Streptomyces cloning

08/09/21

PCR of Streptomyces

Tm = 50℃

Protocol (for one reaction)

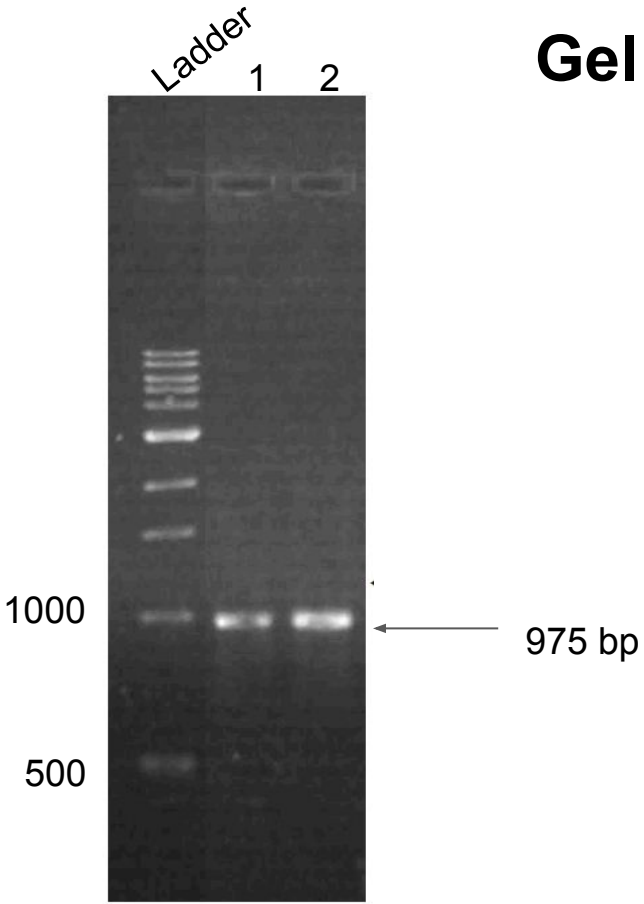
KOD 10X buffer	5 uL
MgSO4 (25mM)	2.5 uL
dNTPs	2.5 uL
KOD Polymerase	0.5 uL
10uM FP	1.5 uL
10uM RP	1.5 uL
Template(10 ng)	1 uL
Water	35.5 uL
Total	50 uL

Reaction Temp:

95℃	3 min	
95℃	20 s	X 35
50℃	20 s	
70℃	**2 min**	
72℃	5 min	
4℃	hold	

Reaction volume: (50uL each)

Gel Run of PCR product



Annotations:
Ladder: 1 kb NEB Ladder
1 & 2 : ST chitinase gene

PCR Clean Up Amplified product of Streptomyces

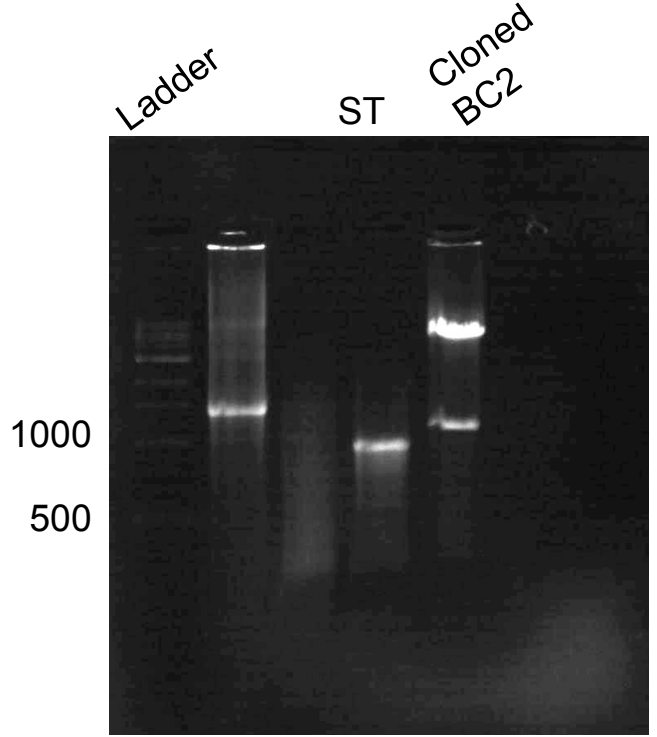
Nanodrop Result:

S.No.	Concentration (ng/uL)	A260/A280	A260/A230
ST	102	1.9	0.07

16/09/21

Restriction Digestion of Streptomyces and pet28a

BamH1 HF	0.4ul
HindIII HF	0.4ul
Cutsmart	4ul
Template	1 ug
Water	Upto 40 ul



16/09/21

GEL ELUTION OF DIGESTED ST & pet28a

Nanodrop Result:

S.No.	Concentration (ng/uL)	A260/A280	A260/A230
ST	10.4	1.73	1.18
pet28a	17.9	1.80	0.66

17/09/21

Ligation of digested pet28a and Streptomyces

Calculation:

50 ng of Vector → 3uL
27.34 ng of ST (1:3) → 3ul

Protocol:

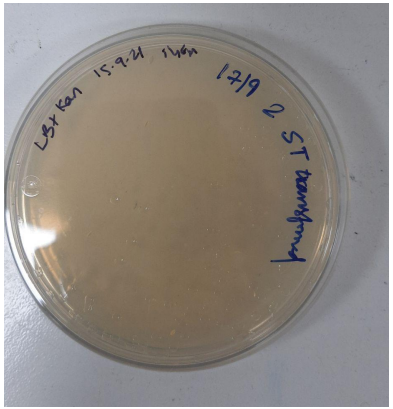
T4 DNA Ligase	0.5ul
10X buffer	2ul
Vector	50ng
Gene	3ul (according to ligation calculator)
Water	Upto 20ul

Kept at 23℃ for 1 hr

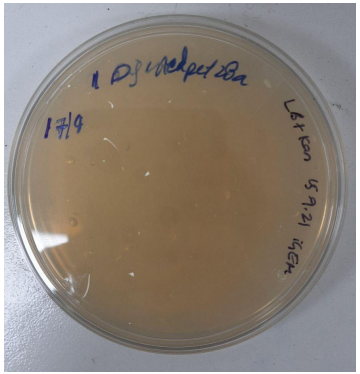
17/09/21

Transformation of ligated product into DH5α competent cells

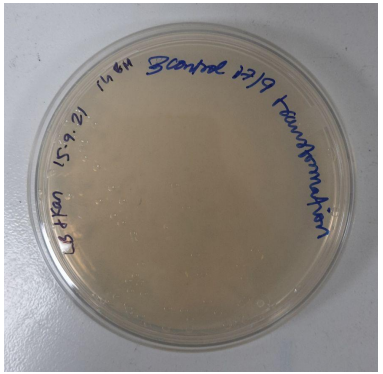
1. Ligated ST
2. Digested pet28a
3. Control - no vector



ST 8-10 colonies



Digested pet28a- no colony



Negative control- no colony

COLONY PCR

18/09/21

5X G0 Taq	2uL
dNTPs(25mM)	0.5uL
MgCl2(25mM)	0.5uL
Taq Polymerase	0.5uL
T7 FP	0.25uL
T7 RP	0.25uL
Template	2uL
H2O	4 uL
Total	10uL

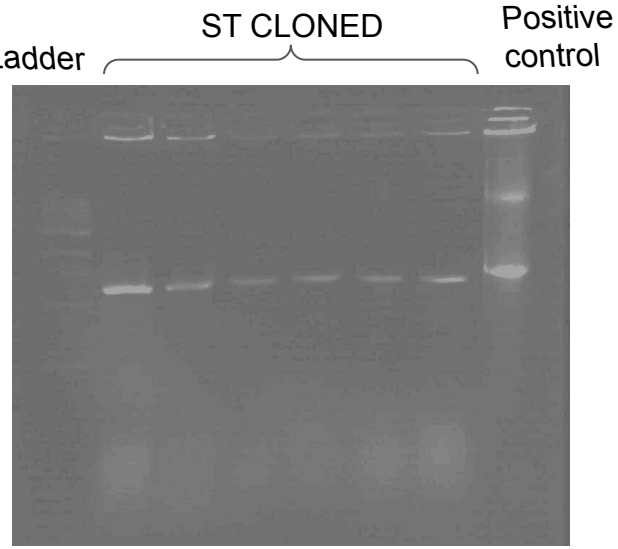
95℃	10 min	
95℃	30 s	X 20
50℃	30 s	
72℃	**3 min**	
72℃	10 min	
4℃	hold	

1. Take 10uL LB add colony take 2ul of this as template
2. To rest 8ul add 80ul LB and keep for incubation till pcr gets over at 37 degree 220rpm.
3. PCR machine should be preheated.
4. Tm = 50 ˚c

GEL RUN OF PCR PRODUCT

Annotations:
Ladder: 1 kb NEB
Colony pcr product of ST cloned in DH5alpha (1.3 kb)
Positive control - Colony pcr product of BC2 cloned in DH5alpha

1.3 kb



BAND AROUND 1.3kB

Result: Colony pcr result shows cloning of ST chitinase in DH5alpha is successful

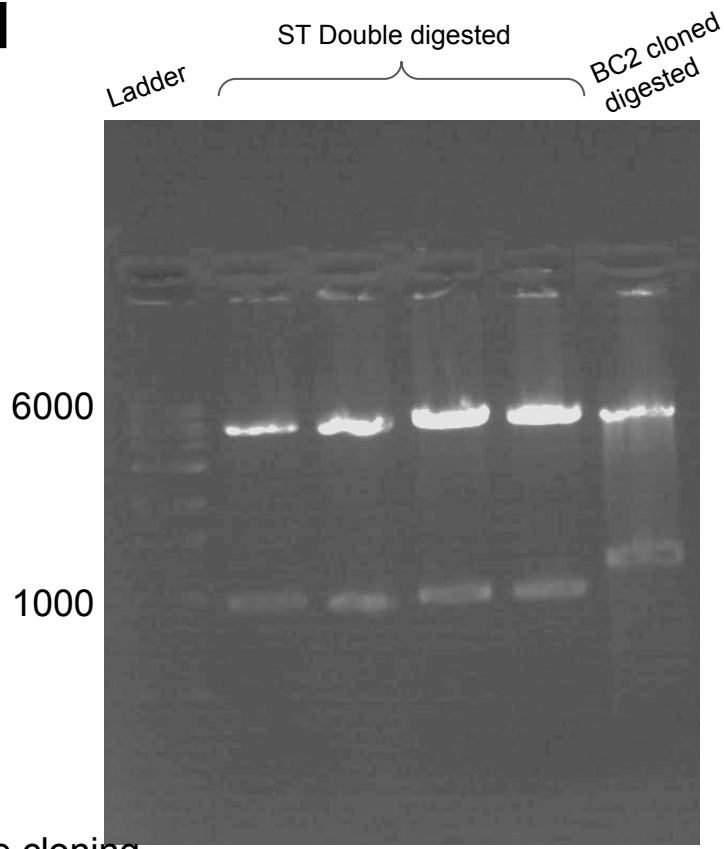
Plasmid isolation of transformed colonies

S.No.	Concentration (ng/uL)	A260/A280	A260/A230
1	51.6	1.64	2.67
2	126.8	1.9	3.18
3	126.9	1.88	2.64
4	113.3	1.88	2.51

RESTRICTION DIGESTION CONFIRMATION

19/09/21

BamH1 HF	0.15ul
HindIII HF	0.15ul
Cutsmart 10X	1ul
Template	Gene- NEB
	Pet28a- 50 ng
Water	Upto 15uL

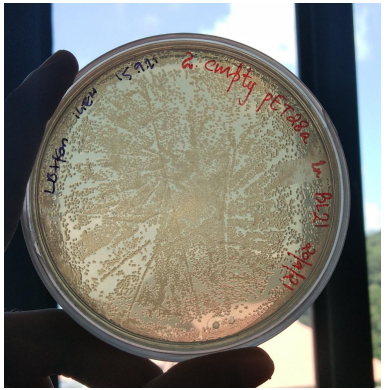


Result: As per restriction digestion confirmation result the cloning of ST chitinase in DH5alpha is successful

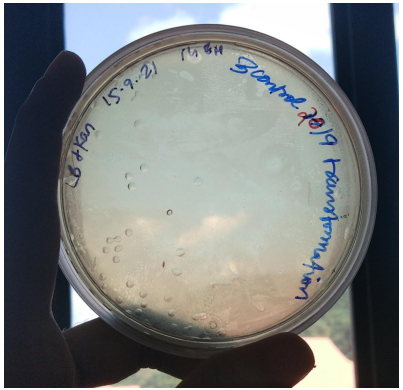
Transformation of cloned ST INTO BL21(DE3)

1. Cloned ST
2. Empty pet28a
3. Control - no vector

ST cloned in BL21(DE3)



Empty pet28a

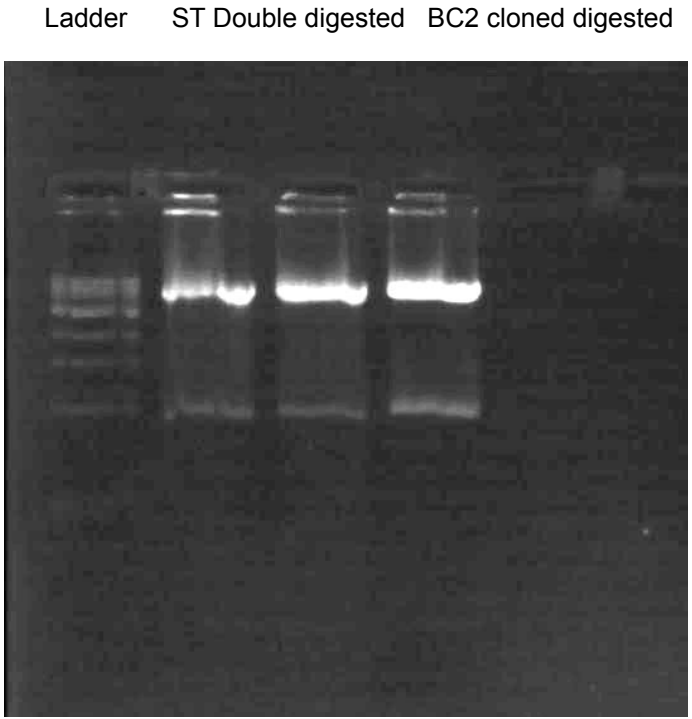


Negative control- no colony

22/09/21

RESTRICTION DIGESTION CONFIRMATION

BamH1 HF	0.15ul
HindIII HF	0.15ul
Cutsmart 10X	1ul
Template	Gene- NEB
	Pet28a- 50 ng
Water	Upto 15uL



Result: Result: As per restriction digestion confirmation result the cloning of ST chitinase in BL21(DE3) is successful

08/10/21
09/10/21

IPTG induction of BC2 cloned plasmid in BL21

Protocol:

- Primary inoculum: Take a 50ml falcon tube, add 10 ml LB broth and 10ul Kan(50 ng/ul). Select a colony from **GLYCEROL STOCK** cloned BL21 cells and dip the tip in lb.
- Incubate for 12 hrs
- Secondary inoculum: (For one sample) Take 100ul of primary inoculum in 50ml falcon tube and add 10 ml LB and 10 ul Kan.
- Keep it in incubation till OD reaches 0.6
- Take **1 L** of secondary inoculum and add IPTG (of required concentration)
- Keep it in incubation at req temperature and time. (for temp less than 34 incubation time is overnight and for temp greater than 34 incubation time is 5hr
- When the incubation period is over, pellet down the cells(centrifuge at 11,000 rpm 4℃ for 15 min) and store it in -20℃

09/10/2021

Ni-NTA Purification Of supernatant

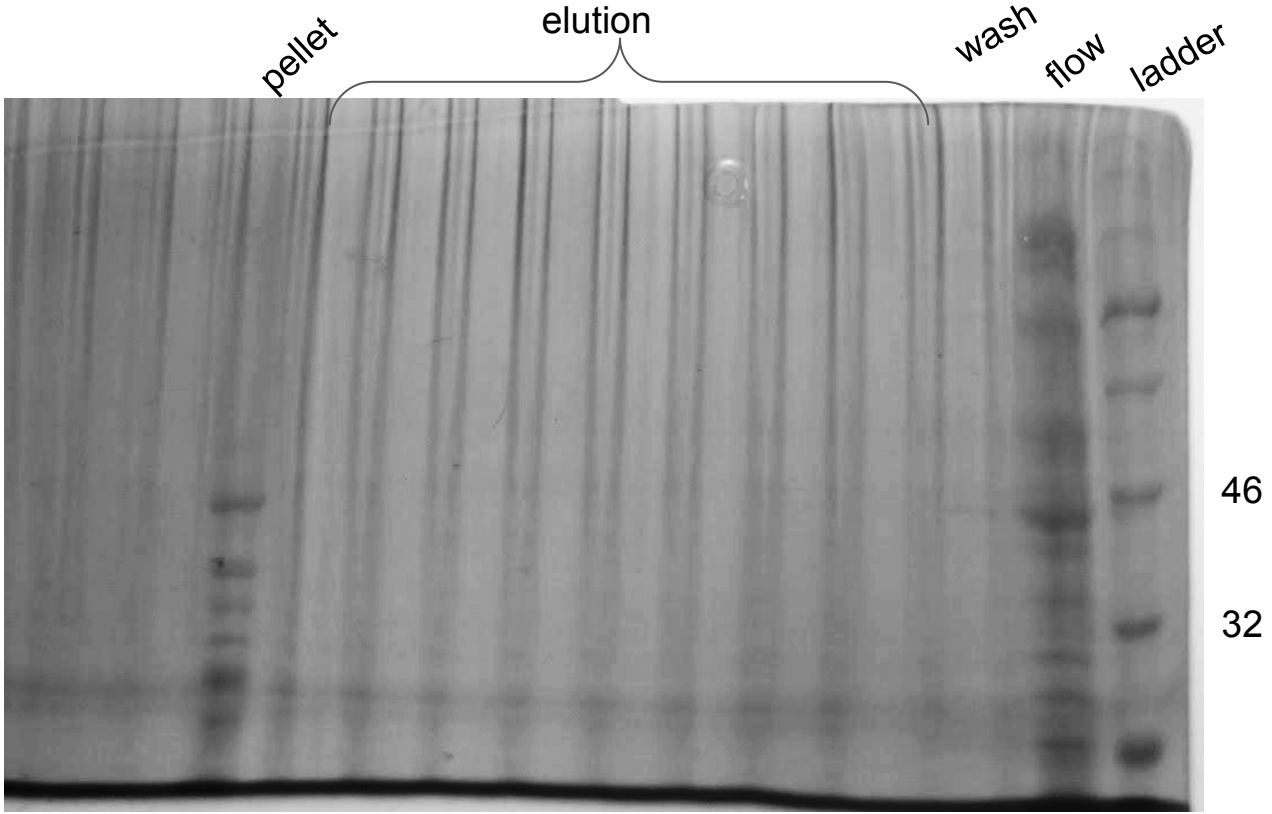
- The flow through (filtrate collected after loading solubilised protein) and wash buffer were collected separately in 50 mL falcon tube.
- The elution flow through was collected in fractions of 1mL in 30, 1.5mL Eppendorf tubes.

SDS PAGE of Purified Protein

09/10/2021

- The collected fractions were run on SDS along with collected wash through and flow through.
- Stacking Gel : 12 %
- Resolving gel : 12 %

SDS PAGE of Purified Protein



BAND CAME IN PELLET

10/10/21

Purification of protein

1. Washing pellet
2. Urea treatment
3. NI-NTA
4. SDS
5. Ethanol precipitation

Washing the pellet

Component of **Wash Buffer**	Stock Concentration	Working Concentration	Volume for 50 mL Wash Buffer
NaPB (pH 7)	1 M	50 mM	2.5 mL
Sodium Chloride	5 M	0.5 mM	5 mL
Triton X	100%	0.5%	250 uL
Autoclaved MilliQ	Make upto 50 mL		

1. **Wash with wash buffer 20ml twice.**
2. **Wash with 20ml 10mM CaCl2.**

Urea Treatment

Component of **Extraction Buffer**	Stock Concentration	Working Concentration	Volume for 70 mL Extraction Buffer
NaPB (pH 7)	0.1 M	50 mM	3.5 mL
Sodium Chloride	5 M	0.5 mM	7 mL
Urea	10 M	8M	56ml
Autoclaved MilliQ	Make upto 70 mL		

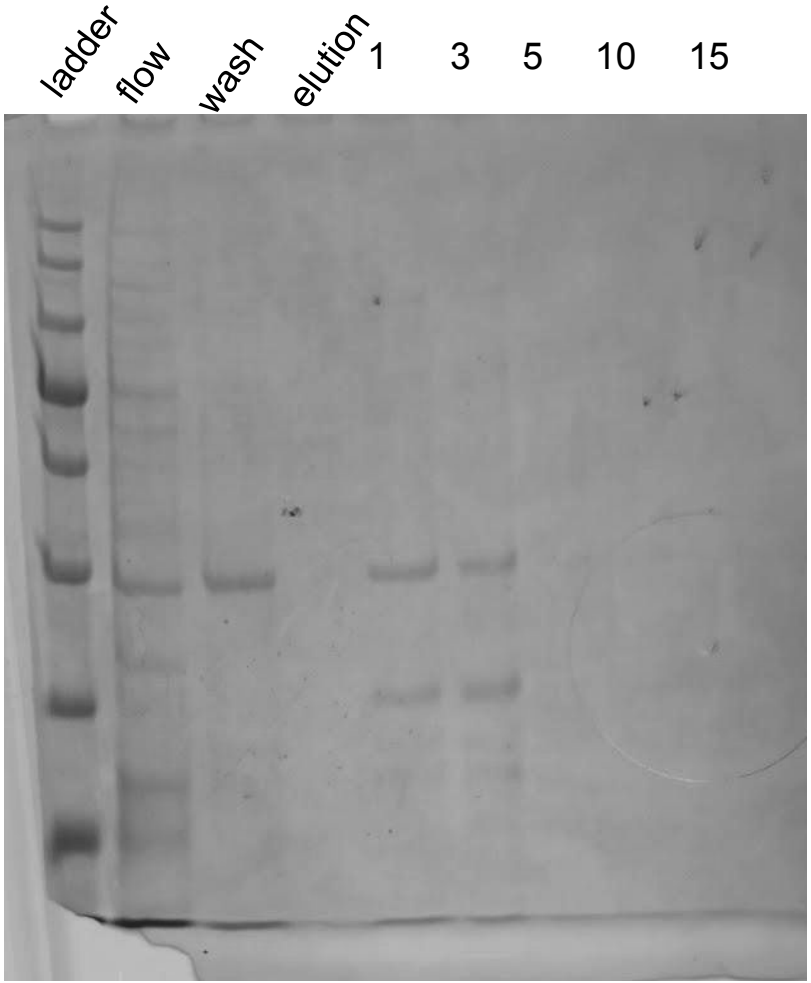
- **Resuspend the pellet in 30ml Extraction Buffer.**
- **Centrifuge it for 1hr 15 min at room temperature 11000rpm.**
- **Collect the supernatant.**

Ni-NTA Purification

- The column is equilibrated with 30ml equilibration buffer.
- Inoculation- 1 hour
- The flow through (supernatant) is passed through Ni NTA column and collected in 50ml falcon tube.
- The column is washed with 50 ml wash buffer and wash flow through is collected in 50 ml falcon tube.
- The column is eluted with 50 ml elution.
- The elution flow through was collected in fractions of 1ml in 30, 2mL Eppendorf tubes.

SDS of purified protein

SDS
12%
STACKING
12%
RESOLVING



GOT BANDS IN 1 & 3 elution fraction

Ethanol precipitation

- Simply add 9 volumes ice-cold ethanol (100%) to one volume of buffer containing the protein in 8M urea.
- Incubate for 2hr at -20°C and then spin down (7000rpm, 1h) the precipitate.
- Wash the pellet 4-5 times with 90% ice-cold ethanol, remove the supernatant as good as possible
- Resuspend the pellet in 1X PBS with 0.1% SDS.

Note: If the protein is precipitating add more volume of buffer.

Concentrating the purified protein

Buffer	Stock solution	Working solution	Vol for 40 ml
NaPB	5M	200mM	1.6 ml
PBS	10X	1X	4 ml
Glycerol	50%	10%	8ml
SDS	10%	0.1%	0.4 ml

- **The protein is concentrated using amicon filter.**

Nanodrop concentration of concentrated protein

Ɛ = 48.150 Mol. mass = 36671.50 kDa

mg/ml	A280	A260/A280
0.749	0.98	0.77