Pertanika J. Sci. & Technol. 31 (3): 1137 - 1155 (2023)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Humic Acid-Amended Formulation Improves Shelf-Life of Plant Growth-Promoting Rhizobacteria (PGPR) Under Laboratory Conditions

Buraq Musa Sadeq[1], Ali Tan Kee Zuan[1]*, Susilawati Kasim[1], Wong Mui Yun[2], Nur Maizatul Idayu Othman[3], Jawadyn Talib Alkooranee[4], Sayma Serine Chompa[1], Amaily Akter[1] and Md Ekhlasur Rahman[1,5]

[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[3]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Melaka, Kampus Jasin 77300 UiTM, Merlimau, Melaka
[4]Department of Plant Protection, Faculty of Agriculture, University of Wasit, Wasit, Iraq
[5]Divisional Laboratory, Soil Resource Development Institute, Krishi Khamar Sharak, Farmgate, Dhaka-1215, Bangladesh

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) is a soil bacterium that positively impacts soil and crops. These microbes invade plant roots, promote plant growth, and improve crop yield production. *Bacillus subtilis* is a type of PGPR with a short shelf-life due to its structural and cellular components, with a non-producing resistance structure (spores). Therefore, optimum formulations must be developed to prolong the bacterial shelf-life by adding humic acid (HA) as an amendment that could benefit the microbes by providing shelter and carbon sources for bacteria. Thus, a study was undertaken to develop a biofertilizer formulation from locally isolated PGPR, using HA as an amendment. Four doses of HA (0, 0.01, 0.05, and 0.1%) were added to tryptic soy broth (TSB) media and inoculated with *B. subtilis* (UPMB10), *Bacillus tequilensis* (UPMRB9) and the combination of both strains. The shelf-life was recorded, and

ARTICLE INFO

Article history:
Received: 01 June 2022
Accepted: 16 August 2022
Published: 31 March 2023

DOI: https://doi.org/10.47836/pjst.31.3.01

E-mail addresses:
buraqmosa1991@gmail.com (Buraq Musa Sadeq)
tkz@upm.edu.my (Ali Tan Kee Zuan)
susilawati@upm.edu.my (Susilawati Kasim)
muiyun@upm.edu.my (Wong Mui Yun)
nurmaizatul@uitm.edu.my (Nur Maizatul Idayu Othman)
jalkooranee@uowasit.edu.iq (Jawadyn Talib Alkooranee)
serinesayma@gmail.com (Sayma Serine Chompa)
amailyakter2554@gmail.com (Amaily Akter)
ekhlasurrahman02@gmail.com (Md Ekhlasur Rahman)
*Corresponding author

ISSN: 0128-7680
e-ISSN: 2231-8526

viable cells count and optical density were used to determine the bacterial population and growth trend at monthly intervals and endospores detection using the malachite green staining method. After 12 months of incubation, TSB amended with 0.1% HA recorded the highest bacterial population significantly with inoculation of UPMRB9, followed by mixed strains and UPMB10 at $1.8x10^7$ $CFUmL^{-1}$, $2.8x10^7$ $CFUmL^{-1}$and $8.9x10^6$ $CFUmL^{-1}$, respectively. Results showed that a higher concentration of HA has successfully prolonged the bacterial shelf-life with minimal cell loss. Thus, this study has shown that the optimum concentration of humic acid can extend the bacterial shelf-life and improve the quality of a biofertilizer.

Keywords: Colony forming unit, formulation, humic acid, PGPR, shelf-life

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) has developed as an alternative method for reducing the use of agricultural chemical fertilisers to promote sustainable farming practices (Saeed et al., 2017; El-Tarabily et al., 2020; Al Raish et al., 2021; Al Hamad et al., 2021; Lahlali et al., 2022; Rizvi et al., 2022). Nowadays, much emphasis is placed on lowering the use of costly inorganic fertilizers and avoiding environmental contamination by reducing nitrogen and phosphate fertiliser doses using biofertilizers (El-Ghamry et al., 2018). Microorganisms commonly used as biofertilizer components include nitrogen fixers, phosphorous and potassium solubilizers, PGPRs, fungi, endo and ectomycorrhizal, cyanobacteria, and other useful microscopic critters (Itelima et al., 2018; Kamil et al., 2018; El-Tarabily et al., 2019; Mathew et al., 2020; El-Tarabily et al., 2021; Alblooshi et al., 2021). In addition, PGPR can directly impact the root's nutrient transportation systems. The most common issue expressed by farmers and inoculant manufacturers is the shelf life of carrier-based inoculants. If short-lived biofertilizers are not utilized or sold before expiration, they will lead to a net monetary loss to the marketing authority (Calvo et al., 2019). Furthermore, the technical restrictions include the possibility of product degradation owing to reduced shelf-life or accidental mutations throughout the fermentation or storage. Therefore, to prolong the shelf-life, an optimized formulation must be developed (Mahalakshmi et al., 2019). Moreover, the formulation should normally include the active component in an appropriate carrier and additives to stabilize and safeguard the microbial cells throughout storage, transportation, and the targeted root zone (Arriel-Elias et al., 2018).

Humic acid (HA) is still soil's most complex and physiologically active component. It is dark brown, water-soluble at higher soil pH levels, and can persist in serene soil for generations (Sootahar et al., 2020). Humic acid amendments contain beneficial effects accelerated absorption of nutrients, decreased toxins, increased water retention, improved microbial growth by providing shelter and carbon sources, and improved overall soil structure (Ekin, 2019; Meng et al., 2021). One of the humic acids' most visible and

significant effects is their contribution to soil microbial communities. Scientists are becoming increasingly aware of the importance of a healthy microbiome to soil habitats. Furthermore, because bad soils and chemicals have devastated bacteria populations, they must be recovered (Pukalchik et al., 2019; Sun et al., 2022). For the past 20 years, scientists have been studying the interactions of humic acid with microbes. Initially, HA was researched primarily as a carbon or micronutrient source or its overall impact on microorganism development (Yang et al., 2021). Moreover, humic acid (HA) is often regarded as the essential component of organic matter, influencing the behaviour of organic contaminants. Due to the huge number of reactive functional groups, there was an extensive range of reaction activities, including surface adsorption, ion exchange, and complexation (Li et al., 2022; Rashad et al., 2022).

The liquid formulations are better than solid formulations because they allow for adding nutrients and extending the bacterial shelf-life (Zvinavashe et al., 2021). It is difficult to design unique formulations, but whether the product is new or improved, it must be steady during transport and storage, easy to handle and use, boost the activities of the organisms in the fields, and be expense and practicable (Young et al., 2006; Berninger et al., 2018). Several studies have shown that mixtures of PGPR could enhance biological control activity for multiple plant diseases through the mechanisms of induced systemic resistance or antagonism (Liu et al., 2018). The isolated bacteria (*B. subtilis*) with humic acid showed good survival with minimal cell loss for up to five months of storage (Young et al., 2006).

Bacillus subtilis cells are rod-shaped, Gram-positive bacteria found in soil and vegetation; they thrive in mesophilic environments (Cho & Chung, 2020). *Bacillus subtilis* generates dormant, extremely resistant endospores in response to environmental challenges or malnutrition. As a result, *B. subtilis* has evolved mechanisms that allow it to thrive under difficult conditions (Luu et al., 2022; Mahapatra et al., 2022). These spores can survive in hostile conditions for many years without necessary nutrition, and it is a type of PGPR with a short shelf-life due to its structural, and cellular components (Luu et al., 2022). Many factors influence a *Bacillus* spore's resistance to heat, strong proteinaceous coats, peptidoglycan cortex, moderate moisture content, and large amounts of di-picolinic acid and divalent cations within the spore core are all present (Durga et al., 2021; Cho & Chung, 2020; Mukherjee et al., 2022). Moreover, *B. subtilis* secretes extracellularly during nutrient scarcity, attempting to kill proteins that affect programmed cell death in siblings, releasing nutrients and allowing a portion of the population to expand. Dormancy can be seen as a risk-aversion strategy since resting structure, and cellular components necessitate energy investment and responsiveness to signals associated with favourable situations (Pashang et al., 2022). Dorner published a method for staining endospores in 1922, and Shaeffer and Fulton refined their approach in 1933 to make the procedure of staining only bacterial endospores speedier. The primary goal of endospore staining is to

distinguish bacterial spores from other vegetative cells and spore formers from non-spore formers (Lindsay et al., 2021).

Therefore, this study was undertaken (1) to develop a biofertilizer formulation consisting of locally isolated PGPRs with an optimized rate of humic acid concentrations as an amendment and (2) to study the shelf-life of these PGPRs for up to 12 months of incubation.

MATERIALS AND METHODS

Pure Colonies Collection of PGPR Strains

Two locally isolated plant growth-promoting rhizobacteria (PGPRs), *B. subtilis* (UPMB10) and *B. tequilensis* (UPMRB9) were collected at the Microbiology laboratory Department of Land Management in the Faculty of Agriculture, Universiti Putra Malaysia. These PGPRs were collected due to their morphological, cultural, and beneficial biochemical characteristics like N_2-fixing ability, solubilizing phosphate and potassium, and producing IAA, siderophore, and pectinase. These PGPR strains were also reported to increase the concentrations of N, P, and K in shoots and storage roots (Ali-Tan et al., 2017; Shultana et al., 2020; Kapadia et al., 2021; Shultana et al., 2021). These PGPRs were grown on nutrient agar (NA) media. Tryptic soy agar (TSA) media was used for sub-culturing these strains. Twenty ml of TSA media was poured into Petri plates, and the new pure colonies of the two strains were assessed by quadrant streaking technique to obtain a single and pure colony. A loopful from the original local strains was taken and streaked into a new Petri plate with TSA media, and each sample was replicated thrice and then incubated at 30°C in the incubator (SD-310 RL, Dasol, Korea) for 24 h.

Collection and Characterization of Humic Acid

Humic acid technical purchased from Sigma-Aldrich brand purity of ≥ 98 mol.% with code: 102098564 53680-50G products of Switzerland. Humic acid, which is black in colour and low solubility in water, has a pH of 6.2; its soluble in alkali but insoluble in acid and adjusted to seven by using tryptic soy broth media (TSB), which has a pH of 7.23, an organic C component of 30–50%, hydrogen (about 5%), nitrogen (about 3%) and a CEC of 70–166 me/100g. Humic acids are not single molecular formula compounds. Figure 1 illustrates that humic acid was generally aromatic in structure, with amino acids, amino sugars, peptides, and aliphatic molecules connecting the aromatic groups. Humic acid's hypothesized structure consists of free and bound phenolic hydroxyl groups, quinines, oxygen and nitrogen bridges, and carboxy groups (Nardi et al., 2021). Model structure of humic acid according to Stevenson and Schnitzer (1982):

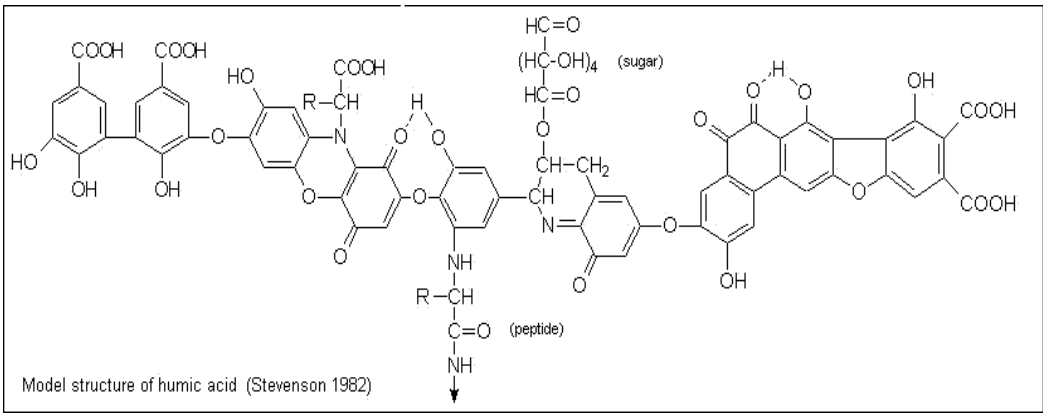


Figure 1. Model structure of humic acid (Stevenson & Schnitzer, 1982)

Shelf-Life Experimental and Treatments and Total Bacterial Population During Shelf-Life Period

A laboratory experiment was conducted with twelve treatments and three replications. The detailed treatments were as follows:

T1 = 0% Humic acid + UPMB10
T2 = 0% Humic acid + UPMRB9
T3 = 0% Humic acid + mixed strain
T4 = 0.01% Humic acid + UPMB10
T5 = 0.01% Humic acid + UPMRB9
T6 = 0.01% Humic acid + mixed strain
T7 = 0.05% Humic acid + UPMB10
T8 = 0.05% Humic acid + UPMRB9
T9 = 0.05% Humic acid + mixed strain
T10 = 0.1% Humic acid + UPMB10
T11 = 0.1% Humic acid + UPMRB9
T12 = 0.1% Humic acid + mixed strain

The microbial activity focuses on obtaining the population of bacteria in four Humic acid concentrations (0, 0.01, 0.05 and 0.1%) with two bacteria strains (*B. subtilis* (UPMB10), *B. tequilensis* (UPMRB9) and the mixture of both). Microbial development was recorded of this bacterium in laboratory circumstances for 12-months at a temperature of 30°C.

Procedure: 0, 0.01, 0.05 and 0.1 gm HA were weighted using an electric weighing machine (Precisa 1620 C) and transferred to 500 ml Erlenmeyer flasks. Tryptic Soy Broth (TSB) was prepared separately by dissolving 30 g of the media in 1 L distilled water, and about 100 ml TSB was transferred to each flask. The liquid formulations were autoclaved at 121°C for 15 minutes. After cooling at room temperature, one loopful (approximately

1 x 10^6 CFU) from UPMB10 and UPMRB9 cultures was taken and transferred into the broth media, then incubated under constant shaking at 150 rpm for 24 h at 33°C; each sample was replicated thrice. These conditions were essential so that both bacteria achieve a stationary stage of their respective growth curves at the time of their integration into the formulations. The bacterial growth population was determined by the serial dilution method 1/10 dilution series. One hundred microliters of each dilution were added to TSA Petri plates; the sample was spread evenly over the agar surface using the sterile glass spreader, and the plates were incubated in an incubator (DS-310RL, Dasol, Korea) at 33°C for 24 hours. The procedure was repeated three times for each humic acid concentration monthly for up to twelve months.

Measurements of Optical Density (OD) at Monthly Intervals for Up to 12 Months

Optical density (OD) was detected using a UV-visible spectrophotometer and checked at monthly intervals for up to 12 months. All treatments during this period were stored at room temperature at 30°C; the procedure was attempted using an 80% volume spectrophotometer cuvette. About 3.5 ml of samples were transferred from each treatment of PGPR-HA formulations (0, 0.01, 0.05, and 0.1%) and tryptic soy broth (TSB) for blank value to measure the absorbance at 600 nm wavelength. All measurements were repeated at least three times. In addition, bacterial growth trend was proposed to measure the optical density value for *B. subtilis* (UPMB10), *B. tequilensis* (UPMRB9), and mixed strains at 0, 6, 12, 18, and 24-hour periods at 30°C for 0.1% humic acid (HA) and control (without humic acid) to see the growth line trend at the 6-hour interval for 24 hours.

Endospore Stain Protocol by Using Malachite Green

According to the Schaeffer and Fulton (1933) method for staining endospores, 0.5 g of malachite green was dissolved in 100 ml of distilled water, and 2.5 g of safranin was dissolved in 100 ml of 95% ethanol. From 24 hours and 12-month-incubated cultures, endospores were detected using malachite green by the endospore staining method. The culture was spread on a slide and dried at room temperature naturally before being heat-fixed and wrapped in blotting paper. The slide was loaded with malachite green solution, which was steamed into the cells and spores using the burner's steaming light. This heating stage stained the vegetative cells and endospores; once the heating was complete, cool, rinse the dyes, and wash gently with water. Next, add safranin to the slide to colour the vegetative cells. Remove excess water, dry with a clean towel, and allow the slide to air dry. A stained smear was observed with low magnification (10X) and then switched to 100X with oil immersion. The endospores appeared green, and the vegetative cells were brownish-red or pinkish (Shen & Zhang, 2017).

Statistical Analysis

All data obtained were subjected to Analysis of Variance (ANOVA) using Statistical Analysis System (SAS) version 9.4. Means were compared by Significance Differences (LSD) at the 5% probability level. Data were subjected to ANOVA using (SAS 2004).

RESULTS AND DISCUSSION

Effect of Humic Acid Concentrations on Bacterial Population

Data presented in Figure 2 showed the bacterial population and viability in humic acid (HA) amended formulation with different strains (*B. subtilis* (UPMB10), *B. tequilensis* (UPMRB9), and mixed strain) can be enhanced by the highest concentration of HA for up to 12 months of incubation at 0.1% HA under 30°C room temperature. At 6 months of incubation, HA formulations at 0.1% and 0.05% concentrations for UPMB10, UPMRB9, and mixed strain, respectively, showed significantly highest population, then started to decline after six months of storage. While 0% HA and 0.01% HA concentration, the maximum population was recorded at 3 months of incubation by UPMB10, UPMRB9,

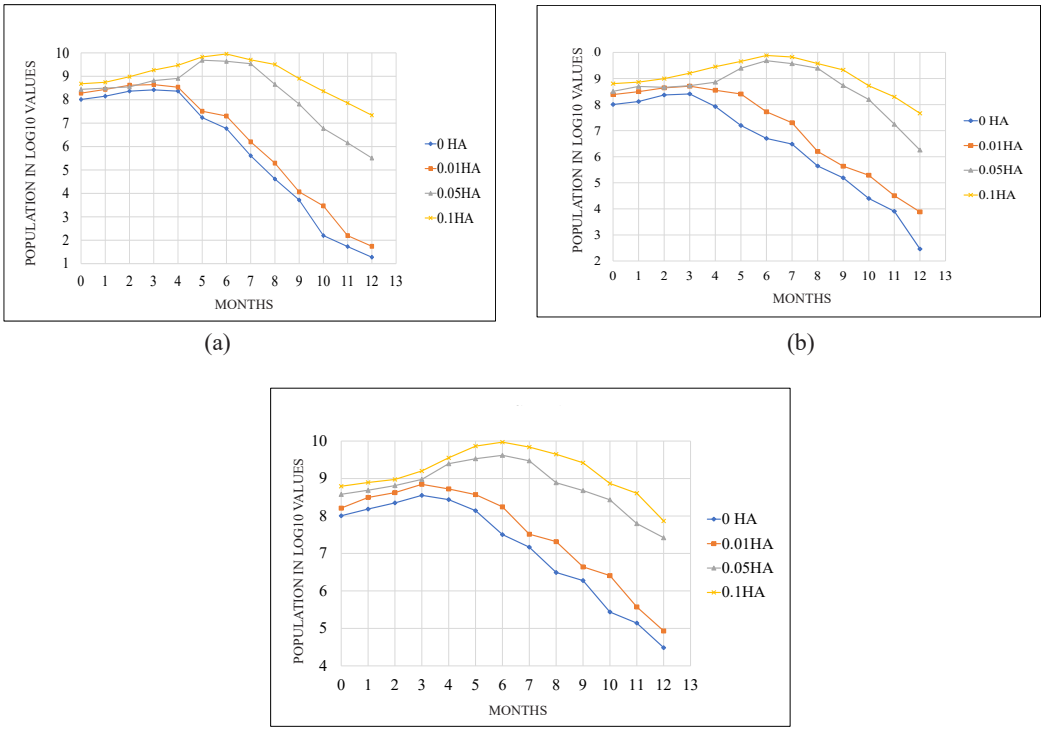


Figure 2. Effect of HA concentrations on bacterial population with three replications in (a) UPMB10, (b) UPMRB9, and (c) mixed strains for up to 12 months interval

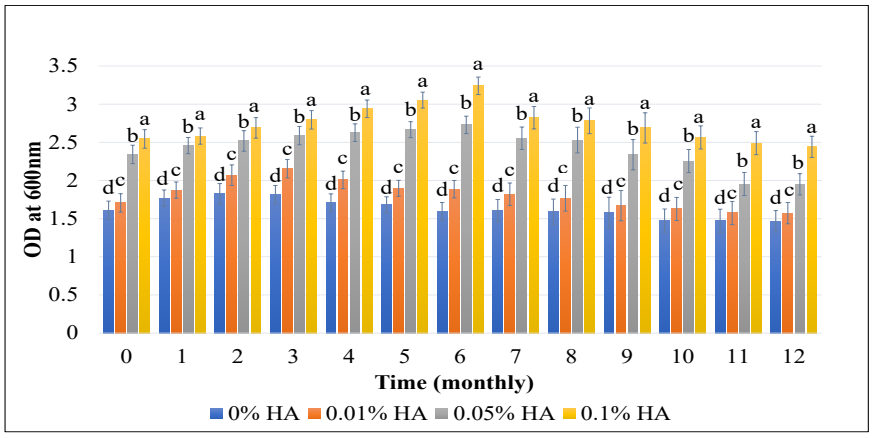
and mixed strain, respectively, then started to decline after three months of storage. After 12 months of incubation, tryptic soy broth (TSB) amended with 0.1% of HA recorded the highest population significantly at 2.8 x10^7 CFU mL^{-1} (log 7.8) by mixed strain, followed by the UPMRB9 and UPMB10 at 1.8 x10^7 CFU mL^{-1} (log 7.6) and 8.9 x10^6 CFU mL^{-1} (log 7.3), respectively, followed by 0.05% HA compared to 0% and 0.01% HA.

Tryptic soy broth (TSB) media with humic acid as an amendment demonstrated an excellent combination as a new formulation for the bacteria shelf-life. Results showed that a higher concentration of 0.1% HA has a better shelf-life after 12 months of storage, and the bacterial strain showed excellent vitality with minimal cell loss from UPMRB9 at 1.8 x10^7, followed by UPMB10 and mixed strains at 8.9 x10^7 and 18.7 x10^5, respectively. At the same time, the lowest concentration was observed at 0% HA and 0.01%HA. A similar finding was made by Young et al. (2006), in which they reported that humic acid could enhance the viability of the encapsulated bacteria and observed minimal cell loss upon storage for five months. The steady growth of microbial activity is noticed in every treatment (bacterial formulations) due to increasing cell count and a maximum extent until its reduction.

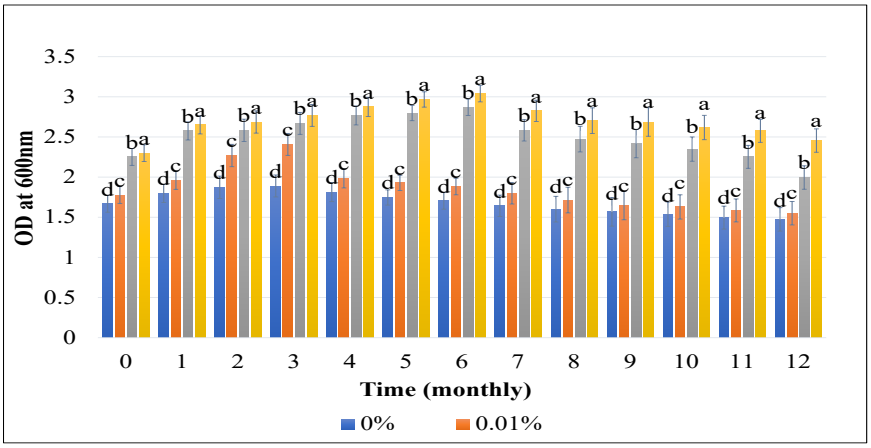
This finding is illustrated by the time the microbial cells have adapted and grown, even if kept at temperatures other than the optimum. Since the cells are not killed by their metabolism, they can slowly take the nutrients in the formulations. Cellular activity decreases as a result of cell death if it is diminished (Mendoza-Labrador et al., 2021). We believe that one of the main reasons is that humic acids essentially offer better habitation and nutrition for beneficial bacteria, which explains why microbial communities thrive in the presence of humic acids (Li et al., 2019; Morawska et al., 2022). Many strategies have been postulated earlier to discuss the impact of humic acid on bacteria, such as changes in surface charge, chemical reactions, and steric impact caused by HA coating. Moreover, HA can also play a crucial function in altering the agglomeration of mineral particles caused by HA smears (Hong et al., 2015). Tikhonov et al. (2010) reported that the humic acid in live cells implies that it may be possible to use their energy more efficiently. ATF stores energy for cell regeneration, growth, and reproduction.

Effect of Humic Acid on Bacterial Optical Density During Shelf-Life

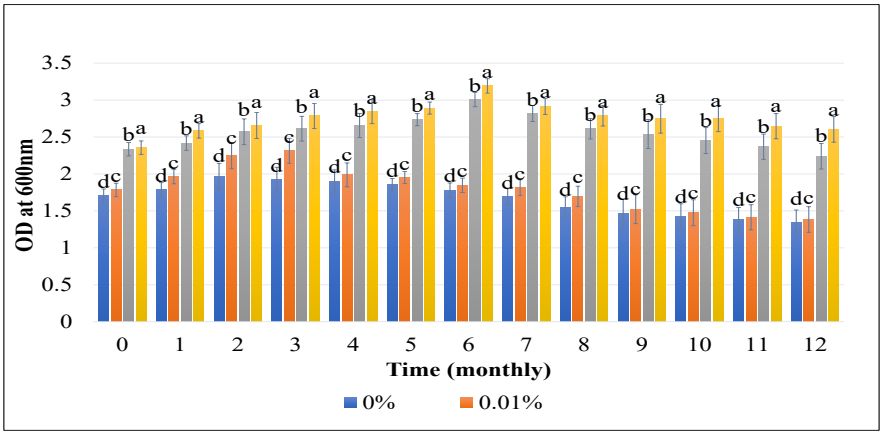
At monthly intervals for up to 12 months, 0.1% of humic acid (HA) significantly stimulated the optical density measurements (OD), followed by 0.05% compared to 0% and 0.01% for PGPRs shelf-life. The results show a significantly highest OD (600nm) in 0.1% HA after 12 months of incubation at (2.60) from a mixed strain followed by *B. tequilensis* (UPMRB9) and *B. subtilis* (UPMB10) at (2.46) and (2.44), respectively, followed by 0.05% HA. While 0% HA and 0.01% HA were recorded as the lowest OD readings (Figure 3).



(a)



(b)



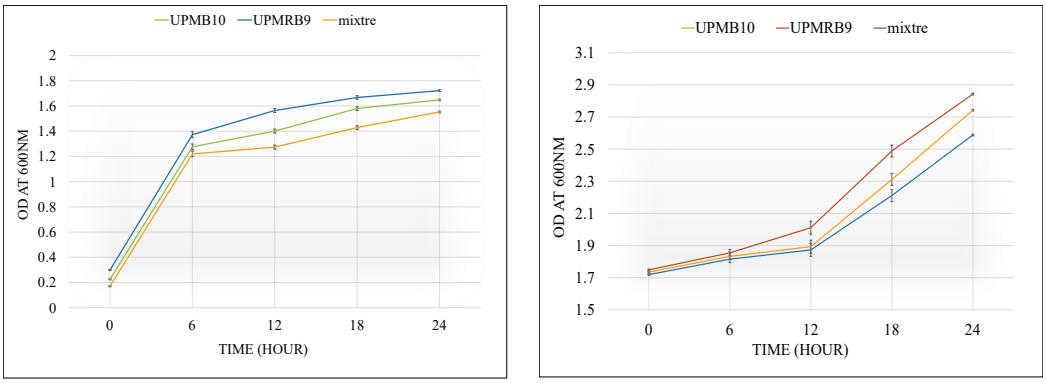
(c)

Figure 3. Optical density at 600 nm monthly intervals for 12 months of incubation in (a) UPMB10, (b) UPMRB9, and (c) mixed strain with 4 concentrations of HA and 3 replications. Means with the same letter do not significantly differ at α = 0.05 LSD.

Among all formulations, 0.1% humic acid was the best formulation for maintaining the survival of bacterial growth throughout the incubation period. After 12 months of storage and in UV-visible spectrophotometer analyses, the optical density (OD) at 600 nm has demonstrated *B. subtilis* (UPMB10), mixed strain, and *B. tequilensis* (UPMRB9) at (3.24) (3.19) and (3.04), respectively. The degradation rate depended on the time. During the first 6 months, there was an increase in bacterial growth. While from six months of storage onwards, the degradation rate of bacterial growth was slow because, in this period, the survival and development of microorganisms are affected by various variables. The inherent, intrinsic characteristics or variables are nutrient content, moisture content, pH, available oxygen, and temperature (Awulachew, 2021). Although the microbe's nutrient requirements are quite organic, the following are important: water, energy source, carbon-nitrogen supply, vitamins, and minerals. For each treatment (bacterial formulation), a progressive increase in microbial activity is noted as the number of cells increases, reaching a maximum point before decreasing (Timmis & Ramos, 2021; Bhakyaraj et al., 2022). This phenomenon is explained by the time required for microbial cells to adapt and proliferate, even when stored without agitation or at temperatures other than optimal. Since their metabolism is not disrupted, the cells continue to ingest the nutrients from the formulas, albeit at a slower rate (Arriel-Elias et al., 2018; Tapia et al., 2020). So, the degradation rate shows when the microbe's nutrient requirements start to decline.

Bacterial Growth Curves With and Without Humic Acid

Cell growth of individual bacterial strains and mixed strains was checked at 600 nm using the UV-visible spectrophotometer. An increase in optical density (OD) has been demonstrated in humic acid as an amendment. The increased bacterial growth in *Bacillus subtilis* (UPMB10), *B. tequilensis* (UPMRB9), and mixed strains with HA was gradually at the first (lag phase) at (1.83), (1.85), and (1.81) respectively, but then became drastic at12 hours on-wards at (2.74), (2.84) and (2.58), respectively. Whereas, without humic acid OD of the bacterial growth in UPMB10, UPMRB9, and the mixed strain was drastically increased from 0–6 hours of incubation and gradually became constant afterward at (1.64), (1.72) and (1.55), respectively (Figure 4). However, the final optical density (OD) was much higher than without HA. UPMB10 and UPMRB9 with and without humic acid were checked under the microscope after 24 hours of incubation, as shown in (Figures 4 & 5).



(a) (b)

Figure 4. Bacterial growth curve (a) without Humic acid, and (b) with Humic acid for 24 hours with three replicates

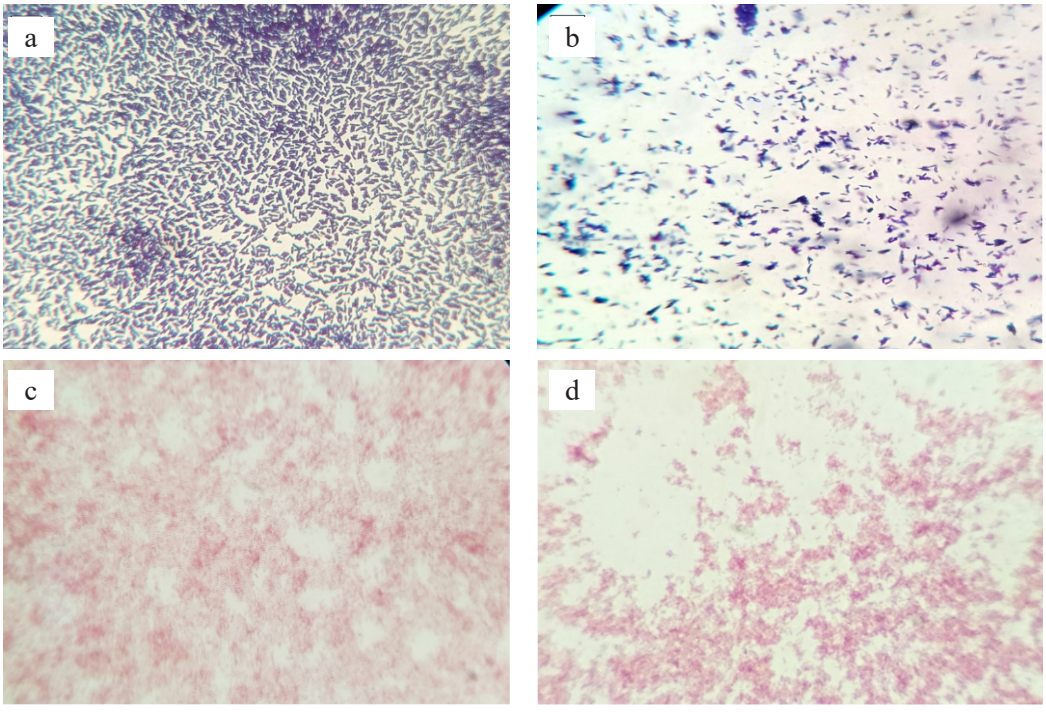


Figure 5. (a) UPMB10 with Humic acid, (b). UPMB10 without Humic acid, (c) UPMRB9 with Humic acid, and (d) UPMRB9 without Humic acid

Humic acid formulation with the concentration of 0.1% amended with tryptic soy broth (TSB) shows that the bacterial growth from *B. subtilis* (UPMB10), *B. tequilensis* (UPMRB9), and the mixed strain was significantly higher than without humic acid for 24

hours (Figure 4). The bacteria's response to HA in the medium varies, and these variances are visible even at the strain level (Tikhonov et al., 2010). The significant impact of humic acid is enhancing bacterial development, which helps to improve microbial growth by providing a carbon source. Moreover, due to its large size, humic acid serves as a food supply for microbes and a source of microflora. Vassilev et al. (2020) reported that Humic compounds had been shown to increase or decrease the populations or actions of various microbial species. In addition, *B. subtilis* encapsulated with HA ensured higher viability of the immobilized biostimulant (Nagpal et al., 2022).

Bacillus subtilis Endospores Detection After 12 Months of Shelf-Life

After 12 months of storage, *B. subtilis* species developed endospores that were detected following the Endospore staining method using malachite green. *Bacillus subtilis* (UPMB10) and mixed strains were used to detect spores. After 24 hours of incubation, results showed that UPMB10 strain, but after 12 months of incubation, there were vegetative cells with pink colour and endospores with dark green colour for both UPMB10 and mixed strain as shown in Figure 6.

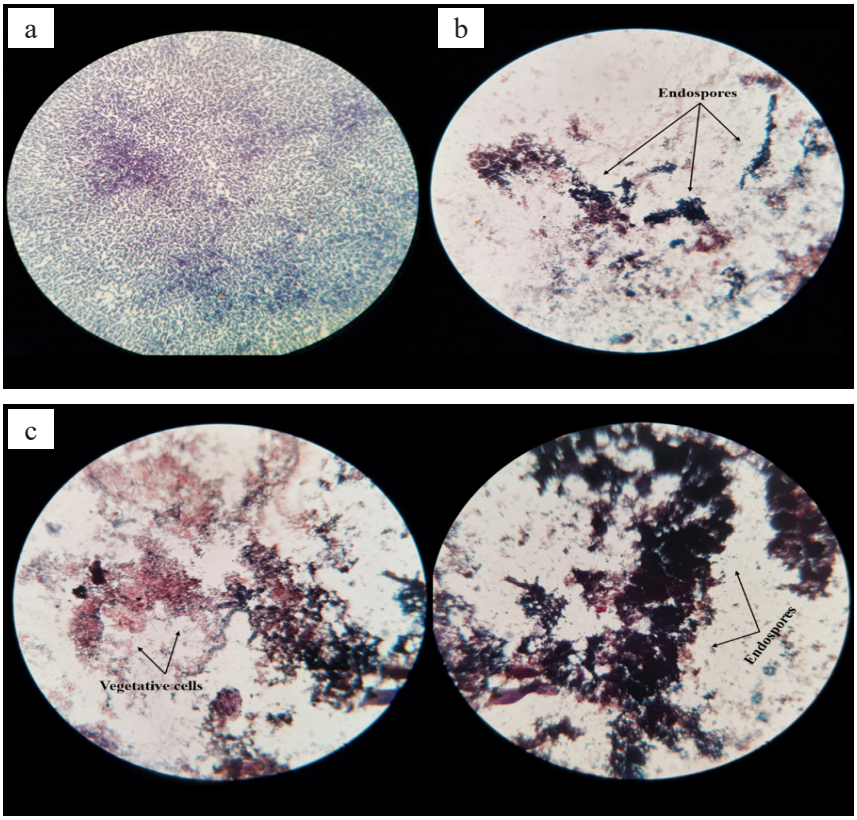


Figure 6. Spores' detection from *Bacillus subtilis* (UPMB10). (a) After 24 hours, (b) 12 months of storage, and (c) mixed strain after 12 months of storage

Endospore stain allows the bacterium to be dormant and highly resistant cells to preserve the cell genetic material during extreme stress. Endospores can survive environmental assaults that normally kill bacteria (Wrangham, 2019). When examined under a microscope, the results of endospore staining in both *B. subtilis* (UPMB10) and the mixed strains of bacteria reveal a dark green colour, indicating the presence of endospores. An endospore is a structure that can withstand harsh environmental conditions such as dryness, heat, and acidity (Rattray et al., 2021; Silaban et al., 2020). Endospores are extremely thick and refractile due to their low water content (Tehri et al., 2018). Bacteria with endospores are extremely difficult to colour, necessitating specialized staining. Spore-producing bacteria are stain-resistant. Bacteria that produce spores will significantly bind to the dye component malachite green. Moreover, it cannot be stained using safranin (Silaban et al., 2020). Połaska et al. (2021) found that the genus Alicyclobacillus contains a group of Gram-positive producing highly resistant endospores during unfavourable environmental conditions.

According to these findings, humic acid could support the combination of two different PGPR strains by demonstrating greater overall biocontrol and plant growth promotion compared to the individual PGPR strains. Microbes in a senescent stage appear to continue some metabolic activity to preserve viability and protect against stress conditions (Haruta et al., 2015; Dehsheikh et al., 2020; Pota et al., 2020). The response of the bacteria to the presence of HA in the medium is various, and these differences are manifested even at the level of the strains. Thus, the capability for the growth of humic acids is widespread among bacteria. It was shown for the first time that the mixed bacteria were compatible with the addition of HA, which provides additional evidence for symbiotic growth between the bacteria strains for a long period (Tikhonov et al., 2010; Lipczynska-Kochany, 2018).

CONCLUSION

In conclusion, as an amendment with an optimum concentration of 0.1% HA, humic acid significantly increased bacterial growth population and the optical density at monthly intervals for up to 12 months of incubation compared to 0% HA. A higher concentration of humic acid has a better shelf-life. The bacterial strain population showed excellent vitality with minimal cell loss. That may relate because humic acids can provide shelter and carbon sources for bacteria. Survival and development of microorganisms in the liquid formulation are affected by various variables: nutrient content, moisture content, pH, available oxygen, and temperature. Thus, the optimum concentration of humic acid can be used to prolong the bacteria's shelf-life and improve the quality of a biofertilizer. In this regard, the use of humic acid as an amendment for PGPR shelf-life in this study provided various advantages over the existing enrichment chemicals, demonstrating the grounds for the current investigation.

ACKNOWLEDGEMENTS

The authors sincerely acknowledge grants of the Fundamental Research Grant Scheme (FRGS) (FRGS/1/2020/STG01/UPM/02/6) by the Ministry of Higher Education, Malaysia.

REFERENCES

Al Hamad, B. M., Al Raish, S. M., Ramadan, G. A., Saeed, E. E., Alameri, S. S., Al Senaani, S. S., AbuQamar, S. F., & El-Tarabily, K. A. (2021). Effectiveness of augmentative biological control of Streptomyces griseorubens UAE2 depends on 1-aminocyclopropane-1-carboxylic acid deaminase activity against *Neoscytalidium dimidiatum*. *Journal of Fungi*, *7*(11), Article 885. https://doi.org/10.3390/jof7110885

Al Raish, S. M., Saeed, E. E., Alyafei, D. M., El-Tarabily, K. A., & AbuQamar, S. F. (2021). Evaluation of streptomycete actinobacterial isolates as biocontrol agents against royal poinciana stem canker disease caused by the fungal pathogen *Neoscytalidium dimidiatum*. *Biological Control*, *164*, Article 104783. https://doi.org/10.1016/j.biocontrol.2021.104783

Alblooshi, A. A., Purayil, G. P., Saeed, E. E., Ramadan, G. A., Tariq, S., Altaee, A. S., El-Tarabily, K. A., & AbuQamar, S. F. (2021). Biocontrol potential of endophytic actinobacteria against Fusarium solani, the causal agent of sudden decline syndrome on date palm in the UAE. *Journal of Fungi*, *8*(1), Article 8. https://doi.org/10.3390/jof8010008

Ali-Tan, K. Z., Radziah, O., Halimi, M. S., Rahim, K. B. A., Abdullah, M. Z., & Shamsuddin, Z. H. (2017). Growth and yield responses of rice cv. MR219 to rhizobial and plant growth-promoting rhizobacterial inoculations under different fertilizer-N rates. *Bangladesh Journal of Botany, 46*(1), 481-488.

Arriel-Elias, M. T., Oliveira, M. I., Silva-Lobo, V. L., Filippi, M. C. C., Babana, A. H., Conceição, E. C., & Cortes, M. D. C. (2018). Shelf-life enhancement of plant growth promoting rhizobacteria using a simple formulation screening method. *African Journal of Microbiology Research, 12*(5), 115-126. https://doi.org/10.5897/AJMR2017.8787

Awulachew, M. T. (2021). Food product shelf stability overview of sourdough-risen flatbread. *Journal of Food Technology & Nutrition Sciences, 3*(3), 1-3. https://doi.org/10.47363/JFTNS/2021(3)123

Berninger, T., González López, Ó., Bejarano, A., Preininger, C., & Sessitsch, A. (2018). Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microbial Biotechnology, 11*(2), 277-301. https://doi.org/10.1111/1751-7915.12880

Bhakyaraj, R., Arunkumar, D., & Anitha, A. (Eds.). (2022). *Synthetic Microbial Research-Challenge and Prospects.* Darshan Publishers.

Calvo, P., Zebelo, S., McNear, D., Kloepper, J., & Fadamiro, H. (2019). Plant growth-promoting rhizobacteria induce changes in Arabidopsis thaliana gene expression of nitrate and ammonium uptake genes. *Journal of Plant Interactions, 14*(1), 224-231. https://doi.org/10.1080/17429145.2019.1602887

Cho, W. I., & Chung, M. S. (2020). *Bacillus* spores: A review of their properties and inactivation processing technologies. *Food Science and Biotechnology, 29*(11), 1447-1461. https://doi.org/10.1007/s10068-020-00809-4

Dehsheikh, A. B., Sourestani, M. M., Zolfaghari, M., & Enayatizamir, N. (2020). Changes in soil microbial activity, essential oil quantity, and quality of Thai basil as response to biofertilizers and humic acid. *Journal of Cleaner Production*, *256*, Article 120439. https://doi.org/10.1016/j.jclepro.2020.120439

Durga, C. S. S., Ruben, N., Chand, M. S. R., Indira, M., & Venkatesh, C. (2021). Comprehensive microbiological studies on screening bacteria for self-healing concrete. *Materialia*, *15*, Article 101051. https://doi.org/10.1016/j.mtla.2021.101051

Ekin, Z. (2019). Integrated use of humic acid and plant growth promoting rhizobacteria to ensure higher potato productivity in sustainable agriculture. *Sustainability, 11*(12), Article 3417. https://doi.org/10.3390/su11123417

El-Ghamry, A., Mosa, A. A., Alshaal, T., & El-Ramady, H. (2018). Nanofertilizers vs. biofertilizers: New insights. *Environment, Biodiversity and Soil Security, 2*(2018), 51-72. https://doi.org/10.21608/jenvbs.2018.3880.1029

El-Tarabily, K. A., AlKhajeh, A. S., Ayyash, M. M., Alnuaimi, L. H., Sham, A., ElBaghdady, K. Z., Tariq, S., & AbuQamar, S. F. (2019). Growth promotion of *Salicornia bigelovii* by *Micromonospora chalcea* UAE1, an endophytic 1-aminocyclopropane-1-carboxylic acid deaminase-producing actinobacterial isolate. *Frontiers in Microbiology*, *10*, Article 1694. https://doi.org/10.3389/fmicb.2019.01694

El-Tarabily, K. A., ElBaghdady, K. Z., AlKhajeh, A. S., Ayyash, M. M., Aljneibi, R. S., El-Keblawy, A., & AbuQamar, S. F. (2020). Polyamine-producing actinobacteria enhance biomass production and seed yield in Salicornia bigelovii. *Biology and Fertility of Soils*, *56*(4), 499-519. https://doi.org/10.1007/s00374-020-01450-3

El-Tarabily, K. A., Ramadan, G. A., Elbadawi, A. A., Hassan, A. H., Tariq, S., Ghazal, E. W., Gamar, M. I. A., & AbuQamar, S. F. (2021). The marine endophytic polyamine-producing Streptomyces mutabilis UAE1 isolated from extreme niches in the Arabian Gulf promotes the performance of mangrove (*Avicennia marina*) seedlings under greenhouse conditions. *Frontiers in Marine Science*, *8*, Article 710200. https://doi.org/10.3389/fmars.2021.710200

Haruta, S., & Kanno, N. (2015). Survivability of microbes in natural environments and their ecological impacts. *Microbes and Environments*, *30*(2), 123-125. https://doi.org/10.1264/jsme2.ME3002rh

Hong, Z., Chen, W., Rong, X., Cai, P., Tan, W., & Huang, Q. (2015). Effects of humic acid on adhesion of *Bacillus subtilis* to phyllosilicates and goethite. *Chemical Geology*, *416*, 19-27. https://doi.org/10.1016/j.chemgeo.2015.10.017

Itelima, J. U., Bang, W. J., Onyimba, I. A., Sila, M. D., & Egbere, O. J. (2018). Bio-fertilizers as key player in enhancing soil fertility and crop productivity: A review. *Direct Research Journal of Agriculture and Food Science, 6*(3), 73-83.

Kamil, F. H., Saeed, E. E., El-Tarabily, K. A., & AbuQamar, S. F. (2018). Biological control of mango dieback disease caused by *Lasiodiplodia theobromae* using streptomycete and non-streptomycete actinobacteria in the United Arab Emirates. *Frontiers in Microbiology*, *9*, Article 829. https://doi.org/10.3389/fmicb.2018.00829

Kapadia, C., Sayyed, R. Z., El Enshasy, H. A., Vaidya, H., Sharma, D., Patel, N., Abd malek, R., Syed, A., Elgorban, A.M., Ahmad, K., & Zuan, A. T. K. (2021). Halotolerant microbial consortia for sustainable

mitigation of salinity stress, growth promotion, and mineral uptake in tomato plants and soil nutrient enrichment. *Sustainability, 13*(15), Article 8369. https://doi.org/10.3390/su13158369

Lahlali, R., Ezrari, S., Radouane, N., Kenfaoui, J., Esmaeel, Q., El Hamss, H., Belabess, Z., & Barka, E. A. (2022). Biological control of plant pathogens: A global perspective. *Microorganisms 2022, 10*(3), Article 596. https://doi.org/10.3390/microorganisms10030596

Li, X., Liu, H., Yang, W., Sheng, H., Wang, F., Harindintwali, J. D., Herath, H. M. S. K., & Zhang, Y. (2022). Humic acid enhanced pyrene degradation by Mycobacterium sp. NJS-1. *Chemosphere*, *288*(Part 3), Article 132613. https://doi.org/10.1016/j.chemosphere.2021.132613

Li, Y., Fang, F., Wei, J., Wu, X., Cui, R., Li, G., Zheng, F., & Tan, D. (2019). Humic acid fertilizer improved soil properties and soil microbial diversity of continuous cropping peanut: A three-year experiment. *Scientific Reports, 9*(1), Article 12014. https://doi.org/10.1038/s41598-019-48620-4

Lindsay, D., Robertson, R., Fraser, R., Engstrom, S., & Jordan, K. (2021). Heat induced inactivation of microorganisms in milk and dairy products. *International Dairy Journal*, *121*, Article 105096. https://doi.org/10.1016/j.idairyj.2021.105096

Lipczynska-Kochany, E. (2018). Humic substances, their microbial interactions and effects on biological transformations of organic pollutants in water and soil: A review. *Chemosphere*, *202*, 420-437. https://doi.org/10.1016/j.chemosphere.2018.03.104

Liu, K., McInroy, J. A., Hu, C. H., & Kloepper, J. W. (2018). Mixtures of plant-growth-promoting rhizobacteria enhance biological control of multiple plant diseases and plant-growth promotion in the presence of pathogens. *Plant Disease*, *102*(1), 67-72. https://doi.org/10.1094/PDIS-04-17-0478-RE

Luu, J., Mott, C. M., Schreiber, O. R., Giovinco, H. M., Betchen, M., & Carabetta, V. J. (2022). Nε-Lysine acetylation of the histone-like protein HBsu regulates the process of sporulation and affects the resistance properties of *Bacillus subtilis* spores. *Frontiers in Microbiology*, *12,* Article 782815. https://doi.org/10.3389/fmicb.2021.782815

Mahalakshmi, S., Vijayapriya, M., & Pandeeswari, N. (2019). Studies on developing PGPR consortium with improved shelf life. *Journal of Pharmacognosy Phytochemistry, 8*(2S), 545-548.

Mahapatra, S., Yadav, R., & Ramakrishna, W. (2022). *Bacillus subtilis* impact on plant growth, soil health and environment: Dr. Jekyll and Mr. Hyde. *Journal of Applied Microbiology*, *132*(5), 3543-3562. https://doi.org/10.1111/jam.15480

Mathew, B. T., Torky, Y., Amin, A., Mourad, A. H. I., Ayyash, M. M., El-Keblawy, A., Hilal-Alnaqbi, A., AbuQamar, S. F., & El-Tarabily, K. A. (2020). Halotolerant marine rhizosphere-competent actinobacteria promote *Salicornia bigelovii* growth and seed production using seawater irrigation. *Frontiers in Microbiology*, *11*, Article 552. https://doi.org/10.3389/fmicb.2020.00552

Mendoza-Labrador, J., Romero-Perdomo, F., Abril, J., Hernández, J. P., Uribe-Vélez, D., & Buitrago, R. B. (2021). Bacillus strains immobilized in alginate macrobeads enhance drought stress adaptation of guinea grass. *Rhizosphere*, *19*, Article 100385. https://doi.org/10.1016/j.rhisph.2021.100385

Meng, F., Huang, Q., Yuan, G., Cai, Y., & Han, F. X. (2021). The beneficial applications of humic substances in agriculture and soil environments. In *New Trends in Removal of Heavy Metals from Industrial Wastewater* (pp. 131-160). Elsevier. https://doi.org/10.1016/B978-0-12-822965-1.00007-6

Morawska, L. P., Hernandez-Valdes, J. A., & Kuipers, O. P. (2022). Diversity of bet-hedging strategies in microbial communities-Recent cases and insights. *WIREs Mechanisms of Disease, 14*(2), Article e1544. https://doi.org/10.1002/wsbm.1544

Mukherjee, S., Pandey, V., Parvez, A., Qi, X., & Hussain, T. (2022). Bacillus as a Versatile Tool for Crop Improvement and Agro-Industry. In M. T. Islam, M. Rahman & P. Pandey (Eds.), *Bacilli in Agrobiotechnology* (pp. 429-452). Springer. https://doi.org/10.1007/978-3-030-85465-2_19

Nagpal, S., Kumawat, K. C., & Sharma, P. (2022). Insights into novel cell immobilized microbial inoculants. In *New and Future Developments in Microbial Biotechnology and Bioengineering* (pp. 289-318). Elsevier. https://doi.org/10.1016/B978-0-323-85577-8.00001-9

Nardi, S., Schiavon, M., & Francioso, O. (2021). Chemical structure and biological activity of humic substances define their role as plant growth promoters. *Molecules, 26*(8), Article 2256. https://doi.org/10.3390/molecules26082256

Pashang, R., Ronan, E., Kroukamp, O., Korber, D. R., Laursen, A. E., Wenk, J., & Wolfaardt, G. M. (2022). Huddling together to survive: Population density as a survival strategy of non-spore forming bacteria under nutrient starvation and desiccation at solid-air interfaces. *Microbiological Research*, *258*, Article 126997. https://doi.org/10.1016/j.micres.2022.126997

Połaska, M., Dekowska, A., & Sokolowska, B. (2021). Isolation and identification of guaiacol producing *Alicyclobacillus fastidiosus* strains from orchards in Poland. *Acta Biochimica Polonica, 68*(2), 301-307. https://doi.org/10.18388/abp.2020_5574

Pota, G., Venezia, V., Vitiello, G., Di Donato, P., Mollo, V., Costantini, A., Avossa, J., Nuzzo, A., Piccolo, A., Silvestri, B., & Luciani, G. (2020). Tuning functional behavior of humic acids through interactions with stöber silica nanoparticles. *Polymers*, *12*(4), Article 982. https://doi.org/10.3390/polym12040982

Pukalchik, M., Kydralieva, K., Yakimenko, O., Fedoseeva, E., & Terekhova, V. (2019). Outlining the potential role of humic products in modifying biological properties of the soil-A review. *Frontiers in Environmental Science*, *7*, Article 80. https://doi.org/10.3389/fenvs.2019.00080

Rashad, M., Hafez, M., & Popov, A. I. (2022). Humic substances composition and properties as an environmentally sustainable system: A review and way forward to soil conservation. *Journal of Plant Nutrition*, *45*(7), 1072-1122. https://doi.org/10.1080/01904167.2021.2005801

Rattray, J. E., Chakraborty, A., Li, C., Elizondo, G., John, N., Wong, M., Radovic, J. R., Oldenburg, T. B. P., & Hubert, C. R. (2021). Sensitive quantification of dipicolinic acid from bacterial endospores in soils and sediments. *Environmental Microbiology*, *23*(3), 1397-1406. https://doi.org/10.1111/1462-2920.15343

Rizvi, A., Ahmed, B., Khan, M. S., El-Beltagi, H. S., Umar, S., & Lee, J. (2022). Bioprospecting plant growth promoting rhizobacteria for enhancing the biological properties and phytochemical composition of medicinally important crops. *Molecules*, *27*(4), Article 1407. https://doi.org/10.3390/molecules27041407

Saeed, E. E., Sham, A., Salmin, Z., Abdelmowla, Y., Iratni, R., El-Tarabily, K., & AbuQamar, S. (2017). *Streptomyces globosus* UAE1, a potential effective biocontrol agent for black scorch disease in date palm plantations. *Frontiers in Microbiology*, *8*, Article 1455. https://doi.org/10.3389/fmicb.2017.01455

Schaeffer, A. B., & Fulton, M. D. (1933). A simplified method of staining endospores. *Science*, *77*(1990), 194-194.

Shen, C., & Zhang, Y. (2017). Staining technology and bright-field microscope use. In *Food microbiology laboratory for the food science student* (pp. 9-14). Springer. https://doi.org/10.1007/978-3-319-58371-6_2

Shultana, R., Kee Zuan, A. T., Yusop, M. R., & Saud, H. M. (2020). Characterization of salt-tolerant plant growth-promoting rhizobacteria and the effect on growth and yield of saline-affected rice. *PLoS One*, *15*(9), Article e0238537. https://doi.org/10.1371/journal.pone.0238537

Shultana, R., Kee Zuan, A. T., Yusop, M. R., Saud, H. M., & El-Shehawi, A. M. (2021). *Bacillus tequilensis* strain 'UPMRB9'improves biochemical attributes and nutrient accumulation in different rice varieties under salinity stress. *Plos One*, *16*(12), Article e0260869. https://doi.org/10.1371/journal.pone.0260869

Silaban, S., Marika, D. B., & Simorangkir, M. (2020). Isolation and characterization of amylase-producing amylolytic bacteria from rice soil samples. *Journal of Physics: Conference Series, 1485*(1), Article 012006. https://doi.org/10.1088/1742-6596/1485/1/012006

Sootahar, M. K., Zeng, X., Wang, Y., Su, S., Soothar, P., Bai, L., Kumar, M., Zhang, Y., Mustafa, A., & Ye, N. (2020). The short-term effects of mineral-and plant-derived fulvic acids on some selected soil properties: improvement in the growth, yield, and mineral nutritional status of wheat (*Triticum aestivum L.*) under soils of contrasting textures. *Plants, 9*(2), Article 205. https://doi.org/10.3390/plants9020205

Stevenson, I. L., & Schnitzer, M. (1982). Transmission electron microscopy of extracted fulvic and humic ACIDS1. *Soil Science*, *133*(3), 179-185.

Sun, S., Abdellah, Y. A. Y., Miao, L., Wu, B., Ma, T., Wang, Y., Zang, H., Zhao, X., & Li, C. (2022). Impact of microbial inoculants combined with humic acid on the fate of estrogens during pig manure composting under low-temperature conditions. *Journal of Hazardous Materials*, *424*(Part D), Article 127713. https://doi.org/10.1016/j.jhazmat.2021.127713

Tapia, M. S., Alzamora, S. M., & Chirife, J. (2020). Effects of water activity (a_w) on microbial stability: As a hurdle in food preservation. In G. V. Barbosa-Canovas, A. J. Fontana Jr., S. J. Schmidt & T. P. Labuza (Eds.), *Water Activity in Foods: Fundamentals and Applications* (pp. 323-355). Wiley. http://dx.doi.org/10.1002/9780470376454.ch10

Tehri, N., Kumar, N., Raghu, H. V., & Vashishth, A. (2018). Biomarkers of bacterial spore germination. *Annals of Microbiology, 68*, 513-523. https://doi.org/10.1007/s13213-018-1361-z

Tikhonov, V. V., Yakushev, A. V., Zavgorodnyaya, Y. A., Byzov, B. A., & Demin, V. V. (2010). Effects of humic acids on the growth of bacteria. *Eurasian Soil Science, 43*(3), 305-313. https://doi.org/10.1134/S1064229310030087

Timmis, K., & Ramos, J. L. (2021). The soil crisis: The need to treat as a global health problem and the pivotal role of microbes in prophylaxis and therapy. *Microbial Biotechnology*, *14*(3), 769-797. https://doi.org/10.1111/1751-7915.13771

Vassilev, N., Vassileva, M., Martos, V., Garcia del Moral, L. F., Kowalska, J., Tylkowski, B., & Malusá, E. (2020). Formulation of microbial inoculants by encapsulation in natural polysaccharides: focus on beneficial properties of carrier additives and derivatives. *Frontiers in Plant Science*, *11*, Article 270. https://doi.org/10.3389/fpls.2020.00270

Wrangham, J. B. (2019, March 24). *Endospore production in response to microbiocides in the microbiologically influenced corrosion implicated genus clostridium*. Paper presented at the CORROSION 2019, Nashville, Tennessee, USA.

Yang, F., Tang, C., & Antonietti, M. (2021). Natural and artificial humic substances to manage minerals, ions, water, and soil microorganisms. *Chemical Society Reviews, 50*(10), 6221-6239. https://doi.org/10.1039/d0cs01363c

Young, C. C., Rekha, P. D., Lai, W. A., & Arun, A. B. (2006). Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. *Biotechnology and Bioengineering*, *95*(1), 76-83. https://doi.org/10.1002/bit.20957

Zvinavashe, A. T., Mardad, I., Mhada, M., Kouisni, L., & Marelli, B. (2021). Engineering the plant microenvironment to facilitate plant-growth-promoting microbe association. *Journal of Agricultural and Food Chemistry*, *69*(45), 13270-13285. https://doi.org/10.1021/acs.jafc.1c00138