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Research Article

# Application of fruit juice for proliferation of *Bacillus* to control fungal phytopathogens

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## Abstract

Plant-based media have recently been of interest as potential natural media for microbial culture. This study was conducted to apply inexpensive and available fruits to culture *Bacillus* antagonizing three fungal phytopathogens including *Phytophthora capsici* GTC 2.6.1, *Rhizoctonia solani* GTC 2.7.1, and *Sclerotium rolfsii* GTC 2.9.1. The results showed that the juice media of dragon fruit, Cavendish banana, watermelon, pineapple, and MT1 seedless guava could all be used to culture two screened antagonistic *Bacillus* strains including B08 and B18. Surveys on the media from watermelon juice indicated that juice concentration (based on mass of fruit pulp), initial pH, concentration of traditional medium added to the juice medium had different effects on the growth of two strains of *Bacillus* sp. B08 and B18. These preliminary results demonstrated the potential application of the juice in biomass production of antagonistic *Bacillus* strains. This approach is certainly safe for the environment and has the potential to improve fruit consumption and reduce the cost of microbial fertilizer production, so it needs more research attention.

***Keywords:*** antagonistic; *Bacillus*; fungal phytopathogen; fruit juice; plant-based media; proliferation

## Introduction

*Bacillus* spp. have been known to be beneficial microorganisms with various plant growth promoting activities such as nitrogen fixation, biocontrol (nematodes, worms, and insects and phytopathogens such as fungi and bacteria), solubilization of insoluble inorganic minerals, stimulate plant defense systems (Saxena *et al*., 2020). *Bacillus* spp. effectively controls many fungal phytopathogens. Many commercial formulations based on *Bacillus* have been successfully applied to control fungal diseases in a variety of crops.

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Until now, *Bacillus* spp. are still cultured in the traditional media for biomass production. Meanwhile, many authors have recently shown that plant-based media have high potential for application for microbial culture. Wastes, by-products, and cheap agricultural products are used to grow microbes by many researchers such as Jadhav *et al*. (2018), García-Sánchez *et al*. (2019), Shareef (2019), and Mohammed *et al*. (2020). These natural media contain sufficient nutrients and are suitable for the growth of microorganisms (Harper *et al*., 2022). Plant-based media are similar to the natural habitats of many microbial communities, particularly endophytes, symbionts, or rhizospheres. Additionally, plant-based media have been proved to be very effective for unculturable microorganisms because they are easier to adapt to than traditional media (Youssef *et al*., 2016; Elsawey *et al*., 2020; Sarhan *et al*., 2020). Besides, some people are of the opinion that plant-based media are an approach to produce microbial products for vegetarians as reported by Heenan *et al*. (2002), Pathak and Martirosyan (2012), and Cichońska and Ziarno (2022).

Application of plant-based media has many advantages over traditional media such as similarity to the natural habitat of microorganisms, helping to exploit low cost and available materials, making use of locally discarded materials, and environmentally friendly (Santos *et al*., 2022). Ibrahim *et al*. (2020a) showed overexpression of activities such as indole acetic acid content and phosphate solubilizing activity of *Streptomyces rochei* DW3 and *Kosakonia radicincitans* DSM 16656 in plant-based media compared with traditional defined media. Also, according to Santos *et al*. (2022), impediments are that natural media have not been studied in detail, evaluations on microorganism strains/species should be made prior to application. The source of plant materials is certainly also influenced by the production region as well as by the season and weather, which makes the quality unstable and heterogeneous. This is difficult for application in microbial cultivation.

To date, the applications of plant-based media for culturing of plant growth-promoting *Bacillus* strains have been limited in literature. Youssef *et al*. (2016) showed that *Bacillus* spp. grow well on agar and liquid media based on cactus (*Opuntia ficus-indica*) and succulent plants (*Aloe vera* and *A. arborescens*). Plant-derived polysaccharides enhance biofilm-forming ability of plant growth-promoting *Bacillus* species and thereby increase root colonization (Beauregarda *et al*., 2013). Suryadi *et al*. (2019) concluded that the medium containing lablab bean mixed with palm sugar can be used to grow Lombok indigenous isolate of entomopathogenic *B. thuringiensis* and retained its toxicity against 3rd -instar *Aedes aegypti* larvae.

There are many fruit crops that are popular in Vietnam and are produced all year such as banana, watermelon, dragon fruit, guava, pineapple, ect. The cultivation area and the yield of these trees increase every year. Juices from many fruits have been previously applied in probiotic fermentation. Juice of many fruits are probably the natural habitat of *Bacillus* spp. as reported by Alwakeel and Al-Humaidi (2008), Aneja *et al*. (2014), and Evelyn *et al*. (2022). This study was carried out to apply a juice-based medium for the proliferation of *Bacillus* strain(s) that antagonize *Phytophthora capsici*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. After screening by dual culture technique on agar plate, the antagonistic *Bacillus* strains were cultured under different conditions such as fruit juice type, juice concentration, pH, added concentrations of traditional medium, and supplement of target fungal biomass.

## Materials and Methods

*Fruit juice-based media*

Fruits that were used for juicing included dragon fruit (*Hylocereus undatus*), watermelon (*Citrullus vulgaris*), pineapple (*Ananas comosus*), banana (*Musa acuminata*) Cavendish, and MT1 seedless guava (*Psidium guajava*). Fruit juice medium was prepared as described by Sarhan *et al*. (2020). The fruits were washed, then peels were removed, and the juice was directly pressed with a juicer to obtain pure juice. The pure juice was

diluted with distilled water to obtain a concentration of 10% (100 g of fruit pulp per liter). Initial pH of the media was adjusted to 7.0 prior disinfected by autoclave according to Ibrahim *et al*. (2020b).

*Microorganisms*

Nineteen strains of *Bacillus* spp. and three strains of fungal phytopathogens including *Phytophthora capsici* GTC 2.6.1, *Rhizoctonia solani* GTC 2.7.1, and *Sclerotium rolfsii* GTC 2.9.1 were provided by the laboratory of Gia Tuong Co., Ltd. *Bacillus* strains were cultured and preserved on Luria Bertani agar slant while pathogenic fungi on Potato Dextrose agar slant.

*Evaluation of the antagonistic efficiency of Bacillus*

A fungus from 3- to 5-day-old slant agar and a *Bacillus* strain from 2-day-old slant agar were inoculated at two opposite points through the center of plates containing Potato Dextrose agar as dual culture technique (Tekiner *et al*., 2019). The points were 1.5 cm from the edge of the dish. Plates that was not inoculated bacteria were the control. All plates were incubated at 30–33 °C until the fungal pathogens on the control plate grew to the opposite edge of the plate. The radii of mycelia in both of the dual culture plate (d) and the control plate (D) were measured in cm to evaluate the inhibition percentage (I) (Equation 1).

$$I\ (\%) = 100(D - d)/D \quad (1)$$

*Bacillus proliferation in fruit juice media*

A loop of *Bacillus* biomass from 2-day-old slant was inoculated into erlen containing 50 ml *of* Luria Bertani broth. The erlens were shaken at 200 rpm at 30–33 °C for 2 days. This obtained culture was then diluted with Luria Bertani broth to prepare a suspension of 0.5 $OD_{600\ nm}$. This bacterial suspension was used as the starter for the experiments on culturing *Bacillus* in fruit juice medium.

For the selection of fruit juice, one ml of the above bacterial suspension was inoculated into erlen containing 50 ml of separate fruit juice media and the culture conditions included 200 rpm and 30–33 °C for 2 days. Each fruit juice medium was a treatment and was triplicated. The obtained cultures was measured cell density using standard plate counting techniques.

The selected fruit juice medium was adjusted to different initial pH before autoclaving. One ml of the starter suspension was inoculated into 50 ml of these different pH media. Each treatment was triplicated. After shaking for 48 h, the influence of pH on the growth as well as pH selection for culture of the selected antagonistic *Bacillus* strain(s) was based on the cell density in the cultures.

The concentration of juice in the medium was adjusted to reach 1, 10, 20, 30, 40, and 50% (10, 100, 200, 300, 400, and 500 g of fruit pulp per liter). Media with different juice concentrations were inoculated with 1 ml of the antagonistic *Bacillus* suspension and the culture conditions were similar. The experiment was repeated three times. The density of cells presented in the obtained cultures was also measured using standard plate counting techniques to select the appropriate juice concentration for the proliferation of the selected strain(s).

Proliferative efficiency of the selected juice medium was compared with four conventional media including Luria Bertani broth, Nutrient broth, peptone meat extract broth, and Tryptic Soy Broth. Then, the traditional medium with the highest cell density would be added to the selected fruit juice medium at different concentrations with the expectation of increasing the proliferation efficiency.

The selected traditional medium was added with different concentrations to the selected juice medium to culture antagonistic *Bacillus* strains. The investigated concentrations were in the range of 0 - 50% to select the appropriate additional concentration.

*Data analysis*

All experiments were arranged in a completely random design and the results were an average of repetitions. Comparisons of means were made using SPSS v.20.1 (IBM, New York, USA) with one-way analysis of variance (ANOVA), Duncan test at a significance level of $P < 0.05$.

## Results

*Screening of Bacillus strains for resistance to fungal phytopathogens*

All nineteen investigated strains of *Bacillus* spp. were able to antagonize all three fungal strains. However, the inhibition percentage varied according to the *Bacillus* strain as well as the fungal strain. The inhibition percentage of *Bacillus* strains with *P. capsici* GTC 2.6.1, *R. solani* GTC 2.7.1, and *S. rolfsii* GTC 2.9.1 ranged 30.67 - 44%, 44.44 - 60.15%, and 38 – 57.33%, respectively (Table 1).

**Table 1.** The inhibition percentage of *Bacillus* strains with *P. capsici* GTC 2.6.1, *R. solani* GTC 2.7.1, and *S. rolfsii* GTC 2.9.1

| ***Bacillus* strains** | **I (%)** | | |
|---|---|---|---|
| | **GTC 2.6.1** | **GTC 2.7.1** | **GTC 2.9.1** |
| B01 | $38.17^{bc}$ | $51.24^{bc}$ | $47.26^{cd}$ |
| B02 | $38.67^{abc}$ | $48.50^{bcd}$ | $49.33^{bcd}$ |
| B03 | $41.83^{abc}$ | $47.00^{cd}$ | $45.90^{d}$ |
| B04 | $37.63^{bc}$ | $50.37^{bc}$ | $50.67^{abcd}$ |
| B05 | $38.67^{abc}$ | $45.90^{cd}$ | $45.52^{d}$ |
| B06 | $38.17^{bc}$ | $51.50^{bc}$ | $52.00^{abcd}$ |
| B07 | $40.30^{abc}$ | $50.37^{bc}$ | $51.85^{abcd}$ |
| B08 | $40.83^{abc}$ | $60.15^{a}$ | $57.33^{a}$ |
| B09 | $39.41^{abc}$ | $53.14^{b}$ | $49.33^{bcd}$ |
| B10 | $39.26^{abc}$ | $52.17^{bc}$ | $52.59^{abcd}$ |
| B11 | $40.67^{abc}$ | $50.96^{bc}$ | $51.17^{abcd}$ |
| B12 | $42.33^{ab}$ | $52.17^{bc}$ | $56.17^{ab}$ |
| B13 | $41.71^{abc}$ | $52.38^{b}$ | $53.83^{abc}$ |
| B14 | $37.33^{c}$ | $52.00^{bc}$ | $52.17^{abcd}$ |
| B15 | $40.44^{abc}$ | $49.83^{bcd}$ | $49.52^{bcd}$ |
| B16 | $40.95^{abc}$ | $48.89^{bcd}$ | $47.00^{cd}$ |
| B17 | $41.00^{abc}$ | $53.50^{b}$ | $51.50^{abcd}$ |
| B18 | $44.00^{a}$ | $53.71^{b}$ | $53.33^{abc}$ |
| B19 | $30.67^{d}$ | $44.44^{d}$ | $38.00^{c}$ |

In the same column, means followed by the same letter(s) indicated insignificant differences (Duncan test, $p < 0.05$).

*Effects of fruit juice media on the growth of two selected antagonistic Bacillus strains*

The two selected *Bacillus* strains grew well in all five surveyed fruit juice media. There was no statistically significant difference in the cell density in the obtained cultures (Table 2). The *Bacillus* sp. B18 cultures had higher cell densities than the *Bacillus* sp. B08 cultures in each respective fruit juice medium.

**Table 2.** The cell density in the cultures of the selected antagonistic *Bacillus* strains after cultivation in different fruit juice media

| Fruit juice media | Log CFU/ml | |
|---|---|---|
| | ***Bacillus* sp. B08*** | ***Bacillus* sp. B18*** |
| Dragon fruit | 8.10 | 8.13 |
| Cavendish banana | 7.97 | 8.25 |
| Watermelon | 7.94 | 8.19 |
| Pineapple | 8.19 | 8.32 |
| MT1 seedless guava | 7.95 | 8.07 |

*: There were no insignificant differences at P < 0.05.

*Effects of pH of the medium on the growth of the selected antagonistic Bacillus strains*

The *Bacillus* sp. B08 strain had good growth in all media with pH 6.0 – 8.0 and the cell density had no statistical difference (Table 3). However, for the *Bacillus* sp. B18 strain, the culture from the medium with pH 7.5 had the highest cell density while the cultures from the remaining investigated pH had the same cell densities and were significantly lower than that of pH 7.5.

**Table 3.** The cell density in the cultures of selective antagonistic *Bacillus* strains after cultivation in watermelon juice media with different initial pH

| Initial pH | Log CFU/ml | |
|---|---|---|
| | ***Bacillus* sp. B08*** | ***Bacillus* sp. B18** |
| 6.0 | 8.41 | $8.10^{b}$ |
| 6.5 | 8.43 | $8.13^{b}$ |
| 7.0 | 8.50 | $7.98^{b}$ |
| 7.5 | 8.46 | $8.49^{a}$ |
| 8.0 | 8.45 | $8.16^{b}$ |

In the same column, means followed by the same letter(s) indicated insignificant differences (Duncan test, p < 0.05).

*: There were no insignificant differences.

*Effects of watermelon juice concentration on the growth of the selected antagonistic Bacillus strains*

The change in the % concentration of watermelon juice (calculated by the mass of the fruit pulp in one liter) affected significantly on the growth of both of *Bacillus* sp. B08 and B18. The cell density in the cultures was positively correlated with the concentration of watermelon juice in the range of 1 - 20% (Table 4). When the concentration of watermelon juice in the range of 20 - 40%, the cell density in the cultures was not significantly different. The cell density tended to decrease when the concentration of juice in the medium was 50%.

**Table 4.** The cell density in the cultures of the selected antagonistic *Bacillus* strains after cultivation in media with different concentrations of watermelon juice

| Juice concentration (%)* | Log CFU/ml | |
|---|---|---|
| | ***Bacillus* sp. B08** | ***Bacillus* sp. B18** |
| 1 | $7.36^{c}$ | $4.00^{c**}$ |
| 10 | $8.30^{b}$ | $6.87^{b}$ |
| 20 | $8.43^{ab}$ | $7.67^{ab}$ |
| 30 | $8.60^{ab}$ | $8.16^{a}$ |
| 40 | $8.64^{a}$ | $8.11^{a}$ |
| 50 | $8.59^{ab}$ | $6.79^{b}$ |

In the same column, means followed by the same letter(s) indicated insignificant differences (Duncan test, p < 0.05).

*: calculated by the mass of the fruit pulp in one liter of medium.

**: Too few to count.

*Comparison of the proliferative efficiency of watermelon juice and traditional media*

The selected bacterial strains were cultured in liquid media including Luria Bertani broth, nutrient broth, peptone meat extract broth, and Tryptic Soy Broth for comparing the proliferative efficiency of watermelon juice medium with conventional media. The cell density in the obtained cultures of *Bacillus* sp. B08 from 20% watermelon juice medium was statistically significantly lower than all four surveyed traditional media (Table 5). Meanwhile, *Bacillus* sp. B18 has the best growth in peptone meat extract broth followed by nutrient broth. The growth of *Bacillus* sp. B18 was equivalent in three media including 20% watermelon juice medium, Luria Bertani broth, and Tryptic Soy Broth.

**Table 5.** The cell density in the cultures of the selected antagonistic *Bacillus* strains after cultivation in watermelon juice medium and conventional media

| Media | Log CFU/ml | |
|---|---|---|
| | ***Bacillus* sp. B08** | ***Bacillus* sp. B18** |
| 20% watermelon juice | $8.40^{c}$ | $7.57^{c}$ |
| Luria Bertani broth | $9.14^{a}$ | $7.11^{c}$ |
| Nutrient broth | $8.95^{b}$ | $8.28^{b}$ |
| Peptone meat extract broth | $8.99^{ab}$ | $9.05^{a}$ |
| Tryptic Soy Broth | $9.03^{ab}$ | $7.25^{c}$ |

In the same column, means followed by the same letter(s) indicated insignificant differences (Duncan test, $p < 0.05$).

*Effects of combination of traditional and watermelon juice media on the growth of the selected antagonistic Bacillus strains*

The medium containing 20% watermelon juice supplemented with Lubria Bertani broth and peptone meat extract broth was used to culture two strains of *Bacillus* sp. B08 and B18. The concentrations of the traditional media varied from 10% to 50%. The combination of the traditional medium to the 20% watermelon juice medium enhanced the growth of both *Bacillus* sp. B08 and B18 in all treatments. However, the different concentrations of Lubria Bertani broth did not lead to a difference in the final cell density of *Bacillus* sp. B08 (Table 6). Meanwhile, the addition of peptone meat extract broth from 10% to 30% significantly increased the cell density in the final culture of *Bacillus* sp. B18. Cell density in the final cultures of *Bacillus* sp. B18 was almost unchanged at the higher added concentrations of peptone meat extract broth. Therefore, the medium containing 20% watermelon juice supplemented with 10% Lubria Bertani broth was suitable for culturing *Bacillus* sp. B08 while this juice medium was supplemented with 30% peptone meat extract broth was suitable for culturing *Bacillus* sp. B18.

**Table 6.** The cell density in the cultures of the selected antagonistic *Bacillus* strains after cultivation in 20% watermelon juice medium combined conventional media

| Concentration of traditional media (%) | Log CFU/ml | |
|---|---|---|
| | ***Bacillus* sp. B08*** | ***Bacillus* sp. B18**** |
| 0 | $8.40^{b}$ | $7.57^{d}$ |
| 10 | $8.98^{a}$ | $8.09^{cd}$ |
| 20 | $8.91^{a}$ | $8.35^{bc}$ |
| 30 | $9.17^{a}$ | $9.16^{a}$ |
| 40 | $9.09^{a}$ | $8.80^{ab}$ |
| 50 | $9.09^{a}$ | $8.49^{abc}$ |

*: 20% watermelon infusion supplemented with Luria Bertani broth.

**: 20% watermelon infusion supplemented with Peptone meat extract broth.

In the same column, means followed by the same letter(s) indicated insignificant differences (Duncan test, $p < 0.05$).

## Discussion

The antagonistic efficiency varied according to the surveyed *Bacillus* strain, which was similar to many previously reports by researchers such as Mojica-Marín *et al*. (2008), Rios-Velasco *et al*. (2016), Margani *et al*. (2018), Rajkumar *et al*. (2018), Kumari *et al*. (2021), and Moon *et al*. (2021). For example, Mojica-Marín *et al*. (2008) showed that the antagonistic efficiency of 16 strains of *B. thuringiensis* on *R. solani* was 34.44 - 66.66%. With *S. rolfsii*, five strains of *Bacillus* spp. in Kumari *et al*. (2021) had the inhibition percentage of 29.63 - 58.44% and 30 strains of *B. subtilis* in Rajkumar *et al*. (2018) had the inhibition percentage of 11.98 - 64.04%. Meanwhile, Anjum *et al*. (2019) recorded two strains of *B. subtilis* inhibiting the growth of *P. capsici* strain at 41.56 and 54.36%, respectively. In addition, the results also indicated that the antagonistic efficiency also depended on each specific phytopathogenic fungus of *P. capsici* GTC 2.6.1, *R. solani* GTC 2.7.1, and *S. rolfsii* GTC 2.9.1 as discussed by Rios-Velasco *et al*. (2016) and Moon *et al*. (2021). For example, Moon *et al*. (2021) showed that the antagonistic efficiency of *B. velezensis* CE 100 reached 54.6 - 74.3% depending on each strain *Phytophthora* spp. Previously, Margani *et al*. (2018) also reported the inhibition percentage of five strains of *Bacillus* spp. reached 30.33 – 58% on another strain of *R. solani*, depended on each bacterial and fungal strain. According to Tekiner *et al*. (2019), *Bacillus* sp. B08 had a high efficiency and a medium efficiency to control *R. solani* GTC 2.7.1 and *S. rolfsii* GTC 2.9.1, respectively (Table 1). *Bacillus* sp. B18 was low effective to control *P. capsici* GTC 2.6.1 and less effective than the strains reported by Anjum *et al*. (2019) and Moon *et al*. (2021) and thereby the detection for strains with higher antagonistic efficiency was essential.

To date, fruit juice has not been studied much for proliferation of microorganisms in the production of microbial fertilizers. However, the juices of many fruits were used to ferment *Lactobacillus* probiotics for human. For example, watermelon juice was fermented for probiotics of *Lactobacillus* spp. (Naga Sivudu *et al*., 2014; Lani *et al*., 2022). The fermented pure watermelon juice had a final cell density of *Lactobacillus* spp. of $10^8$ - $10^9$ CFU $ml^{-1}$ (Lani *et al*., 2022). *L. rhamnosus* reached $10^7$ – $10^9$ CFU $ml^{-1}$ in the fermented banana juice (Mukisa and Birungi, 2018). Probiotics from pure pineapple juice fermented by *Bifidobacterium lactis* and *Lactobacillus* spp. contained the cell density of $10^9$ and $5x10^9$ CFU $ml^{-1}$, respectively (Nguyen *et al*., 2019). Nawangsih *et al*. (2021) obtained a probiotic product from the juice of 25% pink guava with *Lactobacillus* spp. $4.9x10^{11}$ CFU $ml^{-1}$ meanwhile Nurainy *et al*. (2022) harvested *L. casei* 10.93 log CFU $ml^{-1}$ from red guava supplemented with cinnamon and *Caesalpinia sappan* wood extract. The fermented dragon fruit extract contained *L. acidophilus* at 9 log CFU $ml^{-1}$ (Maryati and Susilowati, 2018).

A few studies have applied plant-based media to culture *Bacillus*. Two strains of *B. thuringiensis* were cultured in aqueous media including soybean meal extract + starch, soybean flour + food grade barley flour, peanut flour + starch, and peanut flour + food grade barley flour and the cell density ranged $1.5x10^7$ – $2.1x10^8$ CFU $ml^{-1}$ (Shojaaddini *et al*., 2010). Magarelli *et al*. (2022) obtained cultures of *Bacillus* spp. 7.91 – 8.98 log CFU $ml^{-1}$ by cultivation in *Opuntia ficus-indica* stem juice medium. Devidas *et al*. (2014) used the basic mineral medium supplemented with 10 g $l^{-1}$ of ripe banana pulp for *B. thuringiensis* and showed the similar growth compared to Luria Bertani broth. This study proved the potential of fruit juice-based media for culturing *Bacillus*. The obtained cultures had similar cell densities compared with the above authors (Table 2). Low-cost and local availability were other advantages for all five of these fruits. Watermelon was easily to obtain juice and a higher amount of juice than the remaining four fruits (detail data not recorded) should be selected as the most potential medium for further studies.

*Bacillus* spp. are present in a wide range of habitats with very different pH. *Bacillus* sp. can survive or grow at extreme pH conditions. Some strains have optimal growth in a wide range of pH. However, most *Bacillus* grows optimally at near-neutral pH. Many strains of *Bacillus* used as probiotics for both plants and animals had the optimal pH for growth in the neutral zone. For example, the nitrogen-fixing *B. subtilis* AS-4

strain had the optimal growth at pH 7.0 (Satapute *et al*., 2012). *B. subtilis* MS21 antagonizing *Gloeosporium gloeosporioide* reached the highest cell density at pH 8.0 (Anjhana and Sasikala, 2017). *Aspergillus flavus*-controlling *B. subtilis* UTB96 strain grew optimally in the pH 7.0 medium (Ghasemi and Ahmadzadeh, 2013). The medium with pH 6.5 was suitable for culturing *B. subtilis* 11A as probiotic for chickens (Cahya *et al*., 2019). Sidorova *et al*. (2020) studied on two strains of *B. subtilis* for biocontrol of phytopathogenic *Fusarium* and showed that pH 6.0 – 8.0 and pH 8,0 were the most suitable for the growth of *B. subtilis* BZR 336 g and *B. subtilis* BZR 517, respectively. Evaluating the influence as well as choosing the optimal pH for bacterial culture has always been of interest to researchers. Changes in the external pH can alter the ionization of nutrient molecules and thus reduce their availability to the organism (Willey *et al*., 2023). Besides, the pH is lower or higher than the optimal pH, bacteria can respond by different mechanisms to balance the cytoplasmic pH for survival and growth. In this study, *Bacillus* sp. B08 grew well at pH 6 – 8 while *Bacillus* sp. B18 had the optimal growth at pH 7.5 (Table 3). Therefore, the medium with a pH of 7.5 was selected to culture both the two antagonistic *Bacillus* strains.

Previously, agar medium containing 80% pure watermelon juice was used to culture *B. subtilis* and *B. megaterium* and these strains grew on this medium as well as on conventional Nutrient agar (Reddy *et al*., 2017). Kavuthodi *et al*. (2015) applied 10% (w/v) watermelon rind extract to form liquid media containing 2.5 - 15% (v/v) of this extract to culture *B. subtilis* BKDS1. The medium containing 12.5% the extract was optimal for the growth and the synthetization of exo-pectinase of *B. subtilis* BKDS1. Until now, watermelon juice from fruit flesh has not been applied in *Bacillus* culture. Recently, Magarelli *et al*. (2022) recorded that *Opuntia ficus-indica* stem juice medium with different concentrations affected the growth of two strains of *B. ambifaria* MCI 7 and *B. amyloliquefaciens* LMG 9814. Accordingly, the growth of these *Bacillus* strains was higher in media containing 25 – 50% (v/v) *O. ficus-indica* stem juice than media containing only 15% (v/v) (juice yield reached 20.2% from raw materials). The *B. cereus* strain grew best in the medium with 7.5% textured soy protein in the investigated concentration range of 0.5 – 10% (Cruz *et al*., 2020). The 2.5% fresh rice leaves extract medium was more suitable for the growth of *Cercospora janseana* than 5%, 10%, and 20%. Thereby, it was shown that the appropriate concentration of extract for culture depended on the plant material as well as the microbial strain. In this study, the low concentration of watermelon juice (1%) was probably not nutritionally sufficient for the growth of two strains of *Bacillus* sp. B08 and B18. Meanwhile, nutrients in medium containing 20% concentration were enough for these two strains to have good growth and therefore the cell density was not significantly increased at the higher concentrations (Table 4). In addition, Hassan *et al*. (2011) reported that watermelon juice had antibacterial activity and thereby the medium containing 50% watermelon juice concentration was probably high enough to inhibit the growth of these two strains. The medium containing 20% watermelon juice concentration was suitable to culture both *Bacillus* B08 and B18 strains for cost control.

Watermelon juice was a natural medium with various macronutrients and micronutrients required for growth of microorganisms. Watermelon juice was rich in carbohydrates, similar to other plant extracts (Adeleke and Oguntuga, 2003; Fish *et al*., 2009; Ozcelik and Yavuz, 2016). It was noteworthy that the carbohydrates mainly consisted of sugars such as glucose, fructose, and sucrose which were easily metabolized. However, the nitrogen content in watermelon juice was relatively low as reported by Adeleke and Oguntuga (2003) and Fish *et al*. (2009). The carbon/nitrogen ratio was higher than 10 in watermelon juice and this ratio was not suitable for the growth of some *Bacillus* strains as discussed by Supono *et al*. (2013), Yuniarti *et al*. (2019), and Kusumaningrum *et al*. (2020). Accordingly, some strains tended to form spores or change metabolic pattern instead of vegetative cell growth at high carbon/nitrogen ratio. Meanwhile, the traditional media had a lower carbon/nitrogen ratio than watermelon juice medium and was probably responsible for the higher growth of *Bacillus* sp. B08 and B18 (Table 5). In addition, the traditional media contained 0.5 - 1%

NaCl while, according to Adeleke and Oguntuga (2003), the low potassium content was 0.0102% in pure watermelon juice. This could be the other reason why the proliferative efficiency of the medium containing 20% watermelon juice was lower than that of the traditional media.

Fruit juice, although it could contain a full range of macro and trace components, may also be too much or too short of certain ingredients for the growth needs of microorganisms. According to Reddy et al. (2017), it may be necessary to supplement certain nutrients to an agar based on 80% pure watermelon juice for the growth of microorganisms, including *Bacillus* strains. The 20% boiling extract of purple-skinned sweet potato and white yam supplemented with 2% glucose was more suitable for the growth efficiency of strain *Pleurotus eryngii* (Andrade et al., 2020). *B. ambifaria* MCI 7 and *B. amyloliquefaciens* LMG 9814 grew better in 25 - 50% (v/v) *O. ficus-indica* juice supplemented with 0.2% sucrose (Magarelli *et al*., 2022). There were cases where no nutritional supplementation was required for microbial growth. For example, 2.5% fresh rice leaves extract was more suitable for the growth of strain *Cercospora janseana* than this medium supplemented with 10% and 20% V8 medium (Uppala *et al*., 2019). As discussed above, in addition to nutritional supplements, the combination could have helped to reduce the carbon/nitrogen ratio in the medium to accommodate the growth of two *Bacillus* sp. B08 and B18.

## Conclusions

Among the 19 strains of *Bacillus* surveyed, *Bacillus* sp. B08 had the highest ability to inhibit the growth of *R. solani* GTC 2.7.1 and *S. rolfsii* GTC 2.9.1 and *Bacillus* sp. B18 had the highest ability to inhibit the growth of *P. capsici* GTC 2.6.1. These two *Bacillus* strains were able to grow in the media containing fruit juice of dragon fruit, Cavendish banana, watermelon, pineapple, and MT1 seedless guava. When watermelon juice was used for cultivation, the suitable conditions for culturing *Bacillus* sp. B08 consisted of 20% (w/v, based on mass of fruit pulp) juice, initial pH 6 - 8, supplemented with 10% Luria Bertani broth. The suitable conditions for culturing *Bacillus* sp. B18 consist of 20% (w/v, based on mass of fruit pulp) juice, initial pH 7.5, supplemented with 30% of peptone meat extract broth. The cultures obtained from these cultivation conditions had the cell densities of 8.98 and 9.16 Log CFU $ml^{-1}$, respectively. It was clear that these available fruit juices had potential applications for culturing antagonistic *Bacillus* growth.

## Authors' Contributions

XTV and KDT performed the experiments, data analysis, and wrote the results. NTN participated in the antagonistic experiments and wrote the first draft of the manuscript. NNN designed the experiments, wrote the protocol, managed the project. All authors read and approved the final manuscript.

**Ethical approval** (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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