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SIMPLE PRESERVATION OF *Paenibacillus alvei* (M312) AS BIOLOGICAL NITROGEN-FIXING INOCULANT FOR OIL PALM

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ABSTRACT

Biofertilisers are increasingly preferred in the oil palm industry due to the fluctuating prices of conventional fertilisers and the need to support sustainable agriculture. *Paenibacillus alvei* M312, an endophytic bacterium capable of biological nitrogen fixation, has been proven to enhance the growth of oil palm seedlings during the nursery stage. However, using fresh culture broth for field applications is impractical due to handling challenges, risk of contamination and shorter shelf life. This study aims to develop a suitable formulation for the M312 liquid inoculant to ensure the bacteria remain viable and can be effectively delivered to the target plants. Four additives such as Polyvinyl Pyrrolidone (PVP), Polyvinyl Alcohol (PVA), Polyethylene Glycol (PEG) and gum Arabic, were tested for their effectiveness in maintaining the viability of the M312 in a liquid formulation. Gum Arabic exhibited the most effective performance in maintaining M312 viability. The M312 liquid inoculant formulated with gum Arabic was stored at various locations under temperature conditions ranging from 3°C to 41°C. After 12 months of storage, the M312 liquid inoculant stored at temperatures between 23°C to 25°C remained viable, with a concentration of 4.8×10^6 CFU mL^{-1} (log_{10}: 6.68). These preliminary findings highlight the potential of gum Arabic to preserve the M312 liquid inoculant and offer guidelines for the future development of commercial nitrogen-fixing inoculants.

Introduction

Nitrogen (N) is an essential macronutrient required for optimal plant growth and development. It is primarily absorbed by plants in the form of soluble nitrate ions (Ikhajiagbe *et al.*, 2022). Nitrogen plays a vital role in several key physiological processes such as chlorophyll formation, protein synthesis, and cell division, all of which are essential for overall plant growth (Fathi, 2022). In oil palm cultivation, nitrogen deficiency causes pale green or yellow leaflets, reduced frond production, slower plant growth and ultimately decreased yield (Kushairi *et al.*, 2013). Inorganic nitrogen fertilisers, mainly urea and ammonium sulphate are widely applied to oil palm and other crops to overcome nitrogen deficiency, as they deliver nitrogen in a readily available form (Behera *et al.*, 2022). While effective in the short term, excessive and prolonged use of these inorganic fertilisers has raised significant environmental concerns. Issues such as disruption of the soil microbial community (Salamat *et al.*, 2021), greenhouse gas emissions, groundwater contamination, and eutrophication of nearby water bodies have been well documented (De la Peña *et al.*, 2023).

In light of environmental concerns and the rising cost of inorganic fertilisers, there is growing interest in biofertilisers as

sustainable alternatives (Supriatna *et al.*, 2023). Biofertilisers not only help reduce environmental pollution but also decrease dependence on inorganic fertilisers (Jaiswal *et al.*, 2023). Various formulations are available to suit different crops and agronomic conditions (Ajeng *et al.*, 2020), each is designed to deliver specific microbial strains that address nutrient deficiencies or enhance plant functions. Among the most established types of biofertilisers are those containing nitrogen-fixing inoculants as their active ingredient. These products include beneficial bacteria capable of converting atmospheric nitrogen into a form that plants can readily absorb (Wagner, 2011). In the context of oil palm cultivation, *Paenibacillus alvei* strain M312 has shown great potential. Razak *et al.* (2019) demonstrated that this nitrogen-fixing bacterium significantly improves the growth of oil palm seedlings at the nursery stage through its biological nitrogen fixation activity.

Nitrogen-fixing inoculants are commercially available in various formulations such as powders, liquids, granules, and seed coatings, depending on the intended application method (Bashan *et al.*, 2014; O'Callaghan *et al.*, 2022). Each formulation presents unique advantages and limitations depending on its physical characteristics and application method. Solid formulations such as powders and granules, are commonly applied to soil (Lobo *et al.*, 2019; Rai *et al.*, 2023). Powders, due to their excellent adhesion, are especially suited for seed treatments (Flynn, 2015), but they are prone to dust generation and degradation under environmental stress (Fadiji *et al.*, 2024). In contrast, granules are dust-free, easier to handle, and more robust under stress due to protective coatings that reduce microbial desiccation (Flynn, 2015; Chaudhary *et al.*, 2020; Sarbani & Yahaya, 2022). However, they are often more expensive to produce due to the higher volume and encapsulation technology required (dos Reis *et al.*, 2024; Saskatchewan Pulse Growers, 2025).

Liquid formulations are gaining popularity due to their ease of use and uniform application, which enhances microbial colonisation and efficacy (Khan *et al.*, 2023). They also allow better control over sterilisation and can maintain high viable cell counts during production, resulting in lower application rates (Herrmann & Lesueur, 2013; Díaz-Rodríguez *et al.*, 2025). Saskatchewan Pulse Growers (2025) and other studies have identified liquid inoculants and seed-coating formulations as more user-friendly and efficient, especially for *Rhizobium* inoculants. Nonetheless, granular and peat-based formulations still offer better microbial protection in harsh environmental conditions, though at the cost of higher material volume and handling requirements (Maitra *et al.*, 2021). One of the key drawbacks of liquid inoculants is their shorter shelf life and the need for proper storage (Rezaei *et al.*, 2023). However, this issue can be mitigated through the inclusion of additives or adjuvants that enhance shelf stability and microbial viability during storage (Chaudhary *et al.*, 2020).

Across all formulation types, the goals remain consistent: Maintaining microbial viability, ensuring storage stability, cost-effectiveness, and facilitating ease of handling during transport and application (Herrmann & Lesueur, 2013). Without proper formulation, microbes in broth cultures can rapidly lose viability and efficacy (Allouzi *et al.*, 2022). Therefore, any successful biofertiliser must be engineered to support long-term microbial survival, resist environmental stress and maintain performance in field application (Yadav & Yadav, 2024). However, one of the greatest challenges to biofertiliser adoption remains ensuring microbial purity and viability throughout the product's shelf life (Soumare *et al.*, 2020).

Currently, most nitrogen-fixing inoculants on the market are liquid formulations (Singleton *et al.*, 2002; Jaiswal *et al.*, 2023). These typically contain a broth culture along with carriers, emulsifiers, thickeners, and protective agents to

support microbial survival and product stability (Rai *et al.*, 2023). Developing a high-quality liquid formulation requires extensive testing to determine the most suitable and effective composition (Sarbani & Yahaya, 2022). Given the complex, time-consuming nature of formulation development and commercialisation (Herrmann & Lesueur, 2013), there is a pressing need for simpler, short-term preservation methods. This is especially important during field trials and efficacy studies, where prolonged microbial viability during transportation is crucial. Therefore, this study aims to develop a straightforward preservation method to maintain the viability of the M312 liquid inoculant for extended periods, facilitating its application in field settings.

Materials and Methods

Microorganism and Culture Conditions

Paenibacillus alvei M312 used in this study was obtained from the Microbial Culture Collection of Beneficial Microbes Laboratory, FGV R&D Sdn. Bhd., Bandar Enstek, Nilai, Negeri Sembilan, Malaysia. This isolate was cultured on Nutrient Agar (NA) at 28°C ± 2 for 24 hours. One loopful of grown isolate M312 on NA was inoculated into growth medium of Nitrogen-free (N-free) medium containing: Glucose 10 g L^{-1}, sodium molybdate 0.0025 g L^{-1}, calcium chloride 0.2 g L^{-1}, magnesium sulphate 0.1 g L^{-1}, potassium dihydrogen phosphate 0.41 g L^{-1}, di-potassium hydrogen orthophosphate 0.52 g L^{-1}, sodium sulphate 0.05 g L^{-1}, and iron sulphate 0.005 g L^{-1}. The culture was shaken using an orbital shaker at 130 rpm and a room temperature of 28°C ± 2 for 24 hours.

Screening of Different Additives

The culture broth in N-free medium was tested with different chemical additives to increase the survival of isolate M312 in a liquid formulation. Chemical additives or polymers, like Polyvinyl Pyrrolidone (PVP), Polyethylene Glycol (PEG), Polyvinyl Alcohol (PVA), and gum Arabic, were individually added to N-free broth. The concentration of the additive used is listed in Table 1. The concentration was based on a literature review on the liquid formulation of *Azospirillum* sp. (Kumaresan & Reetha, 2011; Mohamed *et al.*, 2019). The prepared formulation was stored for 3 months at a room temperature of 28°C ± 2. The viable cell of M312 was monitored for over 3 months at monthly intervals.

Preparation of M312 Liquid Inoculant and Shelf-life Study

The additive that exhibited the highest survival rate of M312 was further studied. A culture broth of M312 in N-free medium was prepared and supplemented with 0.3% commercial grade gum Arabic. These prepared liquid inoculants were then packed into sterile high-density polyethylene bottles (Biradar & Santhosh, 2018) and stored for 12 months at various locations with different temperature ranges (Table 2).

Shelf-life Assessment

The survival of the M312 liquid inoculant was monitored for up to one year by monitoring the viable cells using total plate count analysis and the serial dilution technique. 1 mL of liquid inoculant was serially diluted and plated on NA, then incubated at 28°C ± 2 for 24 hours.

Table 1: The concentration of the additive used

Treatment No.	Additives	Concentration (%)
1	Gum Arabic (Commercial Grade, CG)	0.3
2	Gum Arabic (Laboratory Grade, LG)	0.3
3	PVA	0.5
4	PEG	1.0
5	PVP	2.0

Table 2: Storage location of M312 liquid inoculant

Location No.	Storage Locations	Temperature Range (°C)
1	Chiller	3–10
2	Chemical and media room	23–24
3	Nursery store	26–34
4	Glasshouse	26–38
5	Drying oven	39–41

Application of M312 Liquid Inoculant on Oil Palm Seedlings

20 mL of the M312 liquid inoculant, prepared with 0.3% commercial grade gum Arabic as an additive was sprayed onto 4-month-old oil palm seedlings to validate the endophytic characteristics of M312. Sterilised water was used as a control. One week after application, oil palm leaves were sampled and surface sterilised. The surface sterilisation process followed the methods described by Duhan *et al.* (2020) and Fishal *et al.* (2022) with some modifications. The collected leaf samples were cut into 1.5 to 2.0 cm pieces and rinsed under tap water to remove dust and debris. The leaves were then dipped in 10% sodium hypochlorite for two minutes, followed by sequential immersion in 50%, 70%, 90%, and 99% ethanol for 30 seconds each, and finally rinsed three times with sterile water. The leaf samples were allowed to dry on sterilised filter paper. Once dried, the edges of the leaves were trimmed to 0.5 cm, plated on NA, and incubated at 28°C for 24 hours. The morphology of bacterial colonies grown on NA plates was compared with pure M312 cultures.

Strain Verification

Samples of the M312 liquid inoculant, stored for one year at various locations under different temperature ranges (Table 2) were cultured on NA for microbial identification. The isolates were identified through 16S ribosomal RNA (rRNA) gene sequencing. Genomic DNA (gDNA) was extracted from colonies scraped from agar plates using lysis buffer containing 1% SDS, 0.4 M NaCl, 0.4 M EDTA, 0.5 M Tris-HCl (pH 8.0), and 10 µg mL^{-1} proteinase K. The mixture was vortexed for 15 seconds and incubated at 65°C for 20 minutes, followed by sequential incubations at 37°C for 1 hour and 65°C for 1 hour to ensure complete lysis. The lysate was then vortexed at maximum speed for 10 minutes.

Genomic DNA was purified using the phenol–chloroform–isoamyl alcohol (25:24:1) extraction method, precipitated with isopropanol, washed with 70% ethanol and resuspended in nuclease free water. PCR amplification of the 16S rRNA gene was carried out using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, United States of America) according to the manufacturer's protocol, with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA CTT-3'). The PCR amplicons were cloned into the linearised pGEM5zf vector (Promega, United States of America) via in vivo homologous recombination in *Escherichia coli*. Plasmid DNA was extracted using the Presto™ Mini Plasmid Kit (Geneaid, Taiwan). Bidirectional Sanger sequencing was performed at Apical Scientific Sdn. Bhd. (Seri Kembangan, Selangor, Malaysia) using universal sequencing primers. The resulting sequences were analysed by Standards and Industrial Research Institute of Malaysia (SIRIM) and compared with those in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) for taxonomic identification.

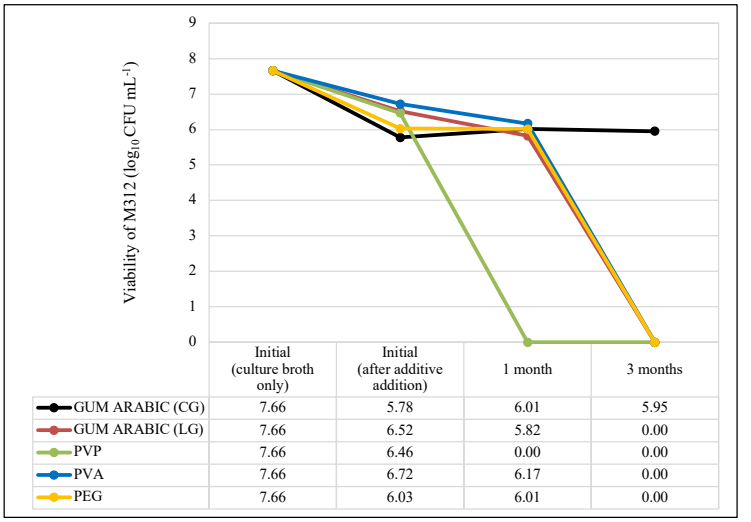
Results

Screening of Different Additives

The impact of various additives on the survival of *P. alvei* M312 over a three-month period is illustrated in Figure 1. The initial concentration of the culture broth before mixing with additives was 4.57×10^7 CFU mL^{-1} ($\log_{10}$: 7.66). One day after mixing with the additives, the viability of M312 was reassessed, showing different concentrations for each additive. The M312 liquid inoculant added with commercial grade gum Arabic showed a significant drop in viability to 6.00×10^5 CFU mL^{-1} ($\log_{10}$: 5.78), whereas the laboratory grade gum Arabic maintained a higher viability at 3.33×10^6 CFU mL^{-1} ($\log_{10}$: 6.52). Additives such as PVP, PEG, and PVA also showed a significant drop to 10^6 CFU mL^{-1}. After one month of storage, the M312 liquid inoculant containing PVP was the only one that failed to maintain viability. After three months of storage, only the M312 liquid inoculant with commercial gum Arabic was successfully preserved and maintained, showing a slight reduction to 9.00×10^5 CFU mL^{-1} ($\log_{10}$: 5.95).

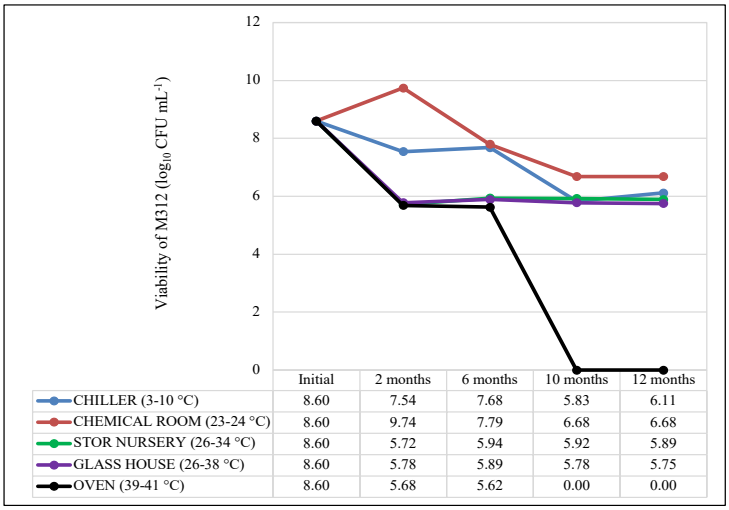
Effect of Storage Temperature on M312 Liquid Inoculant

The most effective additive identified from the screening process was subsequently used in the preparation of the M312 liquid inoculant, which was then stored at various locations with different temperature ranges. Figure 2 illustrates the viability of M312, measured in colony-forming units per millilitre (CFU mL^{-1}), over a period of 12 months under different storage conditions. The M312 liquid inoculant stored in the chemical room (23°C to 24°C) showed the highest initial increase in viability at 2 months but declined thereafter. In contrast, the viability of M312 liquid inoculant stored in the nursery store and glasshouse exhibited a significant initial drop during the first 2 months but remained stable over the 12 months. Storage at temperatures ranging from 3°C to 10°C (in the chiller) provided better stability and viability compared with other conditions. The oven storage at 39°C to 41°C was the poorest result, showing a drastic decline in viability, with no viable cell remaining after 10 months. Thus, for optimal long-term storage of M312, maintaining a temperature range below 38°C is crucial.



	Initial (culture broth only)	Initial (after additive addition)	1 month	3 months
GUM ARABIC (CG)	7.66	5.78	6.01	5.95
GUM ARABIC (LG)	7.66	6.52	5.82	0.00
PVP	7.66	6.46	0.00	0.00
PVA	7.66	6.72	6.17	0.00
PEG	7.66	6.03	6.01	0.00

Figure 1: Viability of M312 liquid inoculant supplemented with different additives



	Initial	2 months	6 months	10 months	12 months
CHILLER (3-10 °C)	8.60	7.54	7.68	5.83	6.11
CHEMICAL ROOM (23-24 °C)	8.60	9.74	7.79	6.68	6.68
STOR NURSERY (26-34 °C)	8.60	5.72	5.94	5.92	5.89
GLASS HOUSE (26-38 °C)	8.60	5.78	5.89	5.78	5.75
OVEN (39-41 °C)	8.60	5.68	5.62	0.00	0.00

Figure 2: Viability of M312 in liquid inoculant stored at different storage conditions

Application of M312 Liquid Inoculant on Oil Palm Seedlings

A week after the application of liquid inoculant, the oil palm leaves [Figure 3 (a)] were collected and processed for endophytic identification. Colonies appeared on the edges of leaf explants on nutrient agar media, as shown in Figure 3. Based on the morphological characterisation, the colonies from the leaf sample [Figures 3 (b) and (c)] resembled the pure culture of M312 grown on nutrient agar [Figure 3 (d)]. No growth was observed on the control leaf [Figure 3 (e)]. This observation demonstrated that the liquid inoculant was successful in delivering strain M312 to the target oil palm seedling. Additionally, the isolate M312 was previously reported as an endophyte by Razak *et al.* (2019).

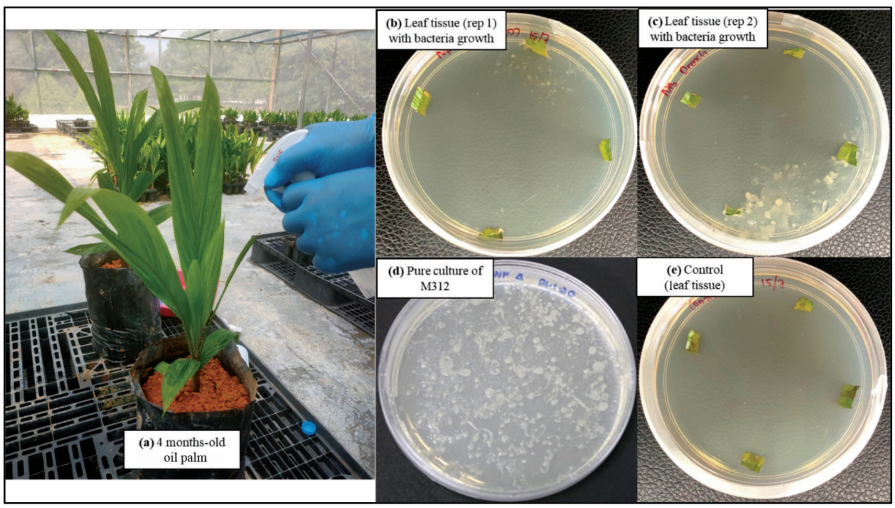


Figure 3: (a) Application of M312 liquid inoculant on oil palm through spraying, (b) bacterial growth from edge of leaf tissue replicate 1, (c) bacterial growth from edge of leaf tissue replicate 2, (d) pure culture of M312, and (e) leaf sample from control palm

Strain Verification

After 12 months, the M312 liquid inoculants stored under different conditions were sampled and identified using 16S rRNA gene sequencing. Strain identification showed 99% similarity to sequences in the NCBI GenBank, with accession numbers provided in Table 3. Isolates from Location 2 (chemical and media room) matched the original strain used in the inoculant preparations. In contrast, isolates from Location 3 (nursery store) and Location 4 (glasshouse) were also identified as *P. alvei*, but with different accession numbers, possibly due to minor sequence variations caused by differing storage conditions. The M312 inoculant stored at Location 1 (chiller) was contaminated and identified as *Bacillus subtilis*, likely introduced during the sampling process. Similarly, the inoculant stored at Location 5 (drying oven) was suspected of contamination, as viability results (Figure 2) indicated that M312 could not maintain viability beyond 10 months of storage at high storage temperatures. The isolate from this location was identified as *Enterobacter cloacae*.

Discussions

Based on these findings, the viability of *P. alvei* M312 was significantly reduced following the addition of various additives, indicating an initial stress response or dilution effect. Although all treatments began with the same concentration of culture broth, survival rates varied, suggesting that each additive offered different levels of protection, likely due to variations in their physicochemical properties and modes of action.

Among all the tested additives, commercial-grade gum Arabic emerged as the most effective. It consistently maintained the viability of M312 over three months, likely due to its natural polysaccharide composition, which can form a protective matrix around the cells, reducing dehydration and mechanical stress (Abdukerim *et al.*, 2023; Kamel *et al.*, 2024). Being plant-derived and environmentally friendly further strengthens its appeal (Mohamed *et al.*, 2025). In contrast, laboratory-grade gum Arabic provided moderate initial protection but failed to sustain viability beyond the third month. This discrepancy may be attributed to differences in purity, molecular weight, or composition, which could influence its effectiveness in microbial preservation. Other additives performed less favourably. PVP failed to preserve cell viability beyond one month, despite its reported antioxidant and stabilising properties (Maitra *et al.*, 2021; Khan *et al.*, 2023). This suggests that PVP alone is not suitable for preserving M312 in liquid formulations. Both PVA and PEG supported only short-term survival. Their initial protective effects may stem from their film-forming ability and water solubility; however, viability declined rapidly, possibly due to oxygen limitation, nutrient depletion, or additive-cell incompatibility over time (Pham Van & Tho Bach, 2014; Jaiswal *et al.*, 2023; Gutierrez *et al.*, 2024).

Table 3: 16S rRNA gene sequence analysis

Location No.	Liquid Inoculants (Storage Locations)	NCBI Database Match	Percentage of Identity (%)
1	Chiller	*Bacillus subtilis* (NR_027552.1)	99
2	Chemical and media room	*Paenibacillus alvei* (NR_113577.1)	99
3	Nursery store	*Paenibacillus alvei* (NR_04209.1)	99
4	Glasshouse	*Paenibacillus alvei* (NR_04209.1)	99
5	Drying oven	*Enterobacter cloacae* (NR_044978.1)	99

Overall, the addition of 0.3% commercial-grade gum Arabic proved to be a simple and effective strategy for preserving M312 in liquid form. This method is particularly valuable for field applications where the timely use of microbial inoculants is critical. Developing an optimal formulation may take time, as various factors must be considered. All additives tested in this study are commonly used in liquid inoculant development. Mohamed *et al.* (2019) previously reported that PVP, PEG, PVA, and gum Arabic support microbial survival and extend the shelf life of *Rhizobium*-based inoculants. Similarly, Elsakhawy *et al.* (2021) highlighted gum Arabic's superiority over PVP when used alone in liquid rhizobia formulations. These additives function as protective agents that enhance microbial stability (Rai *et al.*, 2023). Beyond formulation, other practical considerations must be addressed, particularly production cost, storage and transportation. Maintaining inoculant viability often requires cold storage, which adds a logistical and financial burden (Arbaugh *et al.*, 2022). In this study, the M312 liquid inoculant amended with 0.3% gum Arabic remained stable at room temperature (23°C to 38°C) for up to 12 months, offering substantial advantages in terms of usability, storage and cost-effectiveness.

Unlike many commercial nitrogen-fixing inoculants that rely on multi-component formulations (nutrient carriers, stabilisers, surfactants, additives, or buffering agents) to ensure shelf life (Mohamed *et al.*, 2019; Allouzi *et al.*, 2022; Rezaei *et al.*, 2023), the M312 liquid inoculant achieves comparable or superior stability using a single natural additive. It can be used directly without requiring special preparation or temperature control. Its stability at room temperature reduces the risk of viability loss during routine handling. Moreover, the M312 liquid inoculant can be transported without the need for refrigerated vehicles, simplifying logistics and lowering transportation costs. The ability to store at room temperature also minimises the need for refrigeration infrastructure, leading to additional cost savings and convenience. Collectively, these characteristics make the M312 liquid inoculant a practical and cost-effective solution for agricultural applications, facilitating broader adoption and use.

Application to oil palm seedlings was used to evaluate the efficacy of the M312 liquid inoculant. The results confirmed its successful colonisation, with the M312 isolate detected in leaf tissue even after 10 months, consistent with previous applications using fresh cultures (Razak *et al.*, 2022). Moreover, 16S rRNA gene sequencing verified that the stored inoculum remained viable and genetically stable as *P. alvei* M312, confirming that gum Arabic effectively preserved both its identity and functionality.

Conclusions

This study demonstrates that commercial-grade gum Arabic is an effective single-component additive for preserving the nitrogen-fixing bacterium *P. alvei* M312 in liquid form. The formulation maintained microbial viability and genetic stability for up to 12 months at ambient temperatures (23°C to 38°C), eliminating the need for refrigeration. Its effectiveness is likely attributed to the natural polysaccharide properties of gum Arabic, which form a protective barrier around the cells, reducing dehydration and mechanical stress. This simple, cost-effective and scalable formulation is well-suited for field applications, particularly in resource-limited agricultural settings. The demonstrated stability and practicality of this formulation support its direct use in sustainable agriculture. These findings pave the way for developing a robust, field-ready inoculant with minimal formulation complexity. Future research should focus on optimising aspects such as colour, viscosity and packaging; evaluating field efficacy across diverse soil types; and, further elucidating gum Arabic's protective mechanisms to support large-scale application and commercialisation.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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