Dt. 03/09/2018

A report submitted

on completion of summer project

On

METHYLTRANSFERASE EXPRESSION AND PURIFICATION

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CERTIFICATE OF COMPLETION

This is to certify that **Ms. M. Adishree** of NISER, has successfully completed the summer project for the academic year of 2018 in **chemical ecology** in the institution **NCBS**, TIFR, Bengaluru.

Chairperson

Dr. Chandan Goswami

SBS, NISER reader, Ramanujan fellow

Principal Investigator

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National Centre for Biological Science (NCBS),
Tata Institute of Fundamental Research (TIFR),
Bengaluru.

ACKNOWLEDGEMENT

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My sincere thanks to my mentor, Dr. Kokila.S for her continuous support, patience, motivation, and immense knowledge. I admire her for her earnest efforts to make this project a success.

I thank my fellow lab mates Anupam sir, Praveena mam, Reddy, Enakshi, Neha, Sweta, Aswathi, Rohith, Harsha, and Vishal for the stimulating discussions, for always lending a helping hand, and for all the fun we have had this summer. Without their precious support, this project would not have been a success.

Lastly, I would like to thank my friends and family for their endless love and support in the long run of my life.

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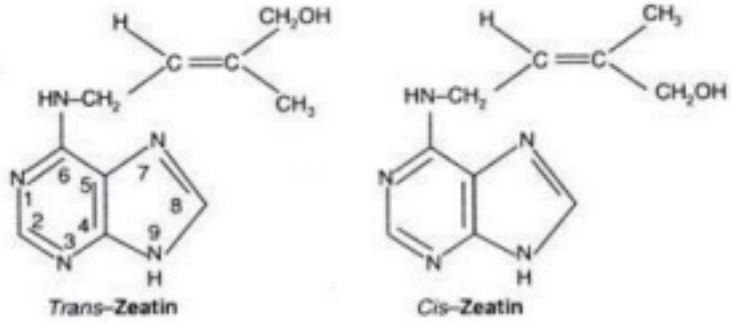
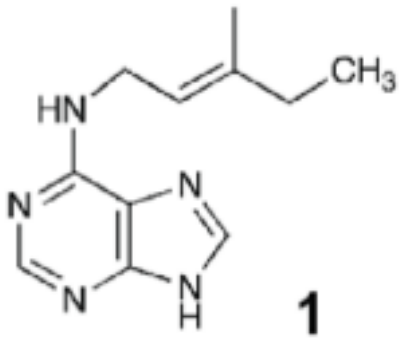
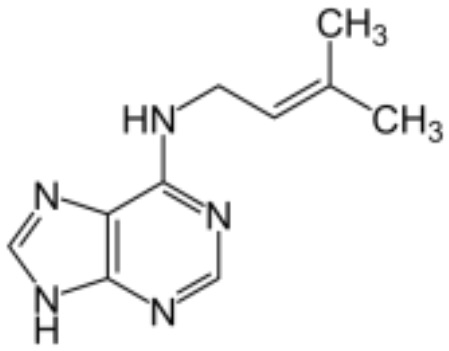
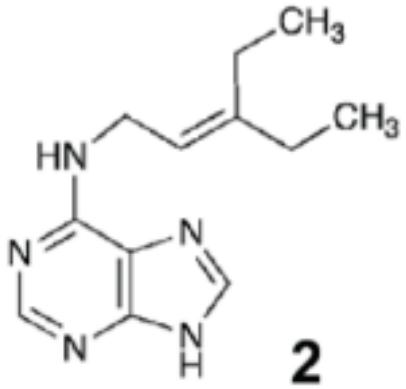
INTRODUCTION

- **Cytokinins (CKs)** are a class of adenine derivative phytohormones that regulate plant growth and development along with auxin.
- Often, phytopathogens mimic phytohormones to disrupt the hormonal balance and to facilitate niche establishment in their host.
- Here, such a case has been addressed: *Rhodococcus fascians* is a gram positive actinomycete that harbors the **fasciation (fas) locus** encoding genes homologous to CK synthesis.
- Methylated cytokinins from *Rhodococcus fascians* contribute to pathogenesis as hormone-mimics. Methyltransferases are S-adenosyl methionine (SAM) dependent enzymes.

Naturally occurring CKs	Methylated CKs
i. Zeatin  *Trans*–Zeatin *Cis*–Zeatin **(tZ) (cZ)**	**i. Monomethylated N6-(Δ2-isopentenyl) adenine**  **1**

ii. Isopentenyl adenine	ii. **Dimethylated N6-(Δ2-isopentenyl) adenine**
	 **2**

Reaction:

(substrate $\xrightarrow{\text{enzyme}}$ product)

Dimethylallyl diphosphate (DMAPP) + adenosine phosphate $\xrightarrow{\text{Isopentyl transferase (IPT)}}$ N^6 Δ^2- isopentyl adenine (iP)

This project involves the purification of methyltransferase, with the sole purpose of characterizing the methylated products and the reaction intermediates associated, using GC-MS & NMR techniques.

COMPETENT CELLS

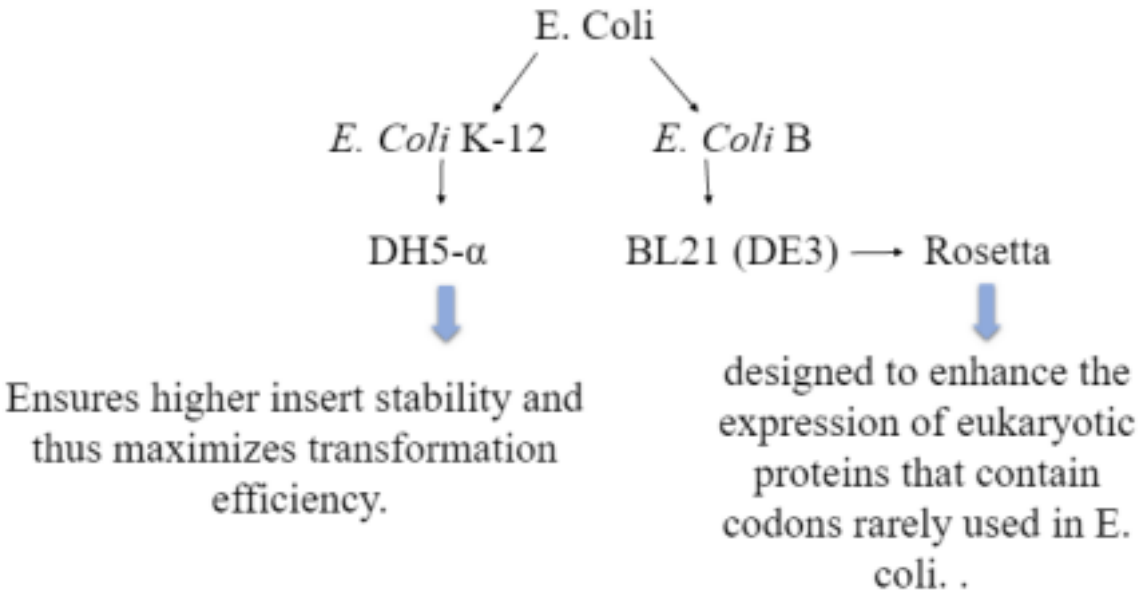
- Competent cells are ready to use bacterial cells that are capable of taking up exogenous DNA from their environment and serve as hosts for the vector with our gene of interest.
- Cells can be artificially made competent by

  i) Chemical induction using Calcium Chloride ($CaCl_2$) treatment.

  ii) Electroporation
- Here, two different types of E. coli competent cells were used:

  – **DH5α cells** as cloning host.

  – **Rosetta cells** as expression host.



TRANSFORMATION

Direct uptake and incorporation of exogenous genetic material by the competent cells is termed **transformation**.

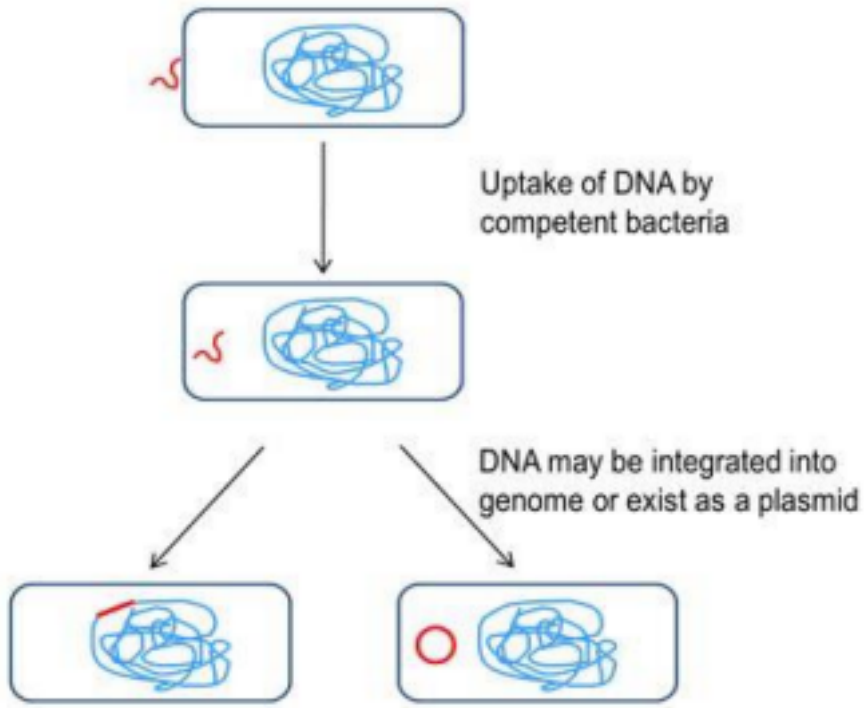


Figure 1**: Process of Transformation**

Here, We have used **Vector pCOLD I (4.407kbp)**

- **His-tagged vector**
- **Cold-shock vector**, i.e. enables extremely efficient protein expression at low temperatures by virtue of a cold-inducible promoter.

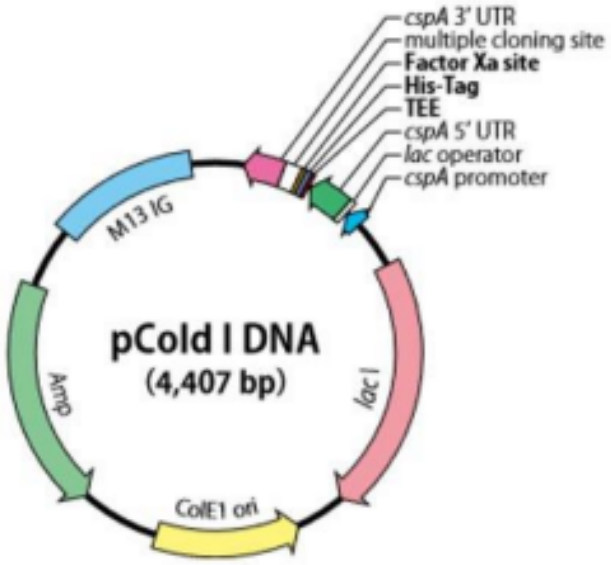


Figure 2:

pCold I DNA

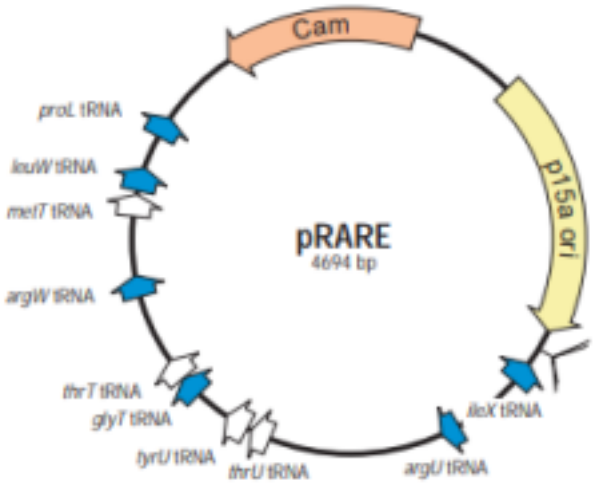
Vector map

And, **insert: MT1 and MT2 (0.8kb)**

Both the vector and our insert were digested with the same two different restriction enzymes.

Restriction enzymes used:	**Nde I**	**Xho I**
Recognition sequence	5'C A T A T G 3' 3'G T A T A C 5'	5'C ↓T C G A G 3' 3'G A G C T ↑C 5'
Cut ends produced:	5'---CA TATG---3' 3'---GTAT AC---5'	5'---C TCGAG---3' 3'---GAGCT C---5'

Antibiotic resistant genes were used as **selectable markers**:

DH5-α cells	**Rosetta cells**
• Only **carbenicillin** used as marker. • Vector pCOLD1 only has ampicillin resistant gene. • Agar plates containing ampicillin are used as growth media.	• **Chloramphenicol and carbenicillin** used as markers. • The added plasmid pRARE in rosetta strain codes for several rare codon tRNAs that carries its own antibody selection marker. • Agar plates containing ampicillin and chloramphenicol are used as growth media. 

Procedure followed for transformation:

1. Competent cells were taken out of -80°C and thawed on ice.
2. Agar plates containing the appropriate antibiotic are taken from storage at 4°C and let warm up to room temperature.
3. 5 µl of DNA mixed with 20-50 µL of competent cells in a microcentrifuge tube.
4. competent cell/DNA mixture incubated on ice for 15 mins.
5. Heat shock was given to each transformation tube by placing them in 42°C water bath for 2min.
6. Tubes were put back on ice for 15min.
7. Added 300 µl LB to the bacteria and grown in 37°C shaking incubator for 45 min.
8. Plated the transformation onto LB agar plates containing the appropriate antibiotic.
9. Plates were incubated at 37°C overnight.

INOCULUM PREPARATION

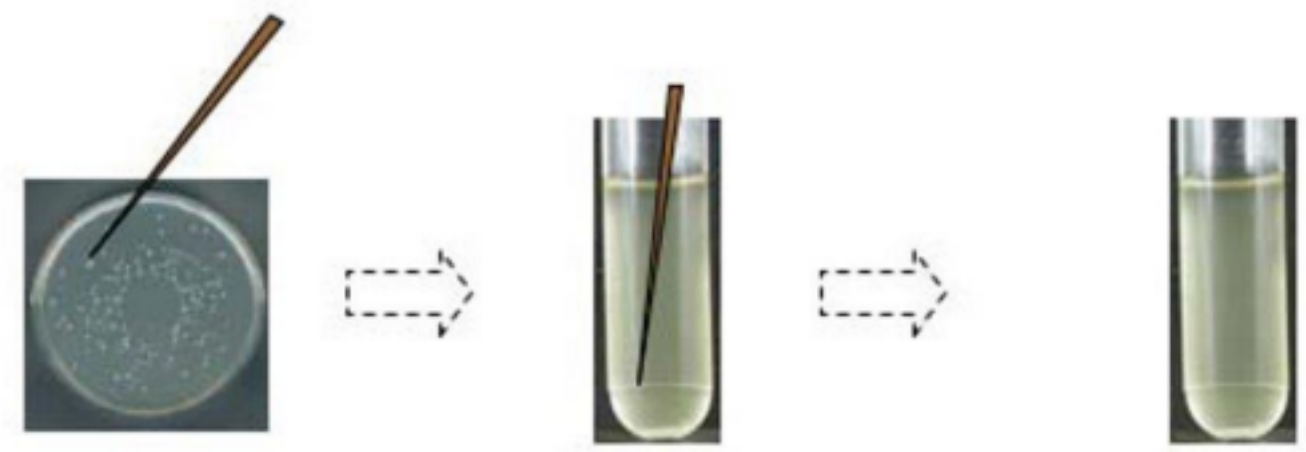


Figure 3: **overnight inoculum preparation**

Procedure followed for inoculum preparation:

1. 5ml autoclaved LB broth taken in sterile cotton plugged tubes and labeled. (3*2=6 tubes)
2. Appropriate antibiotics added.
3. Single colonies were picked with toothpick using forceps (from the overnight transformation plates) and put into respective tubes.
4. These tubes were kept in 37°C shaker overnight.
5. overnight fresh culture taken out the very next day and kept in 4°C for further use.

PLASMID ISOLATION & SEQUENCING

Plasmid was isolated from the DH5α transformed cells (using Qiagen mini-kit).

Procedure followed for plasmid isolation:

1. 1-5ml fresh bacterial overnight culture pelleted by centrifugation at 8000rpm for 3min. at room temperature (15°-20°C).
2. Pelleted bacterial cells resuspended in 250µl Buffer P1& transferred to a microcentrifuge tube.
3. 250µl Buffer P2 added & mixed thoroughly.
4. 350µl Buffer N3 added and mixed immediately.
5. centrifuged for 10 min. at 13,00rpm in a tabletop centrifuge.
6. Obtained supernatant transferred to separate spin columns and then centrifuged for 60sec and discarded the flow-through.
7. Spin columns were washed by adding 500µl Buffer PB and then centrifuged for 60sec and discarded the flow-through.
8. 8. Spin columns were again washed by adding 700µl Buffer PE and then centrifuged for 60sec and discarded the flow-through.
9. 9. 1 min. dry spin done to remove residual wash buffer.
10. Spin columns were placed in clean 1.5ml microcentrifuge tubes.
11. To elute DNA, 50 µl Buffer EB was added to the center of each of the spin columns, let it stand for 1min, and centrifuged for 1 min.

Gel run done to check for our inserts.

Results:

i) MT1

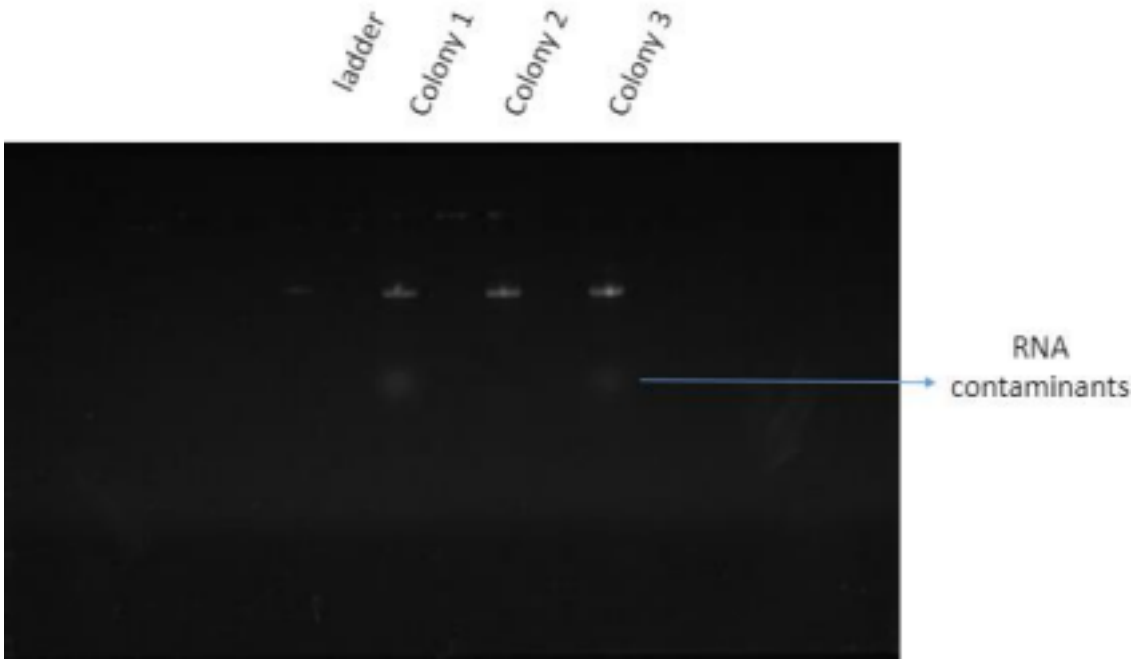


Figure 4: **Gel Docx picture of isolated plasmids with intact MT1 from different colonies.**

Eluted DNA of colony 2 was given for sequencing, as it was RNA free. Sequencing results matched with NCBI database:

Id	Sequence	Showing similarity to (in NCBI)
1	CTACTGCGCTCACCATGTCG (F1)	Rhodococcus-Isopentenyl transferase/Geranyl diphosphate2-C-methyltransferase
2	TGGCTTCGTCGGTGTATCGC (R1)	
3	ATGCCGAACCTCGACGTG(F2)	Rhodococcus-Geranyl diphosphate2-C-methyltransferase
4	CTACAGACGCTCGCACGC(R2)	

ii) MT2

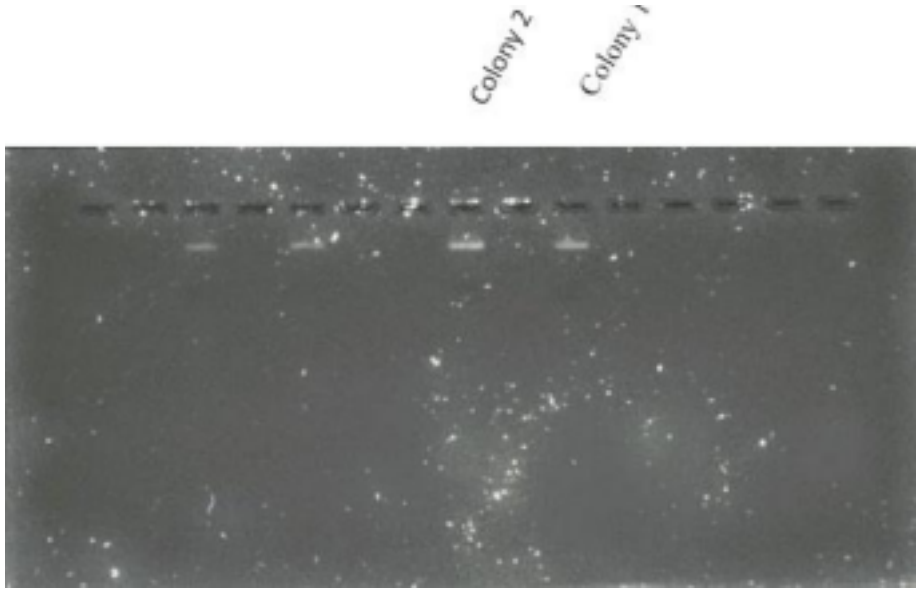


Figure 5: **Gel Docx picture of isolated plasmids with intact MT2 from different colonies.**

Eluted DNA of colony 2 was given for sequencing, as it was higher in concentration (thus the high band intensity). Sequencing results cross-checked with NCBI database:

Id	Sequence	Showing similarity to (in NCBI)
1.	>A2_MT2-F1 TTGGGCCCCGTGAACGCGGG	R. fasciens isopentenyl transferase
2.	>A2_MT2-R1 TGTGGGGCACAGACAATTTG	

PROTEIN EXPRESSION

Seed culture

Seeding the cells into a small culture ensures that the cells we're seeding into your large culture are healthy and replicating well.

Procedure followed to prepare seed culture:

1. 25ml autoclaved LB broth taken in test tube.
2. 25µl of carbenicillin and chloramphenicol each added. (stock = 1µl/ml)
3. 3. 1% of fresh Rosetta overnight culture added.
4. Kept overnight in shaker at 37°C.

Bulk culture

Bulk culture is prepared to increase the cell density and thus quantify protein expression.

Procedure followed to prepare bulk culture:

1. 500ml LB broth autoclaved in 1000ml conical flasks. (5×500ml = 2.5L broth total)
2. 500µl of carbenicillin and chloramphenicol each added to each of the flasks.
3. 3. 1% seed culture added to each.
4. Kept in 37°C shaker for 2.5hrs.

IPTG induction

Isopropyl β-D-1-thiogalactopyranoside, abbreviated IPTG, is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon. Unlike allolactose, the sulfur (S) atom chemically binds with the lac repressor which is non-hydrolysable by the cell, preventing the cell from "eating up" or degrading the inductant; therefore, the IPTG concentration remains constant resulting in constitutive expression of our protein.

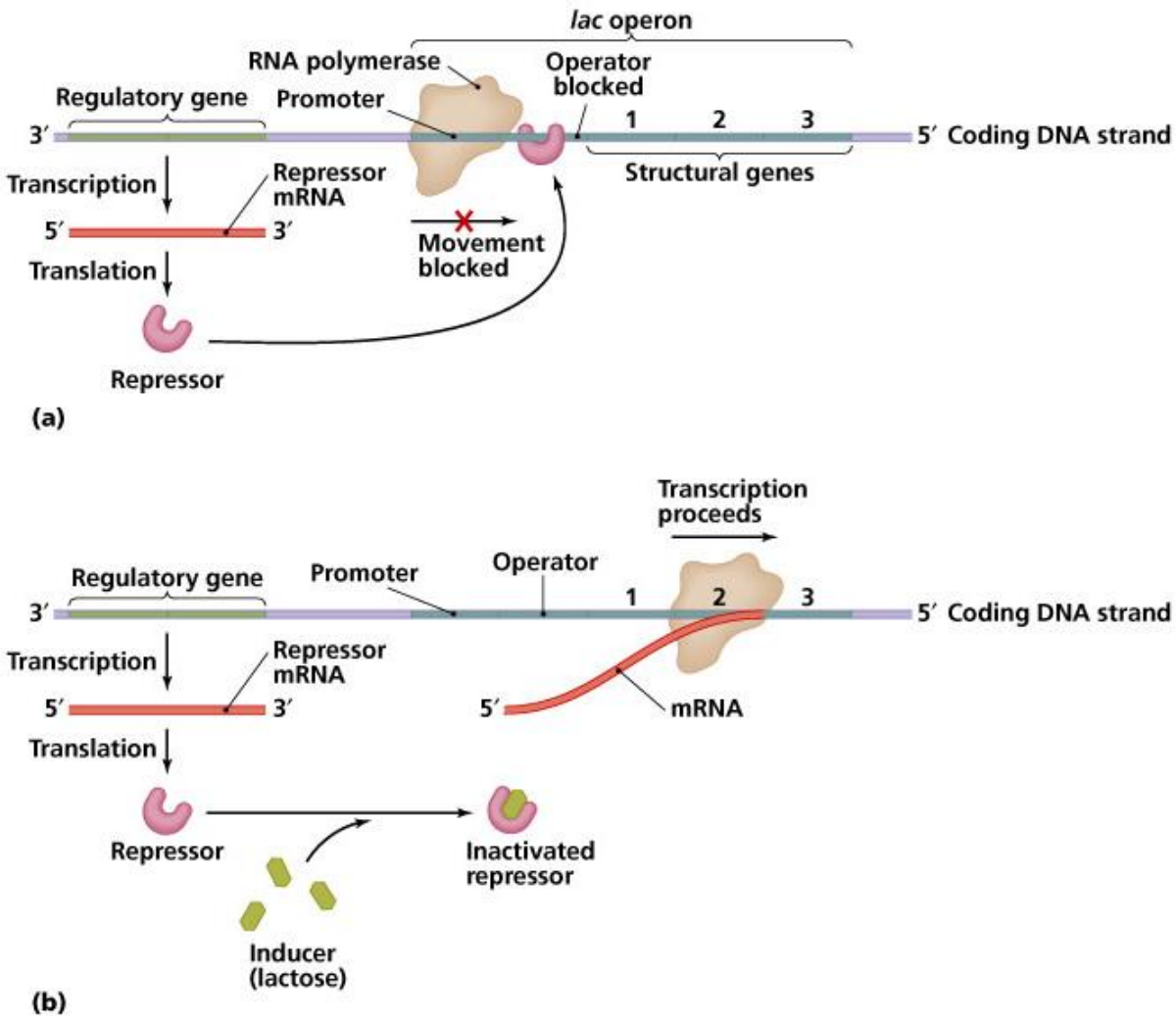


Figure 6: **Diagramatic representation of Inducer and Reprssor activity**

Procedure followed for IPTG induction:

5. After 2.5hrs, bulk culture taken out from 37°C shaker.
6. 2ml taken out from each of the flasks and kept separately as uninduced sample.
7. Rest culture kept on ice for an hour (cold induction).
8. 0.5mM IPTG added to each. (stock = 1M)
9. Kept in 15°C shaker for 20hrs.

(after this, culture can be kept in cold room for further use)

10. 2ml taken out from each and kept separately as induced sample.
11. Culture pelleted by centrifugation at 15000rpm for 30min. at 4°C.
12. pellet stored in -20°C for further use.

SODIUM DODECYL-SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

- SDS-PAGE is an electrophoresis method that allows protein separation by mass.
- Sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) is a detergent with a strong protein-denaturing effect and binds to the protein backbone;
- Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size.
- The use of SDS and polyacrylamide gel largely eliminates the influence of the structure and charge, and proteins are separated solely based on polypeptide chain length.
- The gel used is formed by radical polymerization.

Reagents used	**uses**
Acrylamide	Sieving medium
Bisacrylamide	Cross-linker
TEMED	Catalyst
APS	Radical initiator
Isopropanol	Prevents oxygen exposure
Bromophenol blue	Dye
Coomassie staining solution	Protein sorting and analysis

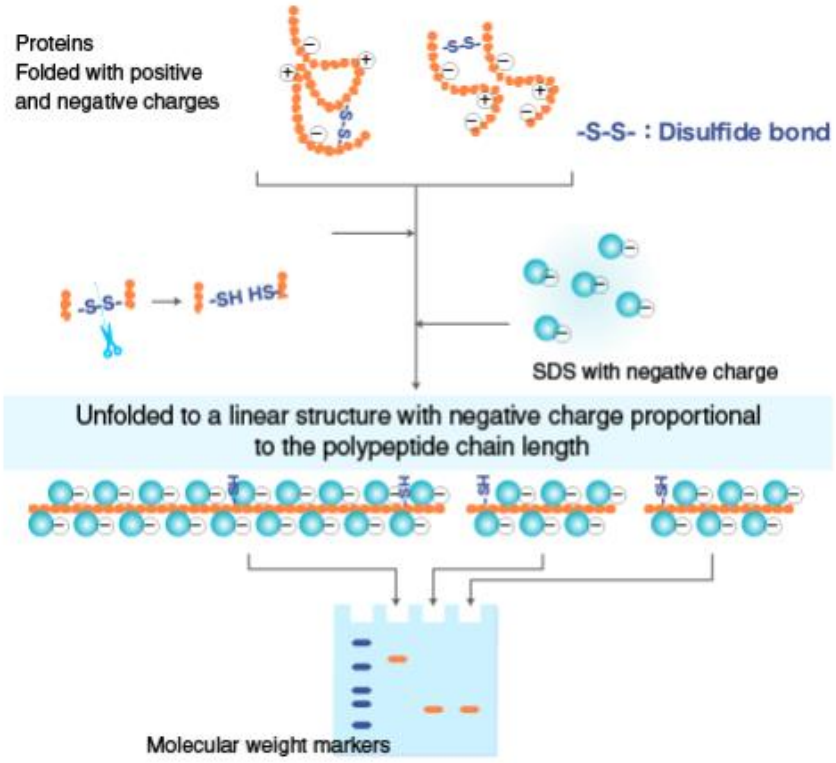


Figure 7: **Mechanism of separation of proteins in SDS-PAGE.**

Gel composition (for 3×1.5mm gels):

	10% resolving gel	**4% stacking gel**
4x buffer	6.25ml	2.5ml
Dd H_2o	10.5ml	5.2ml
30% acrylamide	8.25ml	1.3ml
TEMED	25µl	20µl
10% APS	125µl	100µl
Total volume	25ml	10ml

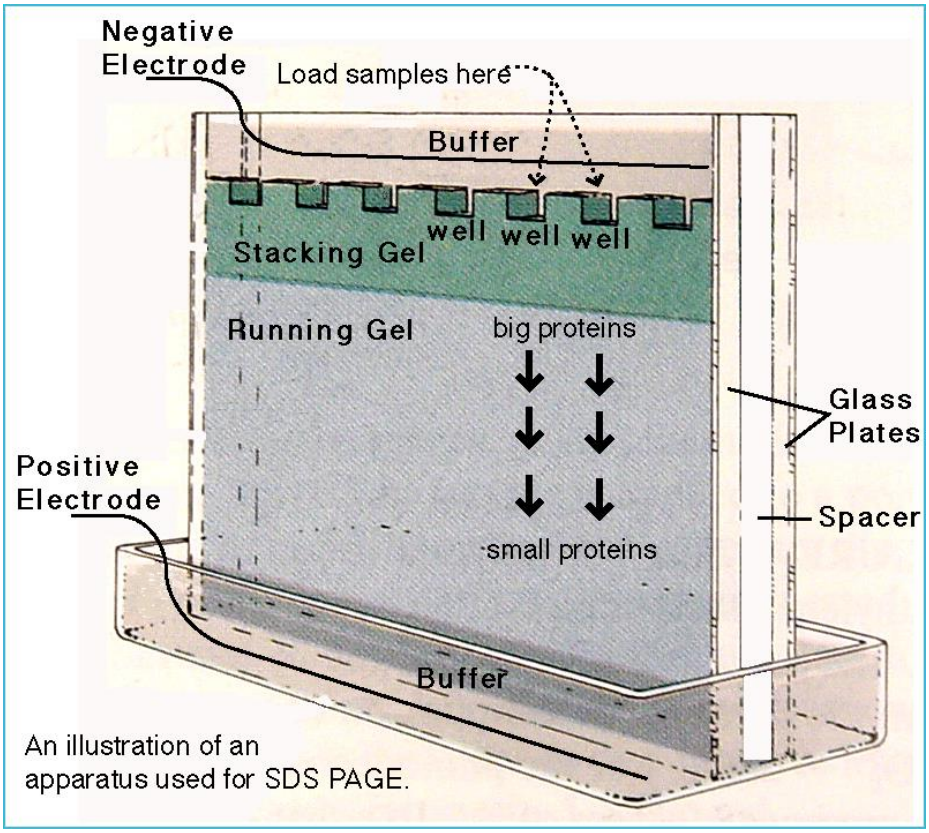


Figure 8: **An illustration of SDS-PAGE apparatus**

Procedure followed to run SDS-PAGE

1. Gels prepared according to requirement.
2. The induced culture vials taken out of the bulk culture before, pelleted by centrifugation at 10,000rpm for 5min. at 4°C.
3. Supernatant discarded.
4. Pellet resuspended in 100µl TE buffer.
5. Required volume aliquoted to separate Eppendorf tubes, mixed with dye and ddH_2o to make up the volume to 25µl. (maximum capacity of wells = 30µl.)
6. Samples loaded into wells and run started at 110V for an hour.
7. Gel removed from the glass plates, put into staining solution and kept on rotator for 30-45min.

8. Staining solution discarded, gel put into de-staining solution and kept on rotator overnight.
9. Picture of the gel taken in uv trans-illuminator.

Results:

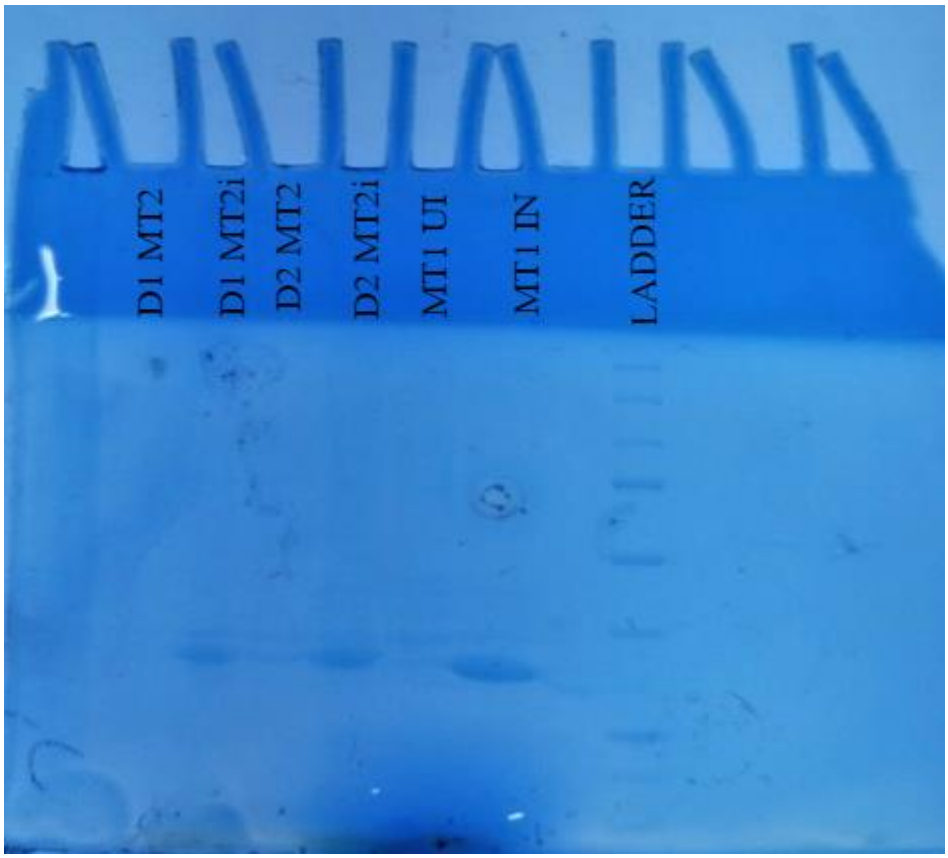


Figure 9: **SDS-PAGE of induced and uninduced samples to check for protein expression.**

MT1 PROTEIN PURIFICATION

STEP 1: Cell lysis

1. Pellet resuspended in equilibrium buffer containing:

	uses
50mM phosphate buffer	maintains pH
100mM NaCl	keeps proteins soluble
100µM PMSF	serine protease inhibitor
10mM imidazole	prevents non-specific interactions
5mM β-ME	reducing agent
1 PIC tablet	Aspartic, cysteine, serine, metalloprotease and aminopeptidase inhibitor

2. **Sonication**: ultrasound (high-frequency) energy is applied to samples, using an ultrasonic probe, to agitate and disrupt the cell membranes.

(30% amplitude, 5sec ON, 5sec OFF for 20-3-min)

STEP 2: Cell lysate

3. Sonicated cells pelleted by centrifugation at 15000rpm for 30min. at 4°C.
4. Pellet stored in 4°C and supernatant (flow through) taken.

NICKEL-NTA AFFINITY CHROMATOGRAPHY

- Ni-NTA Agarose is used for purification of from any 6xHis-tagged vector.
- The resin exhibits high affinity and selectivity for 6xHis-tagged proteins.
- When incubated with an affinity resin containing bound divalent nickel ions, his-tagged vector having our protein of interest binds to the column which is then collected.

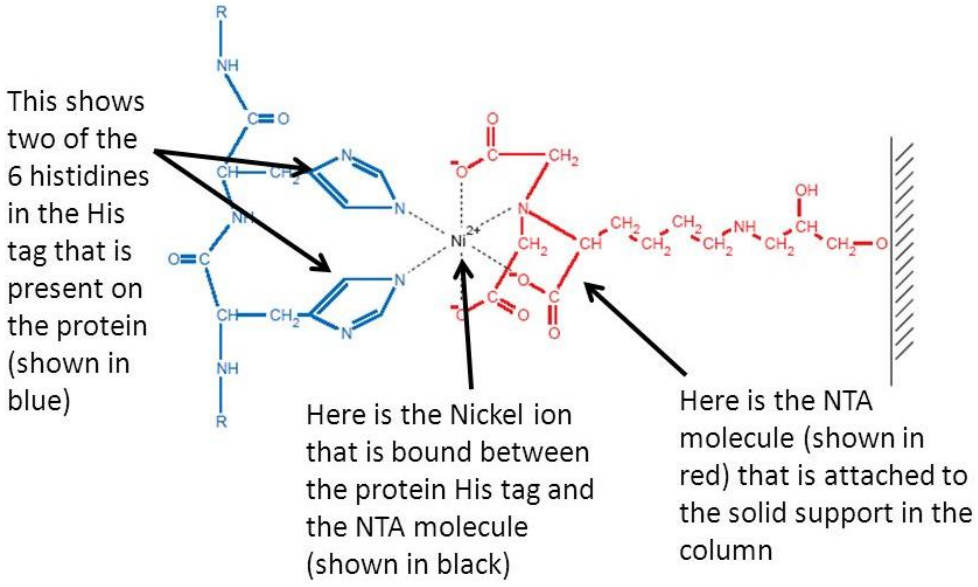


Figure 10: **co-ordination bond formed between nickel-nta and His-tags**.

STEP 3: Column regeneration

Length of column is termed as **column volume (CV) or bed volume**, and 1 CV = 5ml.

Procedure followed for column regeneration:

5. 5cv 0.1M EDTA (0.5ml/min)
6. 20cv ddH_2O (1ml/min)
7. 3cv $NiSO_4$ (0.5ml/min)
8. 10cvddH_2O (1ml/min)
9. 2cv binding buffer (0.5ml/min)

STEP 4: Purification using regenerated column

Procedure followed for protein purification using regenerated column:

10. 10cv equilibrium buffer
11. Cell lysate loaded carefully (0.5ml/min)
12. 5cv wash-A
13. 5cv wash-B
14. 5cv elution buffer, collected in 2ml Eppendorf tubes.

STEP 5: SDS-PAGE of the collected fractions to check for our protein.

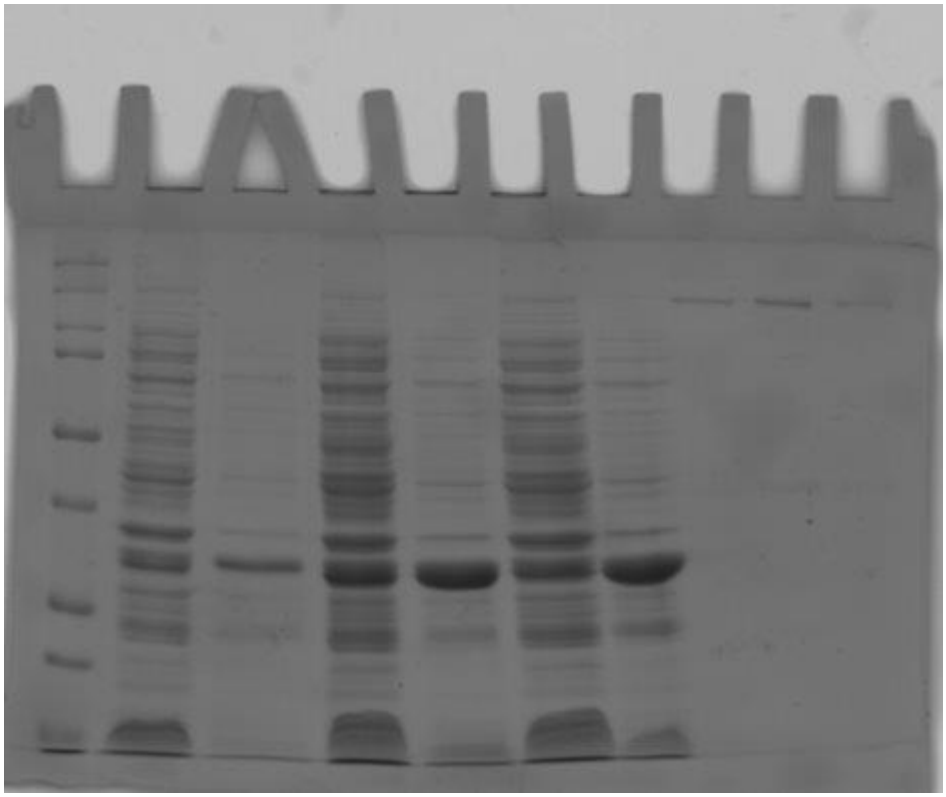


Figure 11: **SDS-PAGE gel picture of eluted fractions**

Well number	Sample
1	ladder
2	Fraction 7
3	Fraction 8
4	Fraction 9
5	Fraction 10
6	Fraction 11
7	Fraction 12

REFERENCES

1. Joseph J. Kieber and G. Eric Schallerb (2002) *Cytokinins.*

2. Venkatesan Radhika, Nanae Ueda, Yuuri Tsuboi, Mikiko Kojima, Jun Kikuchi, Takuji Kudo, and Hitoshi Sakakibara (2015) *Methylated Cytokinins from the Phytopathogen Rhodococcus fascians Mimic Plant Hormone Activity.*