Purifying WT Wheat Chitinase Protein

9/6/21

Aim: Sterilising wheat seed

Protocol:

- Seeds are taken in the 1.5mL eppendorf tube.
- 1 mL 70% ethanol is added to it and after some time ethanol is discarded.
- 20% bleach solution is added to it and after mixing it is discarded.
- Then distilled water is added and discarded 7 times.
- Seeds are stored at 4 °C for 2 days.

11/6/21

Aim: Grow the seeds on petri plate.

Protocol:

- Filter paper is placed in the two 60mm Petri dishes.
- Sterilized grains(10 seeds each) are put there and labeled.
- Enough water is given that the seeds should not float.
- It was covered with petri plate lid,
- Keep the plate at room temperature
- Wait for 7-10 days until germination.

18.06.21

Aim: Induce the chitinase secretion in grown wheat by spraying fungi spore suspension on it.

Protocol:

1) The mycelia and spores of fungi formed on the PDA medium were scraped using a sterile No. 21 blade and placed in a 50 mL sterile conical tube.
2) PBS (20mL) was added to the 50 mL tube and vortexed for 5 min to disperse the spores.
3) A spore suspension was obtained by filtering the PBS containing mycelia and spores twice through three layers of cheesecloth, .
5) Collect the filtrate in a spray bottle.
6) Spray the spore suspension on wheat grown seeds and label it as induced. Take few more seeds and label it as control.
7) Incubate seedlings in high humid condition for 12-24 hrs before RNA extraction

Day : 1

Day : 4

Day : 7

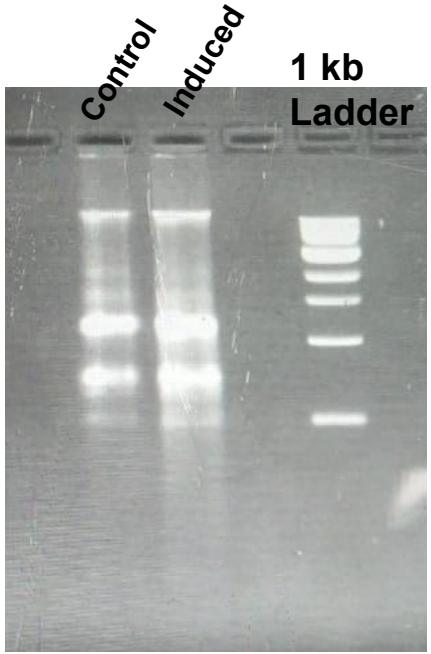
Aim: RNA extraction from the grown seeds

19.06.21

Protocol: TRIZOL Method

- Cell is harvested in a 1.5 ml **RNase free Eppendorf tube**, frozen and grounded in liquid nitrogen.
- Add 1mL of Trizol and mix by vortexing. Incubate at room temperature for 5-10 mins.
- Add 200ul of chloroform and incubate at R.T. for 5 min.
- Centrifuge at 12000**xg** rpm for 15 min at 4degree C
- Separate aqueous phase into new 1.5 ml **RNase free Eppendorf tube**
- Add 500ul isopropanol. Incubate at R.T for 10 min
- Centrifuge at 12000xg rpm at 4 degrees C for 10 min
- Remove supernatant and add 1mL **75% ethanol (diluted using RNase free water)**
- Vortex lightly to dilute the pellet.
- Centrifuge at 7500xg (8000 rpm) for 5 min at 4 degrees C
- Discard the supernatant and **dry the pellet** completely
- **Dissolve completely** in 50ul RNAse free water by continuous pipetting.
- Incubate at 55-60 degrees for 10 min
- Do DNase treatment* and then gel run.

NOTE- we can store RNA at room temperature (1-2 hrs) for gel run else store at -20 degrees



Nanodrop result:

#	ng/uL	A260/A280	A260/A30
Control	208.4	1.95	2.22
Induced	368.2	1.98	2.10

Result: Got the required bands

21.06.21

Aim: cDNA synthesis

Protocol:

- Prepare a buffer:

5x Buffer	4 ul
RT enzyme	1ul
oligoDT	1ul
Random 6mer	1ul
RNA	1 ug
RNase free water	Upto 20ul

- Vortex and Centrifuge ~ 5 seconds.
- Place in Thermocycler, away from outer edges, and run PCR.

General Settings:
a. 95°C for 3 min
b. Set this step to repeat 34X: 94°C for 40 sec 55°C for 30 sec 72°C for 40 sec
c. 72°C for 5 min d. 4°C ∞ hold

- Store products of PCR in freezer until needed.

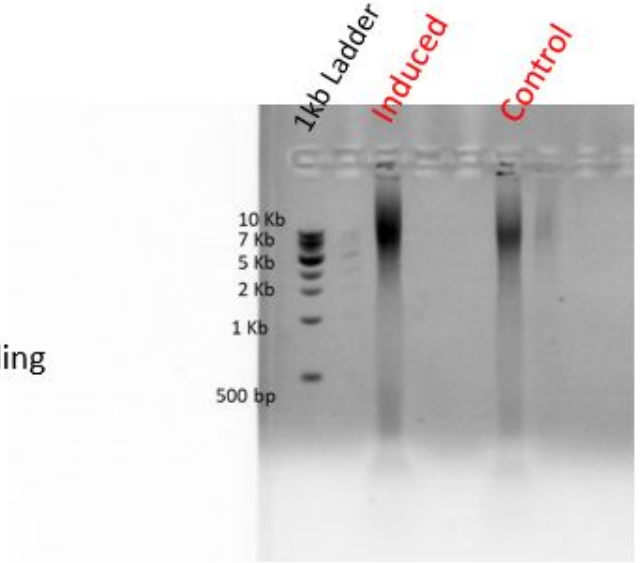
22/06/21

Aim: Trial PCR using Taq polymerase

Protocol:

- 5x GoTaq 4ul
- 10mM dNTP 0.5 ul
- 25mM MgCl2 1ul
- 10mM FP 0.5ul
- 10mM RP 0.5ul
- Taq Pol 1 ul
- Template 1 ul
- Water 11.5 ul
- Total → 20 ul

Tm= 50 °C
1:10 min →
Extension time
30 sec → Annealing time



Remark: Do gradient PCR
If that is also not successful then do PCR of genomic DNA and check whether primers are correct or not.
If primers are fine and then also do not get any band in PCR, synthesize new cDNA

23.06.21

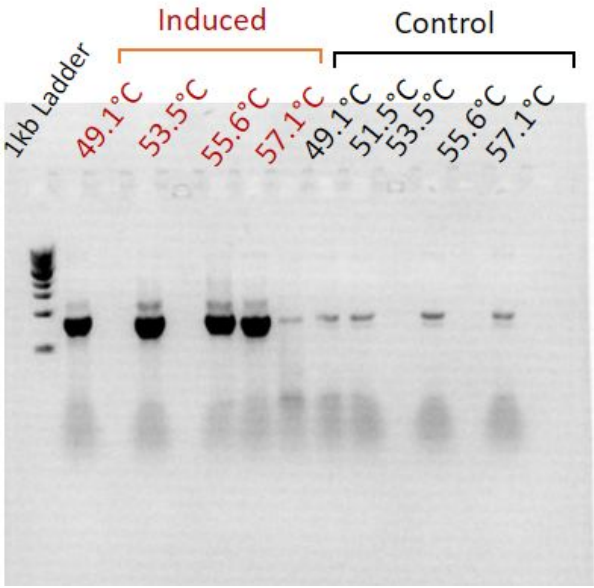
Aim: Gradient PCR using Taq polymerase

Protocol - same as PCR

- Temperature - 49.1°C, 51.5°C, 53.5 °C , 55.6°C, 57.1°C
- 20ul each

Result:

- Inducing sample with spore suspension increased the secretion of chitinase.
- Taq polymerase PCR is successful at 49.1°C, 51.5°C, 53.5 °C , 55.6°C, 57.1°C



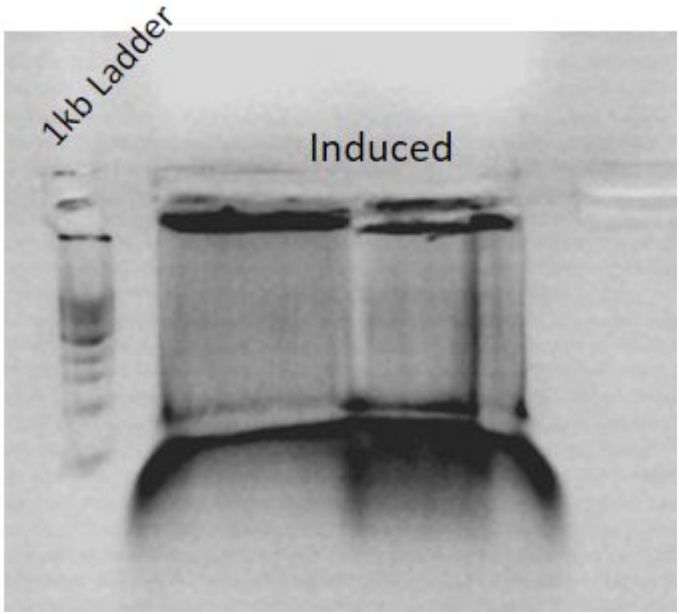
25.06.21

Aim: PCR using high fidelity thermostable DNA polymerase KOD

Protocol:

- 5x GoTaq 4ul
- 10mM dNTP 0.5 ul
- 25mM MgCl2 1ul
- 10mM FP 0.5ul
- 10mM RP 0.5ul
- Taq Pol 1 ul
- Template 1 ul
- Water 11.5 ul
- Total → 20 ul

Temperature 55°C



26.06.21

Aim: Gel elution of PCR product

Protocol:
All centrifugation steps are at maximum speed (≥10,000 x g, ~13,000 rpm) in a conventional, table-top microcentrifuge.

- Excise the DNA band from the agarose gel with a clean, sharp scalpel.
- Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QX1 to 1 volume of gel for DNA fragments 100 bp – 4 kb.
- Incubate at 50°C for 10 min (or until it's completely dissolved) to solubilize the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Check that the color of the mixture is yellow.
- Transfer it to spin column.
- Centrifuge the sample for 30 s and carefully remove the flow through.
- Wash the pellet with 500 µl of Buffer QX1.
- Wash the pellet twice with 500 µl of Buffer PE.
- Airdry: Centrifuge the empty spin column for 2 min
- Take the new collection tube, incubate at 50°C for 15-20 mins (with open cap).
- To elute DNA, add 25 µl of 10 mM Tris·Cl, pH 8.5 or H2O in the center of the spin column.
- Incubate at 50°C for 5 min (DNA fragments 4–10 kb)
- Centrifuge for 30secs
- Transfer the flow through into a new microtubule.

*Wash→ pour the solution in a spin column, centrifuge and remove the flow through.

Nanodrop result:

#	ng/uL	A260/A280	A260/A30
Eluted product	22.5	1.74	1.40

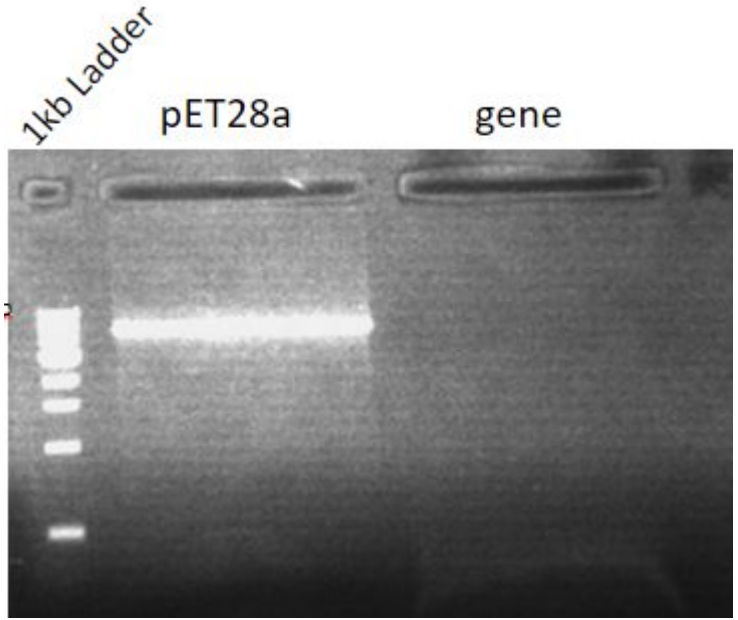
27.06.21

Aim: Restriction digestion of gene and pet28a

Protocol:

Cut Smart buffer	5ul
NdeI	1ul
BamHI	1ul
Template	1ug
Nuclease free water	upto 50ul

Incubate at 37°C overnight.



Result: We can see the band of size 5.329 kb of digested vector. But there is no band of digested gene.

Conclusion: Repeat the digestion of gene.

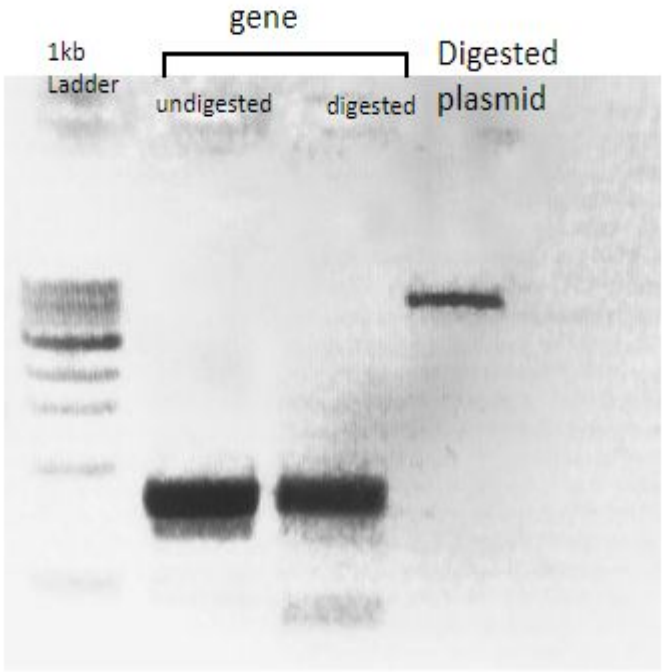
08.07.21

Aim: Restriction digestion of gene and pet28a

Protocol:

Cut Smart buffer	5ul
NdeI	1ul
BamHI	1ul
Template	1ug
Nuclease free water	upto 50ul

Incubate at 37°C overnight.



Result: We can see the band of size 5329 bp and 719bp of digested vector and gene respectively.

09.07.21

Aim: Gel elution of the digested gene and vector

Protocol:
All centrifugation steps are at maximum speed (≥10,000 x g, ~13,000 rpm) in a conventional, table-top microcentrifuge.

- Excise the DNA band from the agarose gel with a clean, sharp scalpel.
- Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QX1 to 1 volume of gel for DNA fragments 100 bp – 4 kb.
- Incubate at 50°C for 10 min (or until it's completely dissolved) to solubilize the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Check that the color of the mixture is yellow.
- Transfer it to spin column.
- Centrifuge the sample for 30 s and carefully remove the flow through.
- Wash the pellet with 500 µl of Buffer QX1.
- Wash the pellet twice with 500 µl of Buffer PE.
- Airdry: Centrifuge the empty spin column for 2 min
- Take the microtube in place of collection tube.
- To elute DNA, add 25 µl of 10 mM Tris Cl, pH 8.5 or H2O in the center of the spin column.
- Incubate at 50°C for 5 min (DNA fragments 4–10 kb)
- Centrifuge for 30secs
- Discard the spin column and store the product in microtube at 4 °C.

*Wash→ pour the solution in a spin column, centrifuge and remove the flow through.

Nanodrop:

Sample	Concentration
Digested Gene	30.8 ng/ul
Digested Vector	11 ng/ul

10.07.21

Aim: Ligation of digested pet28a and wheat chitinase

Calculation:

Mass of insert required at several molar insert:vector ratios in the range needed for typical ligation = 3:1

Vector concentration: 11ng/ul 50 ng of vector = 4.6ul

Gene concentration: 30.8ng/ul 19.59ng of gene = 0.7ul

Protocol :

- T4 DNA ligase 1ul
- 10X Buffer 2ul
- Vector 4.6ul
- Gene 0.7ul
- Water volume upto 20ul

Keep it in overnight incubation at 16°C

11.07.21

Aim: Transformation of ligated product into Competent cell DH5α

Protocol:

- **Control** Add 2.3ul of digested vector (half of the volume used in ligation) in 50ul of competent cell.
- **Cloned wheat chitinase** Add 10ul of ligated product in 50 ul of competent cell.
- Incubate the sample in ice for 30 mins.
- Incubate at 42 °C for 60sec.
- Incubate in ice for 5 min.
- Add 750ul of LB (in Laf).
- Put the sample in incubation shaker for 1hr (speed 197rpm, temperature 37 °C)
- Do micro spin for 2 min
- Do plating **
- keep it on overnight incubation at 37 °C.

**use L rod, first remove excess water from sample, then pipette some sample on LB Agar Kan plate and keep doing spread plating until all the sample solution is absorbed. don't put parafilm.

Cloned wheat chitinase: 35-40 colonies

Control: 15-20 colonies

Result: Got 35-40 colonies for cloned wheat chitinase and 15-20 colonies in control.

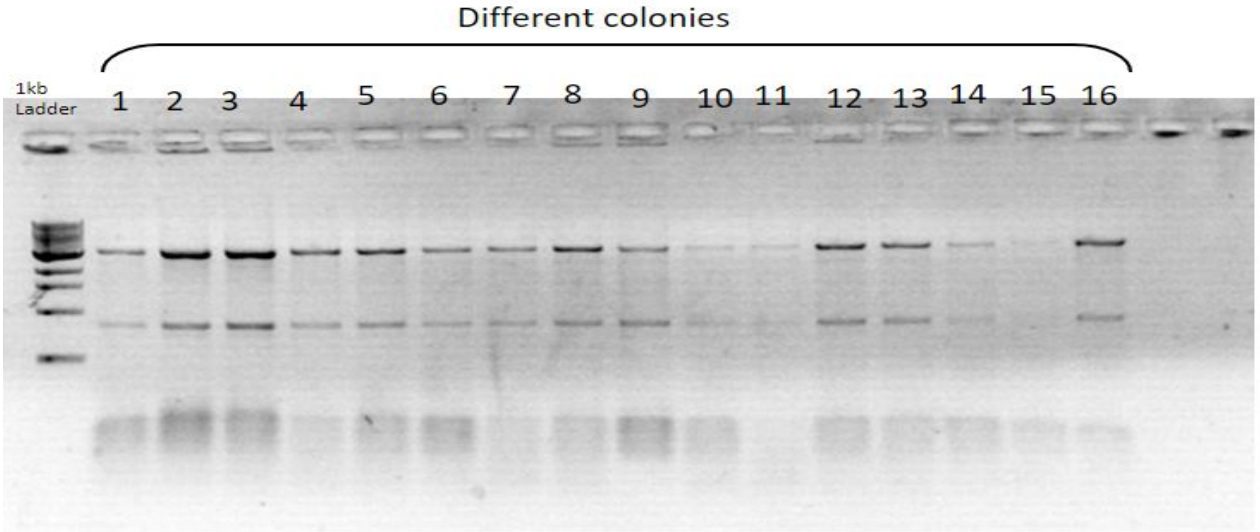
12.07.21

Aim: Colony PCR of transformed colonies

Protocol:

- Take 10ul of water in PCR tube.
- Using same tip, take one colony, resuspend in water.
- Likewise take 16 colonies in each tube.
- Then do Taq PCR using FP of gene and RP of pet28a (T7 RP)

Temperature: 55 °C



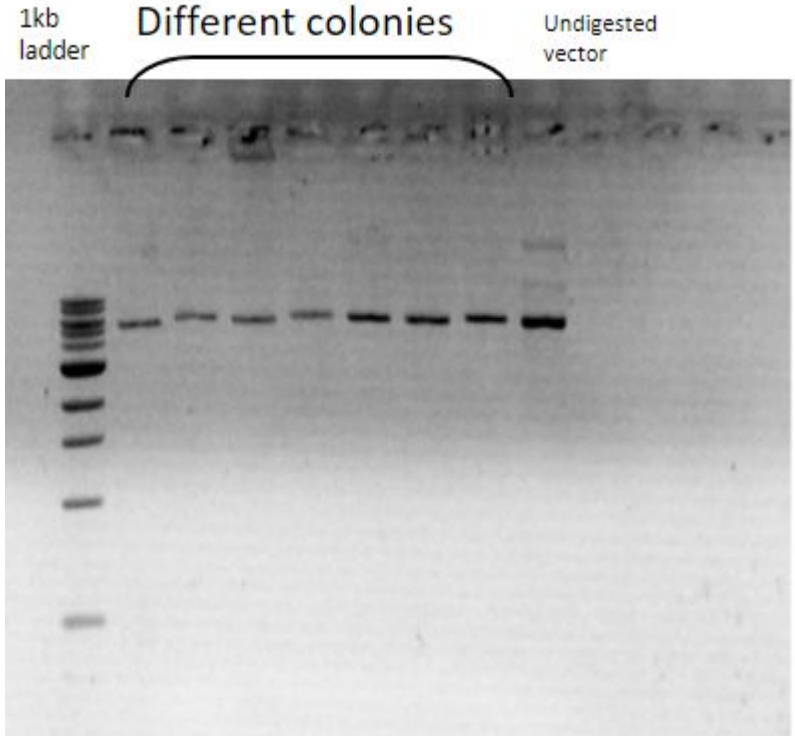
14.07.21

Aim: Digestion confirmation of transformed colonies

Protocol:

Cut Smart buffer	2ul
Pst 1 HF	0.05ul
EcoR 5	0.05ul
Template	1ug
Nuclease free water	upto 20ul

Incubate at 37°C overnight.



Result: Did not get 2 required bands for any colony.

Conclusion: Transformation is not successful.