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Bacillus Species' Gaseous Metabolites Prolong the Shelf Life of Apples and Grapes

Hongyi Shen

Rutgers Preparatory School, NJ 08873, USA

ABSTRACT

In view of the threat to the environment posed by the widely used plant and fruit preservation chemicals in current agricultural practices, and the indispensability of fruit and plant preservatives in ensuring the yield of agricultural products, it is urgent to explore environmentally friendly and efficient preservative alternatives. Against this backdrop, bacterial preservatives stand out as a potential green solution. In this study, 12 strains of Bacillus derived from different ecological niches were carefully screened to evaluate the inhibitory efficacy of their gaseous metabolites against Anthracnose colloidospora, a common fruit pathogen, by bisectional method. The results showed that the bacterial suspension of strain B4 was the most prominent in inhibiting the growth of Colloidosporum anthracnose, and it significantly narrowed the growth circle of pathogenic bacteria, so it was selected as the object for further optimization and application. Subsequently, we designed a practical application test for fruit preservation, in which the bacteria of strain B4 were directly placed in the same environment as the fruit to be preserved, and the observation was continued for five days. By comparing and analyzing the fruit state before and after treatment, we were pleased to find that strain B4 showed a positive effect on inhibiting fruit rot, indicating its great potential as a natural fruit preservative. This discovery not only provides a scientific basis for reducing the use of chemical preservatives, but also contributes to the sustainable development of agriculture and the protection of the ecological environment.

KEYWORDS

Bacillus, Colletotrichum gloeosporioides Penz., Anti-corruption.

1. INTRODUCTION

In recent years, food spoilage has been a big problem worldwide. According to Food and Agriculture Organization(4), about 45% of all harvested fruits, and vegetables are wasted. Most of the losses are caused by bad conditions and pathogens acting on the produce during storage. While many chemicals were invented to prolong their shelf life, they are often harmful to humans, animals, and the environment. Due to the risk, many of these chemicals are completely prohibited in some European countries. (As a result, there is increasing interest in finding new sustainable and eco-friendly alternatives. Biological products, such as plant growth-promoting bacteria (PGPB), offer an alternative to food preservation and controlling diseases affecting produce on its journey to the market(1). PGPB are group of beneficial bacteria that directly and/or indirectly promote the host species resistance, growth, and abiotic stress. These products change the host plants' metabolism, strengthening the plants' resistance and shelf life without negatively affecting other plants, humans, or the environment.(5) One of the PGPB, B. subtilis, is one of the most attractive ingredients for developing plant protection products, as recommended by United States Food and Drug Administration. It has the ability to stop post-harvest gray mold on fruits like strawberry, pears, apple

and tomato.(5) Bacillus spp. has been frequently reported as the biocontrol for fresh cut fruits and vegetables during handling and transportation. Bacillus are aerobic, or facultatively anaerobic, rod-shaped bacteria that form endospores. Most bacillus species grow in decaying organic matter, but they also grow soil and water. Bacillus species are Gram positive when they are young, but some species becomes Gram-negative when they age. Most Bacillus endoscopes are resistant to harsh physical and chemical conditions because of the thick spore coat, which covers up to 50% of the spore's volume. In fact, some bacillus species have unique properties that allow them to thrive in extreme environments ranging from hot springs, arctic soil, desert sand and marine settlements.(1) Research on Bacillus reveals both beneficial and harmful species Certain bacillus are resistant to heat, micas, and radiations so they are used to test heat sterilization procedures. Some bacillus species aer used naturally or artificially to degrade waste products, and some are used as the main ingredient of pesticides. Bacillus has been widely used in the medical, industrial, and agriculture for its benefit to produce large amounts of enzymes, antibodies and other metabolites like Bacitracin and polymyxin. (1) For those reasons, bacillus spp. has potential to prolong the shelf life of produce.

2. MATERIALS AND METHODS

2.1. Preparing LB and PDA Media and LB Broth Liquid Medium

2.1.1. Materials:

LB broth, Agar, 350 ml of pure water, PDW (Potato Dextrose Water), Weighing paper, Electronic balance, Petri dishes, Autoclave, Beaker, clean bench, alcohol lamp, lighter, beaker plastic covering, rubber bands.

2.1.2. Methods:

Fill a beaker with 350ml pure water. Put a weighing paper on a scale to set it equal to zero. Take a clean spoon to scoop out the powder from its containers and measure LB broth powder(8.75g) and 1.5% agar powder (5.25g). Mix them in a beaker two times and add 350ml of pure water. Measure PDW powder (8.75g) and 1.5% agar powder (5.25g) and mix with 350 ml of water in a beaker two times. Cap the opening of the beakers using plastic wraps and rubber bands. Put the 2 LB broth Beakers and the 2 PDA beakers in an autoclave basket and on the top of the other autoclave baskets. Sterilize both media by autoclaving at 121°C and 0.1 MPa for 15 minutes. Take out the hot mixtures and swirl them until there is no settlements on the bottom. Take the mixtures to a clean bench and light an alcohol lamp to make sure e everything near it is sanitary. Wrap the top of the beaker with a paper towel to make sure it doesn't burn the hand while pouring and then pour the media into empty Petri dishes until it covers the whole bottom. Wait until the petri dishes containing the media solidify then we can use it to grow different species.

2.2. Bacillus Inoculation

2.2.1. Materials

Bacillus strains B1-B12, Pipette, TS-200DC incubator, Centrifuge, LB culture, test tubes, test tube caps, autoclave, newspaper, rubber bands, test tube oscillator, centrifuge tubes, salt water.

2.2.2. Methods

Pour LB liquid into test tubes and cap them and cap them. Use rubber bands to tighten 7 test tubes together into a bunch and tighten a piece of newspaper to cover the caps of the test tubes. Put the test tubes into an autoclave basket to kill germs. Using a clean bench, light the alcohol lamp and use a pipette to pick up some bacillus B1-B12 culture from their original petri dishes and transfer them into test tubes of LB liquid medium. Mix the mixture by pipetting it up and down and cap them. Place the test tubes in the TS-200DC incubator to shake and incubate overnight at 37°C. Pipette 1 ml of each

bacillus cultures into four centrifuge tubes. Centrifuge the tubes and separate the supernatant from the pallet and label them. Add 1ml of salt water to the centrifuge tubes with pallets at the bottom and mix them using a test tube oscillator.

2.3. Mold Suspension Preparation

2.3.1. Materials

Colletotrichum gloeosporuoides (mold) dish, salt water, 10ml container tubes, p1000 pipette, microscope with 10x lens, hemocytometer.

2.3.2. Methods

Use a pipette tip to pick up some colletotrichum gloeosporuoides clusters off the supply petri dish and stir them in a 10ml tube filled with salt water. Pipette up and down to mix them as well as shake and flick the tube. Then pipette some of the mixture on a hemocytometer with markings and look through a 10x lens(yellow) on a microscope. From the marking there were, 5*5, 25 boxes and we added the number of dots which are cells on the top left, top right, bottom left, bottom right, and the box right in the middle as A, B, C, D, and E. We calculate the numbers of cells on the markings using the equation (A + B + C + D + E)/5 * 25 * 1000 and estimate the number of cells in the mixture to be 4.6*10^6. The goal is to have 10^5 cells in the mixture so calculated the ratio to be 1:46 we added around 0.2 ml of the mold mixture into a 10ml tube with mixed 9.8ml salt water.

2.4. B1-B12 Bacillus liquid supernatent prevents colletotrichum gloeosporuoides （mold） from growing on petri dish

2.4.1. Materials

PDA Dish, tweezer, 10^5 mold mixture, filter paper, hole puncher, p200 and p20 pipette, airtight seal, B1-B12 mixture.

2.4.2. Methods

Punch little circles from filter papers into a beaker using a hole puncher and disinfect them using an autoclave. Pipette 200 ul of 10^5 colletotrichum gloeosporuoides mixture onto PDA dishes and put two pieces of filter paper on each dish. Add 10ul of Bacillus culture B1-B12 supernatant on each piece of filter paper on the PDA dish, then Seal tight the edges at 28℃. After 24 hours, observe the inhibition zones around the filter paper discs. We find that there is no mold directly around the filter paper discs.

2.5. Experiment of the gaseous metabolites on spoilage bacteria

2.5.1. Materials

two-tier plate culture dish, LB, PDA, agar, 10^5 colletotrichum gloeosporuoides（mold）solution, B1-B12 bacillus, aseptic coating rod, clean bench, vortex oscillator.

2.5.2. Methods

Use the pipette tips to pick up bacillus B1-B12 culture on the petri dishes and stir them in LB tubes. Pipette up and down to mix them. Incubate overnight in a 37℃ shaker incubator to multiply in numbers. Under a clean bench, pipette 100ul of the culture into centrifuge tubes to centrifuge. Remove the supernatant by blotting it above the trash bin. Then mix the settlements with 1 ml of salt water. Press on the vortex oscillator to mix them.

Make a two-tier petri dish, one side is LB and the other PDA. Pipette 10ul Colletotrichum gloeosporuoides (10^5 mold solution) on the PDA side of the petri dish. 100 ul Bacillus bacterial suspension on the LB side and spread with aseptic coating rods. Seal tight the petri dishes and place

them in 28°C cultivation for 2 days. Of all the B1-B12 petri mold dishes, B4 culture petri dishes grew the least amount of mold clusters followed by B6 and B8 culture dishes. The rest of the bacillus cultures petri dishes showed a significant amount of mold clusters. B4 bacillus culture is bacillus amyloplyticus, and B6 is Bacillus subtitles while B8 is just Bacillus. From the results, the B6 petri dish grew the most amount of bacillus bacteria whereas B8 grew the least amount of Bacillus culture and didn't have a little bit more mold than B4 and B6 but didn't have as much mold as the rest of the bacillus culture. Therefore, B8 is the best at filtering based on the ratio of bacillus to mold growing.

2.6. colletotrichum gloeosporioides growth on apples and grapes with bacillus B4 air to test for mold growing

2.6.1. Materials

Grapes, apples, Water, sodium hypochlorite, beaker, basket, p200 pipette, fruit punching rod, aseptic coating rod, colletotrichum gloeosporuoides, cling film.

2.6.2. Methods

Pick out the bad grapes from the good ones. Spoiled grapes, roted grapes and skin fall off are often signs of bad grapes that needed to be removed. Take apples and put them in a bucket and fill each bucket with 2L of water. Add 2% of sodium hypochlorite to each bucket which is 20 ml. Then ut the grapes in a big bin and measure 1L of water and 10 ml of sodium hypochlorite and mix them by swirling. Wait 10 minutes then pour the grapes and apples on separate baskets. Then rinse the fruits 2 to 3 times using tap water. Using a fruit punching rod to remove skin from the apple 4 holes on one face of each apple.

Count the grapes and put 6 of the grapes in each plastic bowls and 3 apples in each baskets. Take B4 culture tubes from the Bacillus culture rack, using a p200 pipette to pipette 200 ul of B4 culture on 12 LB petri dishes. Spread the culture using an aseptic coating rod. Take the petri dishes to 37°C incubator and put them upside down to grow for a few hours. Pipette 10ul of colletotrichum gloeosporuoides into every hole of the apples. In one basket, put three apples pipetted with colletotrichum gloeosporuoides and 6 petri dishes with bacillus B4 petri dishes uncovered facing up. In the other basket, put 3 apples pipetted with colletotrichum gloeosporuoides with 6 petri dishes without bacillus culture facing up uncovered. Wrap the two baskets with cling film. In each grape bowl, tape one petri dish upside down on the lid and place one petri dish with the grapes. Clip tight the bowl and let the grape bowls and apple baskets sit in the constant temperature and humidity cultivation room.

3. RESULTS AND ANALYSIS

3.1. Preparing LB and PDA Media and LB Broth Liquid Medium

The LB and PDA media, along with the LB broth liquid medium, were successfully prepared and solidified in Petri dishes. The media appeared uniform and free of contamination. The successful preparation of sterile and uniform media provided a suitable environment for subsequent experiments, ensuring reliable growth conditions for Bacillus species.

3.2. Inoculating Bacillus and Incubating Overnight on a Shaker

After overnight incubation at 37°C, the Bacillus cultures showed significant growth. The centrifugation process effectively separated the bacterial pellets, which were visibly dense, indicating a high concentration of Bacillus bacteria.

The high concentration of Bacillus bacteria obtained through this method was essential for the effectiveness of the subsequent experiments. The separation of bacterial pellets ensured that only viable cells were used in further testing.

3.3. Preparing a 10^5 Mold Suspension

As shown in Figure 1, the Colletotrichum gloeosporioides suspension was successfully prepared with an estimated concentration of 4.6×10^6 cells/ml. The dilution process was accurately performed to achieve a final concentration of 10^5 cells/ml. The suspension appeared homogeneous, and the cell counts were consistent with the calculated ratio.

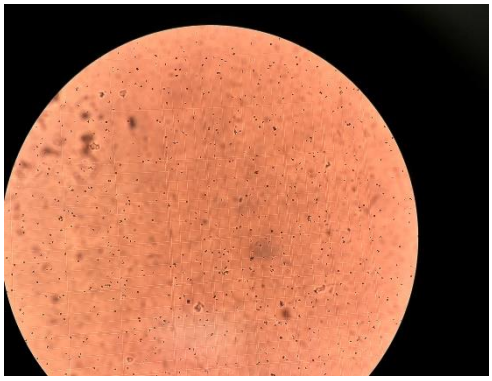

Figure 1 Microscopic Image of Blood Cell Counting Chamber

Achieving a precise and homogeneous mold suspension was crucial for consistent and reliable results in experiments testing the inhibitory effects of Bacillus species on mold growth. The accurate preparation of the mold suspension ensured that each experimental condition received the same initial concentration of mold.

3.4. Bacillus B1-B12 Preventing Colletotrichum gloeosporioides (Mold) from Growing on Petri Dishes

The addition of Bacillus B1-B12 to the PDA dishes significantly inhibited the growth of Colletotrichum gloeosporioides around the filter paper. The areas treated with Bacillus showed less mold growth compared to untreated areas.

These results indicate that Bacillus species can effectively inhibit the growth of Colletotrichum gloeosporioides, highlighting their potential as biocontrol agents for mold on produce. This supports the hypothesis that Bacillus species can serve as eco-friendly alternatives to chemical preservatives.

3.5. Effects of Gaseous Metabolites on Spoilage Bacteria

As shown in Figures 2 and 3, The two-tier Petri dishes with Bacillus B1-B12 cultures showed varying degrees of mold inhibition. The B4 culture exhibited the greatest reduction in mold growth, followed by B6 and B8 cultures. Other Bacillus cultures showed significant mold growth.

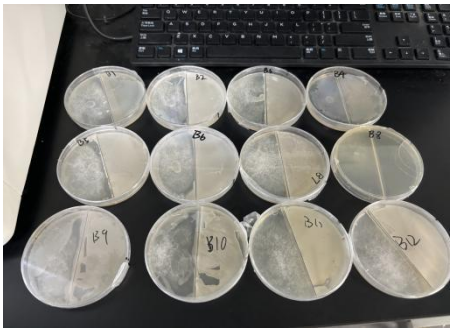

Figure 2 All dishes

The gaseous metabolites produced by Bacillus B4, B6, and B8 have a strong inhibitory effect on spoilage bacteria, suggesting that these Bacillus strains could be effective in extending the shelf life of produce. The B4 culture (Bacillus amyloliquefaciens) was particularly effective, indicating its potential for practical applications in food preservation.

Figure 3 B4, B6 and B8 dish

3.6. Effects of Bacillus B4 Gaseous Metabolites on Apples and Grapes

As shown in Figures 4, the experiment is ongoing. Preliminary observations suggest that Bacillus B4 may effectively inhibit mold growth on apples and grapes when exposed to its gaseous metabolites.

Figure 4 Inhibition of mold on fruit by gaseous metabolites

If the ongoing experiment confirms the preliminary results, Bacillus B4 could be a promising candidate for natural and safe preservation of fruits, reducing the reliance on chemical preservatives and decreasing food waste.

4. CONCLUSIONS

These well-designed experimental series not only validated the significant potential of the microbial community of Bacillus in the field of agricultural preservation, but also revealed the superior performance of a specific strain, Bacillus amyloliquequemile(B4), as a natural preservative. Through a series of scientifically rigorous methods, including bipartite plate assays and practical application simulation experiments, we have explored how the B4 strain can effectively extend the shelf life of agricultural products through a variety of complex and effective mechanisms to inhibit mold growth and control the growth of spoilage bacteria.

In terms of inhibiting mold growth, the B4 strain may effectively inhibit its growth and reproduction by producing a series of metabolites with broad-spectrum antimicrobial activity, such as antimicrobial peptides, bacteriocins or other secondary metabolites, that directly damage the cell wall of molds or interfere with their metabolic processes. In addition, B4 may further consolidate its inhibitory effect on molds through indirect mechanisms such as competing for nutrient resources, occupying ecological niches, and inducing plant defense responses.

More importantly, the results of this study highlight the great potential of Bacillus, especially Bacillus amylolique(B4), as a sustainable and environmentally friendly alternative to traditional chemical preservatives. Compared with chemical preservatives, natural biological preservatives such as B4 not only have higher safety and environmental protection, but also improve the quality and taste of agricultural products to a certain extent. In addition, its production process is relatively simple and the cost is controllable, which provides new ideas and ways for sustainable agricultural development and food safety assurance.

In summary, this study not only provides a solid theoretical basis and experimental basis for the application of Bacillus in the field of agricultural product preservation, but also opens up a broad prospect for the development of more efficient, safe and environmentally friendly natural preservatives in the future.

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