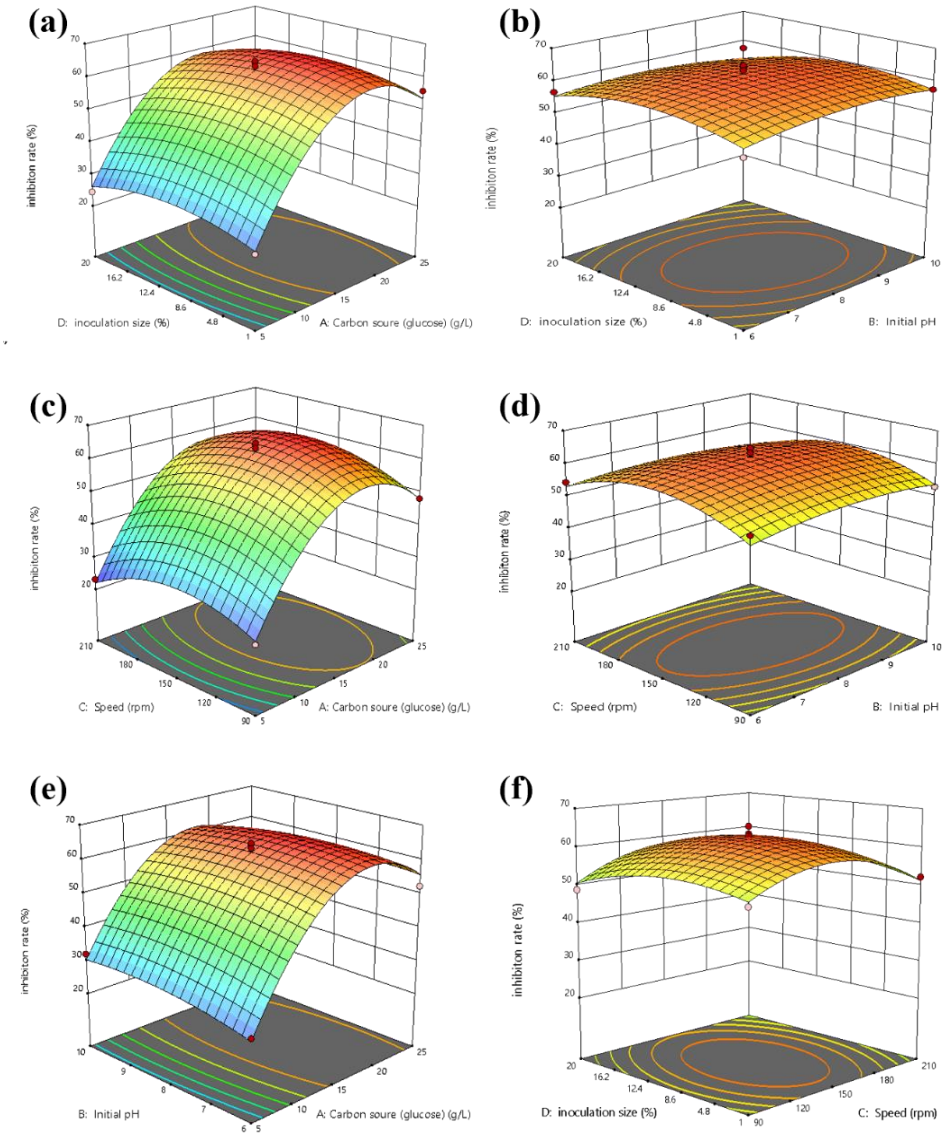

Figure 2.9 Response surface 3D plot and interaction among different factors. A glucose concentration (g /l); B initial pH; C speed; D inoculation size (%) (a) Interaction of DA, (b) Interaction of DB, (c) Interaction of CA, (d) Interaction of CB, (e) Interaction of BA, (f) Interaction of DC.

3.3.3 Validation of the test results

To validate the predictive accuracy of the response surface methodology (RSM) model, an experiment was conducted at the optimal conditions suggested by the model: glucose concentration of 18 g /L, pH 7.4, agitation speed of 149 rpm, and inoculum size of 9.4% (v/v). The observed inhibition rate was then compared to the predicted value of 62.65% to determine the reliability of the model. The results indicated that the antifungal activity of 61.7% exhibited a significant increase from the rate of inhibition observed during the OFAT experiments. endophytic microorganisms were isolated using standard protocol.

3.4 Summary

To optimize the antifungal activity of the X27 endophytic bacteria against *Alternaria tenuissima,* a two-pronged approach was employed. Initially, one-factor-at-a-time experiments were conducted to assess the individual impact of various fermentation parameters.Notably, a 48-hour fermentation period yielded the highest inhibition rate with a 52% inhibition rate., showcasing the importance of a precise incubation time for maximizing antifungal compound production. Similarly, an agitation speed of 150 rpm optimized bacterial metabolism, leading to the highest inhibition rate. An inoculum size of 10% also proved to be crucial, indicating that a specific bacterial population density is required for optimal antifungal compound synthesis. .A glucose concentration of 20 g/L was essential for maximizing antifungal activity, highlighting the critical role of glucose availability in the production of these metabolites with a inhibition rate of 52 % Furthermore, a pH of 8 provided the ideal environment for bacterial metabolic activity, resulting in the highest inhibition rate of 56%. Collectively, these findings underscore the necessity of carefully controlling fermentation parameters to enhance the antifungal potential of X27 bacteria.These experiments revealed that a 48-hour fermentation period, 150 rpm rotary speed, 10% inoculum size, pH 8, and 20 g/L glucose concentration yielded optimal results.

To optimize the antimicrobial performance of the X27 endophyte, a three-factor, three-level Box-Behnken design (BBD) was implemented using Design Expert software, allowing for the investigation of main and interaction effects among the fermentation parameters.The validity of this model was then rigorously assessed through statistical analysis. The close agreement between the predicted R^2 (0.9345) and adjusted R^2 (0.9717) values, with a difference less than 0.2, indicated a strong correlation and excellent fit to the experimental data.

Finally, the insignificant lack of fit, with an F-value of 1.08, confirmed that the model adequately represented the data and that the variation was not due to a lack of fit. These statistical findings collectively demonstrate that the BBD effectively modeled the relationship between the fermentation parameters and the antimicrobial activity of the X27 endophyte, providing a reliable tool for optimizing its production. An experiment was performed using the model's predicted optimal conditions (glucose, pH, speed, inoculum), resulting in an observed inhibition rate of 61.7%. This closely matched the model's predicted value of 62.65%, confirming the model's accuracy and reliability and also shows significant increase from the rate of inhibition observed compared during the OFAT experiments.

4 In vivo efficacy and biochemical index of X27 bacterial extract formulations against *Alternaria tenuissima*

4.1 Introduction

Plant disease control currently primarily relies on chemical pesticide application to manage plant pathogens or vectors of plant pathogens. Unfortunately, the continuous use of these pesticides has proven to be detrimental to the ecosystem and human health. The research community is in continuous search for novel bio-products for sustainable agriculture. The exploration of the endophyte's inherent natural defense system provides an exciting avenue for developing environmentally sustainable strategies for managing postharvest diseases in fruits and vegetables, thereby enhancing food safety and sustainability. Using bacterial endophytes as bio control agents to prevent fungal diseases in fruits is a promising approach to postharvest management and sustainable agriculture. Endophytes, microorganisms that reside within plant tissues without causing harm, have demonstrated significant potential to combat postharvest fungal diseases (Card et al., 2016).

Various direct and indirect mechanisms are involved in the control of phytopathogenic fungi and plant growth promotion by endophytic bacteria. Direct mechanisms involve antibiosis whereby certain bacteria produce antimicrobial compounds such as surfactin, iturin, and DAPG which can inhibit, restrict, or eliminate the growth of pathogens. For example, *B. subtilis* synthesizes fengycin to suppress *B. cinerea* growth on apple fruits. Fungal cell walls are lysed by hydrolytic enzymes derived from various organisms, *Pseudomonas aeruginosa* extra-cellular chitinase regulates *Xanthomonas campestris,* the pathogen responsible for causing black rot disease in cruciferous crops. The mechanism by which *B. subtilis* inhibited *Phytopthora infestans* and *F. oxysporum*, was by effective competition for nutrients and space in potato tubers. Indirect mechanisms are the induction of systemic resistance (ISR) in plants. Plant defenses are classified as innate defenses that are triggered by non-pathogenic microbes and controlled by the jasmonic acid or ethylene pathways. ISR triggered by *Burkholderia gladioli* in saffron against *F. oxysporum.* It also limits pathogen establishment through competition for nutrients and space.

A study by Chen et al. (2019) offers insight into the development of biocontrol in nature (especially *Bacillus* strains) against postharvest pathogens. These strains not only directly inhibit the formation and development of the pathogen *Botrytis cinerea* in and out of the fruit; they also further improve the resistance of fruits by means of the colonization and metabolizing

functions of the strain produced. It was stated that Z-14 cells were the strongest in controlling gray mold with 82.14% disease reduction on strawberry in vivo effects in artificially wounded fruit. The populations of Z-14 and Pnf-4 increased significantly over time in grapefruit tissue. Given their capacity to proliferate in grapefruit tissue, Z-14 and Pnf-4 are significant for biocontrol strategies. It also indicates successful colonization, which may assist with immune system development to create a protective barrier against infection and improve the resistance of the fruit against gray mold.

4.2 Assessment of Antagonistic Strain Against Alternaria tenuissima in Jujube

4.2.1 In Vivo Approach

The pathogen was produced by incubating *Alternaria tenuissima* on potato dextrose agar (PDA) plates at 25–28°C for 7 days. Spores were collected and re-suspended in sterile saline solution to a final concentration of 1×10^{6} spores/mL. The fruit of fresh, healthy jujube fruits of uniform size without injury was chosen for this experiment. Subsequently, the fruits are surface-sterilized and washed with 70% ethanol for 1–2 minutes, rinsed with sterile water, and air-dried. The fruit surface was perforated with 5 mm cork borers to produce uniform wounds (2–3 mm deep). 20 µL of the *Alternaria tenuissima* spore suspension was added to the wounds. Twenty-four hours later, 20 µL of the prepared endophytic bacterial extract is inoculated into the same wound. The experiment is incubated at 25–28 °C, and the entire experiment was repeated three times for each condition. For the evaluation of biocontrol efficacy, the diameters of lesions are recorded and the incidence of Alternaria tenuissima is assessed. The data obtained from the study are quantitatively analyzed to determine the effects of the isolated bacterial endophyte.

The disease severity index was expressed as the percentage of the infected fruit to the total inoculated fruit based on lesion appearance, where:

Table 4.1 Classification of lesion proportion.

Disease severity index	Proportion of lesion appearance
0	no appearance
1	less than 25%
2	25–50%
3	50–75%
4	more than 75%

$$Disease\ severity\ index = \sum \frac{number\ of\ the\ fruit\ within\ the\ scale\ \times\ each\ scale}{total\ number\ of\ the\ fruit\ \times\ the\ highest\ scale} \times 100$$

$$\%Disease\ incidence = \frac{number\ of disease\ \text{inf}e\ cted\ fruits}{total\ number\ of fruits\ assessed} \times 100$$

4.2.2 Determination Method for Jujube Quality Index

(1) Preparation of Sucrose Standard Curve (Anthrone Colorimetric Assay).

Analytical-grade sucrose is dried at 80 °C until a constant weight is obtained for constructing the sucrose standard curve. A precise amount of 1.0 g of the dried sucrose is dissolved in small amount of water. Then, the solution was transferred to a 100 mL volumetric flask, to which 0.5 ml of concentrated sulfuric acid is added. In preparing 1% sucrose standard solution, the flask is filled with distilled water to the 100 mLmark. Then, 1 mLof this 1% sucrose standard solution is accurately transferred into another 100 mL volumetric flask and, the flask is filled to the mark with distilled water to obtain a 100 μg/L sucrose standard solution. Next, a series of 11 test tubes were numbered from 0 to 10. Using the proper concentrations, different amounts of the sucrose standard solution and water are added into the test tubes. A 5 ml portion of 20 mg/ml anthrone reagents is added to each test tube and thoroughly mixed. The vials are then placed in boiling hot water for exactly 1 minute. After the heating period, these test tubes are cooled down at room temperature. The absorbance of the solutions is measured at 630 nm and the blank was used as a reference.

(2) Extraction of soluble sugar.

Two jujubes are randomly selected and peeled, and the pulp is cut into pieces. 0.5 g is accurately weighed and put into a test tube. Distilled water is added at a solid-liquid ratio of 1:10 (g:mL). The tube is sealed with plastic film and extracted in boiling water for 30 min. Filtered the extract into a 100 mL volumetric flask with qualitative filter paper, rinsed the test tube residue repeatedly and diluted to the mark. 1 mLof the sample solution is pipetted into a test tube and1 ml of distilled water are put into another test tube to serve as a blank control tube. Added 5 ml of 20 mg/ml anthrone reagent to each test tube, repeated 3 times for each treatment, shook thoroughly, and immediately put the test tube into a boiling water bath,

ensuring that each test tube is kept at temperature for 1 min. After taking it out, cooled it naturally to room temperature, use the blank as a reference, and measure its absorbance value at a wavelength of 630 nm.

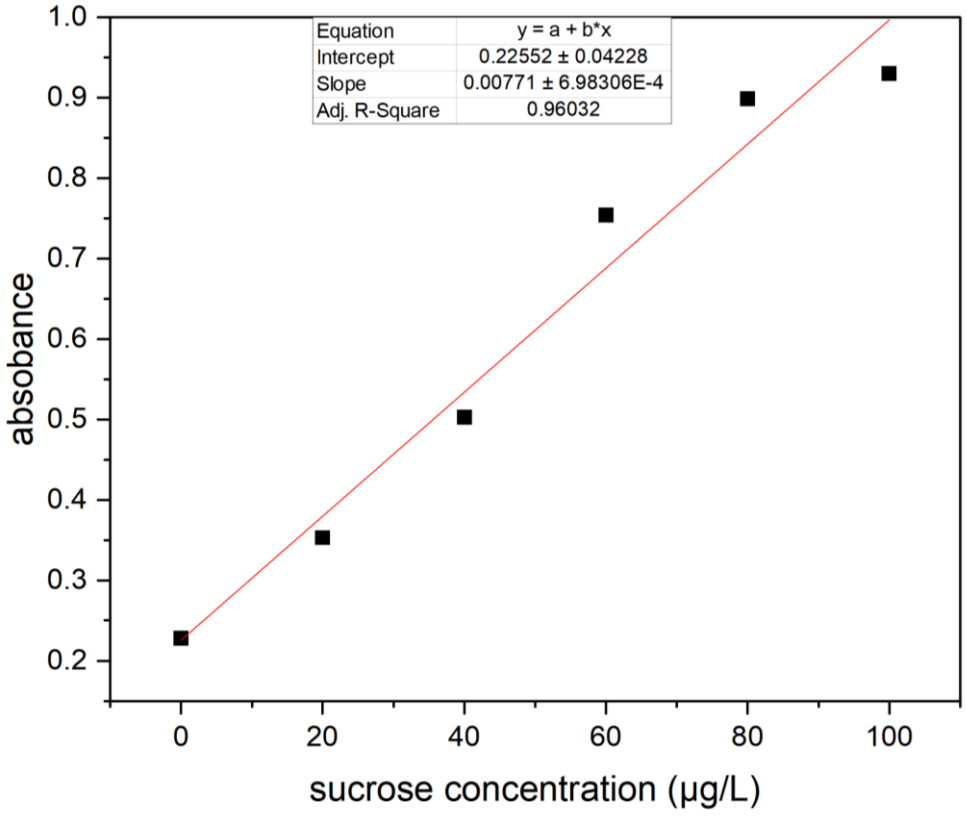

Figure 4.1 standard curve to determine the soluble sugar content

$$\frac{\frac{sugar\ content\ obtained\ from\ the\ regregrssion\ equation(y)}{volume\ of\ the\ absorbed\ sample\ solution} \times extract\ volume \times dilution\ ratio}{sample\ dry\ weight\ \times 10^6} \times 100\%$$

(3) Titratable acid (phenolphthalein indicator method).

Analytically prepared 0.1mol/L of sodium hydroxide and 1% phenolphthalein indicator.

(4) Measurement of Soluble Sugar in Jujube Pulp.

10 g of jujube pulp is ground it with quartz sand to ensure homogenization. The homogenized mixture is transferred to a 100 ml volumetric flask, and the mortar is rinsed with distilled water to transfer all material into the flask. The flask was then filled with distilled water up to the mark and shaken thoroughly. After standing for 30 minutes, the mixture is filtered.A 20 ml aliquot of the filtrate is transferred to a conical flask. To this, 2 drops of the 1% phenolphthalein indicator solution are added. The solution is then titrated with the calibrated sodium hydroxide solution until a faint red color appears that does not fade within 30 seconds. The volume of sodium hydroxide used is recorded. This procedure is repeated three times for accuracy.A blank

control is conducted by titrating with distilled water in place of the filtrate, following the same procedure. According to the consumption of NaOH titration solution, the titratable acid content in jujube is calculated and expressed as mass fraction (%). Calculation formula:

$$\frac{c \times v \times (v_1 - v_0) \times f}{V \times m} \times 100\%$$

Where V is the total volume of sample extract, ml;
The volume of filtrate obtained during titration, ml;
c—— NaOH titrant concentration, mol/L;
V_1 —The volume of NaOH solution consumed by the filtrate was titrated, ml;
V —— The volume of NaOH solution consumed by the distilled water is titrated, ml;
m—sample mass, g; Weight of the sample
f-conversion factor, g/mmol. (Malic acid, 0.067)
convert coefficient

(5) Preparation of Vitamin C Standard Solutions and Absorbance Measurement.

An accurately weighed 0.1 g of ascorbic acid is dissolved in a small amount of distilled water. The solution is then quantitatively transferred into a 100 ml volumetric flask. Distilled water is added to bring the volume to the 100 ml mark, and the solution is shaken thoroughly to ensure complete dissolution. This solution serves as the vitamin C standard stock solution with a concentration of 1.0 mg/L.

To prepare the vitamin C standard solutions, the standard stock solution is first diluted to obtain a 0.1 mg/L concentration of vitamin C. This solution is prepared by pipetting varying volumes of the standard solution into 100 ml volumetric flasks. Specifically, 0, 1, 2, 4, 6, 8, 10, and 12 ml of the 0.1 mg/L vitamin C standard solution are pipetted into separate volumetric flasks. Each flask is then filled to the 100 ml mark with distilled water and shaken thoroughly to ensure uniform mixing. Using distilled water as a reference, the absorbance of each solution is measured at a wavelength of 265 nm. A standard curve is constructed.

(6) Soluble solid content.

The jujube fruits were chopped, and the juice was filtered out. The soluble solids content of the fruits was determined using a handheld refractometer, and the average value was calculated for 3 fruits.

(7) Rotting rate.

The fruits were monitored daily for days to monitor the rate of rotting in the different sample treatments. The formula below was used to determine the rate of rotting in each treatment.

$$\frac{rotten\ fruit\ number}{totalfruit\ number} \times 100$$

(8) Weight loss rate.

The weight of the fruits under the different treatments was measured daily. The weight loss rate was measured using the following formula:

$$\frac{Initial\,weight - final\,weight}{initial\,weight} \times 100$$

4.3 Results and analysis of the in vivo application

4.3.1 The Disease Severity Index (DSI)

Treatment A depicts fruits that were inoculated with only Alternaria tenuissima with a DSI of 70%, demonstrating disease development without any suppression during disease. The second bar, Treatment B, represents the Alternaria tenuissima and bacterial endophyte X27, demonstrating a significantly reduced DSI of 50%.

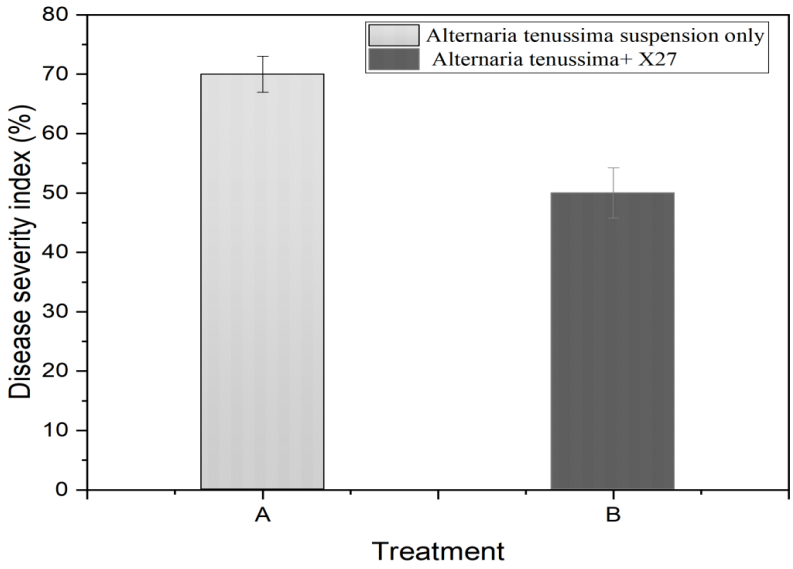

Figure 4.2 The effects of bacterial endophyte X27 on disease severity in jujube fruit infected by pathogenic fungus *Alternaria tenuissima.*

Figure 4.3 In vivo antifungal effect evaluation of isolated bacterial endophyte X27 on jujube fruit. (a) Jujube fruit before in vivo experiment. (b) Control group were treated with *Alternaria tenussima* suspension ($1 \times 10^6\,ml^{-1}$). (c) Treatment group of jujube fruits with *Alternaria tenussima* suspension ($1 \times 10^6\,ml^{-1}$) and bacterial endophyte X27 (d) Untreated group of jujube fruits (control)

After incubation, progressive differences were observed between control and treated jujube fruit. Signs of infection on the fruits were significant shrinkage, wrinkles on the fruit surface and dents on the surface area in single treatments with *Alternaria tenuissima* with dark brown discoloration on the fruit surface area. There was visible microbial growth on the surface; the texture was soft and mushy. In comparison, the control group (untreated) had only a few minor dent marks, along with slightly wrinkled and reddish-brownish skin, and dents around the receptacle of the fruit. Importantly, no visible microbial growth was present at the surface. Finally, fruits co-inoculated with a bacterial endophyte had relatively less dent and were still firm, with reddish-brown around the artificial wound. It is important to note the absence of visible growth of phytopathogens on the surface of treatment.

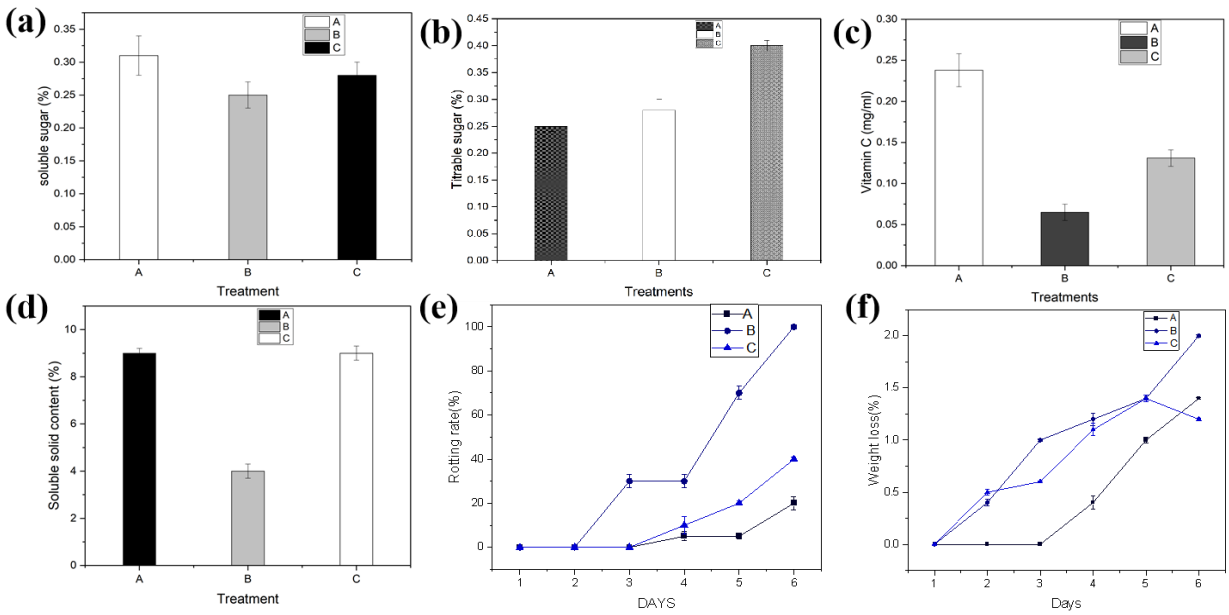

Figure 4.4 Biochemical indexes of jujube fruit under different treatments. (A)Control group (untreated) (B) Treatment group of jujube fruits with *Alternaria tenussima* suspension ($1 \times 10^6\,ml^{-1}$) (C) Treatment group of jujube fruits with *Alternaria tenussima* suspension ($1 \times 10^6\,ml^{-1}$) and bacterial endophyte X27.

4.3.2 Biochemical Analysis and Quality Assessment of Jujube Fruit in Response to Pathogen and Antimicrobial Extract

This study evaluates the biochemical changes in jujube fruit subjected to three conditions: (1) infected by pathogens; (2) no treatment control; (3) infected by pathogens and treated with bacterial X27. The key quality indicator parameters, such as vitamin C levels, titratable sugar, soluble solids content, weight loss, was analyzed.

In Figure 4.4, Treatment B shows a low soluble sugar content of 0.25%. Treatment C improves sugar retention, resulting in a marginally higher percentage of 0.28% as shown in Figure 4.4(a). retains sugar better thus showing a slightly higher percentage of 0.28%. Treatment A showed a sugar solubility percentage of 0.31%. A relatively high titratable acid percentage is observed in treatment C at 0.40% and the lowest is observed in treatment A with a percentage of 0.30 % in Figure 4.4(c). It shows the vitamin C content of jujube as evaluated in treatment A as 0.238 mg/ml and significantly low in treatment B at 0.0649 mg/ml. Treatment C had a vitamin C content of 0.131mg/mL. Soluble solid content is significantly low across all treatments, with both treatment A and C being only 9% and treatment B with only 4%. The weight loss of treated and control jujube fruit showed an increasing trend during incubation. After day 6, treatment B had the highest rotting % of 100%, treatment C had the rotting % of 40% and treatment A had the least rotting % of 20%.

4.3.3 GC-MS analysis of endophytic bacteria X27 compounds

An extract of X27 isolate was analyzed using gas chromatography-mass spectrometry (GC-MS) to identify and quantify the compounds present.The GC-MS examination of the antimicrobial extract revealed a rich array of chemical constituents, many of which are recognized for their antimicrobial efficacy. Among the identified substances, several cyclic dipeptides, heterocyclic compounds, and phenolic derivatives were particularly noteworthy for their documented bioactivity. The analysis, which was conducted under specific GC-MS conditions, revealed approximately 20 distinct compounds, as detailed in Table 4.3. The most abundant compound appears to be at 8.75 min Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), (15.26%).,5-Piperazinedione, 3,6-bis(2-methylpropyl)- (12.1%) at 10.72 min; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (11.99%) at 8.00 min; 2,5-Piperazinedione, 3-(3-methyl)- (5.63%) at 10.93 min. The antifungal activity of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) has been documented in several in vitro studies in *Bacillus* species (Awan et al., 2023). Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) can disrupt fungal cell membranes, interfere with metabolic processes, and inhibit spore germination. Additionally, it has been suggested that this compound acts as a strong antioxidant agent, which can further contribute to its antifungal properties by reducing oxidative stress in fungal cells.Among the first compounds to elute early was benzeneacetic acid, or phenylacetic acid, which is a basic aromatic acid. Research indicates that it can inhibit the growth of bacteria by damaging cell membranes and interfering with crucial metabolic activities(Pan et al., 2022). Thymine, although not inherently antimicrobial, can enhance antibiotic efficacy.

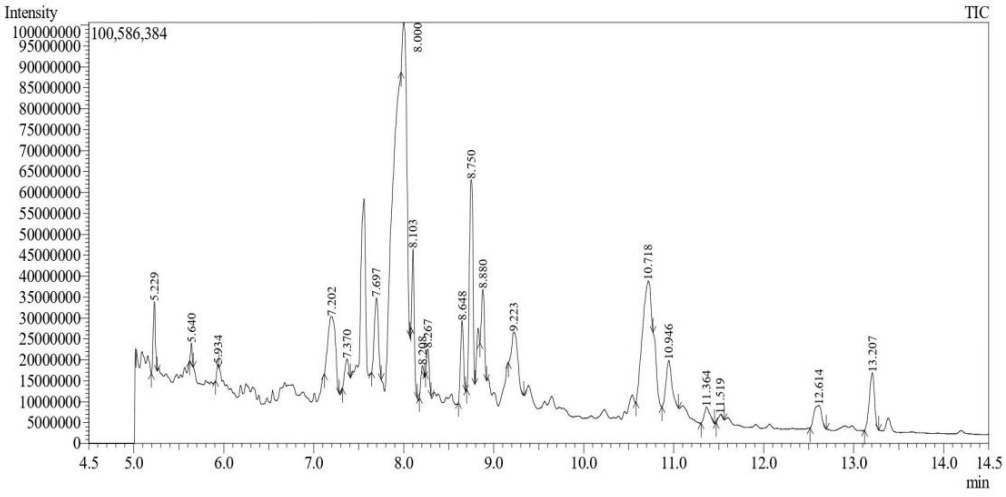

Figure 4.5 Gas chromatography-mass spectrometry analysis of volatile organic compounds (VOCs) produced by bacterial endophyte

Table 4.3 Identification of compounds detected by GC-MS in bacteria X27.

No.	Time (min)	Major constituents	Molecular formula	MW	Peak area (%)
1	5.23	Benzeneacetic acid	$C_8H_8O_2$	136	3.19
2	5.64	4-Amino-2-methoxy-pyrimidine	$C_5H_7N_3O$	125	0.67
3	5.93	7-Cyclohexyl-7-azabicyclo[4.1.0]heptane	$C_{12}H_{21}N$	179	0.32
4	7.19	3-Methyl-2,5-piperazinedione	$C_5H_8N_2O_2$	128	10.26
5	7.37	Thymine	$C_5H_6N_2O_2$	126	1.85
6	7.70	3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	168	6.53
7	8.00	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	11.99
8	8.10	Cyclo(L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$	196	3.89
9	8.21	2,5-Cyclohexadiene-1,4-dione, 2-hydroxy-3,5,6-trimethyl-	$C_9H_{10}O_3$	166	1.17
10	8.27	Cyclo(prolylvalyl)	$C_{10}H_{16}N_2O_2$	196	1.68
11	8.65	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	4.6
12	8.75	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	15.26
13	8.88	Octahydrodipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione-, (5aR,10aR) (isomer 1)	$C_{10}H_{14}N_2O_2$	194	4.04
14	9.23	Lauramide, N-methyl-N-nonyl-	$C_{22}H_{45}NO$	339	4.97
15	10.72	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	$C_{12}H_{22}N_2O_2$	226	12.1
16	10.94	2,5-Piperazinedione, 3-(phenylmethyl)-	$C_{11}H_{12}N_2O_2$	204	5.63

No.	Time (min)	Major constituents	Molecular formula	MW	Peak area (%)
17	11.36	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	1.81
18	11.52	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	0.49
19	13.21	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244	3.57
20	13.38	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	$C_{23}H_{32}O_2$	340	6.1

4.4 Summary

In this chapter, the primary attention is the in-vivo application of bacteria X27 jujube fruit. We evaluate the biological effects of strain X27, including the antibiotic properties. Biochemical index analysis was performed to assess the key physiological and metabolic parameters, providing keen insight into its functional characteristics.he efficacy of the X27 bacterial endophyte in mitigating *Alternaria tenuissima* infection in jujube fruit was evaluated through disease severity assessment and biochemical analysis. Notably, fruits co-inoculated with X27 exhibited a significantly reduced Disease Severity Index (DSI) of 50%, compared to the 70% DSI observed in fruits infected solely with the pathogen, demonstrating the endophyte's suppressive capabilities. Biochemical analysis revealed that X27 treatment (Treatment C) resulted in a marginal improvement in sugar retention and the highest titratable acid content, while significantly impacting vitamin C levels and soluble solid content compared to the pathogen-infected control (Treatment A) and the pathogen-only treatment (Treatment B). Furthermore, X27 treatment reduced fruit rotting significantly compared to the pathogen-only treatment.These results are promising as toward the antagonism research of endophytes and application. To identify the compounds contributing to X27's antifungal activity, a gas chromatography-mass spectrometry (GC-MS) analysis was conducted, revealing the presence of approximately 20 distinct compounds. Among these, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) was the most abundant, a compound known for its antifungal properties through disruption of fungal cell membranes, interference with metabolic processes, and inhibition of spore germination, as well as its antioxidant capacity, suggesting a

multifaceted mechanism for X27's protective effects. The presence emphasize the potential of the identified metabolite as a promising antifungal agent.

5 Conclusion and future outlook

5.1 Conclusion

Various diseases caused by fungi, bacteria, phytoplasma and parasitic plants affect jujube trees and yields. Nearly 30 kinds of jujube diseases, such as rust, anthracnose, white rot, fruit rots, ascochyta spot, fruit shrink, dieback, and witches'-broom, have been known so far. The endophytic microbial community has the ability to influence plant growth and yield, suppress plant- associated pathogens, increase stress tolerance of plants, solubilize phosphate, contribute nitrogen to plants, which is an essential nutrient for many important functions, such as proper growth and development of crops, and thus enhance their ability to withstand the environmental stresses. Overall, endophytes play a crucial role in enhancing plant health, promoting sustainable agriculture, and providing novel biotechnological opportunities. The obtained results from this investigation could be summarized as follows:

Through microbiological techniques, endophytic microorganisms were isolated from jujube fruit with one bacterial isolate, X27, showing a significant inhibitory effect against pathogenic microorganisms *Escherichia coli* and *Staphylococcus aureus and,* most importantly, *Alternaria tenuissima*. Optimization of fermentation conditions for the endophytic bacteria with the high antimicrobial property was performed and the optimal conditions determined were a glucose concentration of 18 g /L, pH 7.4, an agitation speed of 149 rpm, and inoculum size of 9.4% (v/v). This resulted in an overall inhibition rate of 61.7% against *Alternaria tenuissima*. Invivo experiments were carried out and demonstrated a disease severity index of 50% in fruits inoculated with the phytopathogen and endophytic bacteria as compared to the control that had a disease severity index of 100%. The most abundant compounds at 8.75 min Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), (15.26%).,5-Piperazinedione, 3,6-bis(2-methylpropyl)- (12.1%) at 10.72 min; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (11.99%) at 8.00 min; 2,5-Piperazinedione, 3-(3-methyl)- (5.63%) at 10.93 min. In total, endophytic bacterium X27 presents a set of untapped novel agents that have the potential to replace the overuse of synthetic chemicals and enhance plant health and productivity. This study underlines the significance of endophytic microorganisms from jujube fruit as valuable resources for improving the antifungal activity of natural products.

5.2 Future outlook

At the end, by considering the great potential of endophytes, the road map for future research can be designed. The outlook for the use of endophytes is highly promising, with significant

potential in agriculture, environmental sustainability, and medicine; however, there are areas for further improvement and exploration.

Exploration of alternative applications: future research should extend beyond antimicrobial properties to investigate their potential in the field of bioremediation and biopharmaceutical production. The investigations could help harness the full potential of endophytes in agriculture, medicine, and environmental sustainability.

Detailed mechanistic research, encompassing omics analysis and gene expression profiling, can shed light on the fundamental processes that contribute to the observed antimicrobial effects. A deeper understanding of the molecular underpinnings of antimicrobial activity could facilitate the development of targeted strategies to enhance effectiveness and combat resistance mechanisms.

The understanding of the plant-microbe interaction should be given primary importance because, by knowing this interaction better, it could one day lead to the development of crop plants that can interact with endophytes/beneficial microbes more efficiently. Mechanisms backing up the ways of distribution are not clear because endophyte species differ from one plant to the next; they are still a novel field to be explored.

A better understanding of functions encoded by endophytic genomes could help us to have insight into the mechanisms involved in plant-microbe interactions and establish genomic determinants of endophyte lifestyle. Future work could be integrated with screening, testing antagonistic ability in greenhouse and field trials, coupled with biomass production, following the commercialization of biocontrol agents that are ultimately essential to ensure global agricultural security. Eventually, we can move towards achieving our goals of sustainable agriculture.

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