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Protocol
PCR-based Specific Detection of *Bacillus* in Liquid Organic Fertilizer

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Abstract

Rapid molecular PCR-based detection method for *Bacillus* species used in the production of Beyonic® liquid organic fertilizer was carried out based on nucleotide sequence data from the 16S rRNA gene. The method involved sequencing the 16S rRNA gene of several *Bacillus* species and identifying around 16-22 specific nucleotide bases from 5' and 3' ends in the *Bacillus* 16S rRNA gene sequences. One specific primer pair for *Bacillus* detection was determined as follow: 5' - CAT AAG ACT GGG ATA ACT CCG GG - 3' (forward) from positions of 85-107 bp, and 5’ - CCA GGC GGA GTG CTT AAT GC - 3’ (reverse) from positions of 836-854 bp. PCR assay and gel electrophoresis analysis showed that the primer pair was specific to the genus *Bacillus*.

Keywords: bacteria, detection, molecular marker, organic fertilizer, 16S rRNA

Materials and reagents

1. 24-h colony of *Bacillus* spp. From Beyonic® liquid organic fertilizer
2. Kit Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan)
3. Specific Primer Bac-F (5' - CAT AAG ACT GGG ATA ACT CCG GG - 3') (forward) (Hidayat 2016, unpublished data)
4. Specific Primer Bac-R (5’ - CCA GGC GGA GTG CTT AAT GC - 3’) (reverse) (Hidayat 2016, unpublished data)
5. Kit Go Taq® master mix (Promega, USA)
6. Ultra pure water DNA/RNAse free
7. Gel agarose 1% (molecular grade)
8. 50X TAE (Tris-EDTA) Buffer
9. Ethidium bromide (EtBr) 1µL/100 mL
10. DNA ladder 100 bp (Promega, USA)
11. Pipette tips (Eppendorf, Germany)

Equipment

1. 1 set of micropipettes (Eppendorf, Germany) (0.5-10 µL, 2-20 µL, 20-200 µL, and 200-1000 µL)
2. T100™ thermal cycler (Bio-Rad, USA)
3. Mupid-exU (MUPID Electrophoresis System, Japan)
4. Microwave

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5. Control Incubator Shaker (IKA® KS4000i)
6. Centrifuge (Tomy LC-200)
7. Gel Doc Printgraph UV transluminator (Bioinstrument, ATTO)

Procedure

A. Sample preparation and DNA extraction (Geneaid 2017)

1. Transfer bacterial cells (up to 1×10^9) to a 1.5 mL micro centrifuge tube.
2. Centrifuge for 1 minute at 14,000-16,000 × g, then discard the supernatant.
3. Transfer the required volume of Gram+ Buffer (200 µL/sample) to a 15 mL centrifuge tube.
4. Add Lysozyme (0.8 mg/200 µL) to Gram+ Buffer (in the 15 mL centrifuge tube), then vortex to completely dissolve the Lysozyme.
5. Transfer 200 µL of Gram+ Buffer (make sure Lysozyme was added) to the sample in the 1.5 mL micro centrifuge tube then re-suspend the pellet by vortex or pipette.
6. Incubate at 37ºC for 30 minutes. During incubation, invert the tube every 10 minutes.
7. Add 20 µL of Proteinase K (make sure ddH_2O was added), then mix by vortex.
8. Incubate at 60ºC for at least 10 minutes. During incubation, invert the tube every 3 minutes.
9. Add 200 µL of GB Buffer to the sample and mix by vortex for 10 seconds.
10. Incubate at 70ºC for at least 10 minutes to ensure the sample lysate is clear. During incubation, invert the tube every 3 minutes.
11. At this time, pre-heat the required Elution Buffer (200 µL per sample) to 70ºC (for step 24).
12. Add 200 µL of absolute ethanol to the sample lysate and mix immediately by shaking vigorously. If precipitate appears, break it up as much as possible with a pipette.
13. Place a GD Column in a 2 mL Collection Tube.
14. Transfer mixture (including any insoluble precipitate) to the GD Column then centrifuge at 14,000-16,000 × g for 2 minutes.
15. Discard the 2 mL Collection Tube containing the flow-through then place the GD Column in a new 2 mL Collection Tube.
16. Add 400 µL of W1 Buffer to the GD Column.
17. Centrifuge at 14,000-16,000 × g for 30 seconds, then discard the flow-through.
18. Place the GD Column back in the 2 mL Collection Tube.
19. Add 600 µL of Wash Buffer (make sure ethanol was added) to the GD Column.
20. Centrifuge at 14,000-16,000 × g for 30 seconds, then discard the flow-through.
21. Place the GD Column back in the 2 mL Collection Tube.
22. Centrifuge again for 3 minutes at 14,000-16,000 × g to dry the column matrix.
23. Transfer the dried GD Column to a clean 1.5 mL micro centrifuge tube.
24. Add 100 µL of pre-heated Elution Buffer 1, TE Buffer 2 or water into the center of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer 3 or water to be completely absorbed.
25. Centrifuge at 14,000-16,000 × g for 30 seconds to elute the purified DNA.

(**Note**: Standard elution volume is 100 µl. If less sample is to be used, reduce the elution volume (30-50 µL) to increase DNA concentration. If higher DNA yield is required, repeat the DNA elution step to increase DNA recovery and the total elution volume to approximately 200 µL)

B. PCR amplification (Promega 2012, with modification)

1. Add ice cubes into the PCR preparation icebox.

2. Thaw the GoTaq® Green Master Mix at room temperature.
3. Vortex the Master Mix.
4. Spin it briefly in a micro centrifuge to collect the material at the bottom of the tube.
5. Prepare one of the following reaction mixes on ice (25 µL reaction volume) (Table 1):

Table 1. Qualitative assay of IAA production

Component	Volume	Final Concentration
GoTaq® Green Master Mix, 2X	12.5 µL	1X
Forward primer, 10µM	0.25–2.5 µL	0.1–1.0 µM
Reverse primer, 10µM	0.25–2.5 µL	0.1–1.0 µM
DNA template	1–5 µL	< 250ng
Nuclease-Free Water	25 µL	NA

6. Centrifuge the reactions in a micro centrifuge for 5 seconds.
7. Place the reactions in a thermal cycler that has been preheated to 95°C.
8. Perform PCR using the following parameter: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing 59 °C for 30 s, and extension at 72°C for 1.5 min. After the 30 cycles, perform 10 min of final extension at 72°C, then cooling down the reaction at 4°C for 30 min.

C. Gel electrophoresis

C. 1. 1X TAE stock solution preparation

1. Pour 20 mL of 50X TAE solution into 1 L plastic bottle to make 1X TAE stock solution.
2. Bring final volume to 1 L by adding distilled water, then shake the solution gently.

C. 2. Gel casting

1. Add 250 ml of 1X TAE solution in 250 mL flask to make 1X TAE + 1% agarose.
2. Add 2.5 g of agarose.
3. Boil in a microwave for 5 min.
4. Pour hot solution into gel plate.
5. Attach comb to plate.
6. Wait for solution to solidify (10-20 min).
7. Remove the comb slowly and carefully.
8. Place plate with gel into gel box.

C. 3. Samples loading

1. Pour slowly 1X TAE stock solution into gel box until the buffer covers the gel.
2. Pipette 5 µL of DNA ladder into the leftmost well.
3. Pipette 5 µL of each PCR sample into the well.
4. Set the voltage of Mupid-exU system at 100V for 30 min.
5. Visualize the gel using Gel Doc Printgraph UV transluminator (Bioinstrument, ATTO) (Fig. 1)

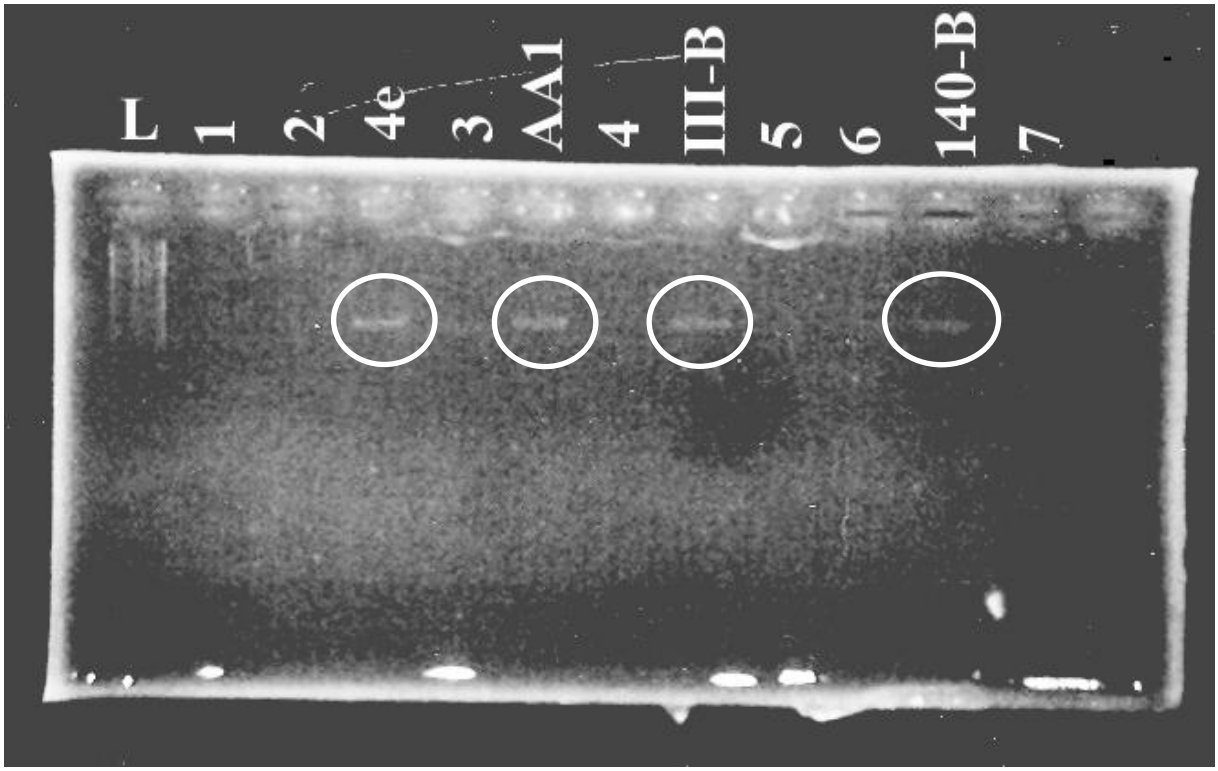

Figure 1. Gel electrophoresis of *Bacillus* detection using a primer pair of Bac-F and Bac-R. (L = DNA ladder; 1 = *Pseudomonas fluorescens*; 2 = *Ps. fluorescens;* 4e = *Bacillus* aff. *cereus*; 3 = *Ochrobactrum intermedium*; AA1 = *B.* aff. *cereus*; 4 = *Brevundimonas diminuta*; III-B = *B.* aff. *cereus*; 5 = *Microbacterium paraoxydans*; 6 = *Pseudomonas aeruginosa*; 140-B = *Bacillus* sp.; 7 = *Brevibacillus* sp.)

Conflict of Interest

The authors state no conflict of interest from this manuscript.

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