MIT_MAHE
EXPERIMENTATION
HANDBOOK
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Contents

General protocols

Media preparation

for *Bacillus subtilis 168* and *Escherichia coli*

Aim: Preparation of a growth medium for bacteria.

Components	Weight for V=500mL
Tryptone	5g
NaCl	5g
Yeast extract	2.5g
Distilled water	Up to 500mL
Agar (for solid media)	7.5g

Antibiotic stock solution:

Components	Stock concentration	Working concentration
Ampicillin (for *E.coli* transformants)	100mg/mL	100µg/mL of media
Spectinomycin (for *B.subtilis* transformants)	50mg/mL	50µg/mL of media
Distilled water	Upto quantity required	-

Storage conditions: -20℃

Procedure for liquid media

1. Add 12.5g of LB broth powder to 500mL of ultra-pure water in an autoclavable flask.
2. Mix until the powder dissolves completely.
3. If solid media is required, add 7.5g of Agar powder to the broth.
4. Seal the flask with a cotton plug.
5. Autoclave at 121℃ and 15psi for a complete cycle.

6. Wait for the media to cool down and leave it at room temperature overnight to check if the media is sterile.
7. Add the antibiotic stocks in the required concentrations (as mentioned above) under the Laminar airflow chamber.
8. Transfer to test tubes and inoculate with required bacteria or store at 4℃ for prolonged usage.

Procedure for Petri plates with LB agar

1. Wait until the agar is warm (but not too hot) and add the appropriate antibiotic according to the working concentration mentioned above.
2. Swirl vigorously (Until dissolution) and immediately pour the plates - about 15 to 20mL of the mixture per plate.
3. Let the plates solidify and store at 4°C for several weeks.

Polymerase chain reaction

Aim: To amplify the quantity of a specific DNA sample.

Principle: PCR is a method of purification or cloning of DNA. It is based on the usage of DNA polymerase and uses the sample DNA to selectively replicate in large quantities.

Materials required: Water, buffer, Taq polymerase, dNTP mix, $MgCl_2$, forward primer, reverse primer, template, DMSO (optional)

Procedure:

1. Take all reagents and thaw on ice.
2. In a thin-walled 0.2 ml PCR tube, add reaction mix into 50 µml.
3. The reagents must be added in the following order:

No.	Components	Quantity
1	Water	To 50 µml
2	Buffer	1x
3	Taq Polymerase	0.05 units/µml
4	dNTP mix	200 µM
5	$MgCl_2$	0.1-0.5 mM
6	Forward primer	0.1-0.5 µM
7	Reverse primer	0.1-0.5 µM
8	Template	200 pg/µL
9	DMSO (optional)	1 to 10% w/v

4. Set the PCR machine to the following guidelines:

Step	Temp	Time	Cycles
Initial denaturation	94° C	5 min	
Denaturation	94° C	30 seconds	30-35
Primer annealing	Tm - 5°C	45 seconds	30-35
Extension	72°C	1 minute per kb	30-35
Final extension	72°C	5 minutes	

5. Analyze the PCR results with gel electrophoresis.

Gibson Assembly

Aim: To ligate and assemble one or more inserts into a vector to create a circular vector containing the inserts of interest.

Procedure:

1. Calculate the mass of each fragment of DNA by using the NanoDrop instrument with absorbance at 260 nm. Conversely, this can be estimated from agarose gel electrophoresis.
2. To calculate the number of pmols of each fragment required for the optimal assembly, which is based on the length and weight of the fragment, we can use the online tool NEBioCalculator or the following formula.

   pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

   50 ng of 5000 bp dsDNA is about 0.015 pmols.

   50 ng of 500 bp dsDNA is about 0.15 pmols.

3. Keep ice ready and handy to place tubes on ice.
4. Set the reaction tubes on ice

Assembly type	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* (x µL)	0.2–1 pmols* (x µL)	10 µL
2X Gibson Assembly Master Mix	10 µL	10 µL	10 µL
Milli-Q water	10-XµL	10-XµL	0
Total Volume	20 µL**	20 µL**	20 µL

* Optimal efficiency for cloning is 50-100 ng of a vector with 2-3-fold molar excess inserts. If the size is less than 200 bps, use 5 times more inserts. The total volume of unpurified PCR fragments in the Gibson Assembly reaction should not exceed 20%.
** Control reagents are provided for 5 experiments.
*** Additional Gibson Assembly Master Mix may be required if greater numbers of fragments are assembled.

5. The samples must now be incubated in a thermocycler for 15 minutes at 50°C (When 2 or 3 fragments are being assembled, 60 minutes when 4-6 fragments are being assembled). Post incubation, store samples on ice (or at –20°C) for subsequent transformation.

6. The competent cells can now be transformed with 2µL of the assembly reaction, following the transformation protocol.

Restriction digestion and ligation

Aim: To transfer DNA fragments from one plasmid to another.

Note: The following procedure is based on materials for NewEngland BioLabs

a) Restriction digestion:

Materials required: Restriction Endonucleases (NEB), Restriction Digest Buffer (e.g. CutSmartTM Buffer), Plasmid DNA

Procedure:

1. Prepare a 25 µl reaction:

Component	Amount
Restriction Endonuclease	1 µl for each used enzyme
CutSmartTM Buffer	3 µl
Nuclease-free water	Up to 25 µl

2. Incubation for 1h at 35°C (or at a different temperature, dependent on enzyme)
3. Heat inactivate restriction enzymes at 65°C to 80°C (dependent on restriction enzyme) for 20 min

b) Restriction ligation

Materials: T4 DNA Ligase Buffer (10x), T4 DNA Ligase, Vector DNA, Insert DNA, Nuclease-free water

Procedure:

It is important to know the sizes of the target vector and the insert. A molar ratio of vector: insert of 1:3 is used for cohesive end ligations generally. For ligations of DNA fragments with blunt ends, a higher molar ratio of 1:4 or 1:5 can be used.

1. Prepare a 20 µl reaction mixture(T4 DNA Ligase should be added last).

Component	Amount
T4 DNA Ligase Buffer	(10x) 2 µl
Vector DNA	Dependent on vector size, e.g. 50 ng Insert DNA Dependent on the insert size
Insert DNA	Dependent on the insert size
Nuclease free water	To 20 µl
T4 DNA Ligase	1 µl

2. Gently mix by pipetting up and down.
3. Incubate at 16°C overnight or for 1 hour at room temperature (for blunt ends: overnight at 16°C or 2h at room temperature).
4. Heat inactivate Ligase at 65°C for 20 min.
5. Chill on ice and possibly transform 1µl of the reaction to 50µl competent cells.

Calibration of spectrophotometer

Aim: To calibrate a spectrophotometer.

Procedure:

1. Warm the spectrophotometer by keeping it on for about 45 minutes.
2. Select a wavelength to calibrate.
3. A dedicated blank is needed for the machine depending on the type of filter that is used. However, if your machine's standard came with a blank, use it. If your machine did not come with a blank do not insert anything, i.e., leave the cuvette holder empty.
4. Take a blank reading.
5. Insert the NIST (National Institute of Standards and Technology) calibration standard, close cover, and record reading.
6. Compare data obtained to the data on the certificate of calibration.

Troubleshooting:

1. If your values from the blank and the NIST calibration standard do not match, check the uncertainty of the calibration standard used. The uncertainty values can be found on the Certificate of Calibration.
2. If the values still do not match, check the owner's manual for the photometric accuracy tolerance. Add the tolerance to the filters to get the new extended tolerance. (If the photometric accuracy tolerance is not listed in the manual, call the spectrophotometer manufacturer).
3. If the values still do not match, the problem either lies with the calibration standards of the spectrophotometer. Test the same out with a standard from another machine to verify.

Transformation

(Protocol provided by the IISER Tirupati team)

Stock solutions:

A. 10x MC media for 100 ml stock

Components	**Quantity (100 ml)**
Potassium phosphate dibasic (K_2HPO_4)	10.7 g
Potassium phosphate monobasic (KH_2PO_4)	5.2 g
Glucose (Dextrose)	20 g
Sodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$)	0.88 g
1000x Ferric ammonium citrate (2.2% stock)	1 ml
Casein Hydrolysate (Oxoid)	1 g
Potassium glutamate monohydrate	2..2 g
ddH_2O	Make up to 100 ml

1. Mix all contents in half volume of ddH_2O and then make up the volume.
2. Filter sterilize the entire media using screw cap filters (0.22 µm PVDF filters)
3. Store 10 ml aliquots in 15ml falcon tubes at **-20 ºC.** 10 ml of 10xMC is enough for ~ 100 transformations.

B. 1000x Ferric ammonium citrate (2.2% stock) for 10 ml stock

Components	**Quantity (10ml)**
Ferric ammonium citrate	0.22 g
ddH_2O	Make up to 10 ml

1. Filter sterilise using screw cap filters (0.22 µm PVDF filters)
2. Wrap in foil as it is light-sensitive.
3. Store at 4 ^{0}C.

C. 1 M $MgSO_4$ for 10 ml stock

Components	**Quantity (10ml)**
$MgSO_4$	1.2036 g
ddH_2O	Make up to 10 ml

1. Filter sterilise using screw cap filters (0.22 µm PVDF filters)
2. Store at 4 ^{0}C.

D. 10 mg/ml Tryptophan 10 ml stock

Components	**Quantity (10ml)**
L-Tryptophan	0.1 g
1 M KOH	1 ml
ddH_2O	Make up to 10 ml

1. Filter sterilise using screw cap filters (0.22 µm PVDF filters)
2. Wrap in foil as it is light-sensitive.

3. Store at 4 ^{0}C.

E. 1 M KOH for 10 ml stock

Components	**Quantity (10ml)**
KOH pellets	0.56 g
ddH_2O	Make up to 10 ml

1. Filter sterilise using screw cap filters (0.22 µm PVDF filters)
2. Wrap in foil as it is light-sensitive.
3. Add water slowly and keep mixing the falcon tube as it is an exothermic reaction.

Day 1: Streaking

1. Streak out small inoculum on media with an appropriate antibiotic to extract single colonies.

Day 2:

1. Pick a single fresh colony from the plate (not more than 12 hours old) to inoculate the following solution in a 15ml falcon tube.
   - ➔ 1 ml of 1x MC i.e. 100 µl of 10x MC
   - ➔ 3 µl of 1M $MgSO_4$
   - ➔ 4 µl of 10mg/ml Tryptophan
   - ➔ 900 µl of ddH_2O
2. Incubate on shaker/roller drum at 35 ^{0}C for 4 hrs
3. At 4 hrs, prepare 4 x 1.5 ml microcentrifuge tubes with 4 µl of linearized plasmid and 1x 1.5 ml microcentrifuge tubes for control (no DNA).
4. At 4.5 hrs, add 200 µl of cells from the 1x MC culture into all 5 microcentrifuge tubes.
5. Incubate all 5 microcentrifuge tubes for 2 hours at 35 ^{0}C.
6. Prewarm the plates with selective media.
7. Plate the entire 200 µl from all 5 microcentrifuge tubes on 5 different plates.
8. Incubate at 35 ^{0}C and check for transformants after 6-8 hrs.

Gel Electrophoresis

Aim: To visualize and analyze DNA fragments.

Procedure:

1. Take 50 X TAE buffer and dilute the necessary amount to make 1 X TAE.
2. To 50ml of 1 X TAE add 0.5 g of agarose to make 1% agarose gel.
3. Bring the mixture to a boil by using a microwave until the agarose is completely dissolved and a clear solution is obtained.
4. Place the electrophoresis combs set in a way so that it is about 2 cm away from the cathode.
5. When the temperature reaches 60 ^{0}C, pour the agarose solution into the central part of the tank carefully to avoid air bubbles. The thickness should not exceed 0.9 cm and ideally should be around 0.5-0.9 cm. Do not disturb the gel. Keep at room temperature to solidify.
6. Pour the prepared buffer into the gel tank till the level stands at 0.5-0.8 cm above the surface.
7. Lift the combs carefully making sure the walls stay intact.
8. Connect the electrophoresis apparatus to a power supply. Make sure that the red wire is connected to the anode and the black to the cathode. Set voltage to 50V.
9. Load samples into wells leaving the first well for the standard ladder. Add Standard DNS ladder to the first well.
10. Turn on the power supply.
11. Once the tracking dye reaches 3/4th of the gel from the well, switch off the power supply. This will take around an hour.
12. A 1X staining dye must be prepared by diluting 6 X dye with distilled water. About 50 ml of 1 X staining dye is needed for one experiment. Hence make 8ml of 6 X dye to 48ml with distilled water.
13. Transfer the gel carefully into the tray which has the 1X staining solution. Immerse the gel completely.
14. To ensure uniform staining, shake the tray intermittently every 10-15 minutes or place the tray on a rocker for about an hour.
15. Pour out the staining dye after the set time into a container as the dye can be reused. The gel must be de-stained by washing under tap water. The DNA should be visible as a dark band against a light background.

Delivery methods

Foliar spray

(note: the bacteria must be transformed with the integrative plasmid consisting of spectinomycin resistance for detection)

Aim: To prepare a foliar spray for application on crops with a suspended microbial inoculum.

Principle: The foliar spray technique involves using a misting apparatus to apply a suspended solution of any kind upon the foliage, shoots, and branches of the plant. The technique has been documented by Sethuraman and Balasubramanian (2010) to be effective in terms of uptake and colonization of selected microbes (bacteria, fungi, etc.) in their use as biopesticides and plant-growth-promoting rhizobacteria. Specifically, the method is known to be effective in terms of reported (and required) colony-forming units (CFU/mL) for *Bacillus sp., Streptococcus sp.* and multiple other fungal specimens used to treat plant diseases and insect infestations. We discuss the protocol for strains of *Bacillus subtilis* in the subsequent procedure.

The technique consists of 3 essential steps:

a. Preparation of microbial culture in the appropriate nutrient medium

b. Suspension of colonies in the appropriate inoculum

c. Preparation of spraying apparatus

Procedure:

Preparation of microbial culture in the appropriate nutrient medium:

1. Prepare 5mL of nutrient broth in the test tube with the selected species of bacteria (*B.subtilis*) after following appropriate protocol involving sterile conditions as necessary, and incubate the same for growth over a course of 24 hours at 25°C on a shaker at approximately 200rpm.
2. Pipette out 0.1 mL over nutrient agar medium and prepare and follow spread plate culture protocol, assigning one plate for each type of strain.

Suspension of colonies in the appropriate inoculum

3. Select specific strain for further preparation and resuspend in nutrient broth, which is subjected to centrifugation at 5000rpm for 10 mins to pelletize the cell suspension.
4. Following this, cells are now dissolved and suspended in sterile phosphate-buffered saline (PBS) solution (0.2g/L, NaCl, 1.44g/L NaH_2PO_4, and 0.24g/L KH_2PO_4). Thoroughly mix suspension to ensure even suspension of all cells and particles within the inoculum.
5. Using growth data of the bacterial strain at OD_{600}), the number of cells in the suspension is measured and appropriated at ____ CFU/mL(Depending on the expression of CRY toxin and LC). Dilute the solution using PBS to obtain the desired concentration of bacterial suspension.

Preparation of spraying apparatus

6. Obtain appropriate misting apparatus and add inoculum obtained to the same for approximately 15-25mL per plant, after having calculated appropriate volume.
7. To enhance the wetting characteristics of the solution, add 0.2% of Silwet L-77 (surfactant co-polymer) and shake to thoroughly allow the bacterial suspension to coat with the same.
8. Apply on leaves, shoots, and branches (if any) of the selected crop and wait for 15 days. Following this, a sample plant is rinsed under tap water.
9. Individual 2 cm stem and root segments are cut from sample plant with a sterile scalpel and placed for 5 minutes into sterile test tubes with 10 mL of aqueous H_2O_2 solution (20% v/v) solution amended with 0.05% (v/v) Triton X-100.
10. Surface-disinfected pieces are aseptically transferred through three washes of 30 mL of sterile water and triturated with 10 ml of 0.02 M-phosphate buffer pH 7.0 using autoclaved mortars and pestles.
11. 1 mL of the resulting suspensions is then spread-plated with six replications. Bacterial populations are estimated by counting the colonies after incubation of 48 hours at 28◦C. Observe and tabulate the same.

Expected results and discussion:

Foliar spray technique was successfully performed at desired CFU/mL for endophytic colonization of *Bacillus sp.* at varying concentrations based on point of application and migration of suspension by vascular uptake.

Seed treatment + Soil drench technique

Aim: To prepare crop seeds for transplantation from nursery using microbial inoculum treatment and soil drench technique.

Principle: For plants that have a predominantly large rhizosphere, the foliar spray technique would not suffice for successful and sufficient colonization. In this case, a pre-germination procedure can be followed that allows for sufficient colonization and uptake of the microbial suspension in the inoculum. Seed treatment along with soil drench directly allows for the mutual growth of plants and bacterial colonies over the course of germination, thereby making it a suitable technique for endophyte colonization in large-scale nurseries and farms.

The technique consists of 3 essential steps:

a. Preparation of microbial culture in the appropriate nutrient medium

b. Suspension of colonies in the appropriate inoculum

c. Seed disinfection

d. Treatment of seeds and soil with inoculum

Procedure:

Preparation of microbial culture in the appropriate nutrient medium:

1. Prepare 5mL of nutrient broth in the test tube with the selected species of bacteria after following appropriate protocol involving sterile conditions as necessary, and incubate the same for growth over a course of 24 hours at 25°C on a shaker at approximately 200rpm.
2. Pipette out 0.1 mL over nutrient agar medium and prepare and follow spread plate culture protocol, assigning one plate for each type of strain.

Suspension of colonies in the appropriate inoculum:

3. Select specific strain for further preparation and resuspend in nutrient broth, which is subjected to centrifugation at 5000rpm for 10 mins to pelletize the cell suspension.

4. Following this, cells are now dissolved and suspended in sterile phosphate-buffered saline (PBS) solution (0.2g/L, NaCl, 1.44g/L NaH_2PO_4, and 0.24g/L KH_2PO_4). Thoroughly mix suspension to ensure even suspension of all cells and particles within the inoculum.
5. Using growth data of the bacterial strain at OD_{600}), the number of cells in the suspension is measured and appropriated at ____ CFU/mL(Depending on the expression of CRY toxin and LC). Dilute the solution using PBS to obtain the desired concentration of bacterial suspension.

Seed disinfection:

6. Disinfect the surface of rice seeds by immersion in 95% ethanol for 1 min, and transfer to 3% H_2O_2 for 4 min. Rinse three times in sterile distilled water. Dry the seeds in a flow cabinet for 12 h.
7. After seed disinfection, sterility control is performed by plating 100 seeds per lot on agar and incubating at 25°C for 48 h. If no microbial growth is detected on the plates, the seed samples are considered surface disinfected and used in the subsequent stages of the experiment.

Treatment of seeds and soil with inoculum:

8. Submerge seeds in a tray filled with the inoculum preparation for 2 h before planting.
9. Plant six seeds per pot within the nursery and drench the surrounding soil with 25mL of inoculum 10 days after planting of seeds. Sterilized vermiculite can be added in order to increase nutrient and water retention.
10. When seedlings have emerged 5 days after planting, thin down to four seedlings and transplant seeds to farm from the nursery. Drench soil again with inoculum 10 days after transplantation.
11. 15 days after the planting of seeds, the sample plant is taken and rinsed under tap water to remove adhered vermiculite.
12. Individual 2 cm stem and root segments are cut from sample plant with a sterile scalpel and placed for 5 minutes into sterile test tubes with 10 mL of aqueous H_2O_2 solution (20% v/v) solution amended with 0.05% (v/v) Triton X-100.

13. Surface-disinfected pieces are aseptically transferred through three washes of 30 mL of sterile water and triturated with 10 mL of 0.02 M-phosphate buffer pH 7.0 using autoclaved mortars and pestles.
14. 1 mL of the resulting suspensions is then spread-plated with six replications. Bacterial populations are estimated by hand-counting the colonies after incubation of 48 hours at 28°C. Observe and tabulate the same.

Expected results and conclusion:
Seed treatment and soil drench were successfully performed for the target crop to achieve desired CFU/mL within the rhizosphere, and was tested for colonization of the shoot and other parts of the plant.

Pruned root dip

Aim: To prepare crops for transplantation from nursery using pruned root dip technique.

Principle: Much like the soil drench and seed treatment technique, the pruned root dip method works best with plants that are known to have an extensive root system. The method is a post-germination procedure that takes advantage of the natural vascularity of the plant/crop and is known to establish colonies of the target endophyte well beyond the rhizosphere. Apart from the obvious labor-intensiveness involved in pruning the roots, the method has been reported to be the most effective in terms of reported colonies beyond the point of application i.e., the rhizosphere.

Procedure:

Preparation of microbial culture in the appropriate nutrient medium:

1. Prepare 5mL of nutrient broth in the test tube with the selected species of bacteria after following appropriate protocol involving sterile conditions as necessary, and incubate the same for growth over a course of 24 hours at 25°C on a shaker at approximately 200rpm.
2. Pipette out 0.1 mL over nutrient agar medium and prepare and follow spread plate culture protocol, assigning one plate for each type of strain.

Suspension of colonies in the appropriate inoculum

3. Select specific strain for further preparation and resuspend in nutrient broth, which is subjected to centrifugation at 5000rpm for 10 mins to pelletize the cell suspension.
4. Following this, cells are now dissolved and suspended in sterile phosphate-buffered saline (PBS) solution (0.2g/L, NaCl, 1.44g/L NaH_2PO_4, and 0.24g/L KH_2PO_4). Thoroughly mix suspension to ensure even suspension of all cells and particles within the inoculum.
5. Using growth data of the bacterial strain at OD_{600}), the number of cells in the suspension is measured and appropriated at ____ CFU/mL(Depending on the expression of CRY toxin and LC). Dilute the solution using PBS to obtain the desired concentration of bacterial suspension.

Root pruning

6. Obtain seedlings or crops grown in nurseries and uproot plants when transplanting from the nursery. Wash the roots under sterile water for 1 minute.
7. Using a scissor or an appropriate shearing tool, mechanically remove approximately 50% of the root mass.
8. Prior to transplantation, submerge and allow the cut roots of the plant to sit in the inoculum for 2 minutes to allow for suspension uptake through the plant's xylem system.
9. 15 days after the planting of seeds, the sample plant is taken and rinsed under tap water to remove adhered vermiculite.
10. Individual 2 cm stem and root segments are cut from sample plant with a sterile scalpel and placed for 5 minutes into sterile test tubes with 10 mL of aqueous H_2O_2 solution (20% v/v) solution amended with 0.05% (v/v) Triton X-100.
11. Surface-disinfected pieces are aseptically transferred through three washes of 30 mL of sterile water and triturated with 10 mL of 0.02 M-phosphate buffer pH 7.0 using autoclaved mortars and pestles.
12. 1 mL of the resulting suspensions is then spread-plated with six replications. Bacterial populations are estimated by hand-counting the colonies after incubation of 48 hours at 28°C. Observe and tabulate the same.

Expected results and conclusion:

Pruned root dip technique was successfully performed for target crop to achieve desired CFU/mL within the rhizosphere, and was tested for colonization of the shoot and other parts of the plant.

Testing and comparing the efficacy of each method

Aim: To test the efficacy of different delivery methods in stem and roots.

Apparatus/Chemicals:

- Sterile Scalpel
- Autoclaved test-tubes
- LAF
- Autoclaved mortar and pestle
- Rice samples
- Distilled Water
- H_2O_2 solution (20% v/v)
- Triton X-100 solution (0.05% v/v)
- 7 pH M-phosphate buffer (0.2M)
- LB agar medium containing Spectinomycin

Procedure:

1. 2cm sections of root and stem must be taken from the plants grown with different delivery methods. The sections must be washed with water to remove any external particulate matter.
2. For seed treatment + soil drench, the sampling should be carried out 15 days after planting, and for foliar spray and root prune, the sampling should be carried out 15 days after inoculation.
3. The segments are placed into test tubes containing 10ml H_2O_2 with Triton X-100 solution for five minutes.
4. These disinfected pieces are then transferred to mortar and pestle using 30ml distilled water and triturated with 10ml M-phosphate buffer.
5. 1ml of the resulting suspension is taken and spread plated in LB agar medium containing spectinomycin, with multiple replications for each method of delivery.
6. CFUs were then counted after inoculation for 48 hours at 27°C.

ACC Deaminase

Qualitative test for ACC Deaminase activity

Aim: To screen the ACC deaminase activity of *Bacillus subtilis*

Modified DF minimal salts medium:

Components	Weight for 1L
Glucose	2.0g
Gluconic acid	2.0g
Citric acid	2.0g
KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
$MgSO_4.7H_2O$	0.2 g
Micronutrient solution	10mL
Distilled water	To bring up to 1000mL

Add 3mM of ACC as the only Nitrogen source

Micronutrient solution:

Components	Weight for 100mL
$CaCl_2$	20mg
$FeSO_4.7H_2O$	20mg
$ZnSO_4.7H_2O$	2mg
Na_2MoO_4	1mg
H_3BO_3	1.5mg
KI	1mg
NaBr	1mg
$NiSO_4$	0.2mg
Distilled water	100mL

Additionally:

- 5ml TSB (tryptic-soy broth) medium
- 0.1M Tris-HCl
- $(NH_4)_2SO_4$ (0.2%w/v)
- Petri plates
- Centrifuge

Procedure:

1. Cultivate the Bacillus subtilis 168 in 5ml of TSB medium. Incubate for 24 hours at 28°C in an incubator shaker (120 rpm).
2. Harvest the bacterial cells by centrifugation at 3000 rpm for 5 min.
3. Wash the pellet twice with sterile 0.1M Tris-HCl and resuspend it in 1ml of 0.1M Tris-HCl.
4. Inoculate the bacteria on Petri plates containing the modified DF minimal salt medium which is supplemented with 3mM ACC.
5. Use plates containing only DF minimal salts with ACC as negative control and plates containing DF minimal salts with $(NH_4)_2SO_4$ as a positive control.
6. Incubate all three plates for 72 hours at 28°C.
7. Compare the growth on the ACC supplemented plates with the other two plates.
8. The growth on the ACC supplemented plates indicates that the bacteria used ACC as its sole nitrogen source.

Expected Observation:

All the bacterial cells should be screened for ACC deaminase based on the enrichment method, where ACC is used as the only nitrogen source. The bacterial isolates will grow efficiently on the DF minimal salts medium with the ones supplemented with ACC or (NH)2SO4 compared to the plate with DF minimal salts medium without a nitrogen source.

Quantitative test for ACC Deaminase

Aim: To evaluate ACC deaminase activity of the Bacillus subtilis cells

Procedure:

1. Cultivate the bacterial isolates in 5ml of TSB medium. Incubate for 24 hours at 28°C in an incubator shaker (120 rpm) until it has reached the late-log phase.
2. Centrifuge the culture at maximum speed for 5 min. Discard the supernatant.
3. Wash the pellet twice with sterile 0.1M Tris-HCl (pH 7.5) and suspend it in DF minimal salt medium with 3mM ACC.
4. Incubate this culture for another 36-72 hours at 28 °C in a shaker.
5. Harvest the cells by centrifugation for 5 minutes at 5000 rpm.
6. Wash the pellet twice with 0.1M of Tris-HCl and resuspend it in 200µL of 0.1M Tris-HCl.
7. Add 5% (v/v) toluene and vortex for 30 seconds to labilize the cells.
8. Incubate 50µL of this labilized cell culture with 5µL of 0.3M ACC in an Eppendorf tube for 30 minutes at 28 °C.
9. For the negative control, prepare 50µL of the labilized cell suspension without ACC and 0.1M Tris-HCl (pH 8.5) with 5µL of 0.3M ACC will be the blank.
10. Mix all three samples with 500µL of 0.56N HCl thoroughly by vortexing.
11. Centrifugate them at 12000 rpm for 5 minutes and remove the pellet of cell debris.
12. Transfer 500µL of the supernatant of each sample to a test tube and mix with 400µL of 0.56N HCl and 150µL of DNF solution.
13. Incubate these test tubes for 30 minutes at 28 °C.
14. Add 1ml of 2N NaOH to the samples before measuring their absorbance at 540 nm.
15. Compare the α–ketobutyrate values in each sample with a standard curve of absorbance vs α–ketobutyrate.

Expected Observation:

The ACC deaminase activity is evaluated by quantifying the amount of α–ketobutyrate produced, by the deamination process of ACC by the ACC deaminase enzyme.
After plotting a standard curve between absorbance vs α–ketobutyrate, compare the absorbance values for each sample with the standard curve. This will give the values of α–ketobutyrate which depicts the activity of ACC deaminase on the ACC present.

Control mechanism

Promoter expression

Aim: To check the level of transcription of SigW promoter at different pH conditions.

Procedure:

Bacteria to be transformed with
Circuit: **SigW promoter** – **RBS** – **GFP** - **Double Terminator**
Control: Constitutive promoter – RBS – GFP - Double Terminator

1. Prepare the LB medium and transfer the media to 2 sets of 6 test tubes (one for the experiment circuit and the other for the control circuit) and label them to indicate their pH (5,7,8,9,10,11)
2. Adjust the pH of the test tube according to the label by adding 1N NaOH to raise the pH and 1N HCl to reduce the pH.
3. Using a sterile loop inoculate the transformed *Bacillus subtilis 168* into the test tubes containing LB broth medium and incubate overnight at 35℃.
4. Use the incubated broth to inoculate all the prepared test tubes.
5. Check the GFP intensities using a fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour. Plot graphs to analyze and compare the graphs of the two circuits.

Expected observation:
The transcription of the SigW promoter should be much more at high pH conditions, compared to the constitutive promoter. Additionally, the transcription of the SigW promoter at physiological pH conditions should be much lower than constitutive transcription.

Expected result:

The level of transcription of SigW at physiological pH in which the rice is expected to grow is very low. Therefore there will be no CRY protein present in the plant tissue. The transcription in high pH conditions, like in the gut of the stem borer, is high. Thus transcription will take place only in the guts of the stem borers.

Cry toxin

Procedure:

1. Grow Cry1Ab transformed Bacillus subtilis with 50 µg/ml Spectinomycin for 48 hours at 35°C.
2. Harvest cells (10,000 Xg, 10 min), and resuspend the pellet in 50 ml of buffer (50 mM Tris-HC1,50 mM EDTA, 15% sucrose, pH 8.0, 2 mg/ml lysozyme).
3. Sonicate the cell suspension on ice (3 x 3 min 30 s) and centrifuge (15,000 x g, 15 min).
4. Wash the pellet with ice-cold 2% Triton X-100, 0.5 M NaCl three times, wash five times with 0.5 M NaCl, and two times with distilled water.
5. Briefly solubilize the crystal protein in a 50 mM carbonate buffer, pH 9.5, containing 10 mM DTT for 2 hours at 35°C.
6. Measure the protein concentration of the protoxin using the Coomassie Protein Determination Kit (Pierce).
7. Treat Protoxins with trypsin 1:20 (by mass) for 2 hours at 35°C.
8. Pass the activated toxins through a size exclusion Superdex-200 column (Akta Explorer HPLC, Pharmacia) to eliminate small peptides, trypsin, and DTT.
9. Concentrate and electrophorese peak fractions in denaturing 12% polyacrylamide gel.
10. Store the fractions containing the 65-kDa activated toxin at 4°C until use.

For bioassays for LC50 estimates:

1. Collect Yellow Stem Borer adults from rice fields using pheromone traps and bring them to a greenhouse to oviposit on rice plants.
2. Sterilize and place the egg masses in scintillation vials containing suitable artificial diet.
3. Mix the trypsin-activated Cry1Ab toxins into the liquid insect diet at a temperature below 60°C.

4. Prepare eight replicate vials containing approximately 5 ml of diet for each toxin concentration, plus a control containing toxin-free diet. Use nine toxin concentrations.
5. Approximately 24 h after egg hatch, collect the larvae and introduce them into a vial with an artificial diet containing toxin. Use six Yellow Stem borer larvae per vial.
6. Record Larval mortality after 4 days of incubation at 27°C.
7. Calculate LC50 estimates using POLO-PC (LeOra Software, 1987).

(Note: this data can be utilised with the data obtained from the Promoter expression data to calculate the concentration of bacteria to be delivered to the crop)

Growth curve

(a similar protocol is to be followed for each growth curve)

Common protocol

Preparation of the inoculum:

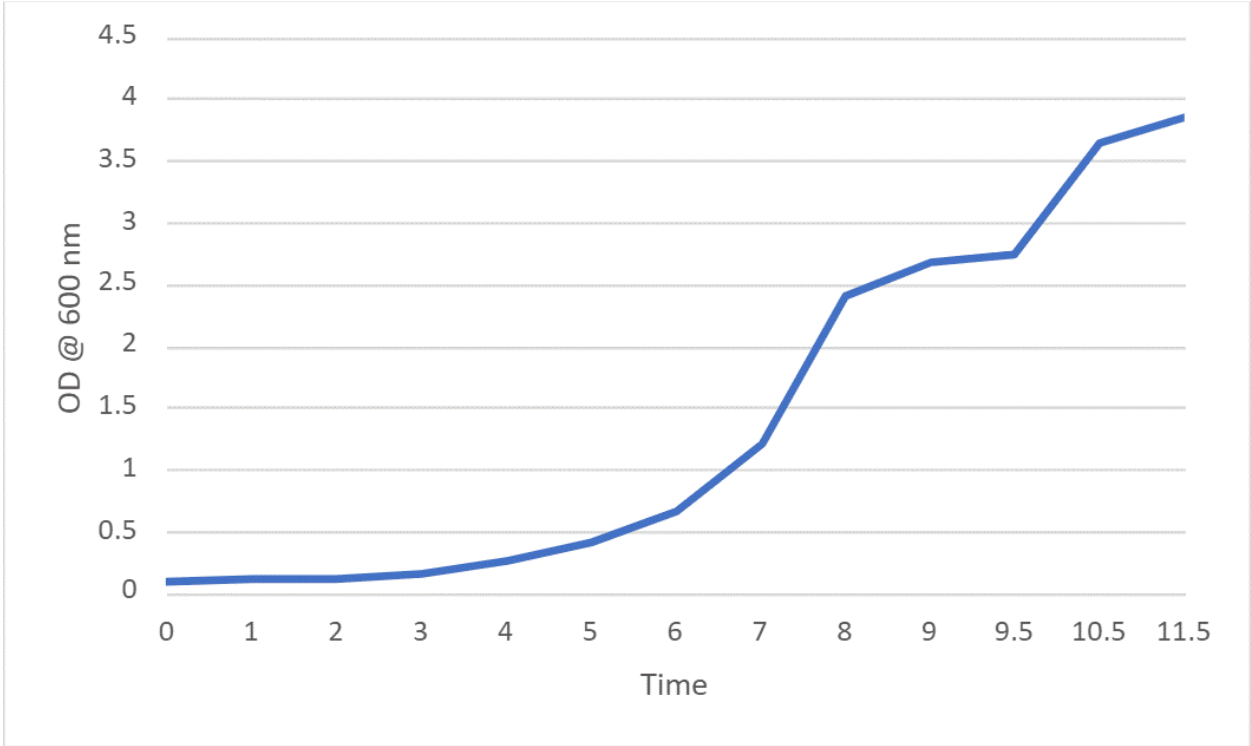
1. Prepare and sterilize 10mL of LB broth in a 50mL conical flask.
2. In the LAF, under sterile conditions, inoculate the flask with one colony of *Bacillus subtilis*.
3. Incubate the flask in a shaker incubator with 170 RPM and 35℃.

Procedure:

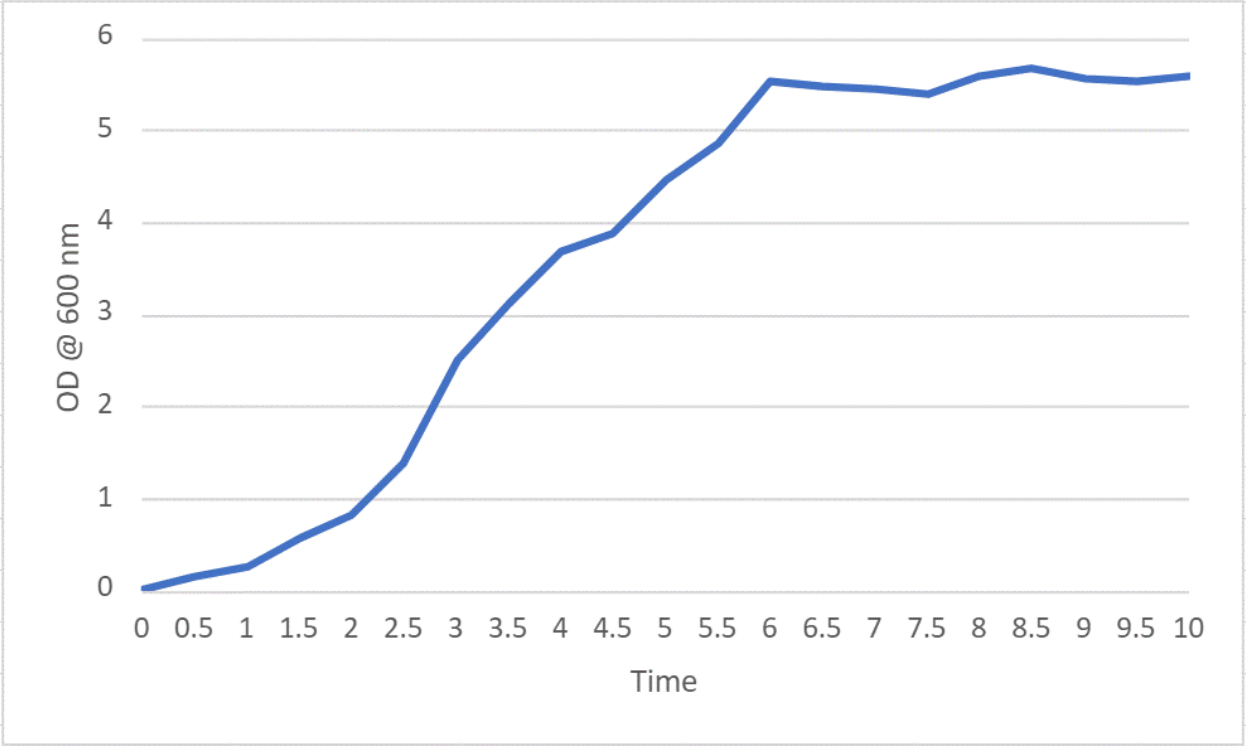
1. Inoculate 250mL of sterilized LB broth (of the appropriate pH) with 2.5mL of inoculum.
2. Utilize 2mL of the broth for the zero-hour reading (using a spectrometer).
3. Take the reading for the inoculum as well (for reference).
4. After the reading is taken, incubate the samples at the appropriate temperature and 170 RPM.
5. Hourly readings must be taken for the sample.
6. Plot the results.

(note: the pH was adjusted using 1 N HCl, 3M NaOH)

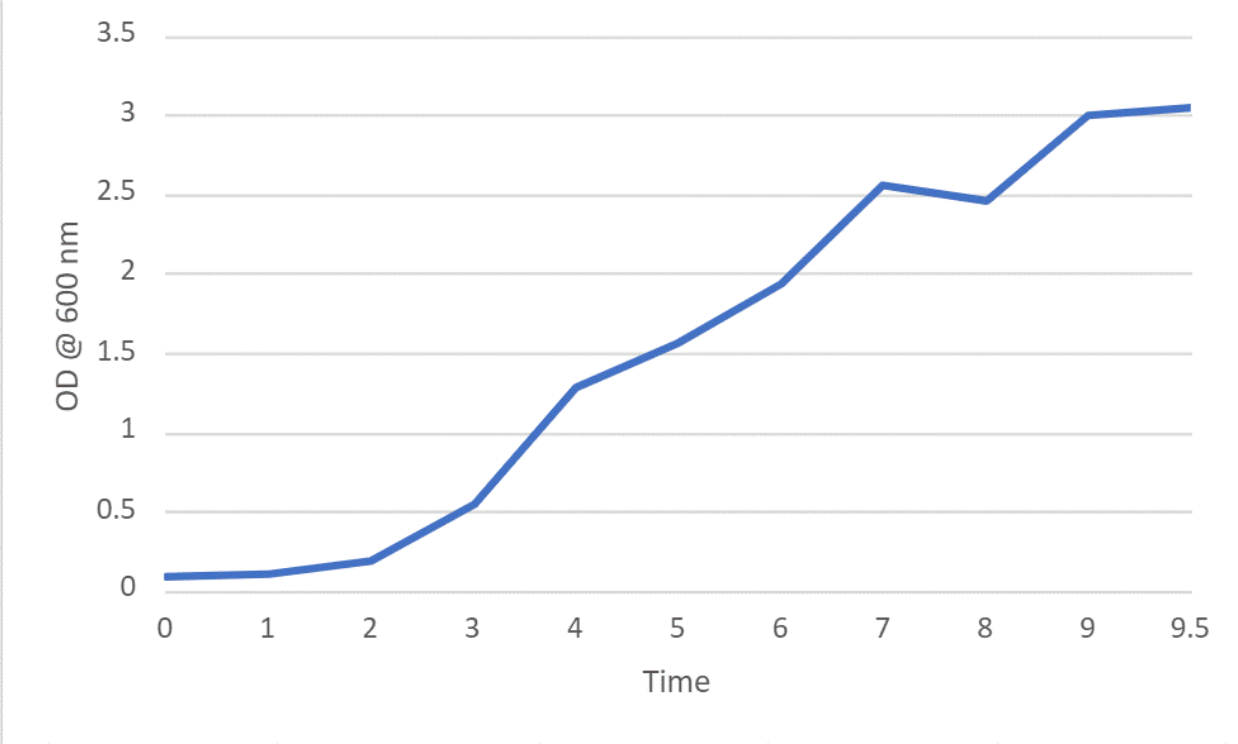
Results:



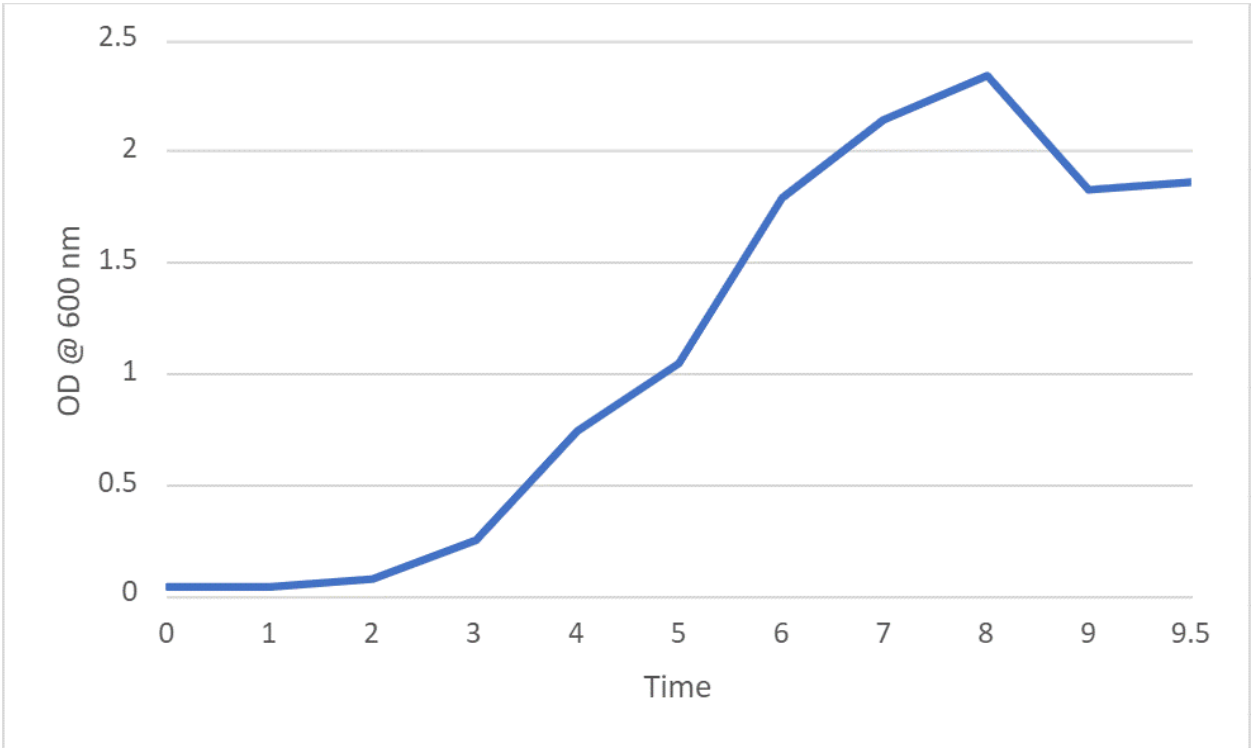
Growth Curve for 25°C,pH 7



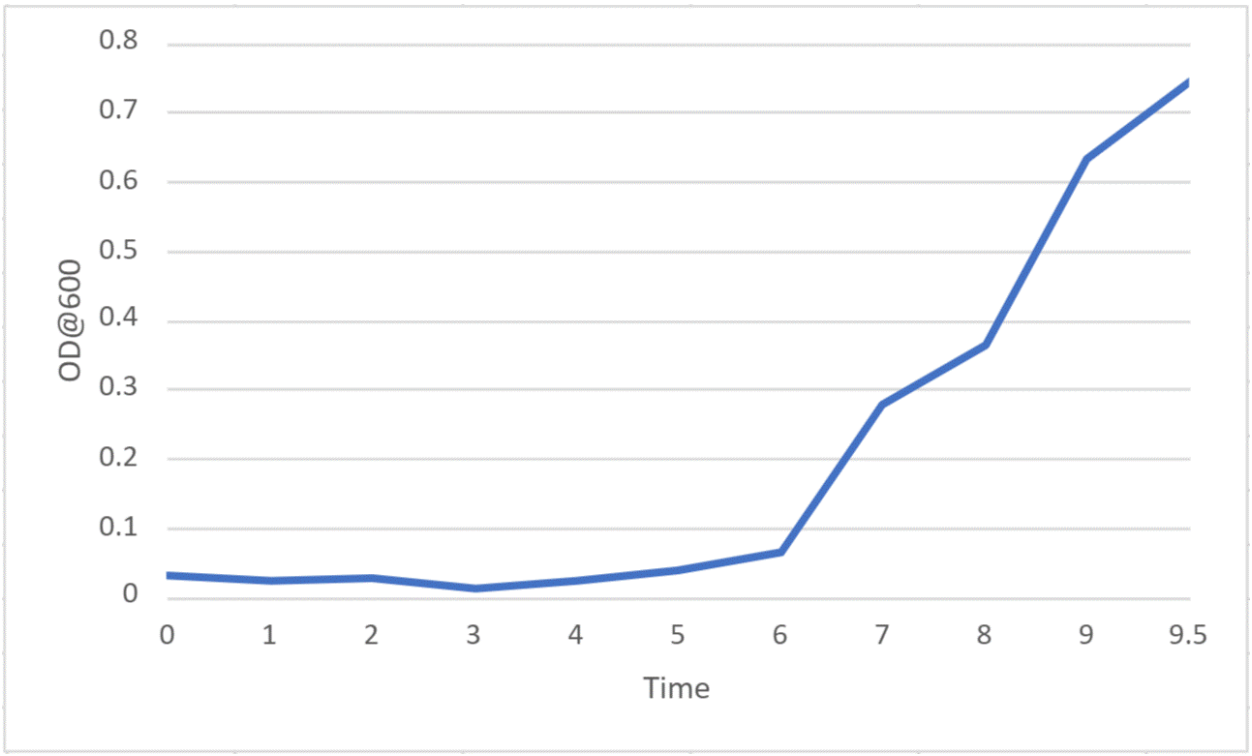
Growth Curve at 35°C, pH 7



Growth Curve at 45°C, pH 7



Growth Curve at pH 5, 35°C



Growth curve at pH 9, 35°C

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General Protocols

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Control Mechanism

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