Jurnal Agrotek Lestari Vol. 12 No. 2, Oktober 2026 P-ISSN: 2477-4790
PP. 95 – 102 E-ISSN: 2721-8945

Cell and Spore Viability of Single Strains and Consortia of *Bacillus* for Biofertilizer in Liquid Media

Viabilitas Sel dan Spora Strain Tunggal dan Konsorsium *Bacillus* untuk Pupuk Hayati Dalam Media Cair

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ABSTRACT

Biofertilizer formulations based on spore-forming Bacillus *support integrated plant nutrient management. This laboratory experiment compared cell and spore counts of four Bacillus species and their consortia across different growth media, as well as the media's acidity and electrical conductivity. The assay tested two molasses-based liquid media for cell proliferation and sporulation of* B. safensis *B1,* B. subtilis *D2,* B. altitudinis *B14, and* Bacillus *sp. E2, and their consortium, formulated with an equal composition of all strains. Batch cultures were performed using 50 mL of tryptic soy broth in a 250-mL Erlenmeyer flask at room temperature for 9 days with agitation at 115 rpm. The cells and spores in each formula were counted at 3, 6, and 9 days after inoculation, and the acidity (pH) and electrical conductivity (EC) were measured on day 9. Medium composition influenced cell proliferation and sporulation differently and varied across all strains and the consortium. Formula A, containing 2% molasses, 10 g peptone, and 3 g beef extract, produced the highest cell and spore populations, followed by Formula B (2 g beef extract) and Formula C. The specific medium for Bacillus (Formula C) supported cell proliferation but poor sporulation. Formula C had the highest pH and EC among the liquid inoculants. This study indicated that sporulation capacity depended on medium composition and Bacillus strain, whereas pH and EC were more determined by the formula. These results suggest that biofertilizer development should consider the medium composition and* Bacillus *strains.*

Keywords: *Acidity, Electrical conductivity, Molasses, Spore, Vegetative cell*

ABSTRAK

Formulasi pupuk hayati berbasis *Bacillus* pembentuk spora mendukung pengelolaan nutrisi tanaman secara terpadu. Tujuan dari percobaan laboratorium ini adalah membandingkan jumlah sel dan spora dari empat spesies *Bacillus* dan konsorsiumnya pada berbagai media pertumbuhan, serta mengukur keasaman dan konduktivitas listrik media tersebut. Pengujian dilakukan pada dua media cair berbasis molase untuk proliferasi sel dan sporulasi *B. safensis* B1, *B. subtilis* D2, *B. altitudinis* B14, dan *Bacillus* sp. E2, serta konsorsiumnya, diformulasikan dengan komposisi yang seimbang dari keempat strain tersebut. Kultur batch

dilakukan menggunakan 50 mL kaldu triptik kedelai dalam labu Erlenmeyer 250 mL pada suhu ruang selama 9 hari dengan pengadukan pada 115 rpm. Sel dan spora dalam setiap formula dihitung pada hari ke-3, ke-6, dan ke-9 setelah inokulasi, sedangkan tingkat keasaman (pH) dan daya hantar listrik (DHL) diukur pada hari ke-9. Proliferasi sel dan sporulasi dipengaruhi oleh komposisi media dan bervariasi pada setiap strain dan konsorsium. Pada hari ke-9, Formula A, yang terdiri dari molase 2%, pepton 10 g, dan beef extract 3 g, menghasilkan populasi sel dan spora tertinggi, diikuti oleh Formula B yang mengandung beef extract 2 g dan Formula C . Formula C, media spesifik untuk *Bacillus*, mendukung proliferasi sel, tetapi sporulasi kurang baik. Nilai pH dan DHL tertinggi ditunjukkan oleh Formula C yang spesifik untuk Bacillus. Studi ini menunjukkan bahwa kapasitas sporulasi bergantung pada komposisi media dan strain *Bacillus*, sedangkan perubahan pH dan EC lebih ditentukan oleh formula yang digunakan. Hasil penelitian ini menunjukkan bahwa pengembangan pupuk hayati perlu mempertimbangkan komposisi media dan strain *Bacillus*.

Kata kunci: Kemasaman, Konduktivitas elektrik, Molase, Sel vegetatif

INTRODUCTION

Biofertilizer containing beneficial rhizobacteria *Bacillus*, aligns with the concept of integrated plant nutrient management (INPM) That prioritizes a multi-nutrient approach that includes inorganic, organic, and biological fertilizers. Biofertilizers function in agriculture by enhancing nutrient availability and storage, and maintaining microbial consortia that support plant growth and crop productivity (Tiwari *et al*., 2022). *Bacillus* is a ubiquitous rhizobacterium easily isolated from agricultural soil; it is a heterotrophic, aerobic bacterium well known as an N-fixer, phosphate solubilizer, and phytohormone producer. A key property of *Bacillus* is its ability to form spores that withstand abiotic stresses in soil, such as drought and nutrient deficiencies (Kuramshina & Khairullin, 2023; Errington & van der Aart, 2022). Inoculation of *Bacillus* biofertilizer in Indonesia has improved the growth and yield of important agricultural crops such as shallots and sweet corn (Gan & Qodri, 2022; Song *et al*., 2025; Anwar *et al*., 2026).

Sporulation is inducible, for example, by nutrient limitation, including carbon, nitrogen, and salt. Currently, several media have been recommended and widely studied to stimulate cell counts during the logarithmic phase. Most *Bacillus* strains form endospores in salt-supplemented media. However, optimization of sporulation-stimulating media containing Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} salts remains limited. These salts increase spore populations and improve spore stability because salt reduces the water content within *Bacillus* cells (Bressuire-Isoard *et al*., 2018; Yuniarti *et al*., 2019).

Bacillus sporulation in specific media also depends on the strain, as strains differ in salinity tolerance (Cui *et al*., 2026). Some *Bacillus* strains can reach 1016 CFU/mL in inorganic media with specific salts, but the population remains low, below 10^7 CFU/mL (Hindersah *et al*., 2025). Meanwhile, Sinnelä et al. (2019) reported *Bacillus* spore counts reaching 10^9 CFU/mL in a medium containing calcium and manganese. *Bacillus* cell counts up to 10^{10} have been reported in molasses-based media when Bacillus is grown with Azotobacter (Hindersah *et al*., 2020). However, inorganic media are more expensive and may not be cost-effective for biofertilizer production.

Including salt in organic-based media is necessary to induce spore growth

while maintaining vegetative cell viability. Molasses is agricultural waste that contains essential nutrients for bacterial growth. Molasses-based media are generally designed to increase bacterial cell counts; however, few studies have examined spore growth in these formulations. Bacillus strains do not form endospores under all cultural conditions.

Determining spore formation in several *Bacillus* growth media is crucial to increasing cell viability during storage. The objective of this study was to observe and compare changes in cell and spore counts of four *Bacillus* strains and their consortia across different growth media, and the acidity (pH) and electrical conductivity (EC) of the media.

METHODS

The *Bacillus* strains, including *B. safensis* B1, *B. subtilis* D2, *B. altitudinis* B14, and Bacillus sp. E2, were isolated through collaboration between PT Pupuk Indonesia and the Faculty of Agriculture, Universitas Padjadjaran. A pure liquid culture of each strain was prepared in tryptic soy broth (TSB) by inoculating 100 mL of TSB with a loopful of colony from the 24-h-old slant, then incubating for 72 h at 30 °C. The pure culture of the *Bacillus* consortium was prepared by mixing equal volumes of all strains 72 h after incubation and homogenizing on a 115-rpm gyratory shaker at room temperature.

Bacillus strains and their consortium were grown in two molasses-based liquid media and one control liquid medium for *Bacillus* production, as follows:

A: Molasses 2%, peptone 10 g, and beef extract 3 g
B: Molasses 2%, peptone 10 g, and beef extract 2 g
C: Peptone 10 g, lactose 5 g, NaCl 5 g, Beef extract 3 g, K_2HPO_4 2 g

The experiment used a completely randomized design. Each strain and its consortium were grown in triplicate on the specific medium described above.

A 50 mL liquid medium was placed in a 250 mL Erlenmeyer flask. The flask was plugged with cotton, then covered with aluminum foil, and sterilized at 121°C for 20 minutes. A 1% (v/v) pure liquid culture of each bacterial strain and the consortium was inoculated separately into sterilized medium (Formulas A, B, and C).

The bacterial cultures were incubated on a shaker at 115 rpm at room temperature (24-27°C) for 9 days. Bacterial growth was assessed daily by measuring vegetative cells and bacterial spores on days 3, 6, and 9 using the serial dilution method (Ben-David & Davidson, 2014) on tryptic soy agar. All measurements were conducted in triplicate.

At the end of the incubation period, we measured the acidity and EC of each Bacillus strain and the consortium culture in different media using a pH meter and an EC meter. Each parameter was measured in triplicate.

All data were subjected to analysis of variance (F test, $p<0.05$). If treatments significantly affected the measured parameter, Duncan's Multiple Range Test (DMRT) was performed at $p<0.05$.

RESULTS AND DISCUSSION

Cell Population

The cell population of all *Bacillus* strains increased continuously from day 3 to day 9 across all media formulations. However, cell counts varied by strain each day. In general, on day 3, Formula A had a lower population than the other formulas. Specific strains reached the highest count, up to 15 $\log_{10}$ CFU/mL in Formula A on day 9.

In Formula A (Table 1), cell densities rose from day 3 to more than 14 $\log_{10}$ CFU/mL on day 9. The lowest population in this formula on day 9 was demonstrated by *B. safensis* B1, while the cultures of the other strain and the consortium reached 14 $\log_{10}$ CFU/mL. A *Bacillus* colony was observed on the TSA

plate after 9 days of incubation (Fig. 1). The E2 strain and the Bacillus consortium were denser than the other strains, indicating a higher cell population in the growth medium.

Table 1. Cell and spore population of four *Bacillus* strains during 9 days of incubation in formula A composed of molasses 2%, peptone 10 g, and beef extract 3

Spesies Bacillus	Cell (log_{10} CFU/mL)			Spore (log_{10} CFU/mL)		
	Day 3	Day 6	Day 9	Day 3	Day 6	Day 9
B. safensis B1	8.95 b	10.30 b	13.06a	4.31 a	6.00 a	4.72 a
B. subtilis D2	10.29 c	11.80 c	14.26b	4.15 a	6.93 b	8.30 b
B. altitudinis B14	8.18 a	11.29 c	14.13b	4.21 a	6.54 ab	9.28 c
Bacillus sp. E2	8.00 a	11.85 c	14.65b	4.35 a	6.70 ab	9.51 c
Bacillus Consortium	8.00 a	10.60 b	14.76b	4.44 a	7.00 b	9.22 c

Average numbers followed by different letters in a column are not significantly different based on DMRT, $p < 0.05$

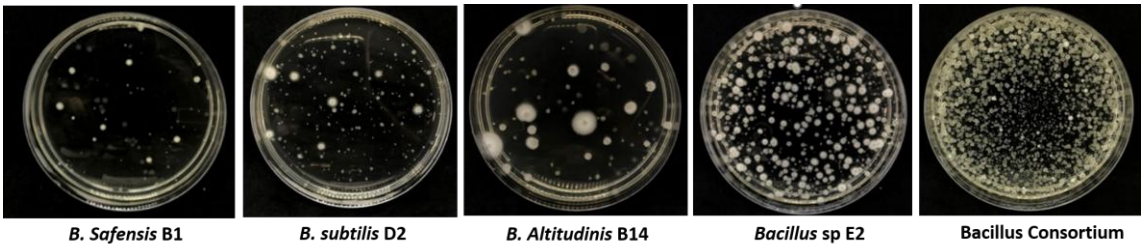


Figure 1. Colonies of Bacillus on TSA at 9 days after incubation for the total plate count

Formula B promoted the highest cell growth (Table 2) compared with formulas A and B (Table 1 and Table 3). Cell populations reached 14.79–15.05 log_{10} CFU/mL on day 9. The *Bacillus* consortium culture showed a higher density than the single culture. Bacillus cell populations in the single-strain cultures were similar, at approximately 14 log_{10} CFU/mL, which is one log lower than in the consortium.

Cell populations in Formula C (Table 3) also increased from day 3 to day 9. However, on day 9, the cell count was generally lower than in the other formulas (Tables 1 and 2).

Table 2. Cell and spore population of four *Bacillus* strains during 9 days of incubation in formula B composed of molasses 2%, peptone 10 g, and beef extract 2 g)

Spesies Bacillus	Cell (log_{10} CFU/mL)			Spore (log_{10} CFU/mL)		
	Day 3	Day 6	Day 9	Day 3	Day 6	Day 9
B. safensis B1	9.11a	12.79a	14.79a	5.90a	5.65b	7.03b
B. subtilis D2	9.67a	12.76a	14.96a	7.06c	6.18c	4.42a
B. altitudinis B14	9.46a	13.21b	15.05a	5.74a	6.11c	8.00c
Bacillus sp. E2	9.80b	13.00b	14.93a	6.08ab	6.47c	4.23a
Bacillus Consortium	9.79b	12.53a	15.38b	6.41b	4.62a	7.07b

Average numbers followed by different letters in a column are not significantly different based on DMRT, $p < 0.05$

By day 9, the cell count of the *B. safensis* B1 culture was the lowest, while those of Bacillus sp. E2 and the Bacillus consortium were one log higher; *B. subtilis* D2 and *B. altitudinis* B4 were 2 logs higher.

Spore Population

The spore counts in the medium did not show growth comparable to that of the cells. The pattern of temporal spore change clearly depends on strains and media (Tables 1-3). On day 3, spore counts for all strains inoculated into all formulas were $<10^6$ CFU/mL. On day 9, spore counts varied by formula and strain.

In Formula A, spore populations increased to 6 log10 CFU/mL by day 6 and further increased by day 9 to 8–9 $\log_{10}$ CFU/mL. However, spore populations in the *B. Safensis* B1 culture decreased to less than 105 CFU/mL by day 9. The highest spore population was observed in *Bacillus* sp. E2, *B. altitudinis* B14, and the consortium.

Spores were already detected on day 3 in all strains grown in Formula B (Table 2). The highest populations at day 3 were observed in *B. subtilis* D2, which decreased by the end of incubation. By day 9, *B. altitudinis* B14 produced the highest spore population (8.00 $\log_{10}$ CFU/mL), followed by *B. safensis* B1 and Bacillus consortium (about 7.00 $\log_{10}$ CFU/mL). However, spores of *B. subtilis* D2 and *Bacillus* sp. E2 declined to below 10^5 CFU/mL by day 9, the lowest among all strains.

The C medium supported only limited sporulation (Table 3) compared to Formulas A and B. Despite a high cell count at day 9, the medium did not support sporulation. The spore counts on day 6 were lower than in Formulas A and B. Only *B. safensis* B1 and *B. subtilis* D2 increased spore populations, while the liquid culture of *B. altitudinis* B14, *Bacillus* sp. E2, and the consortium showed spore counts under 10^5 CFU/mL.

Table 3. Cell and spore population of four Bacillus strains during 9 days of incubation in formula C, a specific *Bacillus* growth medium

Spesies Bacillus	Cell ($\log_{10}$ CFU/mL)			Spore ($\log_{10}$ CFU/mL)		
	Day 3	Day 6	Day 9	Day 3	Day 6	Day 9
B. safensis B1	8.65a	11.60a	12.70a	5.60b	4.10a	7.48c
B. subtilis D2	9.27b	11.74ab	14.53c	6.61c	4.26a	6.70b
B. altitudinis B14	9.85b	12.44b	14.41c	4.76a	4.05a	4.36a
Bacillus sp. E2	9.97b	11.00a	13.70b	4.70a	4.25a	4.73a
Bacillus Consortium	8.98b	11.40a	13.74b	6.16bc	4.16a	4.49a

Average numbers followed by different letters in a column are not significantly different based on DMRT, $p < 0.05$

Acidity and Electrical Conductivity

The acidity (pH) of all media before the experiment was 7, but the EC was not measured. On day 9, the pH of Formula A became slightly acidic, while Formulas B and C were acidic to alkaline (Table 4). The pH of Formulas A and B was similar across the strains, but in Formula C the *B. safensis* B1 and *B. subtilis* D2 cultures were lower than the liquid culture of the other strain.

The *Bacillus* strains determined the EC of Formula A and B but did not affect that of Formula C. Statistical analysis did not yield consistent results among the strains. *B. altitudinis* B14*, Bacillus sp.* E2 and *the Bacillus consortium culture had higher EC in Formula A, while Formula B had the highest EC* in *B. subtilis* D2 and the *Bacillus* consortium. Table 4 showed that the EC of Formula C increased abruptly.

Overall, the pH and EC of all growth media were likely determined by formulation rather than by the Bacillus strain (Table 4).

Table 4. Change in pH and EC in three liquid media inoculated with single and consortia of 4 *Bacillus* strains by day 9.

Spesies Bacillus	Formula A		Formula B		Formula C	
	pH	EC (µS/cm)	pH	EC (µS/cm)	pH	EC (µS/cm)
B. safensis B1	6.98b	8.04a	8.73a	7.84a	8.97a	30.50a
B. subtilis D2	6.08a	7.91a	8.16a	9.01c	8.72a	32.20a
B. altitudinis B14	6.19a	9.18b	8.22a	7.99a	9.21b	29.80a
Bacillus sp. E2	6.24a	8.69b	8.21a	8.57b	9.20b	29.00a
Bacillus Consortium	6.08a	8.42a	8.03ab	9.33c	9.19b	31.70a

Average numbers followed by different letters in a column are not significantly different based on DMRT, $p < 0.05$

DISCUSSION

Comparison of the three formulas shows that medium composition influenced vegetative growth and sporulation differently. The sporulation pattern differed from that of vegetative growth. Although Formula B promoted the greatest cell multiplication on day 6, it did not consistently support high spore production at the end of incubation. In fact, spores of *B. subtilis* D2 and *Bacillus* sp. E2 declined to less than 5 $\log_{10}$ CFU mL^{-1}at day 9. This suggests that conditions favorable for rapid vegetative growth were not necessarily optimal for maintaining spores, which agrees with previous studies (Verma *et al*., 2013; Yuniarti *et al*., 2019; Hindersah *et al*., 2025).

Cell populations and sporulation in Formula A were higher than in Formulas B and C, The highest spore counts across the study were observed in Formula A, reaching about 9 $\log_{10}$ CFU/mL for *Bacillus* sp. E2, B. altitudinis B14, and the consortium. Therefore, Formula A was likely more effective at promoting the transition from vegetative cells to spores.

Some strains produced fewer spores in Formula B, but *B. Altitudinis* B14 maintained sporulation comparable to Formula A. Formula C, which was composed of peptone 10 g, lactose 5 g, NaCl 5 g, Beef extract 3 g, and K_2HPO_4 2 g, did not support good sporulation. Although vegetative populations exceeded 13 $\log_{10}$ CFU/mL for most strains, final spore counts were below 5 $\log_{10}$ CFU/mL in three of the five tested strains. Formula C supported cell proliferation but was less effective for stable spore formation.

Strain-dependent responses were also evident in both cell and spore counts. For example, Bacillus sp. E2 showed excellent sporulation in Formula A but failed to maintain spores in Formulas B and C. The *Bacillus* consortium exhibited a similar cell count in Formula B at day 9, but *B. altitudinis* B14 showed higher sporulation. This suggests that each strain adapts to different nutritional compositions and indicates that sporulation capacity depended not only on medium composition but also on species-specific physiological characteristics (Verma *et al*., 2013; Cui *et al*., 2026)

The consortium responded similarly to several individual strains. In Formula A, the consortium produced high vegetative cell populations and a high final spore density, whereas Formula C showed lower spore counts by day 9. This suggests that strain interactions did not inhibit growth in Formula A but may not have favored sporulation in Formula C.

Bacillus is widely used in biofertilizer development. Both high cell density and spore abundance are desirable in Bacillus-based biofertilizer formulations. Spores ensure that bacteria can survive harsh conditions, mainly drought. Formula A appears to provide the best balance between vegetative growth and sporulation, particularly for *B.*

altitudinis B14, *Bacillus* sp. E2, and the Bacillus consortium.

Based on the growth curve of typical *Bacillus* strains, the stationary and declining phases are reached at 2-4 days and 4-6 days in tryptic soy broth (Salsabila et al., 2024). The logarithmic phase in all formulas was likely extended to day 9 due to nutrient availability. Formulas A and B contained molasses as a C source and peptone and beef extract as N sources, while Formula C used lactose as the C source and beef extract as the N source. Bacillus is a heterotrophic bacterium that relies on organic C and N.

The spore production in Formulas A and B was higher because the sugarcane molasses contained calcium, manganese and sodium that induce sporulation (Bressuire-Isoard *et al*., 2018; Yuniarti *et al*., 2019; Sinnelä et al., 2019). Meanwhile, Formula C contained no additional salt.

Nutrient depletion at day 9 induced sporulation. Manganese is an essential cofactor for enzymes required during the onset and progression of sporulation, while calcium inside the spore provides the signature extreme heat resistance of mature endospores (Yuniarti et al., 2018; Sinnelä et al., 2019).

pH and EC on the final day varied by growth medium. The slight decrease in pH of Formula A compared the pH before inoculation may be related to bacteria producing organic acids (Risanti *et al*., 2025). The increase in EC after Bacillus inoculation was due to increased ionic strength. In this experiment, each growth medium contained a complex molecule that bacteria decomposed into simpler molecules and charged ions, which then underwent chemical dissociation (De Leersnyder et al., 2018).

The pH and viable Bacillus cells in all media met the requirements of the Ministry of Agriculture Regulation No. 216 of 2019 on biofertilizer quality. However, that regulation did not include endospore and EC requirements for liquid inoculants.

CONCLUSION

Among the three formulas, Formula B, composed of molasses 2%, peptone 10 g, and beef extract 2 g, produced the highest cell populations, followed by Formula A and Formula C. Formula A, which contained molasses 2%, peptone 10 g, and beef extract 3 g, produced the highest final spore population, particularly for *Bacillus* sp. E2, *B. altitudinis* B14, and the consortium. Although Formula C, a specific medium for Bacillus, supported vegetative growth, Bacillus sporulation was lower than in the other media. Three formulas inoculated with either a single strain or the consortium showed different pH and EC. Formula A was slightly acidic, while Formulas B and C were alkaline. Formula C has a higher EC than the others. This distinction matters for developing a Bacillus-based biofertilizer, because spores are generally more stable during storage and more tolerant of environmental stress than vegetative cells.

ACKNOWLEDGEMENT

PT Pupuk Indonesia funded this research in collaboration with the Faculty of Agriculture, Universitas Padjadjaran.

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