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# Standardized Protocol for Isolation and Cryopreservation of Cultivable Endophytes From Fresh and Dried *Citrus aurantium* Peels

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## ABSTRACT

Citrus peels, particularly those of *Citrus aurantium*, are agro-industrial and medicinal byproducts that harbor specialized endophytic communities with potential roles in fruit aroma, quality, and biocontrol. However, research studies on these communities have been limited by the lack of standardized, reproducible protocols for recovering cultivable endophytes from both fresh peel and processed materials such as "Zhike," where desiccation creates a stringent selective environment. Here, we present a systematic workflow for isolating, molecularly identifying, and cryopreserving endophytic microorganisms from *Citrus aurantium* peel collected as fresh fruit and dried "Zhike" across production regions. The protocol integrates surface sterilization regimens tailored to distinct physical properties of fresh versus dried tissues, sterility validation, tissue homogenization, and multi-medium cultivation under nutrient-rich and low-nutrient conditions to recover diverse bacterial and fungal isolates. Molecular identification based on 16S rRNA gene and ITS region sequencing resolves taxonomic identities and supports construction of a cryopreserved strain collection representing cultivable citrus peel endophytes. By standardizing key steps from sample preparation through long-term storage, this protocol facilitates studies of endophytes from stressed, low-moisture peel matrices and establishes a microbial resource library for future investigations into plant–microbe–metabolite interactions, bio-aroma (biologically derived aroma compounds) enhancement, endophyte-based biocontrol, and sustainable valorization of citrus processing byproducts.

## 1 | Introduction

Citrus (*Citrus* spp.) is one of the most economically important fruit crops worldwide, contributing essential nutrients, antioxidants, and distinctive aromatic traits that drive both consumer preference and industrial utilization (Liang et al. 2024). Beyond fresh consumption, citrus processing yields large quantities of peel as an agro-industrial byproduct, which is increasingly recognized as a valuable resource rather than waste (Tocmo et al. 2020). Citrus peels are rich in essential oils and phenolic metabolites, including terpenoids, such as limonene, linalool, citral, and nerol, which contribute to the characteristic aroma and functional properties of citrus-derived foods, nutraceuticals, and traditional herbal preparations (Saini et al. 2022). For instance, the dried immature fruit of *Citrus aurantium* (Zhike) is widely used in traditional Chinese medicine for digestive and cardiovascular indications, linking citrus peel chemistry directly to human health and cultural practices (Gao et al. 2022). At the same time, the citrus supply chain faces escalating challenges from biotic stresses, such as Huanglongbing (HLB) and

postharvest pathogens, as well as abiotic stresses that affect fruit development, peel quality, and aroma stability (Munir et al. 2021). These pressures drive the search for sustainable strategies that enhance citrus resilience and value while promoting circular use of peel biomass.

Concurrently, citrus plants are now increasingly viewed as a "holobiont," comprising both the host and its associated microbiota, whose collective genome and metabolism shape plant fitness and product quality (Faddetta et al. 2021). Within this framework, endophytic microorganisms that inhabit internal plant tissues without causing disease have attracted attention as key drivers of nutrient acquisition, growth promotion, disease suppression, and secondary metabolism modulation (Duan et al. 2024). In fruits, endophytes have emerged as promising biological tools for managing postharvest decay and maintaining quality traits, offering low-residue environmentally compatible alternatives to synthetic chemicals (Verma et al. 2022). However, their roles in determining complex organoleptic attributes, such as aroma, are only beginning to be explored.

A growing body of evidence has shown that endophytes can influence volatile organic compound (VOC) profiles that underlie fruit aroma. In strawberry, inoculation with selected endophytic fungi or bacteria altered the production of key esters and terpenes in ripe fruits, even under water deficit, suggesting that microbial partners can reshape aroma formation under stress (Rodríguez-Arriaza et al. 2025). In grape, comparative analyses across vineyard sites revealed that variation in endophytic community structure correlated with differences in berry VOCs, and specific bacterial taxa were positively associated with free aroma compounds (Ren et al. 2024). Similar interactions have been documented in other high-value crops, such as agarwood, where the endophytic fungus *Phaeoacremonium rubrigenum* promoted the accumulation of sesquiterpenoids by modulating host phosphorylation cascades (Liu et al. 2022). Together, these studies suggest that endophytes function as "metabolic co-designers" of fruit quality, with profound relevance for horticulture, food science, and flavor chemistry.

Citrus peel presents a particularly intriguing niche for endophytic communities. The outer cuticle forms a dense hydrophobic barrier that restricts microbial ingress, whereas the inner peel is enriched in VOCs, sugars, and polyphenols, creating selective microhabitats for adapted endophytes (Cajuste et al. 2010; Trivedi et al. 2020). Recent work on the citrus microbiome has focused mainly on roots, leaves, and phloem, particularly in the context of HLB (Blaustein et al. 2017; Trivedi et al. 2012), revealing a diverse citrus-adapted microbiota with promising biocontrol and probiotic potential (Xu et al. 2018; Y. Z. Zhang et al. 2021). However, the endophytic communities of citrus peel—particularly those of processed materials like dried medicinal peels—remain underexplored (Nicoletti 2019). Preliminary studies have isolated bacterial endophytes from citrus peel, including *Bacillus subtilis* and *Pseudomonas* spp., and genomic analyses have shown that these genera harbor the genetic potential to synthesize terpenoids through methylerythritol phosphate (MEP) and mevalonate (MVA) pathways, as well as noncanonical terpenoid gene clusters (Hess et al. 2013; Munir et al. 2020). These microbial metabolites structurally resemble key citrus aroma constituents, suggesting that citrus peel endophytes may act as metabolic co-designers that influence or diversify aroma profiles (Ling et al. 2019; Wang et al. 2023).

Despite these insights, several critical knowledge gaps persist. First, the diversity, cultivability, and functional potential of endophytes inhabiting citrus peel across different species, developmental stages, processing conditions, and geographic regions are still poorly understood (Solanki et al. 2024). The drying process of "Zhike" imposes significant desiccation stress, which likely acts as a selective filter for resilient microbial communities. This is consistent with findings that drying conditions significantly impact the chemical composition and quality of medicinal plants (Blumberg et al. 2025; Ghanem et al. 2012). Additionally, the subsequent storage of dried peels may further reshape the endophytic communities in ways that impact both aroma and bioactivity (Zhu et al. 2025). Second, the lack of standardized reproducible protocols for efficiently isolating and preserving citrus peel endophytes hampers cross-study comparisons and limits the construction of robust microbial resource libraries (Hardoim et al. 2015). Third, although genomic data suggest that bacteria possess biosynthetic gene clusters for terpenoid production (Hu et al. 2023), direct experimental validation linking specific citrus endophytes to volatile modulation remains limited (Pang et al. 2021) This knowledge gap hinders the development of microbiome-based flavor management strategies (X. Zhang et al. 2025).

Addressing these gaps is crucial not only for basic understanding of plant–microbe–metabolite interactions but also for multiple applied domains. A well-characterized citrus peel endophytic microbiome offers multiple translational opportunities across production, processing, and health-related applications. By enabling microbiome-informed cultivation and postharvest management, it may help enhance the consistency of citrus flavor and aroma. In addition, citrus byproducts can be valorized by coupling tailored microbial consortia with green extraction strategies to increase the yield of high-value volatile compounds (Mahato et al. 2018). Such endophytes may also be developed into biocontrol agents that protect fruit while maintaining sensory quality (Moraes Bazioli et al. 2019). Finally, a deeper understanding of peel-associated endophytes can support the rational improvement, quality control, and standardization of traditional medicinal peels. However, progress in both areas depends on establishing well-curated cultivable microbial resources first.

In this context, the present study focuses on the systematic isolation and characterization of endophytic microorganisms from citrus peel, encompassing both fresh fruits and dried "Zhike" collected from multiple production regions. To bridge the gap between metagenomic potential and functional application, we have established an efficient and reproducible workflow that integrates optimized disinfection procedures, tissue homogenization strategies, multi-medium culture, and molecular identification. Unlike conventional isolation methods that often rely on generic sterilization and single-medium cultivation, our protocol introduces specific innovations to enhance efficiency and recovery rates. Specifically, the tailored surface sterilization—incorporating surfactants to penetrate the hydrophobic citrus cuticle—improves isolation effectiveness by minimizing contamination in both fresh and dried tissues.

Furthermore, the multi-medium approach (spanning nutrient-rich to oligotrophic conditions) captures a broader diversity of taxa, including slow-growing species often missed by standard protocols, whereas the integrated molecular sequencing ensures high identification accuracy. This enables the robust construction of a cultivable citrus peel endophyte resource library. Although this workflow was optimized for citrus peel (fresh bitter orange peel and dried immature "Zhike"), the same principles can be extended to other citrus tissues (e.g., seeds, leaves, and flowers) with minor, tissue-specific adjustments. We therefore include practical guidance for tissue-dependent optimization while retaining sterility verification to confirm the endophytic origin of isolates. By providing a standardized protocol and a representative set of bacterial and fungal isolates, this work lays a crucial methodological foundation for advancing citrus microbiome research. More broadly, this resource will support future investigations into plant-microbe metabolic interactions that shape citrus aroma formation. Such knowledge will not only improve fundamental understanding of fruit flavor biology but also enable the development of innovative microbial solutions—such as "bio-aroma enhancement" technologies—to improve citrus flavor and promote sustainable citrus-peel valorization.

In practical terms, our workflow is optimized for speed and reproducibility while maintaining strict endophyte verification. Colonies typically appear within 24–48 h under the incubation conditions used here, and routine purification by repeated streaking can be completed within 3–5 d from first colony appearance (longer for slow-growing isolates). Standardized marker-based identification (16S rRNA for bacteria; ITS for fungi/yeasts), together with unified glycerol cryopreservation, provides a predictable route from citrus tissue to a verified cryopreserved strain library, improving cross-laboratory reproducibility and downstream screening readiness.

## 2 | Isolation and Identification of Endophytic Bacteria and Yeasts

### 2.1 | Sample Preparation and Surface Sterilization

This step is crucial for eliminating epiphytic microorganisms and ensuring that only true endophytes present within internal tissues are isolated. Different protocols were applied for fresh and dried samples to accommodate their distinct states (Figure 1).

#### 2.1.1 | Preparation of Fresh Samples

Healthy, undamaged fresh bitter orange fruits were selected and thoroughly rinsed under sterile water to remove surface debris. In a laminar flow hood, the fruits were washed three times with sterile distilled water and then immersed in 1% (*v/v*) sodium hypochlorite (NaClO) solution for 5–10 min (duration adjusted according to peel thickness), with continuous agitation to ensure direct and thorough contact between the disinfectant and surface microorganisms. Subsequently, the fruits were transferred to 70% (*v/v*) ethanol for 1–2 min (duration adjusted according to sample condition), followed by three rinses with sterile distilled water, each for 1 min.

Sterility check: To verify sterilization efficacy, 100 μL of the final rinse solution was spread onto tryptic soy agar (TSA) plates and incubated at 28°C for 48 h. Plates without microbial growth were taken as evidence of effective surface sterilization; if colonies appeared, fresh samples were used and the surface sterilization process was repeated.

Excess surface moisture was blotted dry with sterile filter paper or removed by air-drying in the laminar flow hood. Sterilized fruits were then aseptically peeled using a sterile scalpel, and the peel was cut into small pieces for subsequent homogenization.

#### 2.1.2 | Preparation and Surface Sterilization of Dried Samples

Dried immature fruits of citrus "Zhike" were selected as the dried peel material. To minimize contamination and ensure that only true endophytes were recovered, a combined ethanol–sodium hypochlorite-surfactant protocol was used for surface sterilization instead of mercuric chloride, considering both biosafety and environmental concerns. All procedures were performed in a laminar flow hood. The dried fruit peels were first cut into small pieces (approximately 0.5–1.0 $cm^2$) using sterile scissors and placed in sterile Petri dishes. To improve wetting of the hydrophobic peel surface and facilitate penetration of the disinfectants into surface irregularities, the peel pieces were pre-rinsed in sterile distilled water containing 0.1% (*v/v*) Tween-20 with gentle agitation (120 rpm) for 5–10 min, and then the solution was discarded. Subsequently, 20 mL of 70%–75% (*v/v*) ethanol was added to each dish, and the tissues were immersed for 30–60 s with occasional gentle shaking. The ethanol was discarded, and 20 mL of sodium hypochlorite (NaClO) solution containing 1%–2% (*v/v*) available chlorine and 0.05%–0.10% (*v/v*) Tween-20 was added. The peel pieces were immersed in this solution for 3–5 min with continuous gentle agitation to ensure thorough contact between the disinfectant and the peel surface. For thicker or more heavily contaminated samples, the upper end of the above ranges (2% available chlorine, 0.10% Tween-20, 5 min) was applied. After NaClO treatment, the peels were subjected to an optional quick rinse in 70% (*v/v*) ethanol for 10–30 s to facilitate the removal and inactivation of residual chlorine. Finally, the tissues were rinsed four to five times with sterile distilled water, each rinse lasting 1–2 min, to completely remove residual disinfectants and surfactant. Sterility check: To verify the effectiveness of surface sterilization, 100 μL of the final rinse solution was spread onto TSA plates and incubated at 28°C for 48 h. Plates without microbial growth were taken as evidence of successful surface sterilization; if colonies appeared, new samples were used and the surface sterilization procedure was repeated with adjusted treatment time or NaClO concentration. After sterilization, excess surface moisture was blotted dry with sterile filter paper or removed by air-drying in the laminar flow hood, and the peel pieces were then prepared for homogenization.


**FIGURE 1** | Citrus samples used for endophyte isolation. (A) Fresh *Citrus aurantium* (bitter orange); (B) Dried "Zhike."

#### 2.1.3 | Applicability to Other Citrus Tissues and Parameter Optimization

The workflow can be extended to other citrus tissues with tissue-specific optimization of sterilization intensity and disruption conditions to balance contamination control and endophyte recovery. In particular, the surface-sterilization intensity (disinfectant concentration and exposure time) should be adjusted according to tissue thickness and permeability, and according to tissue chemistry (e.g., sugar content, essential oils/phenolics, and endogenous antimicrobial compounds) that may affect both microbial survival and contamination risk. Likewise, tissue-disruption conditions should be optimized to achieve sufficient release of endophytes without excessive cell damage.

Importantly, sterility verification should be retained to confirm the endophytic origin of isolates; if sterility controls show growth, repeat sterilization by adjusting treatment time, NaClO concentration, or both.

### 2.2 | Tissue Homogenization and Inoculum Preparation

The blender jar of a high-speed tissue homogenizer was sterilized by rinsing three times with 70% (*v/v*) ethanol, followed by immersion in excess 70% ethanol, and then air-dried in the laminar flow hood prior to use.

Prepared citrus peel pieces were transferred to the sterilized blender jar. Sterile phosphate-buffered saline (PBS, pH 7.4) or sterile physiological saline was added at a ratio of 20 mL solution per 1 g of citrus peel (*v:w* = 20:1) to prepare a dilution. The mixture was homogenized for 1–2 min until a uniform slurry was obtained, taking care not to over-homogenize in order to avoid cell damage and potential bias in the experimental results. The slurry was then allowed to stand at room temperature for 5–10 min to permit the settling of larger debris. After sedimentation, the supernatant (homogenate supernatant) was carefully transferred into 50 mL sterile centrifuge tubes for temporary storage on ice or at 4°C.

After each use, the homogenizer jar was immersed in 70% (*v/v*) ethanol for at least 10 min to ensure thorough disinfection and prevent cross-contamination between samples.

### 2.3 | Isolation of Endophytic Bacteria and Yeasts

#### 2.3.1 | Preparation of Culture Media

To maximize the recovery of cultivable endophytic microorganisms with diverse nutritional requirements and growth rates, the following five solid media were prepared according to the manufacturer's instructions, autoclaved at 121°C for 15 min, and poured into 90 mm sterile Petri dishes after cooling to approximately 50°C–55°C. The formulations of all culture media used in this study are provided in Table 1.

#### 2.3.2 | Inoculation and Incubation

For each sample, a 100 μL aliquot of the prepared homogenate supernatant was pipetted onto the surface of each of the five solid media. Using a sterile L-shaped spreader, the inoculum was evenly distributed across the plate surface to facilitate the

**TABLE 1** | Formulations and purpose of solid media used for isolation of endophytic bacteria, yeasts, and fungi.

| Medium | Key carbon source(s) | Key nitrogen source(s) | Inorganic salts | Agar (g/L) | Final pH (25°C) | Target microorganisms |
|---|---|---|---|---|---|---|
| Tryptic Soy Agar (TSA) | Tryptone, Soytone | Casein peptone, 15 g/L Soybean peptone, 5 g/L | NaCl, 5 g/L | 15 | 7.3 ± 0.2 | Broad-spectrum, general-purpose medium for cultivating diverse bacteria |
| Yeast Extract Peptone Dextrose Agar (YPD) | Dextrose, 20 g/L | Soy peptone, 10 g/L Yeast extract, 5 g/L | — | 14 | 5.8 ± 0.2 | Nutrient-rich medium favoring yeasts and fast-growing fungi |
| 10% TSA | Tryptone, Soytone | Casein peptone, 1.5 g/L Soybean peptone, 0.5 g/L | NaCl, 0.5 g/L | 15 | 7.3 ± 0.2 | Low-nutrient medium favoring slow-growing/oligotrophic bacteria and reducing dominance of fast growers |
| Luria–Bertani (LB) Agar | Tryptone, yeast extract | Tryptone, 10 g/L Yeast extract, 5 g/L | NaCl, 5 g/L | 12 | 7.0 ± 0.2 | Standard medium for fast-growing bacteria (e.g., *E. coli*) and rapid colony development |
| Nutrient Agar (NA) | Peptone, beef extract powder | Peptone, 10 g/L Beef extract, 3 g/L | NaCl, 5 g/L | 15 | 7.0 ± 0.2 | Mild, balanced medium for isolation, purification, cultivation, and enumeration of nonfastidious bacteria |

formation of well-isolated colonies. At least three replicate plates were prepared for each sample–medium combination to enhance the diversity and reliability of recovered endophytic strains.

Plates were sealed with Parafilm, inverted, and incubated at 28°C in the dark for 24–48 h. Plates were examined daily for at least two consecutive days, and colonies with distinct morphological characteristics (e.g., differences in color, shape, size, edge, and texture) were marked and recorded. If no colonies were observed after 48 h, the incubation period was extended as necessary to accommodate slow-growing microorganisms and avoid overlooking rare or slow-growing endophytes.

## 2.4 | Purification and Preservation of Strains

### 2.4.1 | Purification

From the initial isolation plates, single colonies exhibiting clear differences in macroscopic morphology (e.g., variations in color, shape, edge, and texture) were selected using a sterile inoculating loop to cover as many potentially distinct microbial groups as possible. Selected colonies were streaked onto fresh plates of the corresponding medium using the quadrant streaking method and incubated at 28°C, a temperature widely used for cultivating plant-associated and endophytic microorganisms, as it supports robust growth of diverse mesophilic bacteria, yeasts, and fungi while minimizing thermal stress and avoiding selective bias toward fast-growing thermotolerant strains, until well-isolated colonies developed (Figure 2). The streaking process was repeated as necessary until pure cultures displaying uniform colony morphology were obtained.

For strains with high mucilage production or spreading growth patterns, simple streaking was sometimes insufficient for colony separation. In such cases, a combination of streak plating and serial dilution plating was employed to achieve successful purification.

### 2.4.2 | Scale-Up Cultivation

Pure single colonies confirmed by streak plating were used to inoculate liquid culture for biomass accumulation: individual bacterial colonies were aseptically transferred into 10 mL of tryptic soy broth (TSB) and single yeast or fungal colonies were transferred into 10 mL of potato dextrose broth (PDB). Cultures were incubated at 28°C on a rotary shaker set to 180–200 rpm; incubation times were adjusted for each isolate and typically ranged from 24 to 72 h, with growth monitored visually and by optical density ($OD_{600}$) measurements. Cultivation was continued until cultures reached robust growth (mid- to late-exponential phase), at which point aliquots were harvested for downstream DNA extraction, phenotypic assays, or cryopreservation as described below.

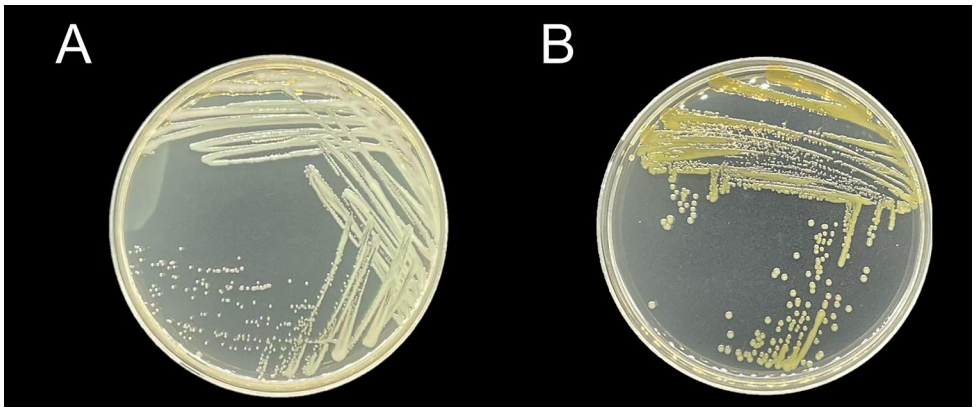


**FIGURE 2** | Isolation of single colonies via streak plating: (A) *Metabacillus* sp.; (B) *Pantoea* sp.

#### 2.4.3 | Long-Term Preservation

For long-term storage, 700 μL of liquid culture harvested when cells had reached mid-exponential phase ($OD_{600} \approx 0.6–0.8$) was mixed with 700 μL of sterile 50% (*v/v*) glycerol in a sterile cryovial, yielding a final glycerol concentration of 25% (*v/v*). Cultures were monitored by $OD_{600}$ to determine growth phase; for some slow-growing strains aliquots harvested at the early stationary phase ($OD_{600}$ up to ≈ 1.0) were used when necessary, but mid-exponential phase ($OD_{600}$ 0.6–0.8) was the standard to ensure high post-thaw viability and reproducibility. The suspension was briefly vortexed or gently pipetted up and down 3–5 times to ensure thorough mixing while minimizing bubble formation. Glycerol stocks were stored at −80°C and repeated freeze-thaw cycles were avoided to prevent loss of viability.

### 2.5 | Molecular Identification of Endophytic Bacteria

#### 2.5.1 | Genomic DNA Extraction From Bacterial Isolates

Well-preserved single bacterial colonies were inoculated into appropriate liquid media and cultured overnight. An aliquot of approximately 1–5 mL of bacterial culture (cell density not exceeding $1 \times 10^9$ cells) was harvested by centrifugation at 12,000 rpm to remove the supernatant. Genomic DNA was extracted using the TSINGKE Bacterial Genomic DNA Extraction Kit according to the manufacturer's instructions, with minor modifications.

Briefly, the bacterial pellet was resuspended in 200 μL Buffer GA. When removal of residual RNA was required, RNase A was added at this step and incubated at room temperature. Subsequently, 20 μL of proteinase K and 220 μL of buffer GB were added, and the mixture was incubated at 56°C for 10–15 min to ensure complete cell lysis. After treatment with absolute ethanol, the lysate was transferred to a spin column, and DNA was allowed to bind to the silica membrane by centrifugation at 12,000 rpm.

The column was then washed sequentially with Wash Buffer A and Wash Buffer B to remove proteins and salt contaminants. After drying the membrane to eliminate residual ethanol, genomic DNA was eluted with 50–100 μL of preheated TE buffer or sterile water and collected by centrifugation. The purified DNA was stored at −20°C until further use.

#### 2.5.2 | 16S rRNA Gene Amplification and Sequencing

The nearly full-length 16S rRNA gene was amplified from the extracted bacterial genomic DNA using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reactions were performed in a total volume of 40 μL containing 20 μL of 2 × T8 High-Fidelity Master Mix, 2 μL of each primer (10 μM), 1 μL of DNA template, and 15 μL of nuclease-free water.

The amplification program was as follows: initial denaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 57°C for 10 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min.

PCR products were examined by agarose gel electrophoresis (300 V, 12 min) to confirm the presence of a single band of the expected size and the absence of nonspecific amplification. Target bands were excised and purified using a commercial DNA gel extraction kit. Purified amplicons (together with the corresponding primers) were submitted to Tsingke (Qingke) Biotechnology Co. Ltd. for commercial Sanger sequencing.

Raw chromatogram data were inspected and quality-checked using Sequencing Analysis 5.2. High-quality consensus sequences were compared against the NCBI nucleotide database using BLAST to infer the taxonomic affiliation of each bacterial isolate. As a representative example of BLAST-based identification and sequence alignment, the isolate *Pantoea eucrina* C-2-1 is shown in Figure 3. The high-quality 16S rRNA consensus sequence was queried against the NCBI nucleotide database using BLAST, and closely related reference sequences (with accession numbers) were selected from the top hits for multiple-sequence alignment. The aligned sequences were then used to infer a 16S rRNA gene phylogeny to confirm the taxonomic placement of the isolate. This example illustrates our criteria for selecting representative BLAST hits and generating a multiple-sequence alignment for taxonomic confirmation.

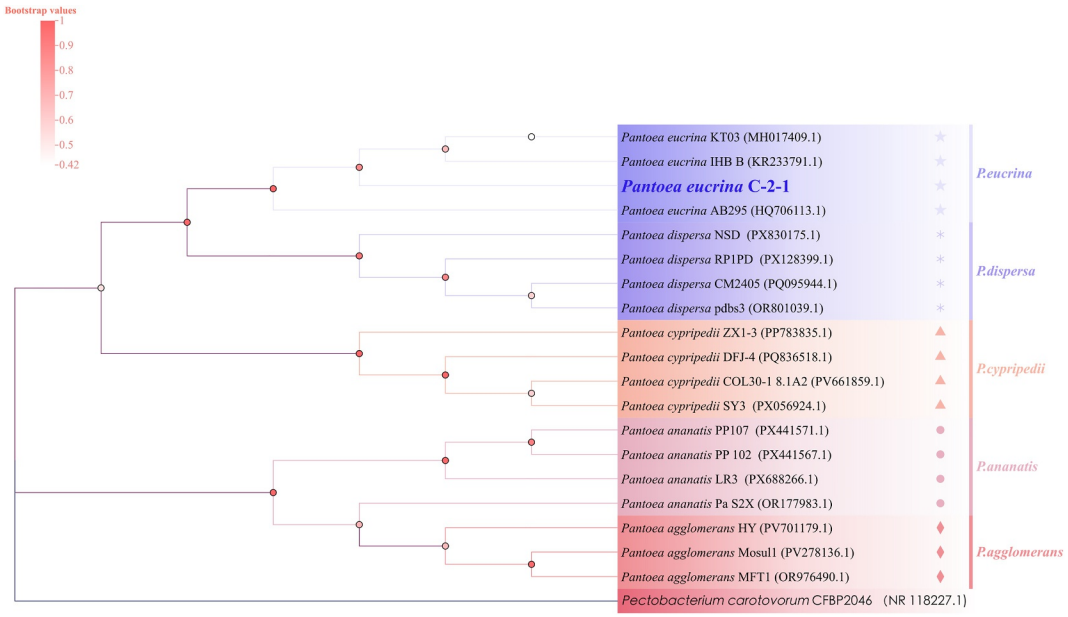


**FIGURE 3** | Representative BLAST-based identification and 16S rRNA gene phylogenetic placement of isolate *Pantoea eucrina* C-2-1. The isolate sequence is highlighted, and reference sequences retrieved from NCBI (accession numbers shown) were selected from the closest BLAST hits for multiple-sequence alignment and phylogenetic inference. Node labels indicate bootstrap support values.

## 2.6 | Molecular Identification of Endophytic Fungi and Yeasts

### 2.6.1 | Genomic DNA Extraction From Fungal and Yeast Isolates

Genomic DNA from filamentous fungi and yeasts was extracted using the TSINGKE Universal Plant DNA Extraction Kit, following the manufacturer's protocol with minor adaptations. Approximately 20 mg of dried mycelium or yeast pellet was ground thoroughly in liquid nitrogen to a fine powder and transferred to a 1.5 mL microcentrifuge tube. A volume of 400 μL Buffer gP1 was added, and the suspension was vortexed vigorously, followed by lysis at 65°C for 10–30 min with intermittent mixing.

Subsequently, 150 μL Buffer gP2 was added, and the mixture was incubated on ice to precipitate proteins and polysaccharide impurities. After centrifugation, the clear supernatant was transferred to a new tube and mixed with an equal volume of absolute ethanol. The mixture was then loaded onto a spin column and centrifuged to allow DNA binding to the membrane. The column was washed successively with Buffer Pw and Wash Buffer to remove residual contaminants.

Following high-speed centrifugation to eliminate residual ethanol, genomic DNA was eluted from the membrane with preheated TE buffer and collected by centrifugation. The resulting high-purity DNA was stored at −20°C for subsequent PCR amplification.

### 2.6.2 | ITS Region Amplification and Sequencing

The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCG-CTTATTGATATGC-3′). PCR reaction components and cycling conditions followed those described for the bacterial 16S rRNA gene amplification in Section 2.5.2, with the annealing temperature and extension time maintained or slightly adjusted as needed according to primer properties and expected amplicon length (approximately 750 bp).

PCR products were analyzed by agarose gel electrophoresis to confirm the presence of single, correctly sized bands. Qualified products were excised, purified using a DNA gel extraction kit, and subjected to Sanger sequencing using the standard dideoxy chain-termination method. The resulting sequences were assembled and manually checked, then submitted to BLAST searches against the NCBI GenBank database to determine the genus- and, where possible, species-level identities of the fungal and yeast isolates. To present the process more clearly, we have drawn a complete process flow chart (Figure 4).

## 3 | Conclusion

In this protocol, we describe a step-by-step workflow for isolating, identifying, and preserving culturable endophytic microorganisms from citrus peel materials, including both fresh fruit peel and processed herbal peel. The procedure integrates practical guidance on sample handling, surface sterilization and sterility validation, selection of cultivation media, preliminary taxonomic identification, and long-term cryopreservation of isolates.

Using this workflow, we were able to recover viable bacterial and fungal endophytes from citrus peel under the tested conditions and to establish an initial working collection of citrus peel-associated strains. This isolate collection, together with the detailed methodological framework provided here,

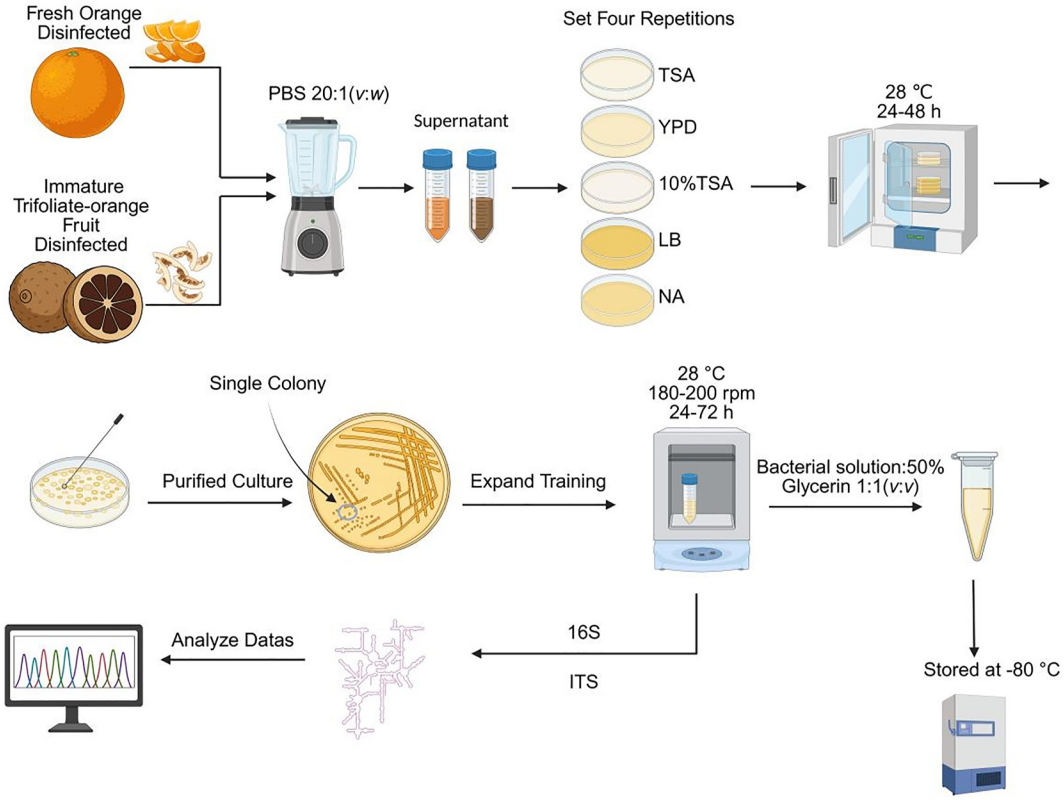


**FIGURE 4** | Flowchart for separating bacteria and fungi.

offers a practical entry point for researchers who wish to obtain and maintain citrus peel endophytes in a reproducible manner.

Although the present work does not evaluate functional traits of these isolates or directly address how endophytes influence citrus peel aroma, nutritional quality, or traditional medicinal properties, the protocol is designed to facilitate such future investigations. For example, individual strains or defined consortia obtained using this method could be subjected to downstream assays, including volatile and nonvolatile metabolite profiling, genome-based functional prediction, or bioactivity testing in food and medicinal contexts.

Overall, this protocol provides an accessible and adaptable methodological foundation for studying culturable citrus peel endophytes. By standardizing critical steps in isolation, verification, and preservation, it aims to support more comparable and experimentally grounded studies on the ecological roles and biotechnological potential of citrus peel-associated microbial communities.

### Author Contributions

**Hujing Cao:** methodology, investigation, data curation, writing – original draft. **Xinlin Huang:** methodology, validation, writing – original draft. **Han Yang:** conceptualization, writing – review and editing, supervision. **Manxi Wu:** data curation, visualization, validation. **Jingping Cao:** resources, formal analysis. **Chongde Sun:** supervision, resources. **Yue Wang:** conceptualization, supervision, funding acquisition.


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### Ethics Statement

The authors have nothing to report.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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