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Carrier Based Liquid Bioformulation of Salt-Tolerant PGPR *Bacillus* Species for Prolonged Survivability

(Bioformulasi Cecair Berasaskan Pembawa PGPR Spesies *Bacillus* Toleransi Garam untuk Kemandirian Berpanjangan)

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ABSTRACT

Salinity has emerged as one of the agricultural plants' most severe environmental stresses. Recently, a plant growth-promoting rhizobacteria (PGPR) is being touted as a means of solving yield and environmental issues worldwide. However, multi-strain salt-tolerant rhizobacteria have a short shelf life due to their structural and cellular components, therefore, they need to be supplemented with a liquid carrier material to serve as a shelter and energy source for the bacteria for longer survival. The present study has been undertaken to develop a liquid biofertilizer formulation from multi-strain salt-tolerant PGPR – UPMR, UPMRE6, and a mixed strain of UPMRB9 and UPMRE6 using an optimum amount of cell protectants, namely glycerol (5 mM), trehalose (10 mM), and polyvinyl pyrrolidone (PVP) at 1%. The shelf-life was assessed through measurements of optical density and bacterial biomass to determine the bacterial population and growth trend at monthly intervals. After three months of incubation, the optical density was the highest in the mixed strain treatment supplemented with trehalose with 1.3% and 2.2% increase relative to the UPMRE6 and UPMRB9, respectively, using the same cell protectants. Similarly, bacterial biomass production was the highest in the mixed strains treatment amended with trehalose (0.025 g/mL), with 13.64% and 38.89% increment followed by UPMRE6 and UPMRB9, respectively. Irrespective of the type of protectants used and PGPR type, the optical density and bacterial biomass generally decreased long incubation period. The results demonstrated that the use of 10 mM trehalose has the potential to extend the bacterial shelf life with the slightest cell loss.

Keywords: Additives; bioformulation; plant growth-promoting rhizobacteria; salinity; shelf-life

ABSTRAK

Kemasinan telah menjadi salah satu tekanan alam sekitar yang paling teruk bagi tanaman pertanian. Sejak kebelakangan ini, rhizobakteria penggalak pertumbuhan tumbuhan (PGPR) digembar-gemburkan sebagai penyelesaian kepada isu hasil dan alam sekitar di seluruh dunia. Walau bagaimanapun, rhizobakteria tahan masin berbilang strain mempunyai jangka hayat singkat disebabkan struktur dan komponen selnya, oleh itu, ia memerlukan tambahan bahan pembawa cecair yang berfungsi sebagai perlindungan dan sumber tenaga demi kelangsungan hidup yang lebih lama. Penyelidikan ini dijalankan untuk membangunkan formulasi baja bio cecair daripada PGPR tahan masin pelbagai strain - UPMR, UPMRE6 dan campuran UPMRB9 dan UPMRE6 menggunakan jumlah optimum pelindung sel,

iaitu gliserol (5 mM), trehalosa (10 mM) dan polivinil pirolidon (PVP) pada kadar 1%. Jangka hayat dinilai melalui pengukuran ketumpatan optik dan biojisim bakteria untuk menentukan populasi bakteria dan trend pertumbuhan pada sela bulan. Selepas tiga bulan inkubasi, ketumpatan optik adalah yang tertinggi dalam rawatan strain campuran ditambah dengan trehalosa dengan peningkatan masing-masing sebanyak 1.3% dan 2.2% berbanding UPMRE6 dan UPMRB9 menggunakan pelindung sel yang sama. Begitu juga pengeluaran biojisim bakteria adalah yang tertinggi dalam rawatan strain campuran yang dipinda dengan trehalosa (0.025 g/mL) dengan kenaikan masing-masing 13.64% dan 38.89% diikuti oleh UPMRE6 dan UPMRB9. Tanpa mengira jenis pelindung yang digunakan dan jenis PGPR, ketumpatan optik dan biojisim bakteria secara amnya mengurangkan tempoh inkubasi yang panjang. Keputusan menunjukkan bahawa penggunaan trehalosa 10 mM berpotensi untuk memanjangkan jangka hayat bakteria dengan kehilangan sel yang sedikit.

Kata kunci: Aditif; formulasi biologi; jangka hayat; kemasinan; rhizobakteria penggalak pertumbuhan tumbuhan

Introduction

The growth and yield of crops can be reduced significantly due to various biotic and abiotic factors. Soil salinity is the main abiotic factor that significantly lowers crop productivity (Zorb, Geilfus & Dietz 2019). The physiological functions of plants, such as seed germination, photosynthesis, membrane transport, antioxidant formation, and ethylene production, are adversely affected when soils contain excess water-soluble salts (saline soils) (Chang et al. 2014). To increase the agricultural productivity of saline soils, salt-tolerant plant growth-promoting rhizobacteria (PGPR) have been used with surprising effectiveness (Arora et al. 2020a, 2020b; Kumar et al. 2021). Some benefits of PGPR as a biofertilizer includes organic matter breakdown, enhanced nutrient availability, phytohormone synthesis, and contribution to abiotic and biotic stress mitigation (Zhang et al. 2021). In addition, it has been well-documented that PGPR strains raise the levels of nitrogen, phosphorus, and potassium in the shoots and roots of rice plants (Ali-Tan et al. 2017; Kapadia et al. 2021; Shultana et al. 2020). A recent study reported that the inoculation of *Bacillus tequilensis* and *Bacillus aryabhattai* strains into rice plants under salinity stress improved the plants' metabolic characteristics and nutrient uptake (Shultana et al. 2021). The PGPR thrives in the rhizosphere through various mechanisms (Mishra, Fatima & Arora 2018; Olanrewaju, Glick & Babalola 2017), including the increased nutrient absorption, induced systemic resistance (ISR), and the generation of phytohormones, siderophores, antioxidants, exopolysaccharides (EPS), osmoprotectants, and enzymes like 1-aminocyclopropane-1-carboxylate (ACC) deaminase under salt stress (Numan et al. 2018). Through the utilization of beneficial bacteria and fungi, many researchers have created bio-fertilizers that can meet the nutrient needs of crops and boost crop output (Aggani 2013; Palai et al. 2021). Biofertilizers are alive or dormant cells administered to soil, seeds or seedlings to lessen salt stress and enhance nutrient availability and uptake from soil (Fasusi, Cruz & Babalola 2021). Plant growth-promoting rhizobacteria act as biological control agents and in phytoremediation processes (such as with *Bacillus cereus, Trichoderma,* and *Pseudomonas*) to protect plants from harmful organisms and heavy metals (Tang, Haruna & Majid 2020).

Bacillus spp. is a type of biofertilizer strain that is more susceptible to potential stress conditions in the field as well as during processing, handling, and storage. One major barrier to achieving agricultural sustainability has been the malfunctioning of beneficial rhizosphere microorganisms, such as PGPR, due to several factors, which include environmental, climatic, and species-specific or niche-specific tripartite soil-plant-microbe interactions (Maheshwari et al. 2015). Another factor that has lowered the projected industrial recognition of using PGPR as biofertilizers is the lack of suitable and commercially viable carriers or cell protectants. Many marketable bio-inoculants do not function under field conditions despite the effectiveness established in greenhouse or laboratory trials due to insufficient and/or poor-quality formulation, including poor compatibility and stability of the carriers (Stamenkovic et al. 2018). Liquid inoculants' formulation includes polymeric additives through their high water-holding capacity, thick consistency, and viscous character and known to extend their shelf life and improve their efficacy in field settings.

A high-quality biofertilizer formulation must increase inoculant cell density throughout the manufacturing and maintain a sufficient extended shelf life while in storage (Sahu & Brahmaprakash 2021).

The microbial cell lose issues must be resolved for a biofertilizer to be successful, and higher-level inoculants must be developed. In this regard, using additives with longer shelf lives in the formulation is becoming increasingly important. *Azotobacter* cells on seeds should be protected from high temperatures and drying out by liquid inoculant formulation additives (Takate & Gaykar 2021). Cells may adhere to seeds more readily due to polymers' sticky qualities, and their viscous nature may cause the inoculant to dry more slowly after their application to seeds (Brahmaprakash et al. 2020). When bacteria are in their dormant growth phase, the cell protectant PVP, one of many polymers, helps bind toxins continuously released into the media (Praveen Biradar & Santhosh 2018). A recent study has investigated the potential of glycerol, a trihydroxy alcohol commonly used to protect cell viability in strain maintenance practices and argued for more research on its use in formulation procedures (Vassilev et al. 2017). Furthermore, researchers discovered that adding 0.5% gly to bacterial cells provided an additional layer of defense against temperature stress (Tamer, Azza & Mohamed 2020). A study by Kumaresan and Reetha (2011) reported an improved survival of *Azospirillum* treated with trehalose due to the trehalose's ability to act as a source of energy and cell protectants. According to Schoebitz, López and Roldán (2013), trehalose extends the shelf life of *Raoultella terrigena* and acts as an excellent desiccation barrier. Furthermore, any bioformulation with a longer shelf life that performs biocontrol and biofertilizer activities simultaneously in a field setting may pave the way for utilizing this technology further marketing (Chakraborty 2020; Ijaz et al. 2019). The present investigation aimed to study the bio-formulation of a multi-strain salt-tolerant PGPR consortium in a liquid inoculant to improve the shelf-life for prolonged cell survivability.

Materials and Methods

Pure colony collection and fermentation of PGPR isolates

Two promising salt-tolerant plant growth-promoting rhizobacteria, namely, *Bacillus tequilensis* (UPMRB9) and *Bacillus aryabhattai* (UPMRE6), were collected from the Soil Microbiology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia (Figure 1). The isolates were selected based on their ability to boost plant development while surviving salt-stress conditions (Shultana et al. 2020). Isolates were sub-cultured on Tryptic Soy Agar (TSA) media to get a single pure colony. The initial, locally isolated salt-tolerant bacterial strains were streaked in a loop onto a brand-new TSA medium plate. The bacterial cultures were kept at 32 °C for 24 h in the incubator (SD-310 RL, Dasol, Korea).

Experimental design, treatments and optimization of bioformulation

This study uses Completely randomized design (CRD) factorial experiments with repetitive three replications. The factors were evaluated as follows: factor 1, three different cell protectants and factor 2 bacterial strains. The optimal concentrations of glycerol (5 mM), trehalose (10 mM), and polyvinyl pyrrolidone (PVP) at 1% and control (Tryptic soy broth only) were used as the most effective cell protectants for a liquid carrier formulation. The beneficial salt-tolerant PGPR strains, *Bacillus tequilensis* (UPMRB9), *Bacillus aryabhattai* (UPMRE6) and the composition of these two bacterial strains *Bacillus tequilensis* (UPMRB9) + *Bacillus aryabhattai* (UPMRE6) were used as factor 2. The treatment combinations were replicated 3 times; consequently, a total of 36 (4×3×3) experimental units were obtained. The detailed treatments were as follows:

T1 = UPMRB9 + TSB; T2 = UPMRB9 + 5 mM Glycerol; T3 = UPMRB9 + 10 mM Trehalose; T4 = UPMRB9 + 1% PVP; T5 = UPMRE6 + TSB; T6 = UPMRE6 + 5 mM Glycerol; T7 = UPMRE6 + 10 mM Trehalose; T8 = UPMRE6 + 1% PVP; T9 = Mixed strains + TSB; T10 = Mixed strains + 5 mM Glycerol; T11 = Mixed strains + 10 mM Trehalose; T12 = Mixed strains + 1% PVP.

Bioformulation

The tryptic soy broth (TSB) medium was used as a base media to produce liquid inoculants. It contained (g/L) 17.0 casein peptone (pancreatic), 3.0 (g/L) soya peptone (papain digest), 5.0 (g/L) sodium chloride (NaCl), 2.5 (g/L) dipotassium phosphate (K_2HPO_4), 2.5 (g/L) glucose, and pH 7.3 at 25 °C. Tryptic soy broth (TSB) was augmented with numerous ingredients to improve the survival of *Bacillus tequilensis* (UPMRB9) and *Bacillus aryabhattai* (UPMRE6) cells in a liquid

bioinoculant. Carrier additives such as glycerol (5 mM), trehalose (10 mM), and polyvinyl pyrrolidone (PVP) at 1% were individually added to 250-mL Erlenmeyer flasks containing 100 mL of tryptic soy broth to homogenize the ideal dosage of the amendments (TSB). The upgraded liquid formulation was autoclaved for 15 min at 121 °C before cooling at room temperature. The two salt-tolerant bacterial strains were grown separately and mixed after reaching the log phase to create a consortium strain for the mixed treatment. Each broth was supplemented with a log-phase culture of *Bacillus tequilensis* (UPMRB9), *Bacillus aryabhattai* (UPMRE6), and the combined strains (*Bacillus tequilensis* + *Bacillus aryabhattai*). Three replicates of each sample were made, and each was incubated at 32 °C for 24 h. The containers were also incubated at 32 °C without any chemical input as a control. The density of the cells in the broth cultures was checked every 30 days for up to 12 months.

BACTERIAL OPTICAL DENSITY (OD) MEASUREMENT

Spectroscopy Measurements: Bacterial optical density (OD) was measured using a UV/visible spectrophotometer (UV 2550 Shimadzu, Kyoto, Japan) at a wavelength of 600 nm. The device logs the spectra, which are averages of three independent observations made using a spectrophotometer cuvette filled with 2 mL of liquid biofertilizer. Sterilized deionized water was used as the reference solution. All measurements were carried out at room temperature using quartz cuvettes with a path length of 1 cm. All treatments were kept at a constant 30 °C room temperature during measurement, and optical densities were recorded at monthly intervals for twelve months.

BACTERIAL BIOMASS (DRY WEIGHT) MEASUREMENT

Centrifugation was carried out for a 10 mL bacterial sample at 10,000 g for 10 min, and the pellets were re-suspended, cleaned, and centrifuged once more to calculate the biomass (dry weight). To produce the pellets in the event of centrifugation, the clear broth was carefully removed. Depending on the oven temperature and pellet thickness, the pellets were subsequently dried for 6-24 h in an air-dried oven at 80 °C to dry the sample completely. The bacterial biomass measurements were taken bimonthly for up to a year.

STATISTICAL ANALYSIS

Statistical tests of all the data for the shelf-life assessment were examined after Analysis of Variance (ANOVA) using the bioinformatics tool R programming software - (R version 4.1.0; 2021-05-18). At a probability threshold of 0.05%, the Least Significant Difference (LSD) test was used to compare means and these tests were carried out in triplicates.

RESULTS

SURVIVAL OF PGPR AND BACTERIAL OPTICAL DENSITY IN DIFFERENT LIQUID FORMULATIONS

The survival and the optical density of efficient salt-tolerant PGPR isolates were considerably enhanced in liquid formulation following the twelve months of observation. The results showed that irrespective of the isolates and protectants used, the bacterial OD was maximum in the first three months of the study and decreased after that with more extended incubation periods. The mixed strain treated with trehalose 10 mM had the highest optical density level in the first three months of the study compared to its counterparts mixed strain treated with glycerol 5 mM and PVP 1% UPMRE6 and UPMRB9 isolates (Figure 2). In the first three months, the mixed strain inoculated with trehalose 10 mM had an OD range of 28.88% followed by UPMRE6 and UPMRB9 with 35.5% and 28.97%, respectively. The control (without amendments) had the lowest OD with a range of 51.36% reduction from the first to the third month. The higher value of OD in the isolates treated with trehalose relative to their glycerol and PVP 1% treated counterparts was due to the combined activities of chemical additives in the medium. The optical density of PGPR strains steadily decreased with the progression of the incubation period. However, despite the decrease in OD with time, the mixed strain treated with trehalose 10 mM increased by 9.6% and 30.8% relative to UPMRE6 and UPMRB9 trehalose-treated isolates, respectively, at the twelve months of the study. The lowest OD was obtained in UPMRB9 and UPMRE6 treated with glycerol 5 mM and PVP 1% with (0.67 and 0.56) and (0.71 and 0.65, respectively) at twelve months of incubation. The addition of glycerol (5 mM), trehalose (10 mM), or PVP (1%) to TSB showed increased survivability of PGPR strains up to the twelve months. In contrast, in control (TSB without any amendment) showed a decreased optical density with increasing incubation time. Thus, compared to TSB, Trehalose 10 mM, glycerol 5 mM, and PVP 1% were more desirable as a medium for developing liquid-based bioformulations of mixed strains than using cell protectants on a single strain.

EVALUATION OF BIOMASS (DRY WEIGHT) OF *Bacillus* spp. ISOLATES UNDER DIFFERENT LIQUID AMENDMENTS

Bacterial cells from the liquid formulations were harvested through centrifugation and oven dried to assess the bacterial biomass of the bacterial isolates. The dry weight of each isolate amended with cell protectants was recorded at two-month intervals for up to a year. The initial biomass significantly enhanced exponentially for the mixed strains amended with trehalose 10 mM, and maximum yield was obtained at the fourth month (0.017 to 0.025 g/mL), followed by UPMRE6 (0.014 to 0.022 g/mL) and UPMRB9 (0.011 to 0.018 g/mL), respectively, which after that decreased gradually with time (Figure 3). Whereas, the minimum dry weight was recorded by the strains UPMRB9 (0.014, 0.014 g/mL), UPMRE6 (0.014, 0.013 g/mL), and mixed strains (0.020, 0.019 g/mL) amended with carrier materials glycerol 5 mM and PVP 1% at the fourth month of incubation. Consequently, the appearance of glycerol and PVP as compatible solutes and osmoprotectants did not inhibit the decline in viable biomass measurement. In this research, the best treatment combination of mixed strain with trehalose 10 mM increased the pellets' dry weight by 22.22 and 57.14% relative to UPMRE6 and UPMRB9 treated with the same amount of trehalose, respectively, at the end of the twelve-month of storage period. When UPMRB9 was amended with TSB, glycerol, and PVP, respectively, the dry weight reduction was seen to be 250%, 75%, and 40% lower. A decrease in dry weight was observed in UPMRE6 treated with TSB alone (80%), glycerol (29%) and PVP (50%), respectively, compared to its trehalose-treated amendment.

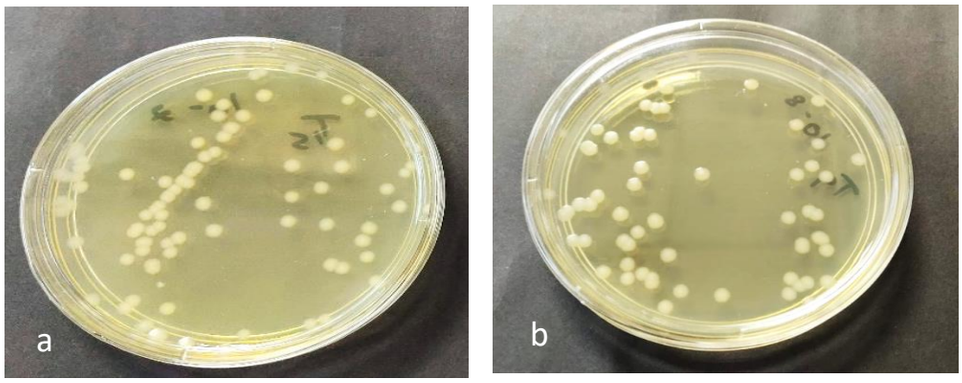

FIGURE 1. Pure colonies of (a) *Bacillus tequilensis,* and (b) *Bacillus aryabhattai* strains

DISCUSSION

The current findings showed that the bacterial optical density started to decrease over time, which could be attributed to the lack of adequate carbon and sucrose, which serve as a food source and help boost nutrient availability for bacterial survival. Trehalose is a disaccharide which can enhance cell tolerance to desiccation, osmotic pressure, and temperature stress by (Gokul et al. 2019). Trehalose's potential protective effect could be due to its stimulation of the synthesis of metabolites to defend against stress (Vanaporn & Titball 2020). Abdel-Gayed et al. (2019) reported that the lower OD values indicate that the formulation was well-protected at a higher level of dissipated oxygen, which steadily decreased, indicating bacterial multiplication and glucose consumption from the culture. Arriel-Elias et al. (2018) studied an experiment and concluded that a formulation containing glycerol, PVP, and molasses effectively boost the growth of bacterial strain species *Pseudomonas fluorescens* and *Burkholderia pyrrocinia* formulation throughout a 180-day shelf-life experiment, which demonstrates the effectiveness microbial activity pattern in the current study. A progressive increase in microbial activity was observed in a study conducted by Buraq et al. (2023), that cell protectants such as humic acid significantly prolonged the plant growth-promoting bacterial shelf-life with minimal cell loss. The decrease in dissolved oxygen is due to increased

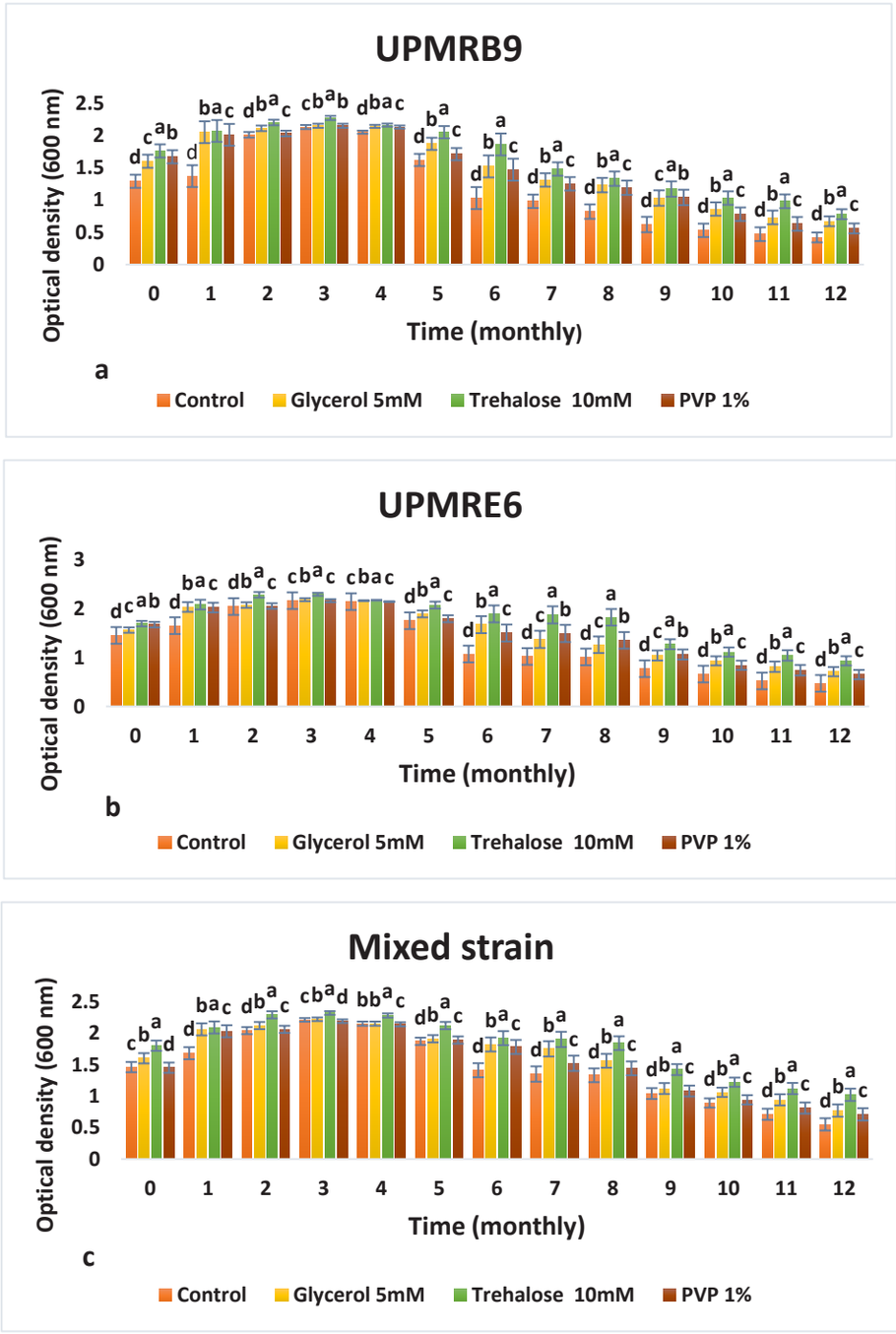

FIGURE 2. Optical density of a) *Bacillus tequilensis*, b) *Bacillus aryabhattai* and c) mixed strains amended with three different additives and control (without additives). Optical density of different treatments measured using spectrophotometers is plotted at 600 nm wavelength, means with different letter significantly differ at $\alpha = 0.05$ LSD

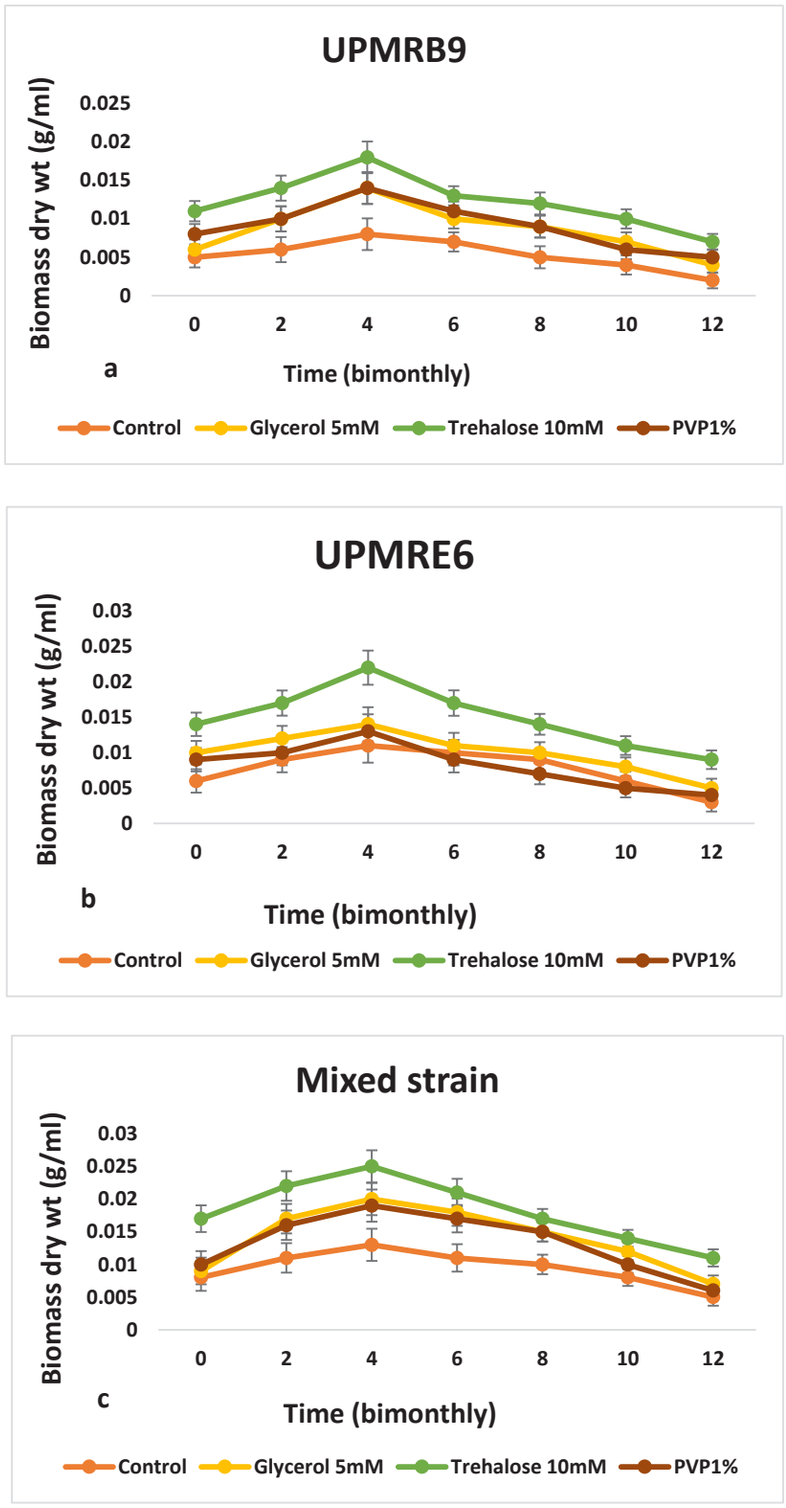

FIGURE 3. Biomass (dry weight) of a) *Bacillus tequilensis,* b) *Bacillus aryabhattai* and c) mixed strains amended with three different additives and control (without additives) at two months interval

O_2 demand for culture growth. The cell protectant contains the necessary amounts of sugar and oxygen for the PGPR's consumption and survival (Tamer, Azza & Mohamed 2020). Another notable study found that bacteria dehydrated with glucose had a lower shelf life than bacteria dried with maltodextrin, trehalose or sucrose (Priour et al. 2021). Our findings were consistent with previous research, which found that the bacterial survival percentages in the liquid formulation of *Exiguobacterium* sp. AO-11 protected in phosphate buffer was higher after 30 °C storage (Chatsuda, Nitchakarn & Onruthai 2021; Muangchinda et al. 2020).

In the present study, the best treatment combination of mixed strain was amended with 10 mM trehalose, recording the highest pellets dry weight at the end of the twelve-months storage period. Although the trehalose-amended treatments were able to maintain their performance as dispersant agents for longer periods of time, the estimated microbial biomass steadily increased with the dry weight of each treatment (bacterial formulation), peaked, and then gradually decreased (Bhakyaraj et al. 2022; Timmis & Ramos 2021). Bacterial biomass analysis showed that three bacterial isolates, *Bacillus* spp. cells*, E. coli* cells, and *P. putida* cells; contain 51.5%, 31.4%, and 48.4% dry matter, respectively (Bratbak & Dundas 1984). The cells' metabolism remains unaffected, so they continue to take up the formulae' nutrients, but at a reduced rate (Arriel-Elias et al. 2018; Tapia, Alzamora & Chirife 2020). It was shown for the first time that the mixed bacteria were compatible with the addition of cell protectants, which provides additional evidence for symbiotic growth between the bacteria strains for a long period (Lipczynska-Kochany 2018; Tikhonov et al. 2010). Li et al. (2011) conducted a study focusing on the appropriateness of trehalose and its synergetic actions with other lyo-protectants in augmenting the viability of liquid formulations. A consortium with an extended shelf life suited for use as prudential bio inoculants was established after researchers studied the impact of Poly vinyl pyrollidone (PVP) concentrations of 1% and 2% on the viability of the PGPR strain (Mahalakshmi, Vijayapriya & Pandeeswari 2019). A recent study found that the highest value of biomass from *Bacillus* spp. was obtained at 11 h, reaching 4.53 g/L; the glucose concentration gradually declined; and full consumption (0 g/L) occurred at 12 h (Abdel-Gayed et al. 2019). Similarly, Riddhi and Vinod (2021) reported the experimental quantities of enzyme action and biomass production from a novel salt-tolerant bacteria, *Bacillus tequilensis,* using response surface methodology to be 8.453 I.U. and 3.213 g/L at adjusted conditions. Previous research discovered that bioformulation medium contains a suitable strain with some cell protectants, extending the shelf life to 19-25 months under stressful conditions (Chandra et al. 2018).

Conclusions

Recently, microbial formulations have surfaced as a potential alternative to help plants endure salt stress conditions. It is now common knowledge that formulations are crucial in facilitating the journey from lab testing to field application for rhizobacteria that promote plant development. However, more effective, and stable formulations may be achieved by integrating carrier materials and microorganism cell protectants as bio-inoculants in a PGPR formulation. This study's approach effectively determined the optimum formulations to prolong and extend the viability of the rhizobacteria *Bacillus* spp., without impairing their beneficial activities during storage. This research demonstrated that for maximal optical density, increased biomass production, and maximized good bioformulation for incubation periods of up to twelve months, a lower trehalose concentration (10 mM) may be given to media containing *Bacillus* spp. mixed strains. Moreover, the study showed that consortium bacterial liquid formulations can be developed at a lower cost, with more excellent stability, a better efficacy rate, economic feasibility, and convenience of use. It is therefore concluded that treating bacterial isolates, particularly *Bacillus* spp. mixed strains with trehalose (10 mM) significantly enhanced and prolonged the bacteria's storage life which peaked at fourth month of the medium. Notwithstanding, the application of the liquid biofertilizer in combination with the cell protectants may significantly contribute to advances in the agriculture industry, under several challenging factors interaction.

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