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*Full Length Article*

# Effects of Growth Medium, pH, Temperature and Salinity on BRIS Soil Plant Growth Promoting Rhizobacteria (PGPR) Growth

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## Abstract

The growth characteristic of plant growth promoting rhizobacteria (PGPR) as affected by growth medium and environmental factors are vigorously studied as basic information for the microbes to be proposed in biofertilizer formulation. PGPRs have been successfully isolated around the world and used as biofertilizer. However, there is still a lack of information and studies about the native BRIS soil PGPR growth characteristics. As BRIS soil is categorized as problematic sandy soil, the PGPR that exists in this area may have superior characteristics that could be used as biofertilizer. This study was conducted to evaluate BRIS soil PGPRs namely UA 1 (*Burkholderia unamae*), UA 6 (*Bacillus amyloliquefaciens*) and UAA 2 (*Enterobacter asburiae*) growth characteristics in an organic molasses growth medium as affected by several environmental factors (pH, temperature, salinity). The concentration of 6% molasses medium was found as the best and economic growth medium for all PGPRs either in single or mix strains (UA 1 + UA 6 + UAA 2) conditions. The UA 6 strain was recorded as the most potential PGPR as it showed the highest growth rate in molasses medium and other diverse conditions of pH (4–9), temperature (20–50°C) and salinity (1–8% $KNO_3$). Mix strains culture followed by UA 1 and UAA 2 also showed a higher growth rate in the tested medium and environment. This information is important for optimum and successful cultivation in the laboratory, effectiveness in biofertilizer formulation and prediction for their growth performance in the field. © 2022 Friends Science Publishers

**Keywords:** Biofertilizer; Molasses medium; PGPR; pH; Salinity; Temperature

## Introduction

Plant growth-promoting rhizobacteria (PGPR) are the microbial inoculant that can be used as biofertilizer, biocontrol agent, bio-pesticide and bio-herbicide (Vessey 2003; Sharf *et al.* 2021). These are reliable substitutes for synthetic fertilizers which are the utmost threat to the environment and deteriorate soil fertility and its health. The microbes in biofertilizer will help the plant in accessing essential nutrients in various actions such as by fixing atmospheric nitrogen, mineralization of elements, production of hormones and movement of nutrients thus increasing soil fertility and plant growth and productivity in a green and sustainable manner. The microbial inoculants in biofertilizers can be introduced to any type of soil, seed or plant (Javaid 2009; Javaid and Bajwa 2011). However, the condition of new introduced environment might have extremes in pH, salinity, temperature and moisture that greatly influence bacterial growth and survival. Thus, the microbes must have the ability to grow and function well in very diverse conditions.

Different type of microbes may have different environmental requirements for their growth which explain why they are found nearly everywhere. Each PGPRs have an optimum growth within a specific pH, temperature and salinity range which may be broad or limited. These specific needs reflect microbial adaptation to their natural and newly introduced environment. Certain conditions such as pH, temperature and salinity can affect bacteria by promoting or blocking their growth and function (Datta *et al.* 2015; Koni *et al.* 2017). The use of complex media in the laboratory seems to be not economically applicable to propagate the isolated beneficial microbes for biofertilizer production due to their high amount of expensive nutrients such as yeast extract, peptone and salts (Batish *et al.* 1990). Thus, an alternative organic medium to propagate the isolated PGPRs rapidly and economically need to be determined. Molasses is a sugar waste product that has been used in a lot of microbiological processes. Molasses is preferable as the medium for microbial growth because of its several

advantages including their effectiveness in extreme temperatures or pH values, higher biodegradability and lower toxicity compared to using chemical substances (Rodrigues *et al.* 2006) and the price is cheaper compared to the complex medium. However, at high concentrations, molasses could cause cell toxicities because of the high value of caramelized and invert sugars (Baei *et al.* 2009).

Two requirements for microbial growth that vary greatly between species are the nutritional and physical factors (Cappucino and Sherman 2005), that affect bacterial adaptation, growth and their secondary metabolites production. The native and local PGPR strains namely *Burkholderia unamae* (UA 1), *Bacillus amyloliquefaciens* (UA 6) and *Enterobacter asburiae* (UAA 2) with multiple beneficial plant growth-promoting characteristics have been isolated from problematic BRIS soil in Besut Terengganu. This study was conducted to determine the growth performance of BRIS soil PGPRs in an organic molasses medium as affected by several environmental factors (pH, temperature and salinity). Understanding these needs is necessary for the successful cultivation of that particular microbes in the laboratory, prediction of their field performance and so do for the biofertilizer formulation and production.

## Materials and Methods

### Preparation of inoculum and measurement of PGPR growth

Three types of PGPRs namely UA 1 (*Burkholderia unamae*), UA 6 (*Bacillus amyloliquefaciens*) and UAA 2 (*Enterobacter asburiae*) isolated from the rhizosphere of *Acacia mangium* tree at BRIS soil in Besut, Terengganu were used in this study either in single or mix strains (UA 1 + UA 6 + UAA 2) culture. Overnight culture of single strain PGPR in the nutrient broth media was used as inoculum. The optical density (OD) of the cell suspension was adjusted to 0.4 A at 600 nm using a UV-VIS spectrophotometer (approximately 3-4 x $10^7$ cells $mL^{-1}$) and used in the subsequent studies.

Room temperature of 26ºC to 30ºC, shaking at 150 rpm and incubation period of 6 days were used for all environmental effects studies. Bacterial growth was measured by serial dilutions and total viable cell number count by the pour plate method. Final dilution (30 $\mu$L) was taken and spread onto nutrient agar medium using the hockey stick. The plates were incubated at room temperature for 24 h. Each colony that appeared on the plate was considered as one Colony Forming Unit (CFU) and calculated using the formula by Sutton (2011).

### Effect of molasses concentration in medium on PGPR growth

Five concentrations of molasses (2, 4, 6, 8 and 10%) in 200 mL $dH_2O$ with pH 7 were prepared in 250 mL conical flask and sterilized at 121°C for 15 min. After cooling, 1 mL of fresh overnight bacterial culture in nutrient broth medium was inoculated in that molasses medium and nutrient broth medium as control. Their growth in molasses medium was calculated and compared to the growth in nutrient broth medium.

### Effect of pH on PGPR growth

Molasses medium (6% molasses in 200 mL $dH_2O$) was prepared with different pH (4, 5, 6, 7, 8 and 9) using 1 *M* HCl and 1 M NaOH in 250 mL conical flask and sterilized at 121°C for 15 min. After cooling, 1 mL of fresh overnight bacterial culture in nutrient broth medium was transferred into the molasses medium and incubated.

### Effect of temperature on PGPR growth

Fresh overnight culture (1 mL) of bacterial inoculum in nutrient broth medium was transferred into molasses medium (6% molasses in 200 mL $dH_2O$) in a 250 mL conical flask. A set of molasses medium without any bacteria inoculation was used as a control. The medium pH was 7 and the temperature for incubation was adjusted to 20, 30, 40 and 50°C respectively using the incubator (Jeio Tech ISF-7100R).

### Effect of $KNO_3$ concentration on PGPR growth

Molasses medium (6% molasses in 200 mL $dH_2O$) with different concentration of $KNO_3$ (0, 1, 2, 4 and 8%) were prepared in 250 mL conical flask. The pH and salinity of the medium were recorded. The salinity of the medium was measured indirectly by testing the electrical conductance (EC) using Horiba LAQUAtwin EC-22 and the units are in mS/cm. Potassium nitrate is an electrolyte that when dissolved in water will become sodium ions ($K^+$) and nitrate ions ($NO_3^-$) to form salt water. It means that the more $K^+$ and $NO_3^-$ in the medium the more the conductivity or salt in the medium. The total dissolved solids (TDS) is a measurement of the salt amount in water or total concentration of dissolved matter in the water sample including all dissociated inorganic and organic anions and cations and undissociated dissolved species (Neil and Cox 2000). The mass of dissolved solids in the medium was measured in mg $L^{-1}$ and estimated as TDS derived from the EC reading using a conversion factor; TDS = EC. $f$, where $f$ = 0.65 (conversion factor) to estimate the volume of evaporated water (Singh and Kalra 1976).

### Experiment design and statistical analysis

The experiments were arranged in a completely randomized design (CRD) with 3 replicates. Data were analyzed using Analysis of Variance (ANOVA) from SPSS version 21.

Multiple comparisons were done using Tukey's multiple comparison.

## Results

### Effects of molasses concentration on PGPR growth

The sugar cane molasses medium does support BRIS soil bacterial growth. All PGPRs either in single or mixed strains grow in 2 to 10% molasses medium (Fig. 1). Increasing molasses concentration has increased bacterial growth. The highest growth for all PGPRs was recorded at 8% molasses but the results were not significantly different ($P < 0.05$) from the growth in 6% molasses medium. A higher concentration of molasses (10%) decreased 1.28% to 4.35% growth of all bacteria strains. The strain of UA 6 ($Log_{10}$ CFU $mL^{-1}$ 12.09) recorded the highest growth in 8% molasses medium followed by mix culture ($Log_{10}$ CFU $mL^{-1}$ 11.97), UA 1 ($Log_{10}$ CFU $mL^{-1}$ 10.98) and UAA 2 ($Log_{10}$ CFU $mL^{-1}$ 10.91). Meanwhile, inoculation in nutrient broth medium produced lower growth compared to the results in 4, 6, 8 and 10% molasses medium for all types of bacteria either in single or mixed form (Fig. 2). Inoculation in 8% molasses medium has increased for an average of 17% microbial growth compared by using nutrient broth medium.

### Effects of pH on PGPR growth

All PGPRs can grow in molasses medium at pH 4–9 (Fig. 3). However, the optimum pH for all bacterial isolates growth was pH 6–7. The highest growth for all microbes was at pH 7 but the result was not significantly different ($P < 0.05$) from pH 6 for UA 1 and UA 6. This means that there was not much difference in bacterial growth between pH 6 and 7. The highest growth was recorded by UA 6 in all pH conditions followed by mixed strains, UAA 2 and UA 1. The results also showed that all PGPRs were more acid-tolerant compared to an alkaline condition. It is because bacterial growth was found to be higher in acidic conditions (pH 4–6) compared to alkaline conditions (pH 8–9). Starting from pH 8–9, growth for UA 1 dropped drastically while the growth of UA 6, UAA 2 and mix strains decreased slowly. UA 6 showed a significantly different ($P < 0.05$) growth to UA 1 and UAA 2 in all pH conditions. UA 6 and UAA 2 were also tolerant to high pH (pH 8 and 9) compared to UA 1.

### Effects of temperature on PGPR growth

All PGPRs can grow in temperatures ranging from 20°C to 50°C (Fig. 4). Generally, bacterial growth was high at 30°C for all strains either in single or mix form. However, the growth result for UA6 and mix strains were also high at 40°C. The growth results also showed that all PGPRs were more tolerant to high temperatures (30–50°C) compared to low temperatures (20°C). Based on the results, the most

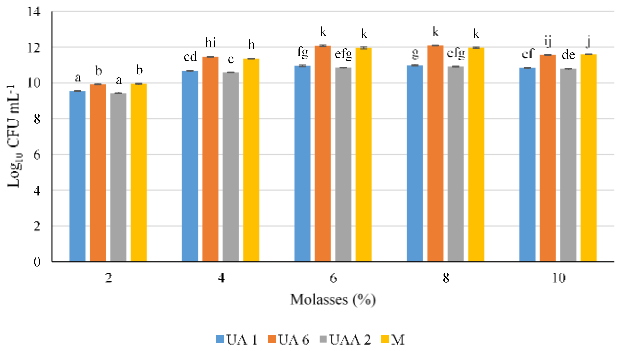


**Fig. 1:** Growth of BRIS soil PGPR in different concentration of molasses medium at 6 days after inoculation. Means with different letters show significant difference at $P < 0.05$ Tukey's multiple comparison, n = 3. Bar indicates standard error of the treatment's mean

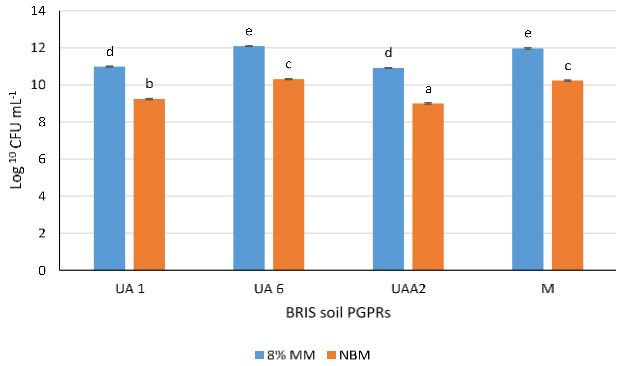


**Fig. 2:** Growth of BRIS soil PGPR in 8% molasses medium (8% MM) and nutrient broth medium (NBM) at 6 days after inoculation. Means with different letters show significant difference at $P < 0.05$ Tukey's multiple comparison, n = 3. Bar indicates standard error of the treatment's mean

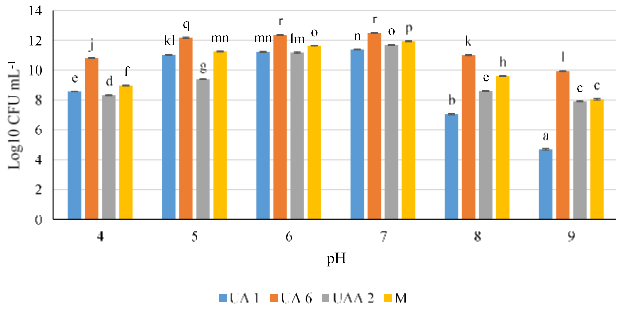


**Fig. 3:** Growth of BRIS soil PGPR in different pH of 6% molasses medium at 6 days after inoculation. Means with different letters show significant difference at $P < 0.05$ Tukey's multiple comparison, n = 3. Bar indicates standard error of the treatment's mean

optimum growth temperature for all PGPR strains either in single or mixed form was at 30°C. The strain UA 6 showed the highest growth at 30°C with $log_{10}$ 12.31 CFU $mL^{-1}$ followed by mix culture with $log_{10}$ 12.20 CFU $mL^{-1}$, UA 1 with $log_{10}$ 11.65 CFU $mL^{-1}$ and UAA 2 with $log_{10}$ 11.62

**Table 1:** The pH, electrical conductance (EC) and total dissolve solids (TDS) value for different concentration of $KNO_3$ in 6% molasses medium

| KNO3 (%) | 0 | 1 | 2 | 4 | 8 |
|---|---|---|---|---|---|
| pH | 4.81 | 4.81 | 4.82 | 4.83 | 4.87 |
| EC (mS $cm^{-1}$) | 1.80 | 9.70 | 16.60 | 27.40 | 43.20 |
| TDS (mg $L^{-1}$) | 1.17 | 6.31 | 10.79 | 17.81 | 28.08 |

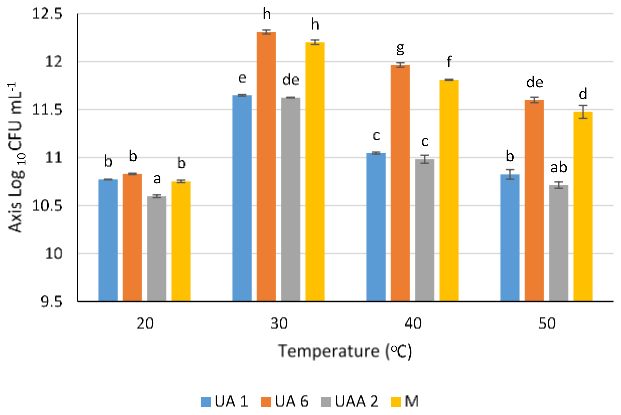


**Fig. 4:** Growth of BRIS soil PGPR in different temperature (°C) of 6% molasses medium at 6 days after inoculation. Means with different letters show significant difference at $P < 0.05$ Tukey's multiple comparison, n = 3. Bar indicates standard error of the treatment's mean

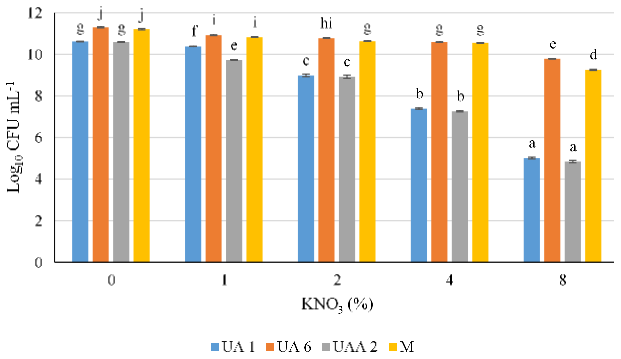


**Fig. 5:** Growth of BRIS soil PGPR in different salinity (concentration of $KNO_3$) in 6% molasses medium at 6 days after inoculation. Means with different letters show significant difference at $P < 0.05$ Tukey's multiple comparison, n=3. Bar indicates standard error of the treatment's mean

CFU $mL^{-1}$. All bacteria showed the lowest growth rate between $log_{10}$ 10.60–10.83 CFU $mL^{-1}$ at 20°C. The strain UA 6 and mix strains showed a significantly ($P < 0.05$) high growth rate compared to UA 1 and UAA at the temperature of 30–50°C.

### Effects of $KNO_3$ concentration on PGPR growth

Potassium nitrate is an ionic salt, a natural source of nitrate and has been used as a constituent for several different purposes including fertilizer. Table 1 shows the pH, EC and TDS of 6% molasses medium supplemented with different percentages of $KNO_3$. Addition and increasing the $KNO_3$ concentration increased the medium EC and TDS. However, the pH medium was not affected and showed only a little increase with the increment of $KNO_3$. All PGPRs showed significant ($P < 0.05$) growth differences in the molasses medium with different concentrations of $KNO_3$ (Fig. 5). Generally, the higher $KNO_3$ concentration (4–8%) in the medium decreased bacterial growth. UA 6 and mix culture showed a slow growth decrease while UA 1 and UAA 2 showed a rapid decrease with the increment of $KNO_3$ concentration. However, the PGPRs still showed high growth (UA 1 with $Log_{10}$ 5.01, UA 6 with $Log_{10}$ 9.78, UAA 2 with $Log_{10}$ 4.84 and mix strains with $Log_{10}$ 9.25 CFU $mL^{-1}$) at such high EC of high $KNO_3$ concentration (8%) in 6% molasses medium. The growth result at higher $KNO_3$ concentrations such as at 8% $KNO_3$ also showed that UA 6 was extremely tolerant to high ionic conditions while UA 1 and UAA 2 were weak in that condition.

## Discussion

UA 1, UA 6 and UAA 2 either in single or mixed strains culture showed a good growth performance in molasses medium. The highest bacterial growth was recorded in 8% molasses medium. However, the result was not significant to the use of 6% molasses medium. Therefore, it was concluded that 6% molasses medium was the best and most economic bacterial growth medium for all PGPRs that were used in this study. As in the biofertilizer industry, bacterial fermentation must be competitive with chemical synthesis. Thus, the potential PGPR strain that will be considered for biofertilizer formulation depends on whether it can be economically produced or not. It is because the fermentation medium can reach up to 30% of the microbial fermentation cost (Hofvendahl and Hahn-Hägerdal 2000). Cane molasses is the by-product of the manufacture of sucrose from sugarcane that contains more than 46% of invert total sugar (Curtin 1983). It is cheaper compared to other chemical-based growth medium. According to Sutigoolabud *et al.* (2005), molasses contains a high percentage of total sugar (38.8%) with glucose (3.8%), fructose (7.9%), sucrose (27.7%) and reducing sugar (23.5%). Besides the carbon and nitrogen source, molasses also contains other nutrients such as manganese, iron, calcium, potassium, magnesium, succinic acid, malic acid, citric acid, vitamin B6 and selenium (Aslan *et al.* 1997; Sutigoolabud *et al.* 2005; El–Enshasy *et al.* 2008).

The nutrient content in molasses makes it suitable to be used as bacterial growth medium as bacteria need a source of energy from carbon and other required nutrients and trace elements for their growth. This study has found that increasing molasses concentration could increase PGPRs growth until the use of 10% molasses was seen to decrease bacterial growth compared to using a lower

concentration. At 10% molasses, all bacteria strains showed a slight growth decrease of around 1–3%. According to Baei *et al.* (2009), molasses at high concentrations could cause cell toxicities because of the high value of caramelized and invert sugars. The result is in agreement with Singh *et al.* (2011) that found the growth decrease of *Rhizobium meliloti* MTCC-100 in more than 10% molasses.

Other than the need for an energy source of carbon and other nutrients, a PGPR must have a permissive range of physical conditions such as temperature, pH and salinity to grow in nature, laboratory or other environments such as in biofertilizer. PGPRs in this study could grow at the pH range 4–9 and showed an optimum growth at pH 6–7. This is in agreement with Cappucino and Sherman (2005) that stated the specific pH range for bacteria is between 4 and 9 and the optimum pH is 6.5– 7.5. It was also found that all PGPR strains were more tolerant to acidic conditions (pH 4–6) compared to alkaline conditions (pH 8–9). The Malaysian soil pH is generally between 4 to 5 (Shamsuddin *et al.* 2011). The acid tolerant characteristic showed by the isolated bacteria makes them suitable to be used for the soil in this country. According to Demoling *et al.* (2007), acidity could affect several steps in the development of the symbiosis relationship including the exchange of molecular signals between the legume and the microsymbiont, and relatively few rhizobia can grow well at pH less than 5. Thus, these PGPRs could be an alternative for the use of rhizobium species.

Different species showed different reactions towards different pH. Some bacteria can grow well in acidic pH while some are in alkaline conditions. Most of the beneficial microbes face several abiotic stress conditions including low pH, salinity, temperature fluctuations, osmotic and oxidative stresses, availability of nutrients and water when they are released to the field. Moreover, the soil pH conditions may affect microbial community structure, their dynamics growth and functional activity, ecosystem processes and interactions with plants (Chowdhury *et al.* 2022). The successful colonization of PGPRs is determined by their ability to tide over the stress condition while retaining their viability and efficacy. The isolated BRIS soil PGPRs in this study showed a higher growth rate in acidic conditions (pH 4–6) compared to alkaline conditions (pH 8–9) and the highest growth rate at pH 6–7. These results were in agreement with many studies by other researchers on the viability and functionality of *Bacillus, Burkholderia and Enterobacter* species that are most optimum in pH 6–7 (Weisskopf *et al*. 2011; Singh *et al.* 2021; Chowdhury *et al.* 2022).

Microbial growth also depends on the environmental temperature that could affect their cellular enzyme activity. The most optimum temperature for all PGPRs was recorded at 30°C. In addition, this type of PGPRs can grow at high temperatures (40–50°C) and the results also showed that the isolated BRIS soil PGPRs preferred to grow at higher temperature (30°C) compared to a lower temperature (20°C). Every bacteria require a certain temperature range for its optimum growth and metabolism. Zvidzai *et al.* (2015) reported that *Enterobacter asburiae* grows and produces cellulase enzyme optimally at pH 6 and temperature 40°C. While the report by Monteiro *et al.* (2016) stated that *Bacillus amyloliquefaciens* 629 colonize plant with more efficacy at 28°C and produces lipopeptides surfactin at an optimal temperature of 15°C. The PGPRs in this study showed high growth in temperature ranging from 25–35°C and their growth was considered high at 40°C and 50°C. This is an interesting characteristic of the PGPRs for biofertilizer production and application in soil for agriculture purposes that usually fluctuates to high and increase temperature, especially BRIS or other types of soil.

The high salinity medium in this study has decreased the growth of the BRIS soil PGPR isolates. However, all the bacterial strains were able to survive and grow in higher medium salinity (up to 8% $KNO_3$). The result is in agreement with Egamberdieva *et al.* (2017) that found salinity reduced bacterial growth but some bacterial strains can grow in a high salinity environment. Soil salinity could also cause a serious problem for crop production because it suppresses plant growth. Salt stress affects plant physiology which leads to reduced plant nutrient uptake and growth (Singh *et al.* 2011). Some plant beneficial microbes are tolerant to various abiotic stresses such as drought and salinity (Vardharajula *et al.* 2011; Hashem *et al.* 2016). The bacterial salinity tolerance can be utilized for fertilizer formulation incorporating an active component of chemical fertilizer with microbes to produce a multi group fertilizer (Muhammad *et al.* 2015; Goenadi *et al.* 2018). Moreover, previous studies by other researchers have suggested that the use of PGPR has significantly decreased plant stress because of soil salinity. PGPR colonization can also improve plant tolerance toward other abiotic stress like drought, injury and metal toxicity (Shrivastava and Kumar 2014). Various strains of PGPR from different genera such as *Rhizobium, Pseudomonas, Bacillus, Burkholderia* and *Enterobacter* have been reported to improve the host plant tolerance to abiotic stress environment (Grover *et al.* 2011). Thus, the role of PGPR in the management of biotic and abiotic stresses is gaining importance.

## Conclusion

The 6% molasses medium was the best alternative growth medium for UA 1, UA 6 and UAA 2 either in single or mix strains culture. All PGPR can grow at different pH (pH 4–9), different temperatures (20–50°C), different salinity (0–8% $KNO_3$). This study also indicates the superiority of BRIS soil PGPRs especially UA 6 which was more tolerant to acidic conditions (pH 4), high temperature (50°C) and high salinity (8% $KNO_3$). UA 6 has also shown the highest growth performance in molasses medium and different environmental factors followed by, mixed strains culture, UA 1 and UAA 2. A lot of further studies could be done to

evaluate the bacterial interactions between environmental factors (pH, temperature and salinity) dependencies for their further growth, physiological and biochemical investigations.

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## Author Contributions

ZM, AJZ and RO contributed to the conception and design of the experiments. ZM conducted experiments, collected, analysed the samples and data and preparing the manuscript. AJZ and DDZ carried out manuscript editing. KSM and DDZ performed final revision and reviewed the manuscript. All authors read and approved the final version.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Data Availability

All the related data reported in the manuscript will be available as requested.

## Ethics Approval

The authors declare that the research was in accordance with all ethical standards.

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