Chapter 6

Detection of MBP-XisA fusion protein of *Anabaena* PCC 7120 in *E. coli*

6.1 Introduction

In *Anabaena* PCC7120 *nifHDK* operon, encodes nitrogenase enzyme, is interrupted by a 11.2 Kb *nifD* element which gets excised out during heterocyst maturation. XisA protein which encoded by a *xis*A gene, present within *nifD* element has been implicated in the excision (Fig.1).

XisA is a site-specific recombinase belonging to the Integrase (*int*) family of Tyrosine recombinases (Lammers et al, 1986). It acts on a "CGGAGTAATCC" 11 bp target sequence flanking the *nifD* element resulting in its excision. *xisA* gene sequence is known since 1986 but the protein has not been detected so far. Earlier studies done in our laboratory suggests that XisA carries out its recombination by forming double stranded breaks which is different from other members of ***int*** family (Karunakaran, 2000). This mechanism of recombination is known amongst a special class of group II endonucleases known as group F. Thus XisA protein is an interesting enzyme with the structural similarity with Int family recombinases but functionally similar to type IIF restriction endonucleases. Since XisA protein acts on 11 bp target site, it has the potential use in recombinant DNA methodologies as a large base cutting endonuclease.

The present study aims at detection of XisA protein. Since XisA protein is known to be toxic to *E. coli* (Brusca *et al.*, 1989), present strategy employed to overexpress the XisA protein under inducible p*tac* promoter in the periplasm.

6.2 Materials and methods

Please refer chapter titled Material and Methods

6.3 Results and Discussion

6.3.1Cloning of *xisA* gene

The *xisA* without promoter was amplified using forward primer 5' CGAATTCGGATCCATGCA AAATCAGGGTCAAGACA 3'and reverse primer 5' TGAGTCGACTGATCAAAG CATTGAGCAGAT 3'using plasmid pMX25 as a template **(Fig 1).** The amplified product was cloned in the two vectors pMC71AGm and pTZ57R. The *xisA* gene form T vector (pTZ57R) was subcloned in the expression vectors pMALp2 and pEG-KT. Cloning of *xisA* ORF in the expression vector was confirmed by colony PCR (**Fig 2**) and restriction digestion. pMALp2-xis plasmid was used for the expression and purification of the XisA.

6.3.2 Expression of MBP-XisA fusion protein in *E. coli*

pMALp2 (control) and pMALp2-Xis were transformed in *E. coli* BL21 and the transformants were confirmed by restriction digestion analysis (**Fig. 3**). Hind III digestion of pMALp2-Xis released 900 bp fragments whereas pMALp2 linearized the plasmid. However, ScaI partially digested pMALp2 and no digestion was seen in case of pMALp2-Xis.

Expression of MBP-XisA fusion protein was done in 10 ml LB at 37°C. Fusion protein could not be detected in SDS-PAGE of total cell protein preparation at around 97.6 KDa. In order to increase the protein amount expression of MBP-XisA fusion protein was carried out using 100 ml LB at 37°C at from total cell protein. After SDS-PAGE analysis around 3-4 μg protein could be detected at around 97.6 KDa (when compared to marker) which was absent in controls (**Fig. 4**).

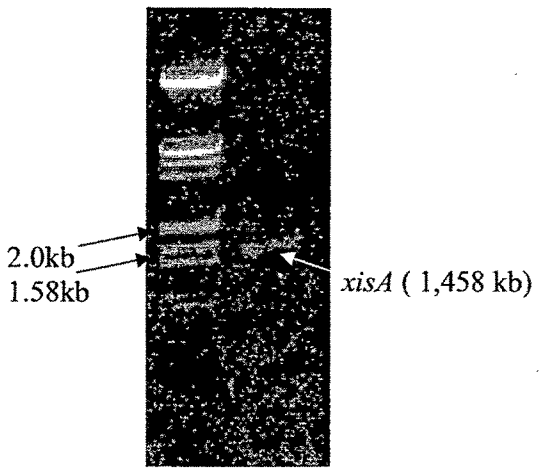

Fig. 1 Amplification of *xisA* without promoter. Lane 1 Lambda DNA cut with EcoR1/HindIII, Lane 2 PCR product of *xisA* without promoter.

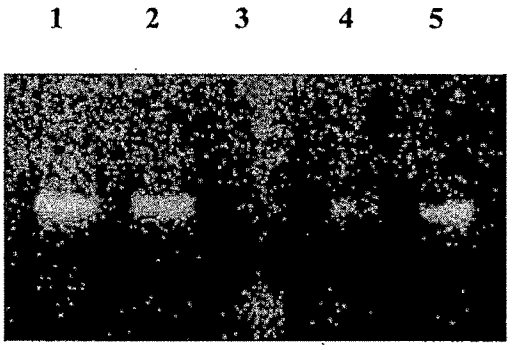

Fig. 2 Colony PCR of *xisA* without promoter. Lane 1 PCR product of *xisA* without promoter using pMX25, Lane 2 Colony PCR of pEG-KT-xis, Lane 3 Colony PCR of pEG-KT-xis, Lane 4 Colony PCR of pMALp2-xis, Lane 5 Colony PCR of pMALp2-xis.

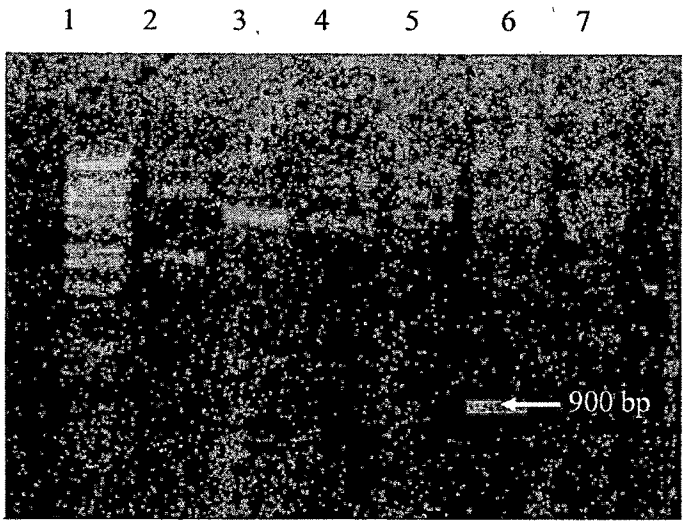

Fig. 3 Digestion of pMalp2 and pMalp2-Xis with HindIII and ScaI. Lane 1- λBst EII Marker; Lane2- pMalp2, undigested; Lane3- pMalp2, HindIII digested; Lane4- pMalp2, ScaI digested; Lane5- pMalp2-Xis, undigested; Lane6- pMalp2-Xis, HindIII digested; Lane7- - pMalp2-Xis, ScaI digested.

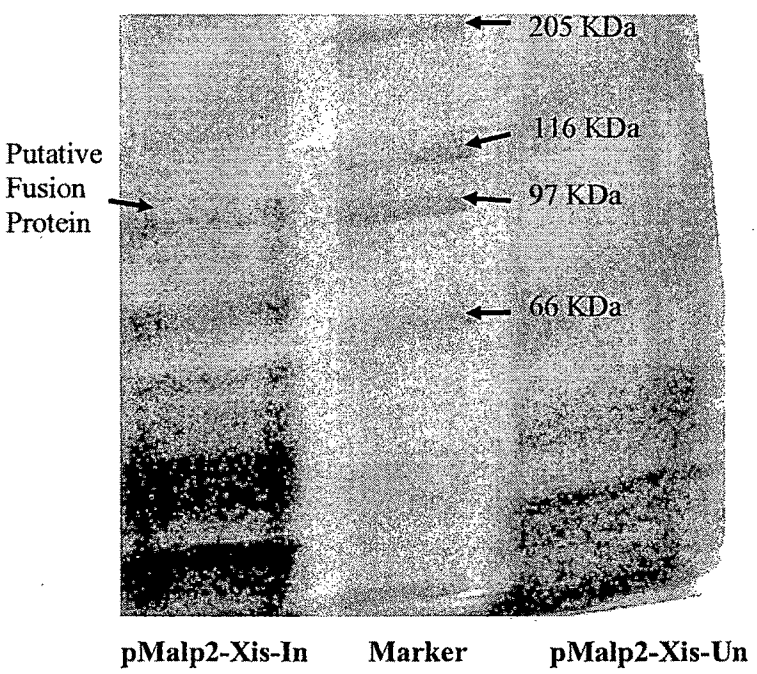

Fig. 4 Protein profile of total cell extract of *E. coli* pMALp2-Xis grown in Luria Broth at 37 C.

Since MBP acts as a signal to export protein into periplasm, fusion protein protocols was standardized MBP fusion protein extraction. Extraction of periplasmic protein was carried out at 37 C from 100 ml culture, however very little or sometimes no protein could be detected at around 97.6KDa (**Fig. 5**).

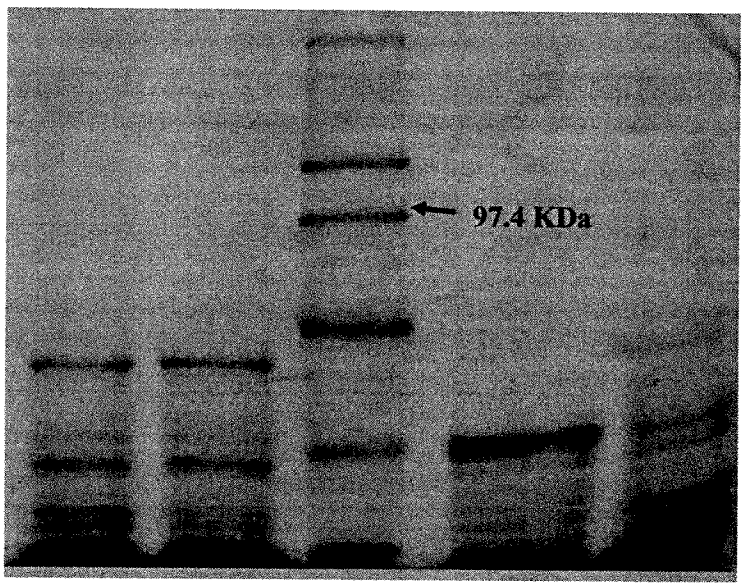

Fig. 5 Protein profile of periplasmic extract of *E. coli* pMALp2-Xis and pMalp2 grown in Luria Broth at 37 C.

It appears that protein was getting lost may be due to high proteolytic activity at 37 C. Hence, the extraction was performed by lowering the temperature to 30 C and 25 C. Small increase of protein amount was observed at 30 C but at 25 C higher amount of fusion protein around 97.6 KDa was detected (when compared with marker) which was absent in controls (**Fig. 6 & 7**).

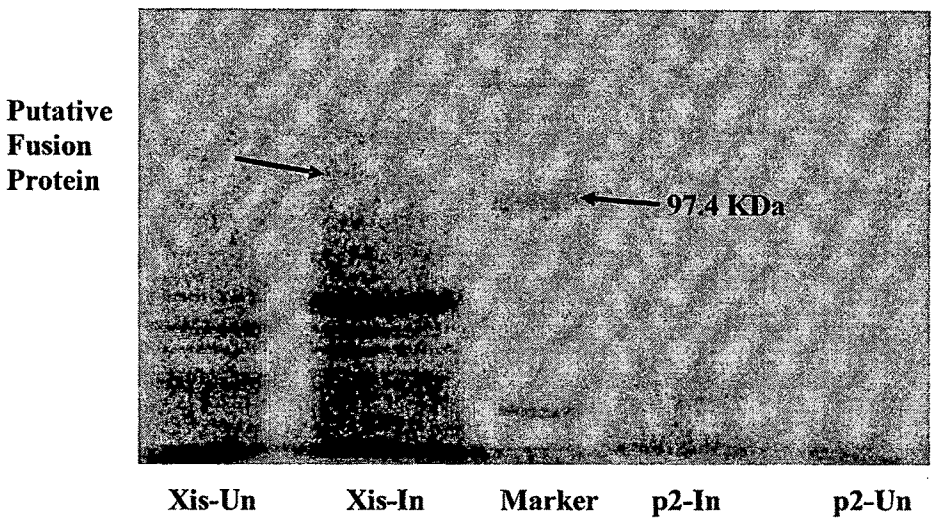

Fig. 6 Protein profile of total cell extract of *E. coli* pMALp2-Xis and pMalp2 grown in 100 ml Luria Broth at 25°C.

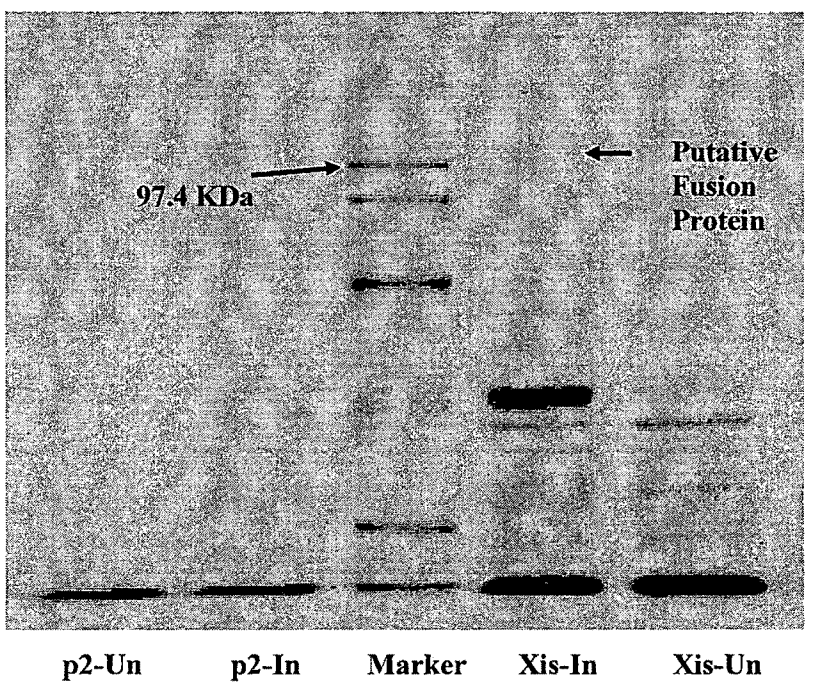

Fig. 7 Protein profile of periplasmic extract of *E. coli* pMALp2-Xis and pMalp2 grown in 100 ml Luria Broth at 25°C.

In order to concentrate, the protein was extracted by TCA precipitation and higher amount of protein was detected around 97.6 KDa in pMalp2-Xis in the periplasm as well as from total cell protein preparations (**Fig. 8 & 9**).

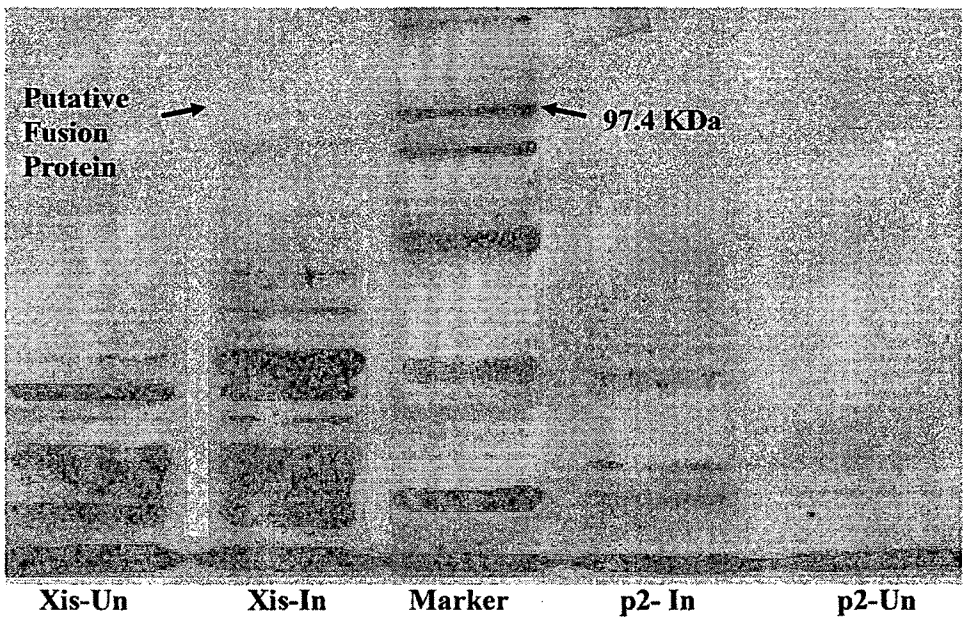

Fig. 8 Protein profile of TCA precipitated periplasmic extract of *E. coli* pMALp2-Xis and pMalp2 grown in 100 ml Luria Broth at 25 C.

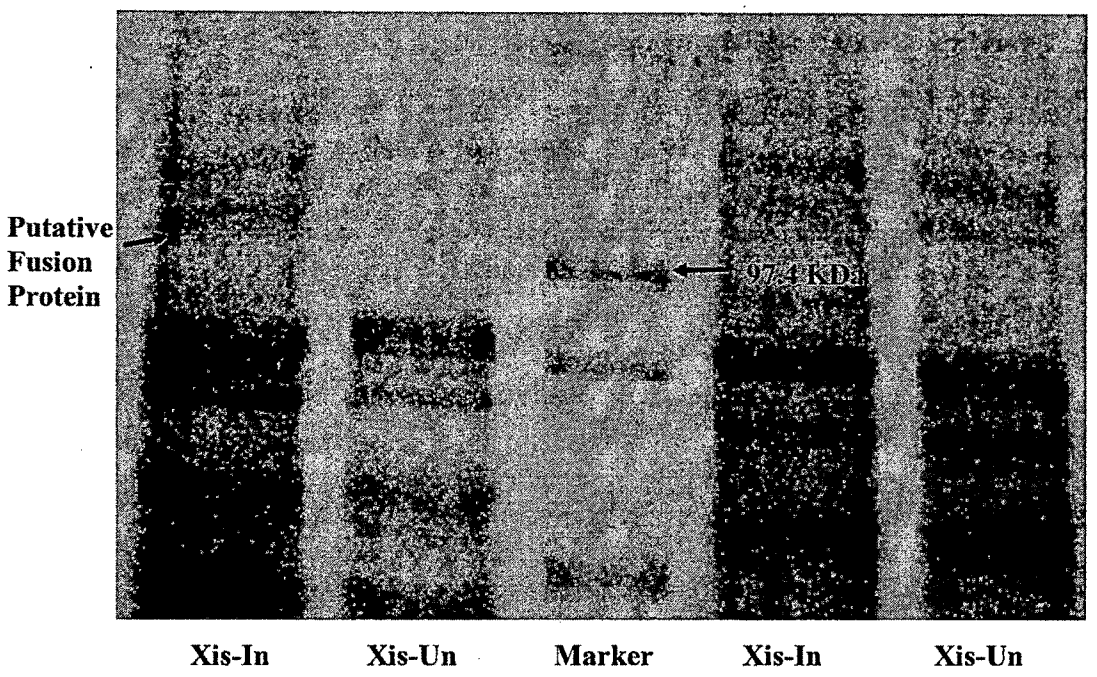

Fig. 9 Protein profile of TCA precipitated Total cell extract of *E. coli* pMALp2-Xis and pMalp2 grown in 100 ml Luria Broth at 25°C.

The results suggest that *E. coli* MBP-XisA transformants showed a protein at around 97.6 KDa corresponding to the expected size of MBP-XisA fusion protein. This protein was not seen in cells containing pMalp2 vector that were used as controls. The gels also showed very prominent protein in both pMalp2 and pMalp2-Xis at around 42 KDa corresponding to the size of MBP. In case of periplasmic proteins the number of proteins was much less as compared to total cell extract.

Thus, XisA fusion protein could be detected by various methods. The XisA protein is expression is low but can be further enriched by affinity column using amylose resin which can be further used for the characterization of XisA.