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Optimizing nitrogen-fixing bacteria viability and plant growth-promoting traits through liquid media composition

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ABSTRACT

Developing effective liquid biofertilizers depends on compatible microbial strains, suitable carrier formulations, and proper scaling-up conditions. The study aimed to assess the effects of N and P enrichment on a molasses-based liquid carrier formula and various agitation conditions on the cell viability of nitrogen-fixing bacteria, *Azotobacter, Priestia,* and *Bacillus.* All selected strains, isolated from the strawberry rhizosphere, showed mutual growth without inhibiting nutrient agar, indicating their suitability for forming a consortium. The liquid carrier, formulated with 1% molasses, 0.1% NH_4Cl, and 0.2% phosphate buffer, produced the highest bacterial populations, reaching 17.3 log CFU/mL in N-free Ashby's agar and 16.4 log CFU/mL in Tryptic Soy Agar after nine days of incubation. This liquid carrier formulation also maintained a more stable pH (8.1) and lower electrical conductivity (1.5 mS/cm) compared to other treatments, suggesting these conditions are optimal for sustained bacterial activity. All agitation speeds (115–135 rpm) showed similar growth curves, with OD reaching a maximum of 3.9–4.0 on day six. However, 135 rpm resulted in the most significant growth rate (slope 0.638, R^2 0.926) and higher levels of indole-3-acetic acid, gibberellin, zeatin, and phosphatase activity. These results demonstrate that carrier formulation enhances population growth and stability. This optimized process provides a promising approach for developing a multi-strain liquid carrier biofertilizer for strawberry cultivation.

Keywords: bacterial population, liquid biofertilizer formulation, nitrogen-fixing bacteria, molasses, strawberry.

INTRODUCTION

Soilless cultivation systems are becoming more popular in modern agriculture for premium horticultural crops, including strawberries. (Rathod et al., 2021). The main benefit of soilless media is reducing the risk of disease transmission through soil-borne pathogens (Boonen et al., 2017); and it allows for easier disease control, nutrient availability, and below-ground conditions, which lead to improved plant productivity (Lakhiar et al., 2025). Moreover, it supports environmentally friendly agriculture. Agricultural by-products (e.g., coconut fiber, rice husks, peat moss, compost) offer a sustainable plant cultivation method (Sahoo et al., 2023). Plant nutrients in a soilless media system are supplied through inorganic fertilizers through fertigation (Othman et al., 2019). The fertilizer is more effective in providing macro- and micronutrients to the roots (Ikegaya et al., 2020). However, the fertigation system only supplies nutrients; long-term use of inorganic fertilizer accumulates the chemicals in the substrate and changes the planting medium's conditions (Lee et al., 2018), especially the acidity.

Nitrogen or P scarcity hinders strawberry growth and development more than potassium (K) limitation, since they are directly involved in

photosynthesis and forming reproductive structures, including flowers and fruits (Rashid et al., 2020). N deficiency reduces shoot biomass and alters the shoot-root (S/R) ratio across growth stages. In contrast, P deficiency elevates N content and reduces K content, and has a significant impact on total soluble sugar (Jiang et al., 2023).

Nutrient deficiencies often manifest as visible symptoms, which also reflect underdeveloped root systems (Barreto et al., 2017). Adequate nitrogen availability supports growth and enhances indole-3-acetic acid and jasmonic acid content while reducing root salicylic acid concentrations (Zhang et al., 2023). These hormonal changes are closely linked to the composition and activity of the root-associated bacterial community. Applying fertilizers at an optimal nutrient ratio increases the abundance of beneficial bacteria, which correlates with improved strawberry growth (Bai et al., 2023).

In sustainable agriculture, reducing the use of inorganic fertilizers is recommended. Application of beneficial microbes stimulates growth, increases resistance, and helps plants adapt to stress conditions (De Tender et al., 2016). Additionally, they produce phytohormones like indole-acetic acid, cytokinins, and gibberellins that stimulate cell division (Gejji and Varma, 2025; Joshi et al., 2021; Katel et al., 2022; Li et al., 2021). Nitrogen-fixing bacteria (NFB), including *Azotobacter* and *Bacillus*, have been shown to enhance strawberry growth (Hindersah et al., 2021). The *Priestia megaterium* (formerly *Bacillus megaterium*) shows the ability to fix N in N-free medium (Li et al., 2022). All bacteria produce phytohormones that promote plant growth and exopolysaccharides for adaptability under stress conditions (Fitriyani et al., 2025; Kurniati et al., 2024). However, biofertilizer composed of N-fixing bacteria for soilless strawberries is still limited.

Biofertilizer formulation is essential in supporting microbial cell proliferation, biological function, and its effectiveness in enhancing plant growth (Alami et al., 2018; Neneng, 2020). Carrier medium determines the cell viability in liquid or solid biofertilizer. Liquid biofertilizer has high viability of bacteria (Duary and Sahoo, 2021). The use of agricultural waste, such as molasses for liquid biofertilizer formulation, is sustainable to enhance the yield compared to other carrier-based materials (Allouzi et al., 2022). However, the molasse enrichment with N and P is needed to optimize bacterial growth. Moreover, agitation speed and aeration determine the cell viability and are reported to affect biofertilizer quality (Hamouda, 2016). Integrating soilless planting and biofertilizers can effectively boost plant growth and healthier fruit, and promote environmentally friendly cultivation (Singh et al., 2023; Dhawi, 2023). Nonetheless, biofertilizer formulation is needed before introducing them to strawberry cultivation. Therefore, this study was conducted to analyze the effect of N and P enrichment, as well as various agitation, on the cell viability of molasse-based formula of liquid biofertilizer composed of *Azotobacter, Priestia,* and *Bacillus*. The findings are expected to contribute to sustainable strawberry production strategies.

MATERIALS AND METHODS

Sub-culture and cell culture preparation

The study used *Azotobacter salinestris* and *Priestia megaterium* isolated from strawberry potted soil (Fitriyani et al., 2025). In addition, *Bacillus subtilis* and *Azotobacter chroococcum* were obtained from the Soil Biology Laboratory at the Faculty of Agriculture, Universitas Padjadjaran. Their characteristics are presented in Table 1. The pure cultures were maintained on N-free Ashby's medium for *A. chroococcum* and *A. salinestris*, and on tryptic soy agar for *P. megaterium* and *B. subtilis*. Three days before experimentation, each strain was sub-cultured at 30 °C in respective medium. *Azotobacter* cultures were grown in Ashby medium (20 g mannitol, 0.2 g dipotassium phosphate, 0.2 g magnesium sulfate, 0.2 g sodium chloride, 0.1 g potassium sulfate, 5 g calcium carbonate, 15 g agar; pH 7) while *P. megaterium* and *B. subtilis* were cultured in tryptic soy broth (17 g casein peptone, 2.5 g dipotassium hydrogen phosphate, 5 g sodium chloride, 3 g soya peptone, pH 7.1).

A slant was scraped and transferred into 250 mL of Ashby's broth or tryptic soy broth and incubated with shaking (25–27 °C) for three days to obtain an active inoculum with a final bacterial population of 6.6×10^7 CFU/mL.

Compatibility test

The compatibility test of *A. salinestris* (AS), *A. chrococcum* (AC), *B. subtilis* (BS) and *P. megaterium* (PM) were performed on nutrient agar (5 g peptone, 5 g sodium chloride, 1.5 g peptone, 1.5 g yeast extract, 15 g agar). One loopful of an isolate

Table 1. Plant growth promoter properties of bacterial Isolates

Bacteria	Phosphatase (µg/mL)	Nitrogenase activity (µmol/mL/g/h)	EPS production (g/L)	IAA (µg/mL)	Cytokinin (µg/mL)	Gibberellin (µg/mL)
P. megaterium	-	4.23	4.4	31.29	1.39	21.73
A.salinestris	-	1.54	4.4	15.98	1.38	12.41
A. chroococcum	-	0.74	6.4	0.52	0.97	0.41
B. subtilis	0.18	-	10.8	0.96	0.85	0.35

was streaked horizontally, and another isolate was streaked vertically to form an intersection point. The plates were incubated at 37 °C for 48 h, and inhibition zones were recorded.

Formulation of liquid carrier biofertilizer

The formulation process consisted of three sequential experiments: 1) N and P enrichment on molasse-based media composition, 2) determination of initial inoculant concentration, and 3) evaluation of agitation speed:

1) Media composition – a completely randomized design (CRD) with six treatments and four replications was applied:
   - A_1 = molasse 1%
   - A_2 = molasse 2%
   - A_3 = molasse 1% + 0.1% NH_4Cl
   - A_4 = molasse 2% + 0.1% NH_4Cl
   - A_5 = molasse 1% + 0.1% NH_4Cl + 0.2% Na_2HPO_4
   - A_6 = molasse 2% + 0.1% NH_4Cl + 0.2% Na_2HPO_4

   Inoculants were prepared by suspending the slant cultures in 10 mL of 0.85% NaCl and mixing them at a 1:2:2:2 ratio (BS:AS:PM:AC). A 1% (v/v) mixed inoculant was added to a 100 ml medium and incubated at 23–27 °C on a 115-rpm gyratory shaker. The bacterial population using the serial dilution plate method, pH, and EC were monitored for up to nine days using Ashby's agar (*A. chroococcum* and *A. salinestris*) and tryptic soy agar (*P. megaterium* and *B. subtilis)*.
2) Initial inoculant concentration – a CRD with four inoculant levels (0.1%, 0.5%, 1.0%, and 1.5%) and six replications was used. Inoculants were added to 50 ml of selected formulations from the first experiment (A3 and A5) in a 100 ml flask, and the flask was shaken at 115 rpm (24–27 °C). Inoculants were prepared as previously described. Bacterial populations, pH, and EC were measured for seven days.
3) Determination of agitation speed – a CRD with three agitation speeds (115, 125, and 135 rpm) and nine replications was implemented. Mix inoculants (1:2:2:2) were added dropwise into 1 L of medium in a 2-L reactor and incubated at 23–27 °C. Bacterial populations, pH, and EC were recorded for seven days.

Evaluation of plant growth-promoting traits

Phosphate-solubilizing activity was measured using nitrophenyl phosphate disodium (pNPP 0.115 M) as a substrate (Rombola et al., 2014). Nitrogenase activity was quantified using the acetylene reduction assay with an uninoculated control to establish baseline conditions (Montes-Luz et al., 2023). EPS production was determined by the gravimetric method (Hindersah et al., 2017). Lactic acid production was measured through high-performance liquid chromatography (HPLC) extraction (Montero-Zamora et al., 2022). IAA syntheses were measured using Salkowski's reagent assay, while cytokinin (specifically zeatin) and gibberellin (GA_3) concentrations were also determined through HPLC extraction with acetonitrile ranging 0–100 µg/mL (Bhalla et al., 2010; Ganapathy et al., 2022). All experimental procedures were meticulously executed in triplicate.

Data analysis

Data were tested for normality and homogeneity before further analysis. ANOVA was used to evaluate treatment effects at $p < 0.05$, followed by Duncan's multiple range test (DMRT) for mean separation. All analyses were conducted using IBM SPSS Statistics version 27.0.

RESULTS

Compatibility among bacteria

The results of the synergistic test between species showed that none of the isolates exhibited

inhibition (Figure 1). There were no inhibition zones between the isolates; each isolate colony grew well when incubated in the same place. However, the compatibility test results showed that *Bacillus subtilis* (BS) exhibited the largest colony size, as indicated by wider growth areas on the growth medium. This indicates that BS has a stronger competitive ability in nutrient absorption compared to *Azotobacter salinestris* (AS), *Priestia megaterium* (PM), and *Azotobacter chroococcum* (AC). Therefore, its proportion was reduced to ensure balanced microbial interaction in the consortia, resulting in a final formulation ratio of 1:2:2:2 (BS:AS:PM:AC). This formulation aims to optimize synergistic interactions while minimizing competitive exclusion, enabling each isolate to contribute effectively within the formula.

Formulation of liquid carrier biofertilizer

Liquid carrier media formulation

The one-way ANOVA revealed that the bacterial population reached up to 17.3 log CFU/mL in A1, A3, and A5 on Ashby's agar (Table 2). In contrast, on TSA medium, populations reached 16.4 log CFU/mL in A5 and were slightly lower in other treatments (Table 3). At the end of incubation, the average population to Ashby's agar was 17.3 on a log scale, while it was 16.4 in TSA. Using Ashby agar, the highest population of N-fixer bacteria was observed in the A1, A3, and

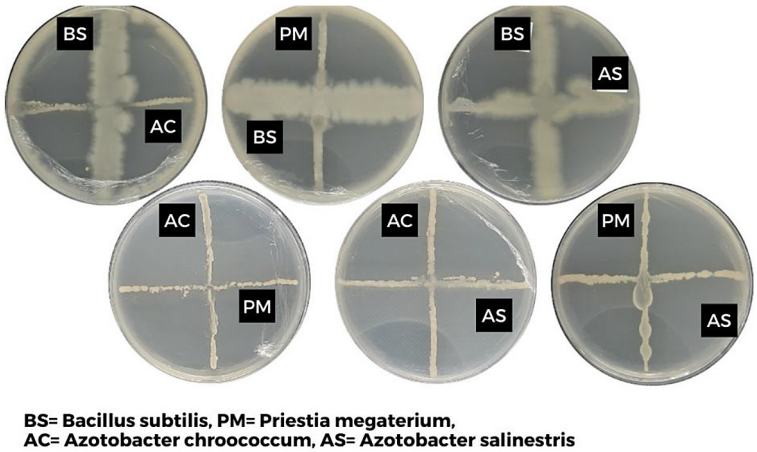

Figure 1. Compatibility test for each isolate on nutrient agar by the streak plate method. Inhibition zones were not observed for a pair of bacteria

Table 2. Population of N-fixer bacteria (Log CFU/mL) using N-free Ashby mannitol agar from different carrier formulations during 9 days of incubation

Liquid carrier formulation	N-fixer bacteria population (Log CFU/mL) during 9 days of incubation									
	0*	1	2	3	4	5	6	7	8	9
A1	6.4 ± 0.0c	7.5 ± 0.2a	9.1 ± 0.3ab	10.5 ± 0.5b	10.9 ± 0.1b	12.5 ± 0.3b	13.7 ± 0.4b	14.9 ± 0.4c	16.1 ± 0.5c	17.3 ± 0.6c
A2	6.2 ± 0.0a	7.5 ± 0.2a	9.1 ± 0.2b	10.1 ± 0.1a	10.8 ± 0.1b	12.2 ± 0.1b	13.4 ± 0.2b	14.6 ± 0.2bc	15.7 ± 0.2bc	16.9 ± 0.2bc
A3	6.4 ± 0.0c	7.4 ± 0.3a	9.0 ± 0.1a	10.3 ± 0.3ab	11.0 ± 0.0b	12.4 ± 0.2b	13.6 ± 0.2b	14.9 ± 0.2c	16.1 ± 0.2c	17.3 ± 0.3c
A4	6.3 ± 0.1ab	7.4 ± 0.3a	9.3 ± 0.1c	10.2 ± 0.1ab	10.3 ± 0.6a	11.9 ± 0.4a	13.0 ± 0.6a	14.1 ± 0.7a	15.2 ± 0.9a	16.3 ± 1.0a
A5	6.3 ± 0.0b	7.4 ± 0.2a	9.0 ± 0.1a	10.3 ± 0.1ab	11.0 ± 0.1b	12.4 ± 0.1b	13.7 ± 0.1b	14.9 ± 0.2c	16.1 ± 0.2c	17.3 ± 0.2c
A6	6.4 ± 0.1c	7.8 ± 0.2b	9.1 ± 0.1ab	10.2 ± 0.1a	10.8 ± 0.2b	12.2 ± 0.1b	13.3 ± 0.2ab	14.4 ± 0.2ab	15.6 ± 0.3ab	16.7 ± 0.3ab

Note: *Inoculation day; A1= molasses 1%; A2 = molasses 2%; A3 = molasses 1% + 0.1% NH_4Cl; A4 = molasses 2% + 0.1% NH_4Cl; A5 = molasses 1% + 0.1% NH_4Cl + 0.2% Na_2HPO_4; A6 = molasses 2% + 0.1% NH_4Cl + 0.2% Na_2HPO_4; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

Table 3. Population of N-fixer bacteria (Log CFU/mL) using TSA medium from different carrier formulations during 9 days of incubation

Liquid carrier formulation	N-fixer bacteria population (Log CFU/mL) during 9 days of incubation									
	0*	1	2	3	4	5	6	7	8	9
A1	6.4 ± 0.0b	7.4 ± 0.2a	9.2 ± 0.2b	10.4 ± 0.4b	10.4 ± 0.4b	12.0 ± 0.6a	13.1 ± 0.7a	14.2 ± 0.8a	15.3 ± 0.9a	16.1 ± 0.3a
A2	6.2 ± 0.0a	7.3 ± 0.3a	9.2 ± 0.1b	10.1 ± 0.1a	10.1 ± 0.1a	11.7 ± 0.1a	12.8 ± 0.1a	13.8 ± 0.2a	14.9 ± 0.2a	16.0 ± 0.2a
A3	6.3 ± 0.1ab	7.5 ± 0.2a	9.0 ± 0.1a	10.2 ± 0.3ab	10.2 ± 0.3ab	11.8 ± 0.3a	12.8 ± 0.4a	13.9 ± 0.5a	15.0 ± 0.5a	16.0 ± 0.6a
A4	6.2 ± 0.1a	7.5 ± 0.2a	9.3 ± 0.1b	10.2 ± 0.1ab	10.2 ± 0.1ab	11.9 ± 0.2a	12.9 ± 0.2a	14.0 ± 0.2a	15.1 ± 0.2a	16.2 ± 0.2a
A5	6.2 ± 0.1a	7.5 ± 0.1a	9.0 ± 0.1a	10.2 ± 0.1ab	10.2 ± 0.1ab	11.8 ± 0.1a	12.9 ± 0.2a	14.0 ± 0.2a	15.0 ± 0.3a	16.4 ± 1.1a
A6	6.3 ± 0.1ab	7.7 ± 0.2b	9.1 ± 0.1b	10.2 ± 0.1ab	10.2 ± 0.1ab	11.8 ± 0.1a	12.8 ± 0.2a	13.8 ± 0.2a	14.8 ± 0.2a	15.8 ± 0.3a

Note: *Inoculation day; A1= molasses 1%; A2 = molasses 2%; A3 = molasses 1% + 0.1% NH_4Cl; A4 = molasses 2% + 0.1% NH_4Cl; A5 = molasses 1% + 0.1% NH_4Cl + 0.2% Na_2HPO_4; A6 = molasses 2% + 0.1% NH_4Cl + 0.2% Na_2HPO_4; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

A5 formula. However, the N-fixer population in all formulas is similar in TSA. Growth patterns showed a steady log-phase increase up to day 9, with the A1, A3, and A5 formulations supporting the highest bacterial populations. A lower concentration enhanced bacterial proliferation and maintained population stability throughout incubation (Aidilfitri et al., 2021).

The pH and EC of the liquid carrier formulations varied during 9 days of incubation (Figure 2). The pH of the media gradually increased during incubation, with A1, A3, and A4 reaching the highest peaks (8.6–8.7), followed by A5 (8.1) and A6 (8.0). The lowest pH was observed in A2 (7.6), indicating greater production and release of the basic gas ammonium into the medium as a result of protein degradation (Makian et al., 2025). EC decreased over time, while formula containing 2% molasses (A2, A4, and A6) exhibited higher peaks (2.1–2.2 mS/cm) compared to 1% molasses (A1, A3, and A5) that had more stable conductivities within 1.5–1.6 mS/cm. The subsequent decline in EC after day six reflected microbial uptake of ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^-, PO_4^{3-}) from molasses, NH_4Cl, and Na_2HPO_4 for biomass formation and metabolic processes.

Among the six formulas, A1, A3, and A5 supported higher populations of N-fixer bacteria in Ashby's agar (Table 2), although all produced comparable populations on TSA (Table 3). A3

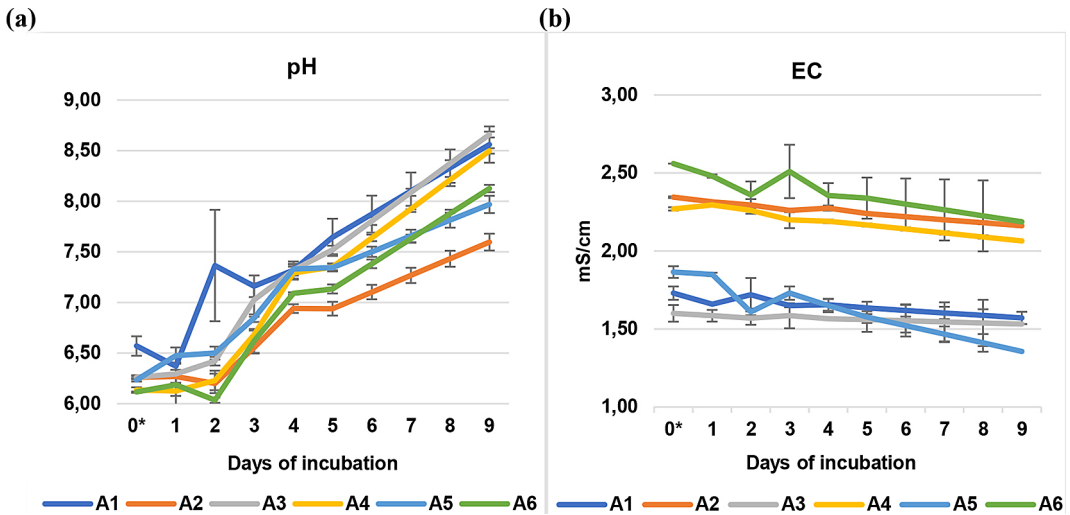

Figure 2. The change of (a) pH and (b) EC (mS/cm) in the liquid carrier formulations during nine days of incubation

and A5 were selected because they showed stable bacterial growth and balanced pH and EC, whereas A1 was excluded because it showed lower nutrient enrichment despite favorable pH trends. In terms of pH conditions, A3 and A5 were slightly alkaline, indicating *Azotobacter, Priestia,* and *Bacillus* were still able to proliferate effectively under mildly alkaline conditions.

Inoculant concentration

Bacterial populations increased throughout incubation across all inoculant concentrations in two liquid carrier formulations, A3 (1% molasses + 0.1% ammonium chloride) and A5 (1% molasses + 0.1% ammonium chloride + 0.2% phosphate buffer). Rapid growth was observed at higher inoculant concentration (1% and 1.5%) within the first two days, which persisted until day 3 before slowing by day 6 (Table 4–7). The lowest concentration (0.1%) reached the stationary phase earlier, particularly in A3, which showed lower overall populations than A5. After seven days, the 1% inoculant concentration consistently produced the highest viable counts on both Ashby's and TSA media. Formulation A5 supported approximately 10–15% greater bacterial growth than A3, indicating the positive effect of phosphate buffer addition on bacterial stability and proliferation (Figure 3, 4). At inoculation, pH values ranged from 4.2 to 5.1, depending on inoculant concentration, increasing steadily during incubation. In A3, higher inoculant levels resulted in a marked rise in pH, reaching a plateau on days 6–7. In A5, pH increased rapidly, peaking at approximately 7.5 by day 4 before stabilizing. These changes suggest active microbial metabolism and buffering effects associated with phosphate enrichment in A5.

Agitation speed

The effect of agitation speed was evaluated using a liquid carrier medium containing 1% molasses, 0.1% ammonium chloride, and 0.2% buffer phosphate, with an inoculant concentration of 1%, over 7 days of incubation (Tables 8 and 9). The 125 rpm treatment supported the most consistent increase until day 6, after which the 135 rpm treatment showed a higher population, reaching 15.0 log CFU/mL on N-free Ashby's mannitol agar (Table 8). A similar trend was observed on TSA medium, where the 125 and 135 rpm treatments had the highest populations of 17.6 and 17.7 CFU/mL, respectively, before slightly declining to 15.6 and 15.8 CFU/mL on day 7 (Table 9). The 135 rpm treatment produced

Table 4. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1% and ammonium chloride 0.1% (A3) using N-free Ashby mannitol agar from different inoculant concentrations during 7 days of incubation

Inoculant	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
Concentration	0*	1	2	3	4	5	6	7
0.10%	5.1 ± 0.1a	6.8 ± 0.3b	8.4 ± 0.0b	12.3 ± 0.0c	12.8 ± 0.3d	14.0 ± 0.3b	16.6 ± 0.1b	16.5 ± 0.1a
0.50%	5.2 ± 0.0b	6.4 ± 0.2ab	8.5 ± 0.0c	11.1 ± 0.1a	11.6 ± 0.1a	12.9 ± 0.5a	16.5 ± 0.1a	17.0 ± 0.1b
1%	5.2 ± 0.1b	6.5 ± 0.1ab	8.3 ± 0.0a	12.0 ± 0.0b	12.4 ± 0.0c	13.6 ± 0.1b	17.0 ± 0.1c	17.5 ± 0.3c
1.50%	5.2 ± 0.0b	6.4 ± 0.2a	8.6 ± 0.0c	11.9 ± 0.0b	11.9 ± 0.0b	12.9 ± 0.5a	16.6 ± 0.1b	16.6 ± 0.1a

Note: *inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

Table 5. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1% and ammonium chloride 0.1% (A3) using TSA medium from different inoculant concentrations during 7 days of incubation

Inoculant	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
Concentration	0*	1	2	3	4	5	6	7
0.10%	5.3 ± 0.1b	6.8 ± 0.3b	8.2 ± 0.0b	12.2 ± 0.0c	12.8 ± 0.3d	12.8 ± 0.3d	16.0 ± 0.0a	16.1 ± 0.1a
0.50%	5.4 ± 0.0b	6.4 ± 0.2ab	8.3 ± 0.0c	12.2 ± 0.0d	11.6 ± 0.1a	11.6 ± 0.1a	16.1 ± 0.0b	17.2 ± 0.1b
1%	5.1 ± 0.0a	6.5 ± 0.1ab	7.9 ± 0.0a	11.9 ± 0.0a	12.4 ± 0.0c	12.4 ± 0.0c	17.0 ± 0.0d	17.1 ± 0.1b
1.50%	5.7 ± 0.2c	6.4 ± 0.2a	8.8 ± 0.0d	12.0 ± 0.0b	11.9 ± 0.0b	11.9 ± 0.0b	16.4 ± 0.0c	15.9 ± 0.3a

Note: *inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

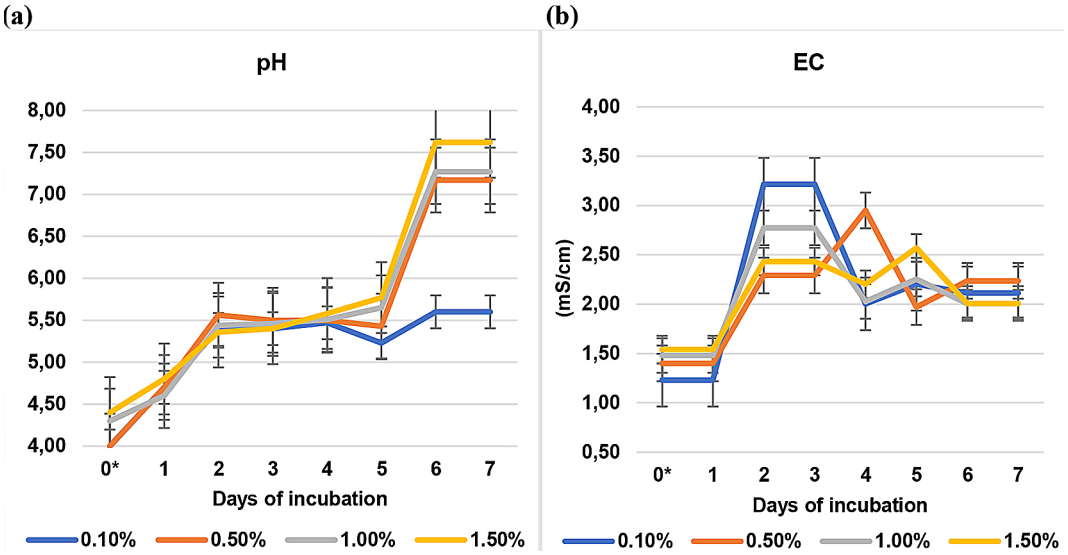

Figure 3. The change of (a) pH and (b) EC (mS/cm) in several inoculant concentrations on media consisted of molasse 1% and ammonium chloride 0.1% formula during 7 days of incubation

Table 6. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1%, ammonium chloride 0.1% and buffer phosphate 0.2% (A5) formulation using N-free Ashby mannitol agar from different inoculant concentrations during 7 days of incubation

Inoculant	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
Concentration	0*	1	2	3	4	5	6	7
0.10%	4.5 ± 0.1a	7.4 ± 0.1a	8.3 ± 0.0b	12.4 ± 0.2a	12.8 ± 0.3d	13.5 ± 0.2a	17.9 ± 0.1a	18.5 ± 0.0a
0.50%	5.1 ± 0.3b	7.5 ± 0.1a	8.2 ± 0.0a	12.8 ± 0.2b	11.6 ± 0.1a	14.4 ± 0.1b	17.9 ± 0.1a	18.6 ± 0.1b
1%	5.4 ± 0.0c	7.4 ± 0.2a	8.9 ± 0.0d	12.6 ± 0.0ab	12.4 ± 0.0c	14.4 ± 0.0b	17.9 ± 0.1a	18.9 ± 0.1c
1.50%	5.7 ± 0.2d	7.3 ± 0.2a	8.7 ± 0.0c	12.6 ± 0.0ab	11.9 ± 0.0b	14.5 ± 0.0b	17.9 ± 0.1a	18.9 ± 0.0c

Note: *inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

Table 7. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1%, ammonium chloride 0.1% and buffer phosphate 0.2% (A5) formulation using TSA medium from different inoculant concentrations during 7 days of incubation

Inoculant	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
Concentration	0*	1	2	3	4	5	6	7
0.10%	5.1 ± 0.0a	6.8 ± 0.3b	8.3 ± 0.0b	12.4 ± 0.0d	13.3 ± 0.3c	13.4 ± 0.5b	16.4 ± 0.1b	17.3 ± 0.0c
0.50%	5.2 ± 0.0c	6.4 ± 0.2ab	8.2 ± 0.0a	12.2 ± 0.0c	11.6 ± 0.1a	12.5 ± 0.4a	16.4 ± 0.0b	16.4 ± 0.1a
1%	5.1 ± 0.0b	6.5 ± 0.1ab	8.8 ± 0.0d	12.1 ± 0.0b	12.4 ± 0.0b	13.0 ± 0.0ab	16.7 ± 0.0c	17.1 ± 0.0b
1.50%	5.3 ± 0.0c	6.4 ± 0.2a	8.7 ± 0.0c	12.1 ± 0.1a	11.9 ± 0.0a	12.4 ± 0.5a	16.3 ± 0.0a	17.4 ± 0.1c

Note: *inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

the highest bacterial population. These results indicate that higher agitation enhanced bacterial growth by maximizing oxygen transfer and nutrient distribution, making 135 rpm the most effective speed (Figure 5).

Optical density data of agitation speeds of 115, 125, and 135 rpm showed an increased growth pattern of nitrogen-fixing bacteria (Figure 6). The rise in OD continued to grow continuously from day 0 to day 6, then slightly declined on day 7. On day 6, the highest OD was 4.7 at 125 and 135 rpm and 4.5 at 115 rpm. A regression study of optical density at various agitator speeds revealed a high coefficient of

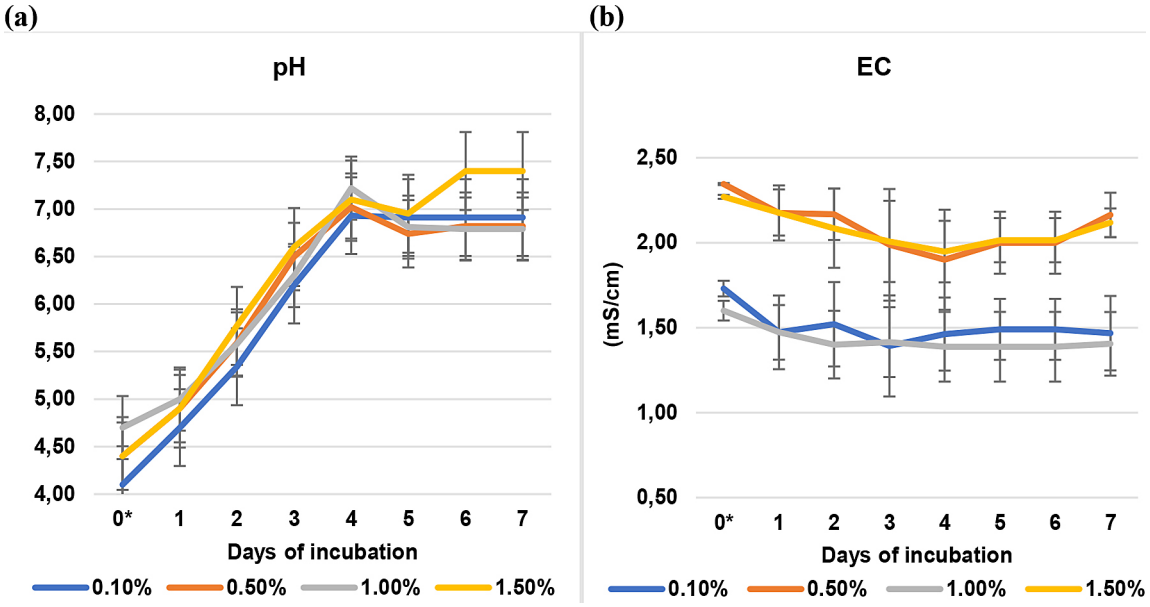

Figure 4. The change of (a) pH and (b) EC (mS/cm) in several inoculant concentrations on media consisted of molasse 1%, ammonium chloride 0.1%, and buffer phosphate 0.2% formula during 7 days of incubation

Table 8. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1%, ammonium chloride 0.1%, and buffer phosphate 0.2% formula using N-free Ashby mannitol agar during 7 days of incubation

Agitator speed (rpm)	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
	0*	1	2	3	4	5	6	7
115	6.0 ± 0.0a	7.9 ± 0.1a	9.9 ± 0.4a	11.7 ± 0.3a	13.5 ± 0.2a	14.8 ± 0.1a	14.6 ± 0.2a	14.8 ± 0.1a
125	6.0 ± 0.1a	8.1 ± 0.1b	10.0 ± 0.1a	11.6 ± 0.2a	13.7 ± 0.2b	14.7 ± 0.1a	14.8 ± 0.1b	14.9 ± 0.2ab
135	6.0 ± 0.1a	7.8 ± 0.2a	10.1 ± 0.4a	11.6 ± 0.4a	13.5 ± 0.2a	14.9 ± 0.2a	14.6 ± 0.2a	15.0 ± 0.1b

Note: *Inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

Table 9. Population of N-fixer bacteria (Log CFU/mL) on media composed of molasses 1%, ammonium chloride 0.1%, and buffer phosphate 0.2% formula using TSA medium during 7 days of incubation

Agitator speed (rpm)	N-fixer bacteria population (Log CFU/mL) during 7 days of incubation							
	0*	1	2	3	4	5	6	7
115	5.6 ± 0.5a	7.9 ± 0.3a	10.4 ± 0.2a	12.2 ± 0.4a	13.5 ± 0.3a	15.1 ± 0.4b	16.9 ± 0.4a	15.6 ± 0.1a
125	6.1 ± 0.4b	8.3 ± 0.2b	10.5 ± 0.0a	12.5 ± 0.2b	13.4 ± 0.2a	15.4 ± 0.0c	17.6 ± 0.2b	15.6 ± 0.2a
135	6.2 ± 0.1b	7.7 ± 0.3a	10.4 ± 0.1a	12.1 ± 0.3a	13.3 ± 0.3a	14.8 ± 0.3a	17.7 ± 0.1b	15.8 ± 0.1b

Note: *Inoculation day; The mean value (n=4) followed by the same letter is not significantly different according to the Duncan Test at the 0.05 significance level.

determination ($R^2 > 0.92$), indicating a strong correlation between the growth curve and high optical density. The agitator speed significantly affected phosphatase activity, phytohormone production, and lactic acid production (Table 10). The results also demonstrated that an agitator speed of 135 rpm was most effective on metabolite production with IAA (39.7 ppm), gibberellin (3.5 ppm), and zeatin (2.8 ppm), and phosphatase activity with 10.4 µg pNP/g/h.

DISCUSSION

The compatibility test result showed that *Bacillus subtilis* exhibited the largest colony size, as indicated by wider growth areas on the growth medium. It indicated that *B. subtilis* has a stronger competitive ability in nutrient absorption than *Azotobacter salinestris, Priestia megaterium,* and *Azotobacter chroococcum.* To prevent *B. subtilis* dominance within the consortium

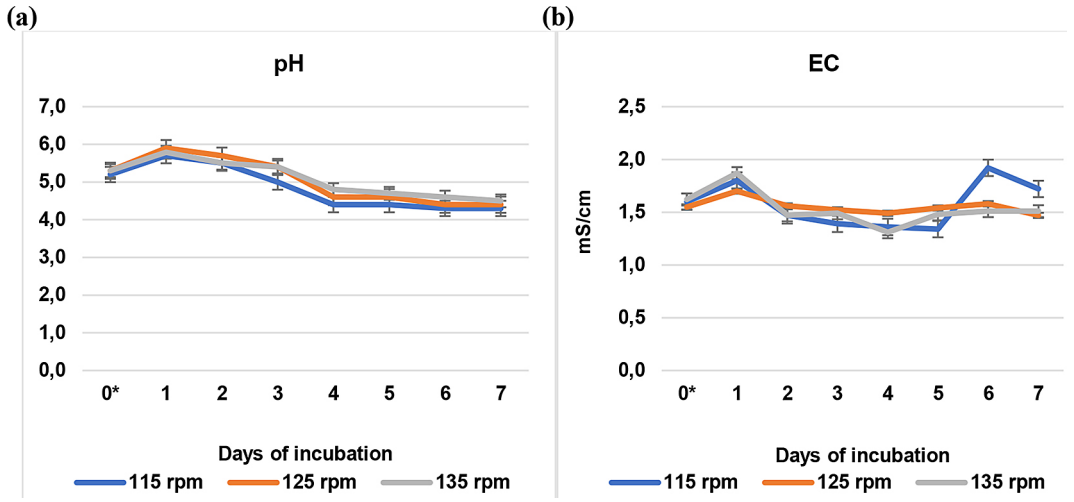

Figure 5. The change of (a) pH and (b) EC (mS/cm) in several agitator speeds on media consisted of molasse 1% and ammonium chloride 0.1%, and buffer phosphate 0.2% formula during seven days incubation

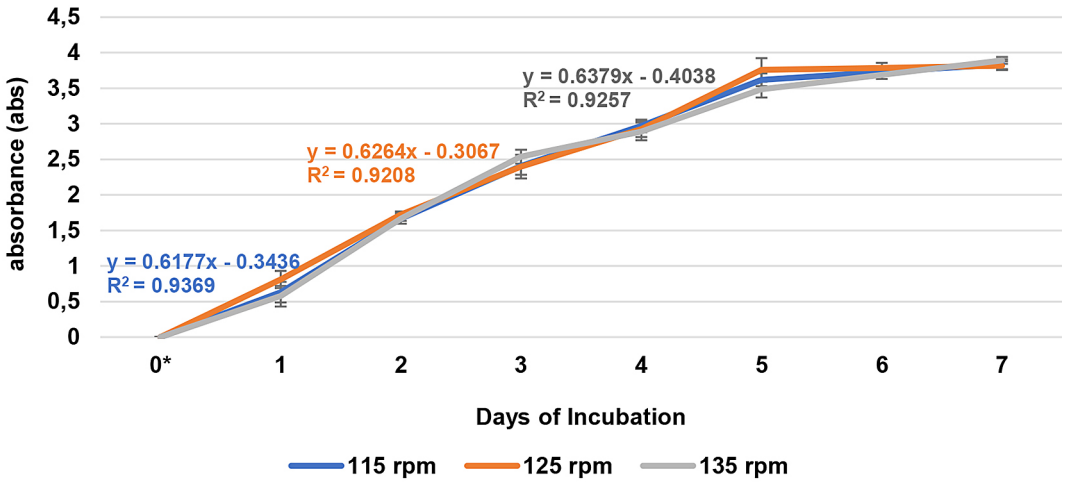

Figure 6. Optical density (abs) on several agitator speeds on molasse 1%, ammonium chloride 0.1%, and buffer phosphate 0.2% formula during 7 days of incubation

Table 10. Plant growth promotor traits of liquid carrier biofertilizer with different agitator speeds

Agitator speed (rpm)	Nitrogenase (µmol/mL/g/h)	Phosphatase activity (µg pNP/g/h)	EPS (g/L)	IAA (ppm)	Gibberellin (ppm)	Zeatin (ppm)	Lactic acid (ppm)
115	0.43 ± 0.04a	4.3 ± 0.4a	0.20 ± 0.08a	27.3 ± 1.0a	1.4 ± 0.1a	1.0 ± 0.0b	43.8 ± 6.7a
125	0.43 ± 0.01a	4.8 ± 0.3a	0.19 ± 0.08a	26.9 ± 1.0a	1.2 ± 0.1a	0.8 ± 0.0a	366.4 ± 1.6c
135	0.44 ± 0.04a	10.4 ± 1.8b	0.16 ± 0.09a	39.7 ± 7.7b	3.5 ± 0.2b	2.8 ± 0.2c	142.5 ± 38.9b

Note: the mean value (n=5) followed by the same letter is not significantly different according to the Duncan test at the 0.05 significance level.

and maintain balanced interactions among the isolates, the selected ratio for the mixed culture was adjusted to 1:2:2:2 (B. *subtilis: P. megaterium: A. salinestris: A. chroococcum*). Rather than competing, these bacteria are characterized by cooperation and shared utilization of nutritional resources within their ecological niche. This formulation aims to optimize synergistic interactions while minimizing competitive exclusion, enabling each isolate to contribute effectively within the formula. The liquid carrier formulation based on molasses, ammonium chloride, and phosphate buffer outperformed other formulations not only because of its higher bacterial growth, but primarily because it provides the most balanced carbon-nitrogen supply and

maintains chemical stability, both of which are critical for sustaining N-fixer bacteria. These results align with earlier research showing that carbon and nitrogen supplementation in balanced proportions can significantly enhance microbial proliferation in liquid-carrier biofertilizer formulations. Molasses is an economical and readily metabolizable carbon source, while NH_4Cl provides a soluble nitrogen source that is particularly important during the initial growth phases (Hindersah et al., 2020). Moreover, buffer phosphate (0.2%) served as a pH stabilizer, maintaining the culture environment within an optimal pH range (7.3–8.0). The addition of phosphate buffer helped maintain pH stability by adsorbing to protein surfaces and regulating their electrostatic stability (Brudar and Hribar-Lee, 2021), thereby preventing the rapid decrease observed in other formulations (Guo et al., 2020). This buffering effect is likely beneficial for sustaining bacterial metabolism over extended fermentation periods. Maintaining near-neutral to slightly alkaline pH has been recommended in inoculant production to preserve cell viability and metabolic activity (Sahu and Brahmaprakash, 2016). EC level is also still moderated and stable, as suitable for bacterial growth without inducing osmotic stress (Lam et al., 2020). The balanced environment ensures that the bacteria receive sufficient carbon and nitrogen for energy, with readily available nitrogen for initiation during early growth, and stable conditions to support enzymatic activity during metabolism (Neneng, 2020). Formula A5 demonstrated the most effective and consistent support for either population growth or physicochemical conditions, indicating the proportion created optimal conditions for viability and functional performance of the consortia.

An initial bacterial concentration of 1% was identified as the most effective because it provides an optimal balance between growth and condition stability. The 1% inoculant performed optimally due to its balance between microbial density and available resources. In lower inoculum levels, there was slower early growth and variability, likely due to longer lag phases and reduced cell-to-cell communication (Nie et al., 2023). Meanwhile, the 1.5% concentration showed signs of resource competition, leading to growth saturation and a decrease in population toward the end of incubation (Bernhardt et al., 2020). The lower concentration exhibited slow growth, whereas the higher concentration led to rapid nutrient depletion, potentially resulting in the accumulation of metabolic waste.

The agitator speed played a crucial role in determining bacterial growth and metabolite production. Among the speeds, 135 rpm was the most effective because it maximized metabolite production by improving aeration, creating a well-aerated, homogenized culture that supports N-fixer bacteria. Improved aeration enhances nutrient uptake, waste removal, and all of which accelerate metabolic activity (Cao et al., 2024). Moderate shear forces at this speed may also activate stress-responsive pathways that stimulate secondary metabolite biosynthesis, including phytohormones and phosphatase enzymes (Rodríguez-Durán et al., 2023). These results align with previous studies, which show that enhancing oxygen availability stimulates enzyme activity and secondary metabolite production (Fàbregas and Fernie, 2022). Lower speed at 115 and 125 rpm provided less aeration and weaker mixing, resulting in lower metabolites output compared to 135 rpm.

These findings clarified that the specific liquid growth media supported N-fixing bacteria in proliferating and synthesizing the essential secondary metabolite. Therefore, this biofertilizer might support strawberry growth in soilless media and, simultaneously, reduce the use of chemical fertilizers.

CONCLUSIONS

The compatibility test showed no inhibition, but demonstrated that *Bacillus subtilis* had the most dominant growth among the tested isolates. Its proportion was reduced to ensure balanced microbial interaction in the consortia, resulting in a final formulation ratio of 1:2:2:2 (*B. subtilis: A. salinestris: P. megaterium: A. chroococcum*). A combination of 1% molasses, 0.1% ammonium chloride (NH_4Cl), and 0.2% buffer phosphate. This formulation (A5) resulted in the highest bacterial population and maintained stable pH and EC levels throughout incubation. The 1% inoculant level was the most suitable for achieving rapid and stable bacterial growth. An agitation rate of 135 rpm yielded the highest bacterial population and significantly increased the production of plant growth-promoting substances, such as IAA, gibberellin, zeatin, and phosphatase enzyme activity.

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