Wet Lab Protocol

SapSyn – iGEM 2025

Green Team

Confirmatory Tests for *Bacillus subtilis* 168:

1. Gram Staining

Aim: To classify *Bacillus subtilis* as Gram-positive or Gram-negative based on cell wall characteristics.

Procedure:

1. Sample Preparation:

Obtain a loopful of *Bacillus subtilis* from a broth culture and spread the same as a smear on a glass slide. Allow slide to dry.

2. Heat Fixing:

Upon drying, heat fix the cells by passing it through the flame of a Bunsen burner 2-3 times. Take care not to overheat the slide.

3. Application of Primary Stain:

Flood the heat-fixed sample with crystal violet stain and let it sit for 1 minute. Then rinse the slide gently with distilled water to remove excess stain.

4. Use of Mordant:

Gram's iodine acts as a mordant; add to smear and ensure thorough application. Let it sit for a few minutes. Rinse the slide with distilled water once again.

5. Decolorization:

Add 95% ethanol dropwise for 10-20 seconds, or till runoff is clear. Rinse the slide immediately after with distilled water, to stop decolourization.

6. Counterstaining:

Flood the smear with safranin stain and let it sit for 30 seconds. Rinse the slide thereafter, with distilled water and air dry thoroughly.

7. Microscopy:

Once dry, add a drop of immersion oil to the smear and observe under the oil immersion lens (100x) of a microscope.

Bacillus subtilis will appear Gram-positive (purple appearance) and rod-shaped.

2. Hot spore staining:

AIM: To visualize and identify the presence of endospores in *Bacillus subtilis* using heat and selective stains.

Procedure:

1. Sample Preparation:

Prepare a thin smear of *Bacillus subtilis* from a broth culture on a clean glass slide.
Air dry thoroughly thereafter.

2. Heat Fixing:

Heat fix the smear by passing it through the flame of a Bunsen burner 2-3 times, smear-side up.

3. Primary Staining:

Place the slide over a steaming water bath/heat source. Flood the smear with malachite green stain and allow it to steam for 5 minutes. Continuously add malachite green to keep the smear moist during the process.

4. Rinsing:

After 5 minutes, remove the slide and let it cool for 1-2 minutes. Rinse the slide gently with distilled water to remove excess stain.

5. Counterstaining:

Flood the smear with safranin and let it sit for 30 seconds. Rinse the slide gently with distilled water and then air dry thoroughly.

6. Microscopy:

Examine the slide under a microscope using an oil immersion lens (100x). Endospores will be visible and appear green, while vegetative cells will appear red.

3. Indole Test

AIM: To differentiate members of the Enterobacteria family.

Procedure:

1. Sample Preparation:

Pipette out a drop of *Bacillus subtilis* broth culture onto clean filter paper.

2. Application of Kovac's Reagent:

Add 1-2 drops of Kovac's reagent directly onto the broth region on the filter paper.

3. Observation of Colour:

A red/pink colour indicates a positive test. No colour change/yellow colour indicates a negative indole test.

4.Methyl Red (MR) Test

AIM: to determine whether *Bacillus subtilis* ferments glucose to produce stable acidic products.

Procedure:

1. Inoculation:

Inoculate MR-VP broth with a loopful of *Bacillus subtilis* from a fresh culture.

Incubate the same at 32°C for 48 hours.

2. Addition of Methyl Red:

Upon incubation, transfer 1 mL of the culture into a separate sterile test tube, and to it add 5 drops of Methyl Red indicator to the test tube.

3. Observation of Colour:

A red colour indicates a positive MR test, indicating the production of stable acidic end products from glucose fermentation.
A yellow colour or no colour change indicates a negative MR test.

5. Voges-Proskauer (VP) Test

AIM: to determine whether *Bacillus subtilis* ferments glucose to produce neutral end products.

Procedure:

1. Inoculation:

The MR broth can be reused for this VP test:

Transfer 1 mL of the *Bacillus subtilis* culture into a clean test tube.

2. Addition of Barritt's Reagents:

Add 15 drops of Barritt's A (alpha-naphthol) Reagent to the test tube.

Add 5 drops of Barritt's B (potassium hydroxide) Reagent to the same test

tube.

Gently shake to mix the reagents.

3. Observation:

Allow the tube to stand for 15-30 minutes.

A reddish colour indicates a positive VP test, demonstrating the production of acetoin as a fermentation product.

No colour change/copper colour indicates negative VP test.

6.Determination of Nitrate Reduction to Nitrite

AIM: To test for the ability of *Bacillus species* to reduce Nitrate (NO_3^-) to nitrite (NO_2^-).

Reagents: Nitrate reagent A is Sulfanilic acid, whereas reagent B is α-naphthylamine.

Procedure:

1. **Inoculation of culture:**

   Inoculate the bacterial suspension (from a fresh culture) with the nitrate broth.

2. **Incubation:**

   Incubate the tube at 35–37°C for 24–48 hours.

3. **Detection of Nitrite**:

   Upon incubation, add 5 drops of Reagent A, and 5 drops of Reagent B.

4. **Observation of colour change:**

   Look for the red colour change within 1-2 minutes. If the colour change is visible, it indicates a positive test for nitrate reduction to nitrite. This indicates that the bacterial species has produced nitrate reductase.

   If no red colour appears, add a small amount of zinc powder to the tube and mix. Appearance of red colour indicates a negative test; no colour change indicates a positive test.

Growth Curves

6) Six-hour Growth Curve

AIM: To analyse the growth characteristics of *Bacillus subtilis* under specific conditions.

Procedure:

1. **Preparation of Overnight Culture**:
   - Inoculate 5-10 mL of LB medium with *Bacillus subtilis* and incubate at 37°C with shaking (180-200 rpm) for 12 hours to reach the stationary phase.
2. **Subculturing into Fresh LB Broth in a Test Tube**:
   - Prepare a fresh test tube with LB broth (e.g., 5 mL).
   - Take 100 µL of the 12-hour culture and inoculate it into the test tube with fresh LB broth.
   - Grow the culture in the test tube until it reaches an OD600 of 0.7.
3. **Inoculation into Flask**:

- Prepare flasks containing 25 mL of fresh LB broth.
- Perform a 1:500 dilution by adding 50 µL of the OD 0.7 culture into the flask with 25 mL of LB broth.

4. **Incubation**:
    - Place the flasks in a shaking incubator at 37°C with shaking (200-250 rpm).
    - Take the initial OD600 reading (time 0 reading) after inoculating the flask, using a spectrophotometer.
5. **Measurement of OD600**:
    - At regular intervals (at every 30 minutes), withdraw 1 mL of the culture from the flask and measure the OD600.
    - Record each of the OD600 value.
6. **Plotting the Growth Curve**:
    - Continue taking OD600 readings until the culture reaches the stationary phase (typically around 12-18 hours).
    - Plot the OD600 values against time to generate the growth curve, showing the lag, log (exponential), stationary, and death phases.

7. 24-hour Growth Curve

1. Preparation of Overnight Culture:

• Inoculate 5-10 mL of LB medium with *Bacillus subtilis* and incubate at 37°C with shaking (180-200 rpm) for 12 hours to reach the stationary phase.

2. Subculturing into Fresh LB Broth (Test Tube):

- Prepare a fresh test tube with LB broth (e.g., 5 mL).
- Take 100 µL of the 12-hour culture and inoculate it into the test tube with fresh LB broth.
- Grow the culture in the test tube until it reaches an OD600 of 0.7.

3. Inoculation into Flask:

- Prepare flasks containing 25 mL of fresh LB broth.
- Perform a 1:500 dilution by adding 50 µL of the OD 0.7 culture into the flask with 25 mL of LB broth

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4. Incubation:

- Place the flasks in a shaking incubator at 37°C with shaking (200-250 rpm).
- Take the initial OD600 reading (time 0 reading) after inoculating the flask.

7. **Measurement of OD600**:
    - At regular intervals (every hour), withdraw 1 mL of the culture from the flask and measure the OD600 using a spectrophotometer.
    - Record the OD600 values at each time point.
    - 0 to 12-hour readings were noted from these 2 flasks with 2-hour intervals. Simultaneously, 12–24-hour readings were noted from the 2 flasks of the previous day.
8. **Plotting the Growth Curve**:
    - Plot the OD600 values against time to generate the growth curve, showing the lag, log (exponential), stationary, and death phases.

8. Transformation of Bacillus subtilis using CaCl2:

AIM: Usage of the Calcium Chloride transformation technique, to enhance competency of *Bacillus subtili*s cells

Procedure:

1. Preparation of Culture:

   Prepare an overnight culture of the bacteria in LB broth. Incubate at 37°C without shaking. 2 hours prior to beginning the procedure, use 1mL of the overnight culture to inoculate 100mL of fresh LB broth. This culture is grown with shaking at 37°C till it reaches roughly $5x10^7$ cells/ml. This corresponds to an OD650 for the culture.

2. Take a 5ml aliquot and transfer to sterile centrifuge tubes. Cool on ice.

3. Pellet the cells by spinning for 5 mins at 500g. Perform the centrifugation at 4°C.

4. Discard the supernatant and resuspend cells in 25mL of chilled 0.1M CaCl2. Leave on ice for 20 mins.

5. Perform centrifugation once again. Observation of a more diffuse pellet is indication of competent cells.

6. Resuspend the cells in 0.2mL of chilled 0.1M CaCl2.

7. Transfer the suspension to a sterile glass bottle/tube.

8. To the tube, add up to 0.1mg of DNA, made up in a standard storage buffer such as TE, to a volume of 100mL. Leave on ice for 30 mins.

9. Transfer to a 42°C water bath for 2 min and return to the ice.

10. Transfer the contents of the tube to 2mL of LB broth in a flask. Incubate with shaking at 37°C for 60-90 mins.

11. Plate 0.1mL aliquots of undiluted, 10^{-1} and 10^{-2} dilutions onto LB plates, to which antibiotics to be used for selection have been added.

12. Incubate overnight at 37°C.

9. Protocol for Detecting TCP2 production in *Bacillus subtilis* 168:

AIM: To detect and confirm the production of the TCP2 protein from a plasmid expressed gene, in *Bacillus subtilis* 168

Procedure:

1. Culture Preparation:

   Inoculate the *Bacillus subtilis* in LB broth. Incubate overnight with shaking.

   Subculture the bacteria into fresh LB broth and grow till mid-exponential phase is reached (determined through OD measurements).

2. Protein Extraction:

   Pellet 10mL of culture through centrifugation process

Suspend the pellet in 1mL of lysis buffer, with lysozyme.

Incubate on ice, then sonicate (approximately 3 pulses)

Centrifuge once again to separate soluble component. Collect both the supernatant and the pellet. Resuspend pellet in lysis buffer if necessary.

3. SDS-PAGE Analysis:

Prepare Agarose gel and SDS-PAGE apparatus.

Mix the protein extract with the loading dye and load sample onto the gel, along with the ladder/marker

Run the gel at 100-120V until dye front reaches the bottom

Stain the gel with Coomassie Brilliant blue (overnight staining might be necessary)

Destain and observe the bands

4. Observation:

A distinct band corresponding to the molecular weight of TCP2 is visible. Its absence is observed in the control.

References

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