# Chapter 2

# Materials and methods

## 2.1 Media

### 2.1.1 Rich Media

Luria Broth
Luria Agar

### 2.1.2 M9 minimal media

5X M9 Salt /liter **(Sambrook and Russell, 2001)**
$Na_2HPO_4.2H_20$ : 64g
$KH_2PO_4$ : 15g
NaCl : 2.5g
$NH_4Cl$ ** : 5g
Distilled water to 1000ml;

After autoclaving add
Glucose : 100mM
1M $CaCl_2.2H_2O$ : 0.1ml
1M $MgSO_4.7H_2O$ : 2ml
Thiamine .HCl : 10mg /Liter
**-Required concentration of $NH_4Cl$ was added from 1M stock solution.
Unless otherwise indicated here M9 minimal medium refers to M9 minimal medium containing 40mM $NH_4Cl$.

### 2.1.3 Antibiotic stock solutions (1000 X for rich medium)

| | |
|---|---|
| Ampicillin/Carbenicillin | : 100mg/ml in $H_2O$. |
| Kanamycin | : 50mg/ml in $H_2O$ |
| Tetracycline | : 25mg/ml in 70% Ethanol |
| Chloramphenicol | : 40mg/ml in Ethanol |
| Trimethoprim | : 60mg/ml ( in Dimethyl formamide) |
| Spectinomycin | : 50mg/ml in $H_2O$ |

For minimal medium, the antibiotic concentrations were used at half the concentration that is used for the rich medium.

X-Gal : 20 mg/ml (in Dimethyl formamide)
IPTG : 100 mM

**2.1.4 Fe source**
For Fe sufficient conditions 30mM and for Fe deficient conditions 0.9mM Ferric ammonium citrate is added.

**2.1.5 For Anaerobic assay**
0.1-0.2 mg/100ml Resazurin and 0.1%Cysteine HCl are added to both M9 and LB.

**2.1.6 Composition of Z Buffer/liter**

| | |
|---|---|
| 60mM $Na_2HPO_4.2H_20$ | : 16.1g |
| 40mM $NaH_2PO_4.H_20$ | : 5.5g |
| 10mM KCl | : 0.75g |
| 1mM $MgSO_4.7H_2O$ | : 0.246g |
| 50mM β-Mercaptoethanol | : 2.7ml |

Adjust the pH to 7.0. Store in refrigerator.
**ONPG**
Ortho-nitro phenyl pyrogalactoside (4mg/ml) in Z-Buffer.

**1M $Na_2CO_3$**

**2.1.7 Nitrogen source**
0.2% Casamino acids

1% Casamino acids

**2.1.8 Protein Molecular weight marker (sigma)**

**Stock no:** SDS-6H (molecular weight range: 30,000- 200,000)

**Content**: 3 mg (approx.) of a lyophilized mixture of 6 proteins of molecular weight shown below

**Sigma high range protein molecular weight marker**

| Approx. Molecular Weight | Approximate Amount in µg (out of 14 µg/well total loaded) |
|---|---|
| 205,000 | 5.14 |
| 116,000 | 2.9 |
| 97,400 | 2.44 |
| 66,000 | 1.65 |
| 45,000 | 1.13 |
| 29,000 | 0.75 |

**2.1.9 For expression of *xisA* following media and antibiotic concentration as well as IPTG concentration was used**

**2.1.9.1 Luria broth:** Luria broth was used for growth of the all *E. coli* strains.

**2.1.9.2 Antibiotics:** Amp 50µg/ml and Chl 34µg /ml

**2.1.9.3 Induction:** 0.5mM IPTG from 1M/ml stock

**2.1.9.4 Sonication Buffer:**

10mM Na-Phosphate Buffer pH 7.8 (1M)
50mM NaCl (5M)
5mM β-Mercaptoethanol (14.33M)
0.1 mM EDTA (0.5M)

PMSF 1mM was added to the supernatant of sonicated sample followed by Centrifugation at 10000rpm for 10 min at 4°C.

**2.1.9.5 Other buffers**

**Low salt Tris buffer**
10mM Tris-HCl Buffer pH 7.4 (1.6M)
75mM NaCl (5M)
0.1 mM EDTA (0.5M)

**High salt Tris buffer**
10mM Tris-HCl Buffer pH 7.4 (1.6M)
1M NaCl (5M)
0.1 mM EDTA (0.5M)

**Low salt phoshhate buffer**
10mM Na-Phosphate Buffer pH 7.8 (1M)
100mM NaCl (5M)
0.1 mM EDTA (0.5M)

**High salt phosphate buffer**
10mM Na-Phosphate Buffer pH 7.8 (1M)
1M NaCl or KCl (5M)
0.1 mM EDTA (0.5M)

**2.1.9.6 PEI Precipitation (10%) 0.5% final concentration**

**2.1.9.7 SDS- PAGE (Sambrook and Russell, 2001)**

A) Monomer solution (30%)

| | |
|---|---|
| Acrylamide | 14.6 gm |
| Bisacrylamide | 0.4 gm |
| Distilled water | till 100 ml |

Store in amber color bottle at 4° C in Dark

B) Separating gel buffer (pH 8.8)

| | |
|---|---|
| Tris base | 9.1 gm |
| SDS | 0.2 gm |
| Adjust to pH 8.8 with HCl | |
| Distilled water | till 50 ml |

C) Stacking gel buffer (pH 6.8)

| | |
|---|---|
| Tris | 3.02 gm |
| SDS | 0.2 gm |
| Adjust to pH 6.8 with HCl | |
| Distilled water | to 50 ml |

D) 10% APS

E) Tank buffer

| | |
|---|---|
| Tris base | 6.0 gm |
| Glycine | 28.8 gm |
| SDS | 2.0 gm |
| Water | till 2 li |

(pH should come to around 8.3)

F) Staining solution (0.025% Commassie Blue R- 250, 40% Methanol, 7% Acetic acid)

G) Destaining solution

(10% methanol, 10 % Acetic acid, add d/w to make up the volume.)

H) Water saturated n- butanol.

I) Sample Loading buffer (2X)

| | |
|---|---|
| SDS | 0.92 gm |
| β-mercaptoethanol | 2 ml |
| Glycerol | 4 gm |
| Tris base | 0.3 gm |
| Bromophenol blue ( 0.1% in water ) | 2ml |

J) Separating gel (15%, 10 ml)

| | |
|---|---|
| 30% Monomer | 5 ml |
| Separating gel buffer [pH-8.8] | 2.5ml |
| Water | 2.3 ml |
| 10% APS | 50 µl |
| TEMED | 2 µl |

K) Stacking gel (3.9%, 5 ml)

| | |
|---|---|
| 30%Monomer | 0.65 ml |
| stacking gel buffer [pH-6.8] | 1.25 ml |
| Water | 3.05 ml |
| 10% A PS | 25 µl |
| TEMED | 3 µl |

L) Sigma high range molecular weight marker

**2.1.9.8 In gel protease digestion protocol and MALDI spotting.**

Solution needed

1. 100% Acetonitrile
2. 100mM Ammonium bicarbonate
3. Resuspension Solution: 5% Acetonitrile + 0.1% TFA (Triflouroacetic Acid)
4. Extraction Solution: 60% Acetonitrile + 1% TFA.

**2.2 Strains and plasmids**

| Strains | Genotype | Reference |
|---|---|---|
| JM101 | *F'traD36lacI$^q$ Δ(lacZ)M15 proA$^+$B$^+$/supE thi Δ(lac-proAB)* | Yanisch-Perron. *et al.* (1985) |
| DH5α | *F'endA1hsdR17($r_k^-$ $m_k^+$)supE44 thi-1 recA1gyrA96(nal$^r$) relA1(lacZYA-argF)U169deoR (Ø80dlacΔ (lacZ)M15* | Hanahan (1983) |
| GM1055x56 | Ch$^R$ | Dr. Jayshree |
| Prat201 | Rf$^R$ | Dr. P. N. Mukhopadhyay |
| BL21 | *E. coli BF$^+$ dcm ompT hsdS ($rB^+$ $mB^+$) gal* | Weiner, 1994 |
| BL21(DE3) | *F$^-$ ompT hsd$_B$($r^-_B$ $m^-_B$) gal dcm (DE3)* | Novagen |
| BL26(DE3) | *F$^-$ ompT hsd$_B$($r^-_B$ $m^-_B$) gal dcm (DE3) with lacZ* | Novagen |
| pMX32 | Km$^R$ Amp$^R$ LacZ$^+$ *xisA*$^-$(defective) | Lammers *et al.*, 1986 |
| pMU575. | Promoterless LacZ Tm$^R$ | Yang & Pittard, 1987 |
| pKK1 | p*xisA* promoter in pMU575 plasmid Tm$^R$ | Karunakaran, 2000 |
| pAM1323 | 834-bp *Eco*RI-*Afl*II containing *ntcA* in pAM504; Km$^r$ Sp$^r$ | Wei *et al.*, 1994 |
| pACYC184 | Low copy compatible vector | New England Biolab |
| pTZ57R | TA cloning vector | MBI Fermentas |
| pTTQ18 | Amp$^R$ plasmid based on pBR322 | |
| pMC71AGm | *nifA* in SalI and Gm$^R$ in BamHI site of pACYC184 | This study |
| pNTMCGm | -89 to 987, N terminal region of *xisA* in ScaI of pMC71AGm | This study |
| pCTMCGm | 732-1575bp of xisA gene | This study |
| pMCXisGm | -89-1575bp of xisA gene | This study |
| pMALp2 | Expression vector | New England Biolab |
| pMALp2Xis | *xisA* cloned in BamHI and SalI site in MCS | This study |
| pEG-KT | Shuttle vector | |
| pEGKTXis | *xisA* gene in BamHI-XbaI site | This study |
| pVN6 | *C* gene in NcoI-BamHI site pET11d | Bindu *et al.*, 1997. |
| pET20b(+) | T7 based expression vector | Novagen |
| pLysE | *lys* gene downstream of *tet* promoter | Novagen |
| pLysS | *lys* gene in antisense frame of *tet* promoter | Novagen |
| pNC1 | Multiple C binding site upsteam of *lys* gene in pLysS | Ramesh *et al.*,1994a |
| pVN6xisA | *xisA* gene in BamHi site downstream of *C* gene in pET11d | This study |
| pETxisA | *xisA* gene in NdeI-BamHI pET20b(+) | This study |

## 2.3 Plasmids

### 2.3.1 pMX25 (31kb)(Lammers *et al.*, 1986)

A 17kb EcoRI DNA fragment of *Anabaena* sp. strain PCC7120 containing the entire 11kb *nifD element* along with the complete *nifK* gene and some region of *nifD* gene was subcloned into unique EcoRI site of the vector pBR322. The mini-mu-transposon carrying the kanamycin gene and β-gal (*lacZ*) gene was inserted inside the 11kb *nifD element* (outside the *xisA* ORF). The pBR322 plasmid component provides resistance to tetracycline and ampicillin. The size of the pMX25 is 31kb.

### 2.3.2 pMXΔ25 (10kb)(Lammers *et al.*, 1986)

Upon rearrangement the 11kb *nifD* element was excised out from pMX25 along with the kanamycin resistance and β-gal (*lacZ*) genes. The rearranged plasmid contains the *Anabaena* region containing *nifK* and the *nifD* genes in the pBR322 plasmid. This rearranged plasmid size is 10kb and designated pMXΔ25.

### 2.3.3 pAn207.65 (Lammers *et al.*, 1986)

A 2.4kb KpnI-HincII fragment containing complete *xisA* gene of A*nabaena* PCC 7120 *nifD* element subcloned into pUC19 multiple cloning site. This plasmid has ampicillin resistance gene and a 11bp target site for excisase A located in the upstream of the promoter region. The size of the pAn207.65 is 5.1kb.

### 2.3.4 pMC71A (Buchanan-Wallaston *et al.*, 1981)

pMC71A is a derivative of the plasmid pACYC184. The *nifA* gene from of *Klebsiella pneumoniae* was inserted into SalI site within the Tetracycline resistance gene of vector. The *nifA* is expressed constitutively under the tetracycline resistance gene promoter. The vector has chloramphenicol resistance gene. The size of pMC71A is 7kb

### 2.3.5 pMU575 (Yang and Pittard, 1987)

It is transcriptional fusion vector carrying trimethoprim resistance gene. The ribosome binding site and the first 55 codons of *galK are* fused to the eighth codon of lacZ. Stop codons are present in all the three reading frames upstream of ribosome binding site, which prevents translational interference from the cloned DNA. The λtL- terminator present upstream of the polylinker prevents transcriptional interference from other promoters.

### 2.3.6 pKK1 (Karunakaran )

The plasmid pAn207.65 carries the *xisA* gene promoter on a 1kb HindIII fragment. The fragment was purified from the gel and ligated to HindIII digested pMU575 plasmid. The recombinant plasmid pKK#1 contains the *xisA* promoter at the multiple cloning site fused to *lacZ* gene. *E. coli* transformed with pKK#1 show blue colonies on X -Gal plates. The presence of the *xisA* promoter is confirmed by the presence of 1kb fragment upon digesting the pKK#1 with restriction endonuclease HindIII. The resultant plasmid has Trimethoprim resistance.

### 2.3.7 pAM1323 (Wei *et al.*, 1994)

It contains 834-bp EcoRI-AflII fragment containing *ntcA* gene and 251 of the upstream region of *ntcA* gene in pAM504, which is having spectinomycin and kanamycin antibiotic resistance gene. pAM504 is pRL444 derivative lacking *luxAB* and containing BamHI-SstI portion of pUC18 cloning site and having kanamycin antibiotic resistance gene (Carrasco *et al.*, 1994).

### 2.3.8 pACYC184 (New England Biolab)

A 4245bp size pACYC184 is an *E. coli* plasmid cloning vector containing the p15A origin of replication This allows pACYC184 to coexist in cells with plasmids of the ColE1

compatibility group like pBR322, pUC19. It is low copy no vector, at about 15 copies per cell, but can be amplified with Spectinomycin; Chloramphenicol cannot be used for amplification due to the presence of the *cat* gene.

### 2.3.9 pTZ57R (MBI Fermentas)

A 2886 bp TA cloning vector form MBI Fermentas vector. It is very convenient vector for cloning of PCR product from MBI Fermentas.

### 2.3.10 pMC71AGm (This study)

In this plasmid Gm resistance gene was cloned in BamHI site from plasmid pGM160. It is a derivative of plasmid pMC71A, in which *nifA* gene is cloned along with some other fragment in plasmid pACYC184. It has two Chloramphenicol and Gentamycin, antibiotic resistance gene.

### 2.3.11 pNTMCGm (This study)

The N-terminus fragment was first amplified from pMX25 using Primer 5 as Forward primer which contain Restriction site Bcl1 at 5' end and sequence from –89 to –70 sequence and Primer 6 is used as Reverse primer it contain sequence from 972-987 and it is followed by termination codon and flanked by Bcl1 RE site. The amplified product was ligated in ScaI digested pMC71AGm.

### 2.3.12 pCTMCGm ( This study )

The C-terminus fragment was first amplified from pMX25 using primer 4 and primer E. Then the PCR product was digested with EcoRI and BclI, the Parent plasmid i.e; pTTQ18 was digested with EcoRI and BamHI and ligated with PCR product. The ligated plasmid will

now have C-terminus under pTaq promoter. Now using primer 4 and primer B the C-terminus under pTaq promoter was PCR amplified. The amplified product was ligated with ScaI digested pMC71AGm.

### 2.3.13 pMALp2 (6.7kb)(NEB)

It is design to create fusions between a cloned gene and *E. coli* malE gene which codes for the maltose binding protein It contains an inducible Ptac promoter position to transcribe a *malE-lacZα* gene fusion the $lacI^q$ gene encodes the *lac* repressor which turns off transcription from Ptac. The gene for ampicillin resistance and the origin of replication are derived from pBR322. The M13 origin is derived from pZ150.

### 2.3.14 pEGKT (10kb)

It is a shuttle vector carrying a two ColE1 and 2μ origins of replication. It can be used to transform *E. coli* as well as *S. cerevisiae*. It is designed to create GST protein fusions where the GST is used as a tag for purification URA3 andLeu2-d are used as selection marker it also has an ampicillin resistance.

### 2.3.15 pVN6 (Paul *et al.*, 1997)

Approximately 7.4 kb derivative of plasmid pET11d carrying C gene of Mu phage in NcoI-BamHI site. C gene is downstream to T7 promoter which is specific for T7 RNA polymerase. This plasmid is C protein expression vector.

### 2.3.16 pET20b(+) (Invitrogen)

The pET20b(+) vector of size 3716 bp, carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of

the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer.

### 2.3.17 pNC1 (Ramesh *et al.*, 1994)

Plasmid pNC1 is derivative of plasmid pLysS, in which multiple cognate sites for C protein binding downstream to promoter of *lys* gene. This plasmid can be used in combination with any pET vector for tight control through regulatory circuits.

### 2.3.18 pLysS (Invitrogne)

pLysS is 4886 bp plasmid constructed by in section of the T7 lysozyme gene into the BamHI site of pACYC184. It is used in λDE3 lysogenic host to suppress basal expression from the T7 promoter by producing T7 Lysozyme, a natural inhibitor of T7 RNA polymerase. Here T7 Lysozyme coding sequence is in the antisense orientation relative to the *tet* promoter, so only a small amount of T7 lysozyme is produced. The construct also contains the weak T7 ϕ3.8 promoter immediately following the lysozyme gene. The p15A origin of replication is compatible with those found in pBR322 and pUC derived plasmids.

### 2.3.19 pLysE (Invitrogen)

pLysS is 4886 bp plasmid constructed by in section of the T7 lysozyme gene into the BamHI site of pACYC184. It is used in λDE3 lysogenic host to suppress basal expression from the T7 promoter by producing T7 Lysozyme, a natural inhibitor of T7 RNA polymerase. Large amount of T7 Lysozyme are produced form the *tet* promoter. The construct also contains the weak T7 ϕ3.8 promoter immediately following the lysozyme gene. The p15A origin of replication is compatible with those found in pBR322 and pUC derived plasmids.

### 2.3.20 pVN6xisA (This study)

The *xisA* gene, amplified form the plasmid pAN207.65 form the second start site is cloned in the BamHI site of plasmid pVN6 which is derivative of plasmid pET11d.

### 2.3.21 pETxisA (This study)

The *xisA* gene, amplified form the plasmid pAN207.65 form the first start site is cloned in the NdeI-BamHI site of plasmid pET20b(+).

## 2.4 Methods

### 2.4.1 Isolation of plasmid DNA

The plasmid DNA isolation was done by the alkali lysis method as described by Brinboim and Doly (1979).

### 2.4.2 Transformation of plasmid DNA

The method of transformation was carried out using $CaCl_2$ method as described by Cohen et *al.* (1972).

### 2.4.3 Restriction enzyme digestion analysis

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 2-4U of the restriction enzymes were used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 20µl. The digestion was allowed to go for overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were photographed.

### 2.4.4 Ligation (Sambrook *et al.*, 1989)

50-100ng of DNA was used in each ligation reaction. Vector to insert ratio was maintained at either 1:1 or 1:2. The ligation reaction was usually done in 15µl volume containing ligation buffer and 0.5-1.0U of T4 DNA ligase. The ligation reaction was carried out at 16°C for 12-16h.

### 2.4.5 Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% Agarose gel in 90mM Tris-Borate buffer at 5v/cm for 2-4h. The gel was stained in 1µg/ml ethidium bromide for 30min at room temperature and the bands were visualized by fluorescence under the UV-light.

### 2.4.6 Monitoring excision of *nifD* element by monitoring rearrangement of pMX25 plasmid in *E. coli*

Rearrangement assay of Lammers *et al.* (1986) was used with modifications. A single colony of *E. coli* containing pMX25 plasmid overnight was grown in 15ml Luria broth with kanamycin, and ampicillin. 0.1ml cells from $10^{-4}$ dilution (diluted in saline) were plated on Luria agar containing ampicillin X-Gal. The 0h plating was done to ensure that there are no whites present in the starting culture. Kanamycin was added to ensure that the rearranged colonies are eliminated.

From this overnight grown culture 1.0 ml was spun at 5000 rpm at 4° C for 5min. The pellet was washed twice with either Luria broth or minimal medium with ammonium chloride and suspended in same volume of Luria broth or minimal medium with ammonium chloride.

1% of (150µl) of the cells was reinoculated either in 15ml of Luria broth, M9 minimal medium with NH4Cl, M9 minimal medium with 0.2% casamino acid with glucose as carbon source, M9 minimal medium with 1% casamino acid with glucose as carbon source and M9 minimal medium with 1% casamino acid without glucose as carbon source, containing only ampicillin. The cultures were allowed to grow for 24 h at 37°C. After 24 h of growth, again the dilutions were made and plated on Luria agar containing X-gal and ampicillin. The excision was calculated using this formula.

$$\text{Excision (\%)} = \frac{\text{Total number of Whites}}{\text{Total number of colonies}} \times 100$$

After counting the number of colonies whites and blues were picked and restreaked on Luria agar containing kanamycin and Luria agar containing ampicillin. The white colonies grew only on ampicillin containing plates but not with kanamycin but blue colonies grew on both plates. Plasmids from white and blue colonies were digested with restriction enzymes to confirm the pattern's resemblance to the rearranged and unrearranged DNA respectively.

### 2.4.7 Effect of $Fe^{3+}$ on the rearrangement of *nifD* element in *E. coli*

To study the effect of $Fe^{3+}$ on rearrangement of *nifD* element, the cells were grown in the minimal medium supplemented with 0.9μM Ferric ammonium citrate (Fe limiting condition) and 30μM Ferric ammonium citrate (Fe excess condition) .The rearrangement assay was same as described earlier.

### 2.4.8 Excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* strain JM101 under anaerobic conditions (Gerhardt *et al.*, 1981).

A single colony of *E. coli* strain to be assayed is inoculated into LB with appropriate antibiotics. The culture was grown overnight. 1% of inoculum was inoculated to either M9 or LB after washing with normal saline. This is added to 1.5 ml of the medium in microfuge tubes containing resazurin (0.1mg/100ml) and Cystine HCl (0.05%). The media is boiled before addition of the last two components to remove any traces of dissolved oxygen. The media is exposed to maximum light for 20 minutes to generate maximum reducing conditions before inoculation of culture. The culture is incubated in dark (to avoid photo degradation of Cystine HCl) at 37°C for 24h with out shaking (to avoid aeration). This is plated on LA plates with X-gal and appropriate antibiotics.

**2.4.9 β-Galactosidase Assay (Miller, 1992)**

The *E. coli* strains were grown overnight in Luria broth, M9 minimal medium with $NH_4Cl$, M9 minimal medium with 0.2 % casamino acid with glucose as carbon source, M9 minimal medium with 1% casamino acid with glucose as carbon source and M9 minimal medium with 1% casamino acid without glucose as carbon source with appropriate antibiotics at 37°C until the cultures reach a density of 2-5X$10^8$ cells/ml (an $OD_{600}$ of 0.28-0.70). The cultures were kept on ice for 20min. 0.05- 0.2 ml volumes cultures was added to the assay medium (Z-Buffer). The cells were ruptured by adding 2 drops of chloroform and 1 drop of 0.1% SDS to each assay mixture. The tubes were vortexed for 10sec.

The reaction was started by adding 0.2ml of ONPG; the contents were mixed for few sec. After sufficient yellow color has developed, the time of reaction was recorded and the reaction was stopped by adding 0.5ml of 1M $Na_2CO_3$ solution. For each tube the optical density was measured at 420nm as well as 550nm.

For *E. coli*, the scattering = 1.75 x $OD_{550nm}$

The enzyme units were calculated as follows:

$$\text{Units of } \beta\text{-galactosidase} = \frac{1000 \times (OD_{420} - 1.75\ OD_{550})}{t \times v \times OD_{600}}$$

t- Time of the reaction in minutes.

v- Volume of the culture used in ml in the assay.

These units are proportional to the increase in the o-nitrophenol per minute per bacterium.

For assaying β-galactosidase activity in stationary phase, the cultures were grown for 24h ($OD_{600}$ of 1.6-2.0), then dilution were made in Z-Buffer (1:10), 0.1-0.2 ml volumes used for assay.

### 2.4.10 Effect of NtcA on the excision of substrate plasmid in the presence of *xisA* gene

A single colony of *E. coli* harboring pMX25 and pAM1323 plasmids was grown overnight in 15ml Luria broth with kanamycin, spectinomycin and ampicillin. Rest of steps of rearrangement assay was performed according to the excision of *nifD* element from substrate plasmid pMX25. M9 minimal medium was used where $NH_4Cl$ used as nitrogen source.

### 2.4.11 Effect of NtcA on the promoter activity of *xisA* gene

The *E. coli* strains harboring plasmid pMU575 & pAM1323 and pKK1-& pAM1323 were grown overnight in Luria broth and M9 minimal medium with $NH_4Cl$ with appropriate antibiotics at 37° C until the cultures reach a density of 2-5X$10^8$ cells/ml (an $OD_{600}$ of 0.28-0.70). The cultures were kept on ice for 20 min and rest of the step was performed according to the β assay of *xisA* promoter using plasmid pKK1.

### 2.4.12 Isolation of Genomic DNA form culture of *Anabaena* sp strain PCC 7120

Isolation of the genomic DNA from *Anabaena* PCC 7120 was done by the method of Xiaoqiang *et al.*, 2000.

### 2.4.13 Amplification of *ntcA* gene form genomic DNA of *Anabaena* sp strain PCC 7120

*Anabaena* sp. strain PCC 7120 genomic DNA was used to amplify *ntcA* gene with following primers, forward primer 5' CGG ATC CTA TTT CTT AAG ATA TTT TCA AGA 3' and reverse primer 5' CGG TCG ACC TGA ATT CTG ACT CCT GCT ATT 3. Forward primer contains BamHI site at 5' end and reverse primer contains SalI site at 5' end. *ntcA* gene amplified by temperature profile 94° C for 5'first program followed by second program

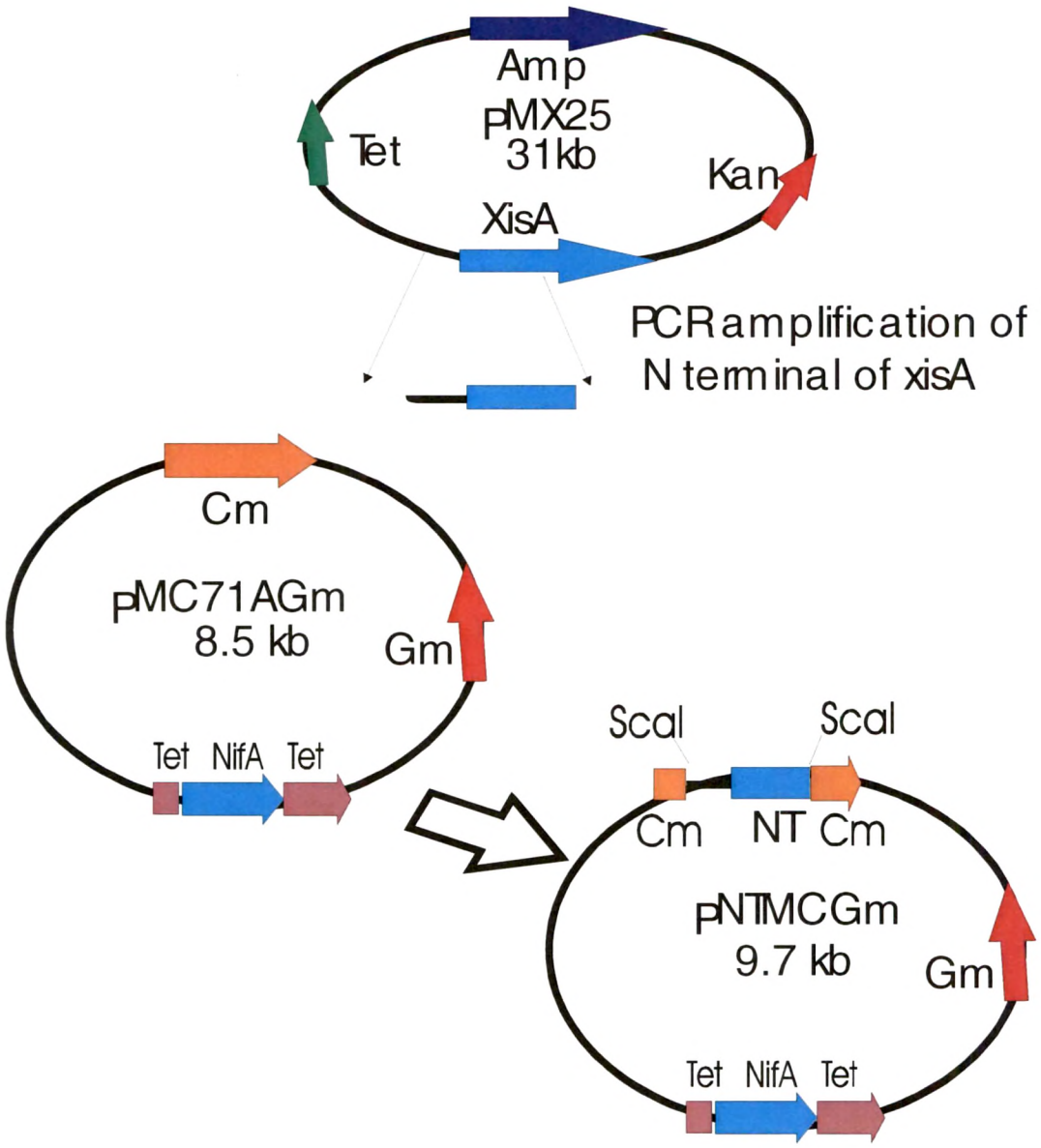
of 94° C for 30 s, 42° C for 30 s and 72° C for 40 s repeated for 5 cycles, third program of 94° C for 30 s, 52° C for 30 s and 72° C for 40 s repeated for 25 cycles and last fourth program of 72° C for 10'.

### 2.4.14 Cloning of *ntcA* gene in pACYC184

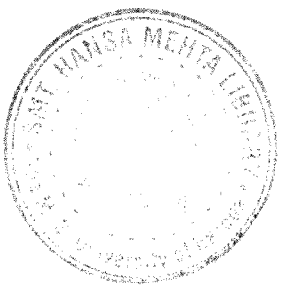
PCR cloning of *ntcA* was unsuccessful in the BamHI and SalI site of the pACYC184 plasmid.

### 2.4.15 PCR Amplification of N terminal region of *xisA* gene

Plasmid pMX25 was used as template for amplification of N terminal region of *xisA*. Forward primer 5' GGT GAT CAT AAT GTT TGC AC T GAG CAG TGT 3' and reverse primer 5' GGT GAT CAT CAG CGT AAA CCA AAA ACT GCT 3' was used to amplify region which include promoter and N terminal region of *xisA*. Both primers contain BclI restriction endonuclease site for to facilitate cloning by restriction digestion of amplified product before ligation with vector. Reverse primer include termination codon at 5' end followed by BclI RE site. PCR was carried out in a Progene (Techne) with Taq polymerase (Bioron gmbh) essentially according to the manufacturer's instruction. All PCR were of 50µl volume and contained 25pmol oligonucleotide primer and a final concentration of 1.5mM $MgCl_2$. Plasmid DNA pMX25 contained 0.01µg concentration was used as template. Reaction was cycled at initial denaturation at 94° C for 1 min followed by 94° C for 30 s, 42° C for 30 s and 72° C for 1min 36 s for 5 cycles and then 94° C for 30 s, 52° C for 30 s and 72° C for 1 min 36s for 25 cycles and finally at 72° C for 10 min for extension. 1,095 bp size amplicon that encodes for 1-287 amino acid residues of *xisA* gene was obtained. As per the schematic diagram N terminal was cloned in the plasmid pMC71AGm.




Schematic representation of the construction of N terminal of *xisA* gene. Tet – Tetracycline resistance gene, NifA- *Klebsiella pneumoniae nifA* gene, Gm- gentamycin resistance gene, Cm- Chloramphenicol resistance gene, Kan- Kanamycin resistance gene, Amp- Ampicilin resistance gene



### 2.4.16 Cloning and screening of positive clone

*E. coli* strain GM1055x56 which is dam$^-$ and dcm$^-$ was used for preparation non-methylated pACYC184 and pMC71A for direct cloning of PCR product containing N terminal region of *xisA*. It was unsuccessful using BclI restriction endonuclease may be due to the inefficiency of ligation. Since Taq DNA polymerase adds Adenine in 70% of the amplicons, therefore, 30% of the amplified product could be ligated with blunt ended vector. Using this approach, N terminal amplicon was cloned at the ScaI site. The recombinant plasmid was transformed into an *E. coli* strain Prat201 kindly provided by Dr. PN Mukhopadyaya, NDDB, Anand. *E. coli* Prat201 strain has rifampicin resistance which helps in the selection of positive clones on the Luria agar containing gentamycin and rifampicin. Colonies containing the N terminal fragment was selected by isolation of plasmid, and then confirmed by restriction digestion and colony PCR.

### 2.4.17 Endonuclease assay

To monitor the endonuclease activity of N terminal of *xisA*, *E. coli* DH5α was used. *E. coli* DH5α harboring substrate plasmid pMX25 was used as a control and while *E. coli* DH5α harboring substrate plasmid pMX32 was used to monitor the endonuclease activity of the either N terminal (plasmid pNTMCGm) of *xisA*.

### 2.4.18 Endonuclease and excision activity of N terminal region of *xisA*

A single colony of *E. coli* strain DH5α containing substrate plasmid pMX32 in combination with plasmid pMC71AGm and pNTMCGm was grown overnight at 37°C in 10 ml Luria broth with ampicillin, kanamycin, and gentamycin. Endonuclease activity and excision activity was performed according to rearrangement assay. 24h plating of *E. coli* culture was done on petri plate containing Luria agar and X-gal, Luria agar along with ampicillin and X-gal, Luria agar along with gentamycin and X-gal and Luria agar along with antibiotics ampicillin and gentamycin and X-gal. Endonuclease activity and rearrangement frequencies were calculated.

### 2.4.19 Expression studies of the N terminal of *xisA*

For expression studies of N terminal of *xisA* gene cells were collected after 24 h followed by 2 h of induction by 0.2 mM IPTG. Here 1 ml of cells was treated with sample buffer and equal amount of protein was loaded in each well of 15% SDS-PAGE gel.

### 2.4.20 Amplification of *xisA* without promoter

The *xisA* gene was amplified with forward primer 5' CGA ATT CGG ATC CAT GCA AAA TCA GGG TCA AGA CA 3'and reverse primer 5' TGA GTC GAC TGA TCA AAG CAT TGA GCA GAT 3'using plasmid pMX25 as a template. Temperature profile of the PCR was carried out in a Progene (Techne) with Taq polymerase (Bioron gmbh) essentially according to the manufacturer's instruction. All PCR were of 50µl volume and contained 25pmol oligonucleotide primer and a final concentration of 1.5mM $MgCl_2$. Plasmid DNA pMX25 contained 0.01µg concentration was used as template. Reaction swas cycled at initial denaturation at 94° C for 1 min followed by 94° C for 30 s, 42° C for 30 s and 72° C for 1min 36 s for 5 cycles and then 94° C for 30 s, 52° C for 30 s and 72° C for 1 min 36s for 25 cycles and finally at 72° C for 10 min for extension.

### 2.4.21 Cloning of amplified *xisA* in plasmid pMALp2

Plasmid pMALp2 was digested with two restriction enzyme BamHI and SalI separately which was used as vector for cloning of *xisA* gene. PCR amplified *xisA* was also digested with BamHI and SalI restriction enzyme which was used as insert in cloning strategy. Ligated mixture was transformed in to the DH5α. Cloning was done in MCS of the plasmid pMALp2; here it will disrupt *lacZ* so it was selected by blue white assay. Positive clones were checked with restriction digestion and isolated in large quantity and again transformed in *E. coli* strain BL21.

### 2.4.22 Standardization of partial purification of MBP-*xisA* fusion

In pMALp2 based protein purification it is very much important to separate periplasmic protein form the cytoplasmic proteins. Once bacterial culture reach at 1 OD at 600 nm at 25°C and 37°C in volume of 10 ml LB, induction of expression of fusion protein was done by 1mM IPTG for 2 h as well as for 3 h, 4 h, like wise. When used as a whole cell it was very difficult to achieve detect protein.

### 2.4.23 Expression of MBP-XisA fusion protein

Protocols were used as given in Riggs *et al,* 1990 with modifications.

### 2.4.24 Expression of MBP-XisA fusion Protein at 37°C from 10 ml LB

Single colony of each pMALp2 and pMALp2-Xis in 10 ml LB having ampicilin inoculated and grown at $37^0$C at 200 rpm on a rotatory shaker till O.D. reached 0.6. Cultures were divided into 2-2 flasks of 5-5 ml each under sterile conditions. 1 mM IPTG (final concentration) was added in one-one flask of each P2 and P2-Xis for induction (Other set of flask was used as control- un-induced). All the flasks were incubated at $37^0$C for 2 hours at 200 rpm on a rotatory shaker. Cells were pelleted and washed with normal saline at $4^0$C. 50µl sample buffer (2X) was added to such samples. Samples were boiled for 5 minutes. 30 µl/well of this treated sample was loaded on 7.5% SDS- PAG and presence of MBP-XisA was monitored around 97.6 KDa.

### 2.4.25 Expression of MBP-XisA fusion Protein at 37°C from 100 ml LB

1% young culture of pMALp2 and pMALp2-Xis in 100 ml LB having Ampicilin was inoculated and grown at $37^0$C at 200 rpm 200 rpm on a rotatory shaker till O.D. reached 0.6. Cells were pelleted and resuspended in 10 ml fresh LB. 1 mM IPTG (final concentration) was added in one-one flask of each P2 and P2-Xis for induction (Other set of flask was used as control- un-induced). All the flasks were incubated at $37^0$C for 2 hours at 200 rpm on a

rotatory shaker. Cells were pelleted and washed with normal saline at $4^0$C. 50µl sample buffer (2X) was added to such samples. Samples were boiled for 5 minutes. 30 µl/well of this treated sample was loaded on 7.5% SDS- PAG and presence of MBP-XisA was monitored around 97.6 KDa.

**2.4.26 Expression of MBP-XisA fusion Protein at 37°C from 100 ml LB and extraction from periplasm**

Osmotic shock with Sucrose Tris-Cl method (Kelleman *et al* 1982).

**2.4.27 Expression of MBP-XisA fusion Protein at 25°C from 100 ml LB**

Protocols were same as above except that the temperature used to induce the protein after IPTG addition was kept at $25^0$C for all the flasks.

**2.4.28 Electroporation** (Sambrook et al 2001 with modifications)

Single bacterial colony was inoculated in 3 ml LB (Amp) and grown overnight at $37^0$C at 200 rpm on a rotatory shaker. 1% of respective culture was inoculated into 100 ml LB and grown till O.D. reached to 0.6. Cells were pelleted at 5,000 g for 5 minutes at $4^0$C. Pellet was washed twice in 15% glycerol (10ml) and incubated at $4^0$C for 30-30 minutes respectively. After centrifugation pellet was resuspended in a final volume 2 ml, 15% glycerol. 200 µl culture aliquots were used for electroporation. 2µl (around 50 ng) plasmid was added to it. A pulse at 2500 mV for 10 µ sec was given. (Eppendorf electroporator was used). 800 µl LB was added to such cells and were grown for 1hour/ 200 rpm/ $37^0$C and cell were plated on plate having appropriate antibiotics.

**2.4.29 SDS- PAGE**

It was performed by Laemmili, 1970 method.

### 2.4.30 Amplification of *xisA* for cloning in pVN6 and pET20(+)

Three primers PSN1 5' GCG GAT CCG CAG CAA AGA GAA C 3', PSN2 5'GCG GAT CCA AGC ATT GAG CAG 3'and PSN3 5' CGC ATA TGC AAA ATC AGG GTC 3' were used for the amplification of *xisA* for two different sizes depending upon the start site. The combination of primers and temperature profile are as under:

To clone *xisA* from first start site, it was amplified using primers PSN3 (forward) and PSN2 (reverse). While for cloning from second start site primers PSN1 (forward) and PSN2 (reverse) was used. Here, plasmid pAN207.65 was used as template.

Following temperature profile was used for the amplification of the *xisA*. First cycle 95°C for 4 min followed by second cycle 95°C for 45 s, 50°C for 45 s and 72°C for 3 min. Second cycle was repeated for 30 times, which was followed by final elongation temperature at 72°C for 15 min.

### 2.4.31 Restriction Digestion

Restriction Digestion of amplified product and vector in which pcr product has to be cloned were digested by either BamHI or BamHI- NdeI depends on the strategy.

### 2.4.32 Dephosphorylation of both the vector (pET20b(+) and pVN6)

Both the vector were treated with 2µl SAP (Shrimp Alkaline phoshphatase) (BM) at final concentration of 1U/µl in 20 µl system. Incubate the system at 37°C for 45 min. Heat inactivate the SAP by incubating it at 70°C for 20 min. Now precipitate the dephosphorylated DNA with ethanol at -70°C and wash the DNA pellet with 70% ethanol and dissolved dried DNA pellet in 10 µl of Double distilled water.

### 2.4.33 Ligation

1:3 vector to insert ratio was taken for the ligation in 10 µl system where 1U/ µl of ligase added.

### 2.4.34 Induction of *xisA* through IPTG

*E. coli* BL26 (DE3) harboring both plasmids was grown for 12–16 h at 37 °C in 5 ml of LB medium containing 20 µg/ml Chloramphenicol and 100 µg/ml ampicillin, and 1% cells were inoculated in to the 10 ml LB medium with both the antibiotics. When it reached to $OD_{600}$ ≈0.8, then XisA expression was induced by adding IPTG to the final concentration of 0.5mM. At the same time 1 ml of cells were harvested and kept at 4 °C until the final induced sample was harvested. After 4 h induced bacteria was harvested and centrifuged to 10 K for 10 min. The induced and uninduced cells were lysed by adding 6X loading dye at final concentration of 1X and then boiled for 10 min. These samples were then centrifuged in the microfuge at highest speed for 10 min and then supernatants were loaded on 10% SDS-PAGE.

### 2.4.35 Standardization of *xisA* purification

The *E. coli* containing both plasmids (pNC1 and pVN6xisA) was grown in 2 l medium of LB broth containing 20µg/ml chloramphenicol and 100µg/ml ampicillin. When cell grown up to $OD_{600}$ ≈0.8. *E. coli* cells were induced by adding IPTG to a final concentration of 0.5mM. After grown at 37°C for 4 h 1ml of 10 samples was removed to check on the SDS-PAGE for the expression of the protein, here cells were centrifuged and mixed with 20µl of 6X loading dye and 100µl of distilled deionised water and then boiled for 5-10 min in BWB and loaded on the 10% SDS-PAGE. Rest of the cells were centrifuged in cold condition and kept at 4° C. Once protein profile confirmed the expression of *xisA* gene rest of the cells were further used for purification. Here cells were suspended in the 10 ml of sonication buffer (10mM Potassium phosphate Buffer pH 7.4, 50mM NaCl, 0.1 mM EDTA, 5mM β-Mercaptoethanol) and sonicated in cold condition rest all process performed at 0-4°

C. The cells were centrifuged at 13K for 20 min at the 4°C to remove the cell debris along with the intact cells. After sonication pellets and supernatant were collected in separate tubes and pellet was again dissolved in the same amount of sonication buffer. 20 µl of sample of both the fraction was used to check the expression of *xisA* gene expression on 10% SDS-PAGE. Then supernatant was added to ultracentrifuge tube and centrifuged at 30,000 rpm for 3½ h. Supernatant was collected in fresh tube and concentration of salt was made to 100mM NaCl. These samples were loaded on SDS-PAGE, *xisA* gene expression was present in the pellet fraction and supernatant fractions. XisA protein was found mainly in the pellet fractions. Supernatant was used for the ammonium sulphate precipitation; here total supernatant was used for the 0-30% ammonium sulphate precipitation. Ammonium sulphate powder was added to the supernatant slowly and mixed in the cold condition in stirring condition. Precipitated protein was centrifuged at 13K at 4°C for 10 min. Pellet collected, dissolved in the buffer A (10mM Potassium phosphate Buffer pH 7.4, 100mM NaCl, 0.1 mM EDTA, 5mM β-Mercaptoethanol) and supernatant was used for the 30-45% ammonium sulphate precipitation, again pellet was colleted and supernatant was used for 45-55% ammonium sulphate precipitation. All the pellets were dialyzed against the same buffer. Final supernatant was also collected; all the samples were used to check expression of XisA in 10% SDS-PAGE. All ammonium sulphate cut samples were checked for the activity of XisA using the two substrate plasmid pMX32 and pBR322NG1. There was no endonuclease activity in the all the fractions.

When 0-60% ammonium sulphate cut precipitated proteins were loaded on the phosphocellulose column where it will acts as a negative charge and acts as a anion exchanger where XisA should bind since it is DNA binding protein, but XisA is not binding to the phosphocellulose column which was shown by the all the fraction collected.

The XisA expression in presence of the regulatory circuit was very low and was not increased by prolonged induction with IPTG as well as different IPTG concentration. In pVN6xisA plasmid *xisA* gene was cloned form the second ATG and gene expression was so low that pVN6xisA can not be used further for the expression and purification.

The other clone pETXisA which contains the *xisA* gene from the first ATG was used for the XisA expression with different plasmid combination in two *E. coli* strains BL21 and BL26.

### 2.4.36 Precipitation of DNA with PEI

The *E. coli* culture was grown in 2l Luria broth and cells were collected through centrifugation at 10,000 rpm for 10 min at 4°C. After adding sonication buffer cells were sonicated, these cells were centrifuged again at 13,000 rpm for 30 min at 4°C. Pellets were collected in separate container. Supernatant was collected in the separate tube and divided in three equal parts. All parts contain low salt in the sonication buffer and in that final salt concentration was made 50mM, 100mM and 200mM respectively. At 4°C Poly Ethene Imine(PEI) was added at 0.5%, final concentrations. All three samples were again centrifuged at 13000 rpm at 4°C for 10 min and pellet and cells were loaded on the SDS-PAGE. Maximum protein was found in the lane in which salt concentration is 100mM and in the supernatant fraction. These samples are further used for the ammonium sulphate precipitation. Here different salt concentration was used for final concentration that is 50mM, 100mM and 200mM NaCl.

### 2.4.37 Ammonium sulfate precipitation of protein

All three supernatant were pooled and dialysed against low salt buffer. This dialysed sample was used for the ammonium sulphate precipitation. Three different percentage of ammonium sulphate, 0-30%, 30-45% and 45-55% were used for the precipitation. After adding ammonium sulphate powder to the supernatant proteins precipitated, the pellet is dissolved in buffer and dialysed against the low salt buffer and loaded on the SDS-PAGE. Where it was found that XisA precipitated in the range of the 30-45% of ammonium sulphate. These fractions were further used to purify the XisA through phosphocellulose column previously equilibrated with the same low salt buffer.

### 2.4.38 Dialysis of protein sample

Dialysis membrane was used to dialysis of samples; here dialysis was done to remove salt ammonium sulfate from the sample which was treated with ammonium sulphate to precipitate the protein against the buffer with low salts.

### 2.4.39 Column preparations

To prepare the phosphocellulse column, take 3 gm solid phosphocellulose was taken and washed with 10 bed volume of distilled water. It is packed in the column and equilibrated with buffer. To activate the column it was first washed with 0.5 N NaOH and then washed with 10 bed volume of water and then again washed with the 0.5 N HCl and then again washed with 10 bed volume of water. Again column material was equilibrated with 100 mM phosphate buffer.

### 2.4.40 Column loading

Phosphocellulose column was washed with 10 bed volume of high salt buffer (10mM phosphate buffer, 1M NaCl of KCl, 0.1 mM EDTA except β-Mercaptoethanol). It is followed by washing of the column material with 10 volume of low salt buffer (10mM phosphate buffer, 100mM NaCl of KCl, 0.1 mM EDTA). Load protein on column and washed with gradient buffer (100mM NaCl & 2 M NaCl, each 45-45ml in gradient mixer, here high salt buffer in outer chamber and low salt buffer in inner chamber) and collect the fractions (1ml/min).

### 2.4.41 Preparation and loading of Phosphocellulose column

Phosphocellulose column was prepared to load the 0-45 % ammonium sulphate precipitated supernatant and pellet. All precipitated product up to 0-45% was pooled for the loading on column. Phosphocellulose column was washed with 10 bed volume of high salt buffer wash which contain 1M NaCl or KCl at the flow rate of 1 ml/min. It was followed by

buffer wash which contains 100mM NaCl. Here buffer which are used should be bubble free and should be sonicated and passed through filter which will remove particulate matter. In gradient mixer, gradient was created by adding 2M (outer chamber) and 100mM (inner chamber) salt containing buffer. If protein contains some particle or not dissolved properly then protein sample was passed through 0.22µm Millex GP filter unite contains PES membrane. 1ml/min flow rate was set and 3ml sample per tube was collected in the sterile tube. After collection of samples column was washed with low salt buffer. All the samples were loaded on the 7% SDS-PAGE. XisA protein went to flow through that means XisA is not able to bind strongly to the column material which is having negative charge, but part of it is binding which is seen in the fraction number 8, 9 and 10.

### 2.4.42 Preparation and loading of heparin column on FPLC

Phosphate buffer, which is not suitable for the FPLC, thus flow through of the phosphocellulose column which contains phosphate buffer was converted to tris buffer pH 7.4. Flow through was dialyzed against 75mM NaCl containing Tris buffer (10mM TrisHCl pH7.4, 75mM NaCl and 0.1mM EDTA). Dialyzed sample was passed through 0.2µm Filter. Heparin column was washed with distilled water to get conductivity zero. Once conductivity reach to zero, then column washed with absolute alcohol to remove any trace protein. Collected flow through was loaded on the heparin column and flow rate was set at 1ml/min. Both high salt buffer and low salt buffer were provided in the separate bottle. FPLC was able to mix it in the form of gradient. Fractions were again loaded with control samples which are different samples from the starting of the purification.

Fractions 3 to 15 contain XisA protein, XisA protein loosely binding to the column materials. Out of 47 fractions of size 3ml, fractions 3 to 15 are initial fractions so XisA protein is unable to bind to the heparin which is again negatively charge material means XisA protein was unable to bind phosphocellulose and heparin. Thus, use positively charge material like Mono Q was necessary to check for binding of the XisA protein.

### 2.4.43 Preparation and loading of Mono Q column on FPLC

Mono Q was used to purify XisA protein form the initial fractions 3 to 15. Here all the fractions from no 3 to no 15 were pooled and loaded on the FPLC. Where Mono Q (1ml) was used as column, it was prepared by washing it with distilled water followed by absolute alcohol and fractions were collected same way as described earlier.

Collected samples were loaded on the 7% SDS-PAGE. Fractions loaded on Lane 6 to 9 contains very high amount of the XisA which can be further purified by diluting the gradient of the salt concentration of the buffer.

### 2.4.44 Activity assay with pMX32 and pBR322NGI

A partially purified fraction of the XisA was used to check the activity of the purified protein with two different substrate plasmids pBR322NGI and pMX32. Standard buffer form New England Bioloabs was used for this activity. Activity was also checked at various temperatures with different fractions.

### 2.4.45 In gel protease digestion protocol and MALDI spotting

Excise strand gel piece into 1mm and transfer in to sterile microfuge. Wash gel with 500µl wash solution [50% acetonitrile, 50mM Ammonium bicarbonate] and incubate it at room temperature for 15 min with gentle agitation [vortex mix in its lowest setting]. Remove solution with pipette. Wash gel two more times with 500 µl wash solution until coommasiae dye has been removed. Dehydrate the gel in 100% acetonitrile for 5 min, when dehydrated the gel piece will be seen opaque with colour and will be significant smaller in size. Remove Acetonitrile with a pipette an then completely dry the gel at RT for 0-20 min in to centrifugal evaporator. While gel is drying prepare protease digestion solution. Typically this is modified sequencing grade Trypsin (product No. V5III, promega, Madison, WI) Resuspend lyophilized Trypsin (20µg/vial) in 1ml of 50mM Ammonium bicarbonate. Aliquot 50µl /tube and store at -70°C. Do not use more than once. Rehydrate the gel with minimum volume of

protein digestion solution. Use 20µl for small gel pulps. Digest at 37°C for 16 h. Spindown sample by centrifugation at 12 K for 30 min. Transfer supernatant (containing tryptic peptides) to sterile microfuge tube. Add 25-50 µl of extraction Solution (60% Acetonitrile, 1% TFA) to gel piece and agitate gently by vortexing at lowest setting. Spin down sample by brief centrifuge at 12K for 30 min and transfer supernatant (containing additional tryptic peptide) to tube. Extract the gels with additional 25-50 µl solution by gentle vortexing. Vortexing and spindown sample and transfer supernatant to the earlier tube. Dry and pooled peptides using centrifuge evaporators to near dryness for some time. Do not heat. Do not dry to extended time. Add 10µl resuspension solution (50% Acetonitrile, 0.1% TFA) to each tube and gently agitate on a vortexer at lowest settings. Spin down sample, spot 0.5µl of α cyano 4 hydroxy cinnemic acid matrix (10mg/ml into 50% acetonitrile, 0.1% TFA). Allow spot to dry completely load peak into voyager. Calibrate using internal tryptic peaks of 842.5 to 2211.1 Dp. Notes: Always use non latex gloves when handling sample. Karatine and latex proteins are proteins are potential source of contaminations.