Chapter 7

Cloning, expression and partial purification of *Anabaena* PCC 7120 XisA protein

7.1 Introduction

Anabaena sp. strain PCC 7120 *xisA* gene contains an open reading frame with two in-frame start codons 117 base pairs (bp) apart and can code for a protein with a calculated molecular weight of 46,000 Da if translated from the first start codon or 41,500 Da if translated from the second start codon (Brusca *et al.,* 1990). Earlier *xisA* gene was expressed under *tac* promoter in *E. coli* DH5α and XLBlue strains. In *E. coli* DH5α, *xisA* expression was toxic and cell grew poorly but in *lac* repressor containing *E. coli* XL1Blue strain grew normally probably because of low level of XisA protein. Expression of *xisA* gene using *Klebsiella pneumoniae* NifA transcription factor resulted in very high increase in the excision frequency in M9 minimal medium (Karunakaran, 2000). Labeling of proteins during the conditions favoring very high excision frequency led to the detection of a 46kDa protein corresponding to the estimated XisA protein. Interestingly, XisA protein also showed restriction endonuclease activity leading to the loss specific target site containing plasmids which was directly correlated with the excision frequency. Thus, lack of success in the detection of XisA protein by overexpression of *xisA* gene could be attributed to the restriction endonuclease activity.

Many strategies have been developed to overexpress toxic proteins and amongst them T7 promoter system is prominent (Paul *et al.,* 1990). Comparison with different systems like T7 promoter using pET system plasmids and regulatory circuit plasmid were analyzed. Restriction endonuclease KpnI (R. KpnI) is toxic at very low level and using regulatory circuit strategy in which the expression was under tight control, enzyme was purified at high level. Alternative strategy involving pLysS and pLysE along with expression vectors of pET also used for purification of toxic protein.

Objectives of the present studies is to overexpress the protein, purification of the protein and to determine the excisase/endonuclease activity. Since XisA protein is toxic expression has been carried out using regulatory circuit and pLysS/pLysE strategies were used. Because two putative start site protein overexpression studies were corresponding to both the start codon.

7.2 Materials and Methods

Please refer chapter materials and methods.

7.3 Results and Discussion

7.3.1 PCR amplification of *Anabaena* PCC 7120 *xisA* gene

Anabaena PCC 7120 *xisA* gene was amplified form two different start sites using two specific forward primers and with a common reverse primer (**Fig 1**).

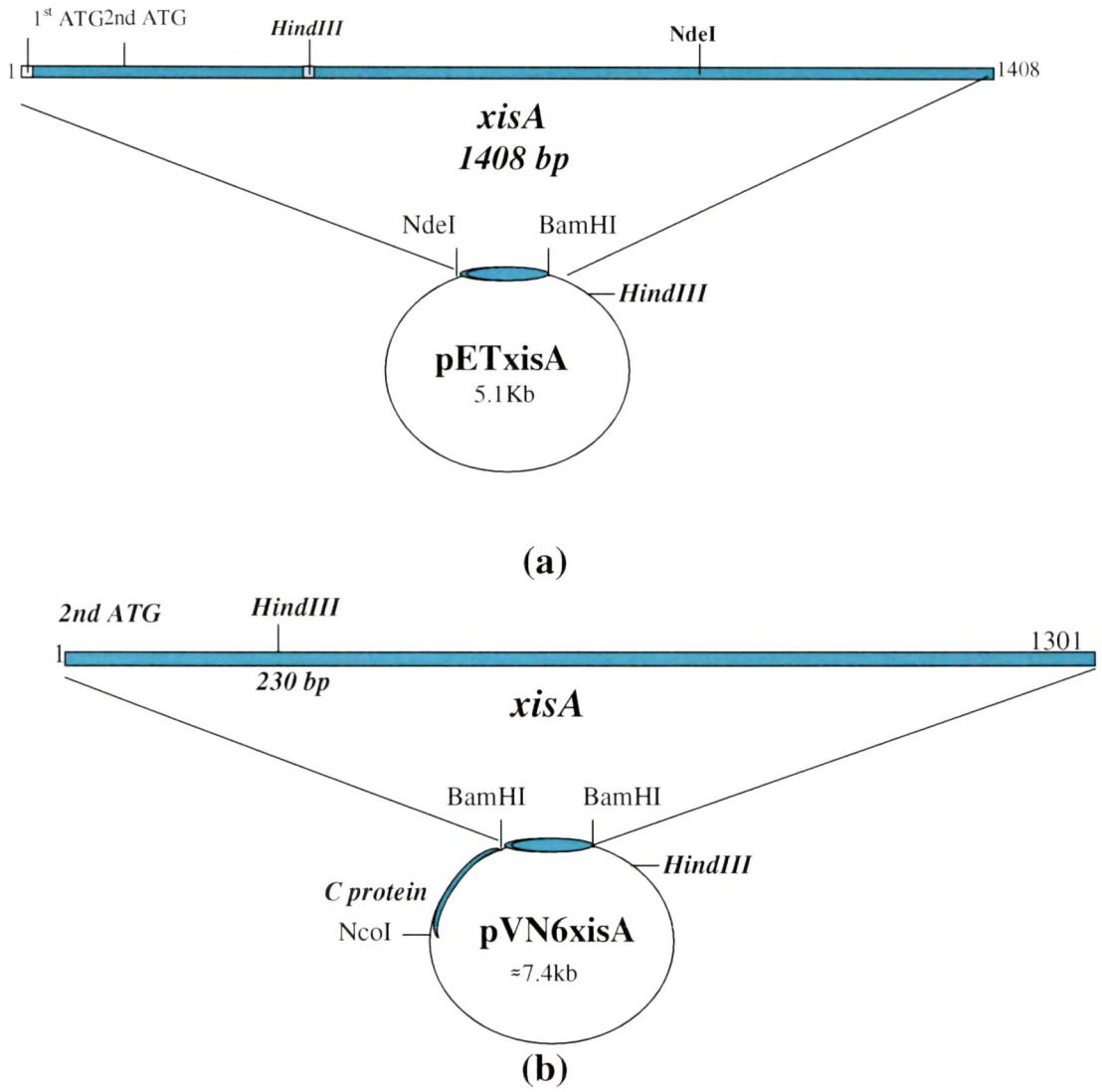

Fig. 1 Cloning of *xisA* in (a) pET20b(+) and (b) pVN6.

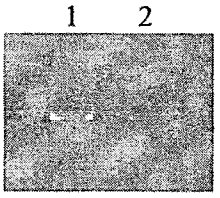

Fig. 2 Amplification of the *xisA* gene. Lane 1 BamHI flanked amplified *xisA*, Lane 2 NdeI-BamHI flanked amplified *xisA*.

For amplification of *xisA* gene from the first ATG, PSN3 primer 5'CGC ATATGCAAAATCAGGGTC 3' was used as a forward primer, while PSN2 primer 5'GCGGATCCAAGCATTGAGCAG 3' used for cloning from the second ATG, and PSN1 primer 5' GCGGATCCGCAGCAAAGAGAAC 3' as reverse primer (**Fig. 2**). Details of the amplification conditions are described in the materials and methods (**2.4.30**).

Amplified products were run in electrophoresis and then gel purified product was used for digestion with either BamHI for *xisA* ORF which has second ATG start point and NdeI and BamHI for *xisA* ORF which has first ATG start point. Digested products were ligated with pET20b(+) plasmid for first start site and pVN6 plasmid for second start site and the schematic representation of the resulting plasmids is shown in **Fig. 1.** BamHI digestion of pVN6xisA plasmid released the fragment of 1.3 kb size (**Fig. 3 & 4**). All positive clones were also confirmed for the orientation with HindIII digestion (**Fig. 5**). In the right orientation HindIII digestion of pVN6xisA releases 1,361 bp whereas in the wrong orientation it will release 520 bp. Clones 2 and 8 are in right orientation whereas clones 1 and 10 are in wrong orientation.

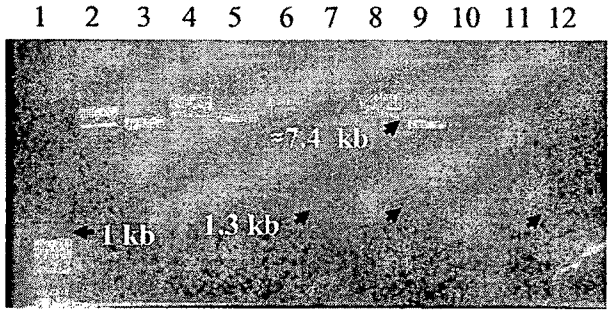

Fig. 3 HindIII digestion of plasmids containing *xisA* gene. 1 Marker 100bp, 2 pVN6 control Uncut, 3 pVN6 control HindIII digest, 4 pVN6xisA1 Uncut, 5 pVN6xisA1 HindIII digest, 6 pVN6xisA2 Uncut, 7 pVN6xisA2 HindIII digest, 8 pVN6xisA8 Uncut, 9 pVN6xisA8 HindIII digest, 10 pVN6xisA10 Uncut, 11 pVN6xisA10 HindIII digest, 12 BamHI flanked xisA PCR product.

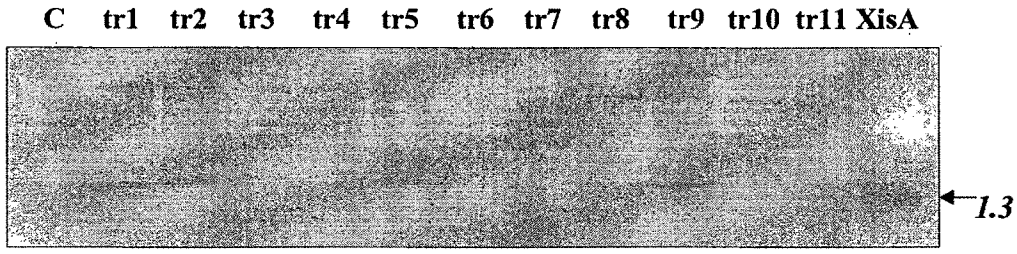

Fig.4 BamHI Digestion of pVN6xisA plasmid. C - pVN6 (control), tr1- tr11 positive clones released 1.3 kb *xisA* gene, XisA – amplified product of 1.3 kb in size.

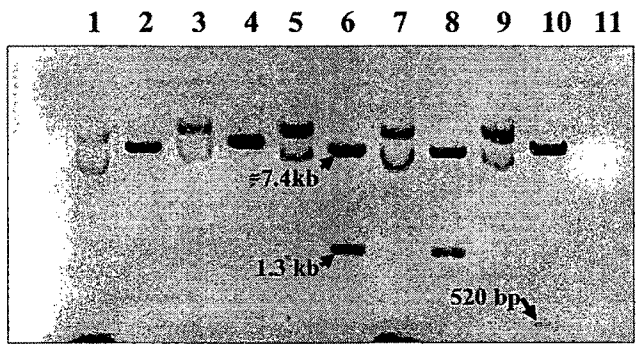

Fig. 5 HindIII digestion of pVN6xisA. Lane 1 pVN6, Lane 2 pVN6 digest with HindIII, Lane 3 pVNxisA1, Lane 4 pVNxisA1 digest with HindIII, Lane 5 pVNxisA2, Lane 6 pVNxisA2 digest with HindIII, Lane 7 pVNxisA8, Lane 8 pVNxisA8 digest with HindIII, Lane 9 pVNxisA10, Lane 10 pVNxisA10 digest with HindIII, Lane 11 1301 bp PCR products

Amplified *xisA* gene product (1,408 bp) was cloned in pET20b(+) plasmid digested with NdeI and BamHI. 1 NdeI site is present in the amplified product of 831bp, hence the amplified product was partially digested with NdeI. Vector has one HindIII site at 173 bp and insert has 338 bp thus HindIII digestion released 1,097 bp products (**Fig. 6**). Release of the 1,097 bp product confirmed the cloning of *xisA* gene form the first start site in the pET20b(+) plasmid.

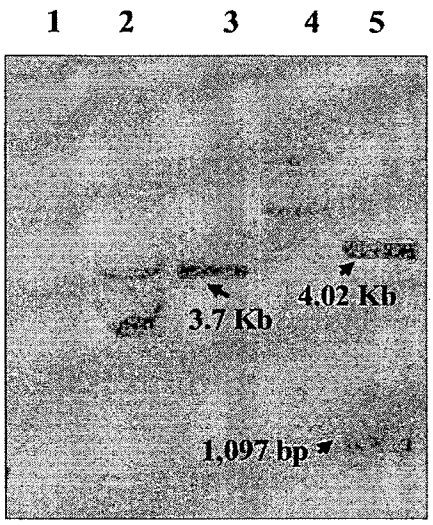

Fig. 6 HindIII digestion of pET20b(+) and pETxisA plasmids. Lane 1 1kb ladder, Lane 2 pET20b(+) undigested, Lane 3 pET20b digested with HindIII, Lane 4 pETxisA, Lane 5 pETxisA digested with HindIII

7.3.2 Expression of *xisA* gene with regulatory circuits

xisA gene was expressed in *E. coli* BL26 (pVN6 and pNC1) cells. *E. coli* cells harboring pNC1 plasmid, a pLysS derivative which contains *lys* gene. Multiple binding sites of C protein are present between the promoter and ORF of the *lys*, which upon C protein binding stops the expression of the *lys* gene under induced condition but in uninduced condition Lysozyme acts on the T4 RNA polymerase. **Fig. 7 & 8** shows the presence of XisA protein at very low amount using regulatory circuit, may be because of the tight regulation of the T7 polymerase and absence of the RBS sequences.

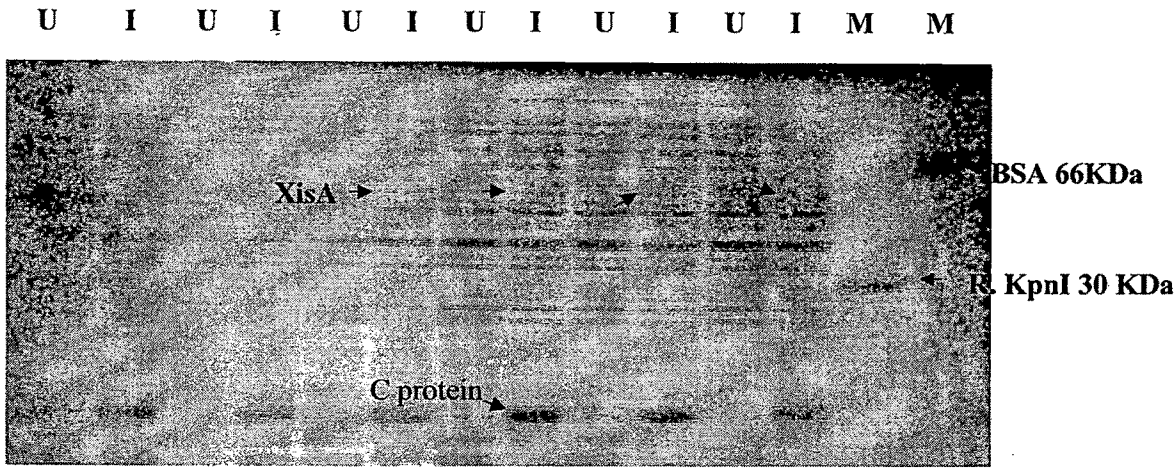

Fig. 7 SDS PAGE profile of expression of *xisA* gene using regulatory circuit

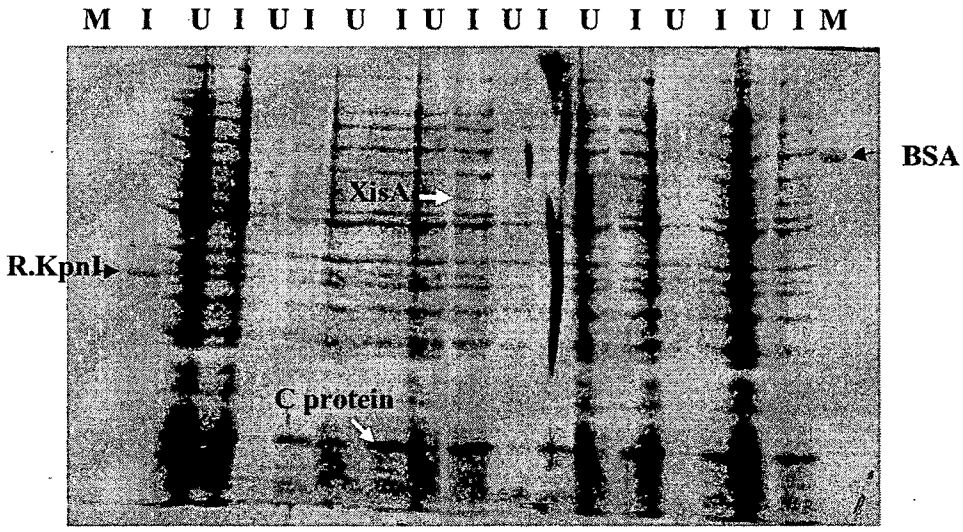

Fig. 8 Induction of the *xisA* gene expression in dicistronic form with C protein of the Mu phage under regulatory circuits in *E. coli* BL26 pNC1 pVN6xisA.

SDS-PAGE profile of the *E. coli* BL26 strain harboring pNC1 and pVN6xisA plasmids, showed poor expression of the *xisA* gene **Fig. 9.** Sonicated cells pellet and supernatant and different percent ammonium sulphate precipitated product was loaded on the SDS-PAGE, here XisA protein was seen in the pellet fraction, it was precipitated with ammonium sulphate with different percentage like 0-30 %, 30-45%, 45-55% and 0-60%. Precipitated protein was loaded on the SDS-PAGE, XisA protein was found in all sonicated pellet and in 0-30% and 0-60% ammonium sulphate sample (**Fig. 10**). Substrate plasmid containing the 11 bp target site was incubated with all samples showed no endonuclease activity (**Fig. 11**). All fractions were collected and loaded on the phosphocellulose column which showed that there was absence of the XisA protein band which suggests that *xisA* gene expression using pVN6xisA plasmid was very low and not suitable for the purification procedure (**Fig 12**).

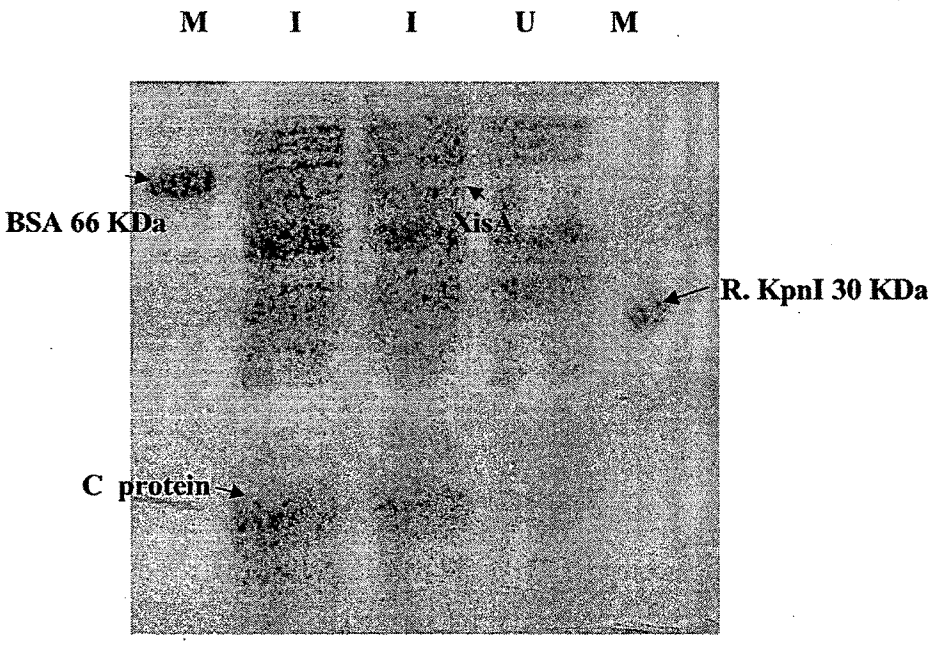

Fig. 9 Induction of the *xisA* gene expression in dicistronic form with C protein of the Mu phage under regulatory circuits in *E. coli* BL26 pNC1 pVN6xisA. M- Marker, U- uninduced, I- Induced.

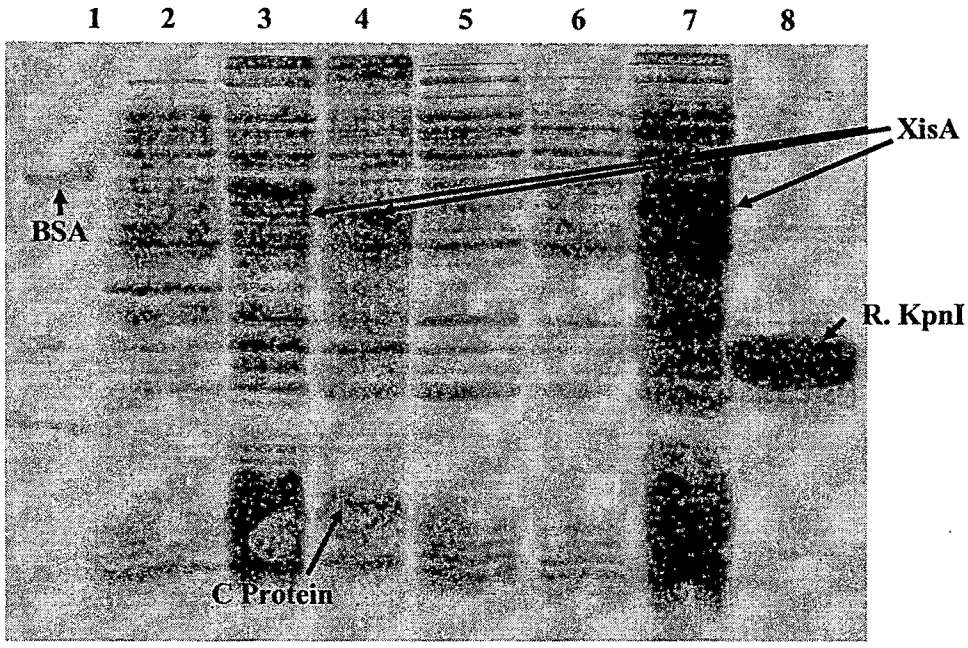

Fig. 10 *xisA* gene expression form BL26 pNC1 & pVN6xisA. Lane 1 BSA Marker 66 K Da, Lane 2 Uninduced cell lysate, Lane 3 Ultra centrifuged pellet, Lane 4 Dial. 0-30% Am. Sul cut, Lane 5 Dial. 30-45% Am. Sul cut, Lane 6 Dial. 45-55% Am. Sul cut, Lane 7 Dial. 0-60% Am. Sul cut, Lane 8 R. KpnI marker36 KDa.

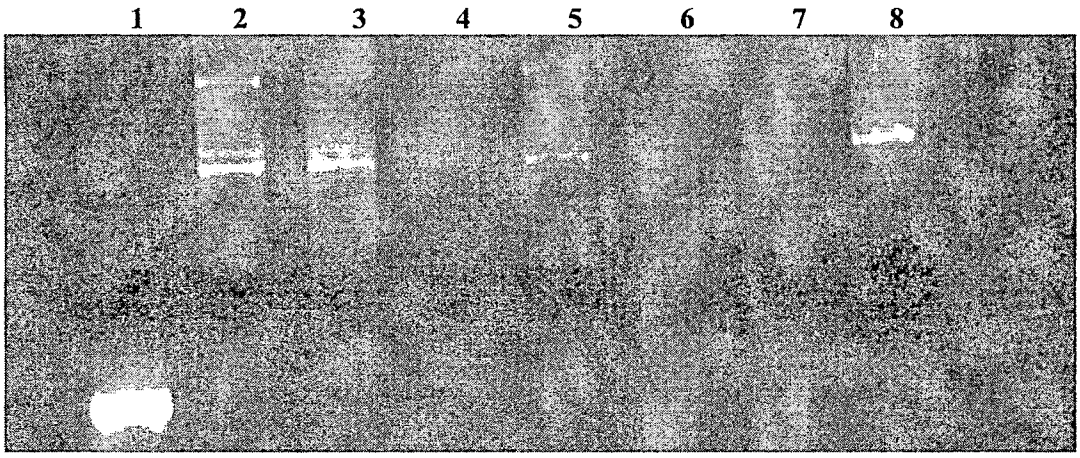

Fig. 11 Activity assay of XisA protein on substrate plasmid pBR322NG1 using low salt buffer of the NEB. Lane 1 1.5 kb DNA, Lane 2 1µl 0-30 % amm. Sulphate cut fraction, Lane 3 1 µl of 1:10 dilution of 0-30 % amm. Sulphate cut fraction, Lane 4 1µl 30-45 % amm. Sulphate cut fraction, Lane 5 1 µl of 1:10 dilution of 30-45 % amm. Sulphate cut fraction, Lane 6 1µl 45-55 % amm. Sulphate cut fraction, Lane 7 1 µl of 1:10 dilution of 45-55 % amm. Sulphate cut fraction, Lane 8 Undigested pBR322NG1.

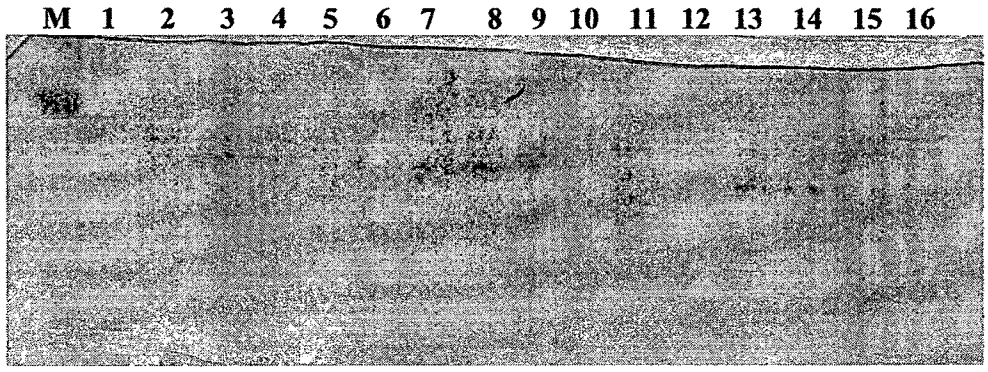

Fig. 12 Phosphocellulose column purification fractions of BL26 pNC1 pVN6xisA.
M: marker, 1-16: fraction of phosphocellulose column

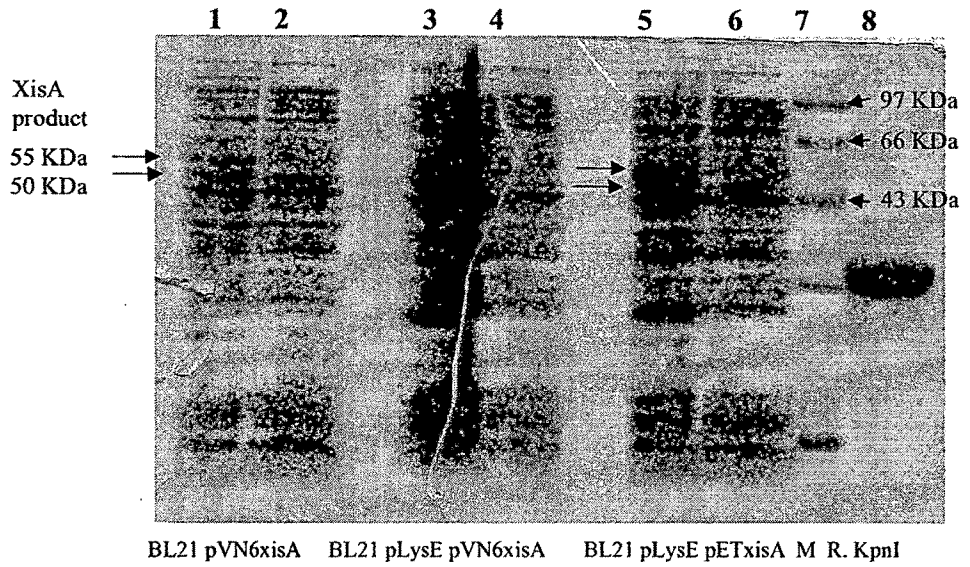

Fig. 13 XisA expression using different combination of plasmid in BL21 (DE3) using plasmid pLysE or without pLysE. Lane 1 Induced *E. coli* BL21 strain containing pVN6xisA plasmid, Lane 2 Uninduced *E. coli* BL21 strain containing pVN6xisA plasmid, Lane 3 Induced *E. coli* BL21 strain containing pLysE & pVN6xisA plasmid, Lane 4 uninduced *E. coli* BL21 strain containing pLysE & pVN6xisA plasmid, Lane 5 Induced *E. coli* BL21 strain containing pLysE & pETxisA plasmid, Lane 6 uninduced *E. coli* BL21 strain containing pLysE & pETxisA plasmid, Lane 7 Standard molecular weight marker, Lane 8 R. KpnI (30 Kda).

Expression of the *xisA* gene was found very poor in the *E. coli* BL26 strain harboring plasmid pNC1 and pVN6xisA. Thus, under normal regulatory circuit strategy protein yield found less and use of alternative strategies was needed. Here, *E. coli* BL21 and BL26 strain were used in different combination of plasmid. *E. coli* BL21 strain carrying plasmid pVN6xisA, pLysE and pVN6xisA, pLysE and pETxisA **Fig. 13**. *E. coli* BL21 strain carrying pLysE and pVN6xisA plasmids showed higher *xisA* gene expression on 7% SDS-PAGE protein profile, thus again used for the more induction with 0.5mM IPTG. After 5 h induction with 0.5mM IPTG resulted in poor expression of *xisA* gene in compare to *E. coli* BL21 strain harboring pLysE and pETxisA combination, where it showed more induction after 2 h on 7% SDS-PAGE (**Fig. 14 & 15**).

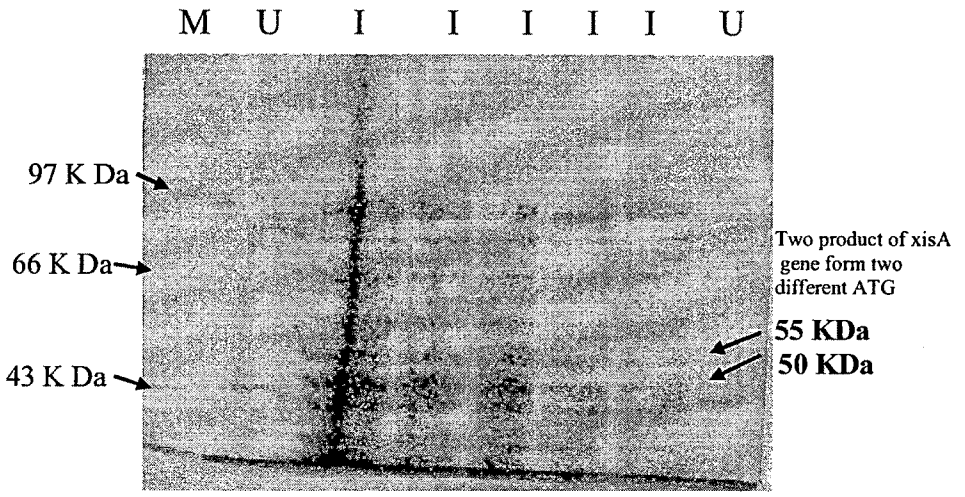

Fig. 14 Expression of XisA using BL21 harboring pLysE pVN6xisA after 5 h of induction with 0.5 mM final concentration of IPTG. M- Marker, U- uninduced, I- Induced.

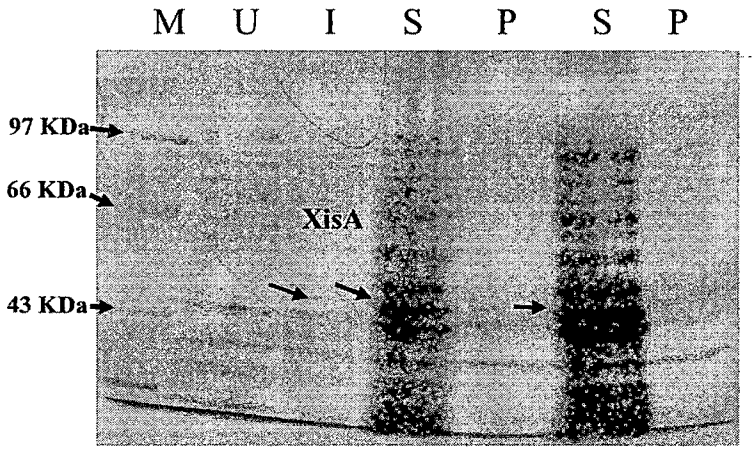

Fig. 15 Protein expression profile of the sonicated supernatant and pellet sample of BL21 pLysE pETxisA. M- Marker, U- uninduced, I- Induced, S- Supernatant, P- Pellet.

E. coli BL21 harboring pLysS and pETxisA plasmids was used for the expression and purification. Cells were grown in 3 l of LB medium. Since XisA protein was found in pellet fraction, cells were sonicated but not centrifuged. Sonicated samples were divided in three equal parts at 50mM, 100mM and 200mM. DNA was precipitated using polyetheneimine (PEI) at final concentration of 0.5% from the stock of 10%. Precipitated samples were centrifuged and pellet fraction was dissolved in the concentration buffer and supernatant and

pellet were loaded on SDS-PAGE (**Fig. 16**). 100mM NaCl gives maximum concentration of XisA protein on the SDS-PAGE. For standardization, all three samples were pooled and dialysed against low salt buffer with 10mM NaCl.

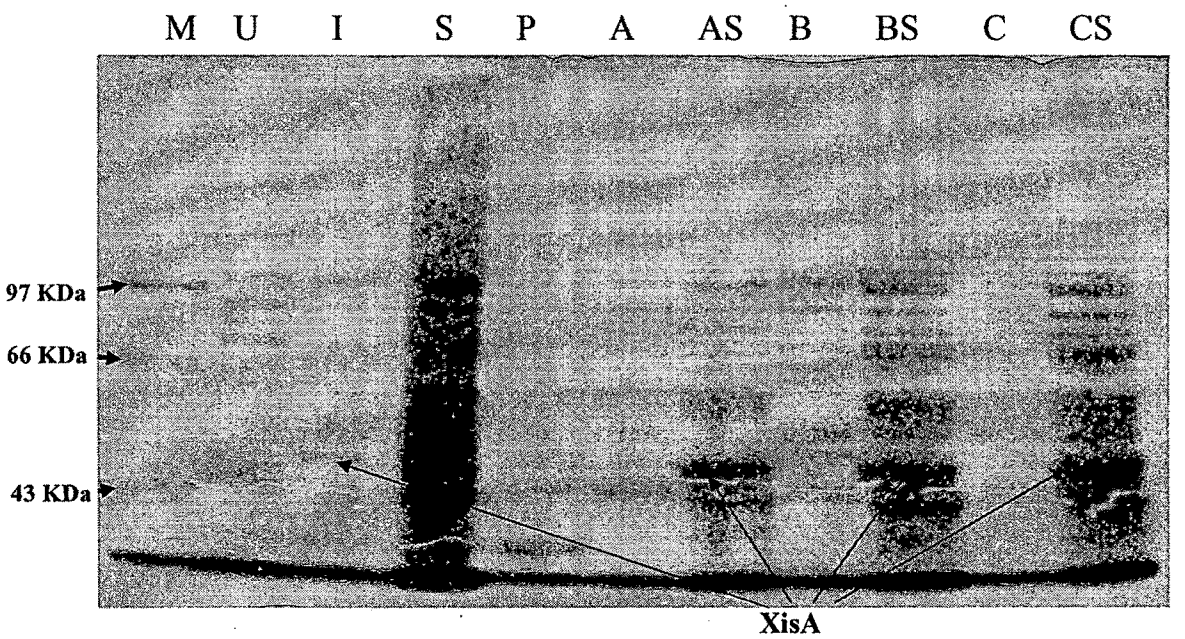

Fig. 16 PEI precipitation in presence of different NaCl concentration BL21 pLysE pETxisA. M- Marker, U- uninduced, I- Induced, S- Supernatant, P- Pellet, A- Pellet of PEI cut in presence of 50mM NaCl, AS- Sup of PEI cut in presence of 50mM NaCl, B- Pellet of PEI cut in presence of 100mM Salt, BS- Sup of PEI cut in presence of 100mM Salt, C- Pellet of PEI cut in presence of 200mM Salt, CS- Sup of PEI cut in presence of 200mM Salt

Samples were treated with different percentage of ammonium sulphate precipitation, 0-30%, 30-45%, 45-55%. Precipitated samples were dialysed with low salt buffer containing 50mM NaCl and dialyzed immediately after precipitation and dissolved in the low salt buffer containing 50mM NaCl. All the fractions from the supernatant and pellets were again loaded on the 7% SDS-PAGE **Fig. 17**. XisA protein was found in high amounts in 30-45% ammonium sulphate precipitated supernatant.

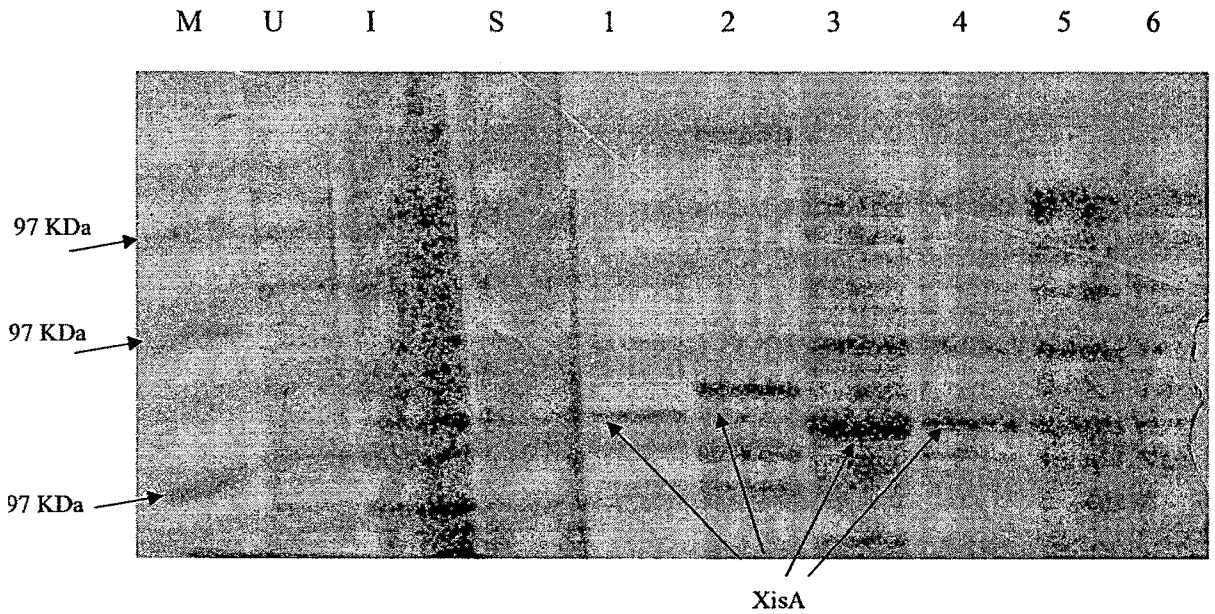

Fig. 17 Different ammonium sulphate precipitation of PEI cut samples of BL21 pLysE and pETxisA. M- Marker, I- Induced, U- uninduced, S- Supernatant, 1- 0-30% ammonium sulphate Sup, 2- 0 -30% ammonium sulphate Pellet 3- 30-45% ammonium sulphate Sup, 4- 30-45% ammonium sulphate Pellet, 5- 45-55% ammonium sulphate Sup 6- 45-55% ammonium sulphate Pellet.

`All samples were pooled and passed through preactivated affinity column with phosphocellulose. Collected fractions were loaded on the SDS-PAGE (**Fig. 18**). XisA binds poorly to the phosphocellulose material and thus maximum amount of XisA protein was present in the flow through and in fractions 8-10. Flow through of the earlier column was loaded on the heparin column (Biorad) with bed volume 5 ml, equilibrated column with the buffer, 30 ml of flow through was loaded on the column and fractions were loaded on the SDS-PAGE. XisA protein was present in earlier fractions and binds strongly to the heparin material (**Fig. 19**).

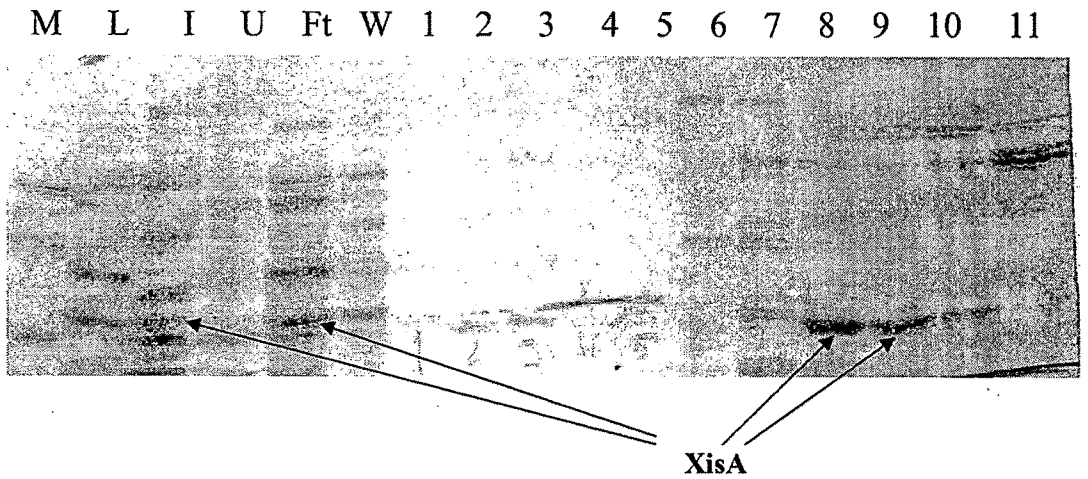

Fig. 18 Protein purification using affinity column chromatography where phosphocellulose as column material. M-marker, L- Load, I-Induced, U-Uninduced, Ft- Flow throw, W-Wash, 1-11 Fraction 1-11.

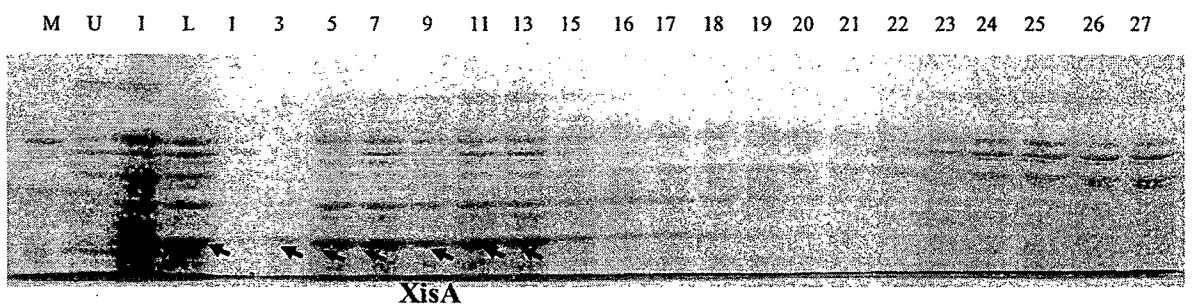

Fig. 19 Heparin column purified early fractions. M Marker, U Uninduced, I Induced, L Load, 1 Fraction1, 3 Fraction3, 5 Fraction5, 7 Fraction7, 9 Fraction9, 11 Fraction11, 13 Fraction13, 15 Fraction15, 16-27 Fraction16-27.

Fractions containing XisA protein were loaded on the MonoQ column, and XisA protein was seen in late fractions which suggest that it binds strongly to MonoQ (**Fig 20**).

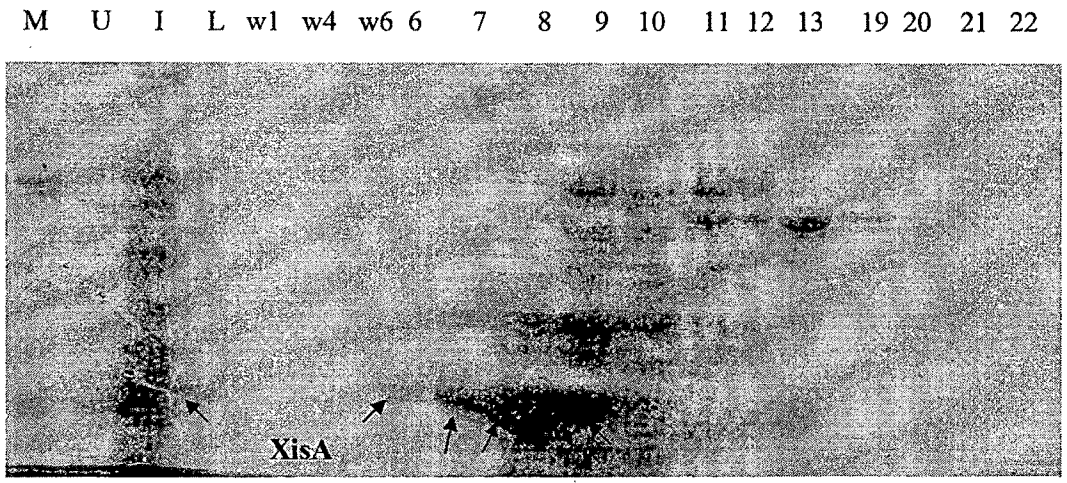

Fig. 20 Mono Q fractions loaded on the 7% SDS-PAGE. M - Marker, U- Uninduced, I – Induced, L – Load, w1,4,6 - wash1,4,6, 7-13,19-22 Fraction

In conclusion, the *xisA* gene was cloned from two start sites in two different expression vectors. In presence of tight control in regulatory circuit strategy, XisA protein concentration was poor. Thus, alternate strategy with the vector carrying the *xisA* gene in pETxisA plasmid was employed with pLysS and pLysE plasmids. The expression analysis was better with *E. coli* BL21 strain. Since DNA was interfering it was removed by PEI precipitation. Centrifugation of sonicated cell extract at higher RPM resulted in the presence of XisA protein in the pellet fraction. Thus, cell extracts were centrifuged at lower RPM to remove the cell debris. PEI precipitation of supernatant of the sonicated sample was done in combination with different salt concentration and 100mM of NaCl concentration and 30-45% ammonium sulphate precipitation was optimal for concentration of XisA protein. Ammonium sulphate precipitated proteins were further purified by phosphocellulose, heparin and MonoQ. Amongst them XisA protein binds strongly to the MonoQ and suitable for purification. *In vitro* excisase activity was absent in purified XisA fraction Further purification and standardization is required for determining the *in vitro* activity of XisA.