Modulation of *Escherichia coli* AlkB function by single-stranded DNA-binding protein

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The Degree of Master of Technology

Department of Biotechnology

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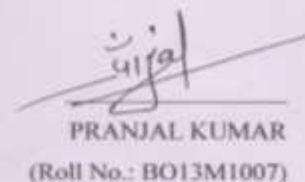

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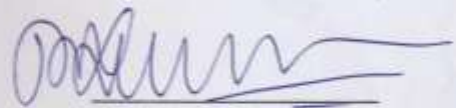

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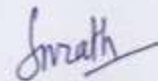

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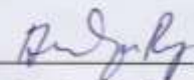

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Abstract

Cells are always get exposed to the external environment and some metabolism also contribute to the change. In the event of changed external or internal environment, the genome gets damaged. If they remain unrepaired it may be toxic for the cells and may cause some alteration/mutation. Organisms are evolved such that they come out of this kind of condition by various DNA repair mechanisms. *Escherichia coli* AlkB is a kind of DNA repair protein that belongs to the Fe(II) dependent 2-OG (2-oxoglutarate) dioxygenase superfamily and involved in the repair of mainly 1-methyl adenine (1-meA) and 3-methyl cytosine (3-meC). In this study, the interaction of single-stranded DNA-binding protein (SSB) and AlkB was tested *in-vitro* and observed positive for the interaction. The *in-vitro* repair activity was performed with 40mer oligo (40mer Adenine and 40mer Cytosine). The *in-vitro* repair activity of purified recombinant GST-AlkB is found to be increased significantly in the presence of SSB, while the ABH3 activity was unchanged in the presence and absence of SSB.

Contents

Declaration.. **Error! Bookmark not defined.**

Approval Sheet .... **Error! Bookmark not defined.**

Acknowledgements...iv

Abstract vi

Chapter 1: Introduction 1

Chapter 2: Materials and Methods 7

Chapter 3: Figures 52

Chapter 4: Result and Discussion...59

References..61

Chapter 1
Introduction

1.1 DNA damaging alkylating agents are cytotoxic:

Every cell's genomes always get exposed to an environmental and/or endogenously generated DNA damaging alkylating agents. Cancer chemotherapy also uses some of the alkylating agents (1). Methylating agents, such as MMS, methylates double-stranded DNA and generate 7-methylguanine (7meG) and 3-methyladenine (3meA) (2). These block the DNA synthesis and are considered to be a lethal lesion. MMS also methylates single-stranded DNA and generates 1-methyladenine (1meA) and 3-methylcytosine (3meC) (3). These sites participate in base pairing, so they are protected in double-stranded DNA, but they get momentarily exposed during cellular processes, such as replication, transcription or recombination. Therefore, MMS causes genotoxicity majorly by generating these main lesions, namely, 3meA, 1meA and 3meC (3, 4).

1.2 Structure of *E. coli* AlkB reveals molecular mechanism of DNA repair:

The *E. coli* AlkB protein eliminates damaged DNA by mechanism of oxidative dealkylation. Comparative structural analysis of the AlkB with no ligand and AlkB with ssDNA having 1-meG (PDBIDs: 3KHB and 3KHC, respectively) unveils many fascinating mechanistic details (5). The protein makes hydrophobic interactions as well as hydrogen bonding and stretches across five nucleotides to maintain the protein-DNA complex. The phosphodiester backbone of ssDNA binds at DNA binding groove (electropositive) created by a threonine at 51-tyrosine at 55 (DNA binding loop), serine at 129, and lysine at 127. This helps in flipping of alkyl-lesions

into substrate binding center where the metal binding residues (histidine at 131, aspartate at 133, and histidine at 187) and substrate binding residues come close to each other. The base substrate binds in the active site patch formed by non-polar stacking interaction of tryptophan at 69th position and histidine at 131st position (5). This interactions help to stabilize the flipped conformation of 1meG in substrate binding center. It guides to the proper positioning of the methyl group of the 1-meG adjacent to the bound metal ion for oxidative repair. The hydroxyl group of threonine at 51st position and backbone amide group of glycine at 53rd position interact directly through hydrogen bonding to the phosphate of the nucleotide next to the 1-meG base towards 5'-end. Three amino acids namely tyrosine at 76th, lysine at 127th and serine at 129th interact additionally with the phosphodiester backbone, while the side chain of tyrosine at 55th interacts with the ribose sugar via hydrophobic group. The common interactions between protein and the phosphodiester backbone or methylated base of the nucleotide provide few opportunities to distinguish ribonucleotide and deoxyribonucleotide substrates, which is homogeneous with the capability of AlkB to function on both RNA and DNA (5).

1.3 Single stranded DNA-binding (SSB) proteins:

Double-stranded (ds) DNA carry all of the needful genetic information, although empirical uses of these information require unwinding of the DNA duplex and this unwinding generates single-stranded DNA (ssDNA) intermediates which may serve as template for numerous cellular functions. Exposure of these ssDNAs presents several complications to the cell. First, ssDNAs are thermodynamically not much stable as dsDNA and it heads to spontaneous generation of duplex secondary

structures that obstruct with genome maintenance processes. Second, ssDNAs are comparatively hypersensitive to chemical and nucleolytic attacks than dsDNA and cause damage to the genome. Cells settle down these possibilities by synthesizing specialized protein namely SSB that bind to the ssDNA structures and provide stability to them (6).

SSBs are crucial proteins found in all forms of life. SSBs bind ssDNA with high affinity and in a sequence-independent manner and, in doing so, help to form the central nucleoprotein complex substrate for various processes such as DNA replication, recombination, and repair. SSBs are found in almost every organisms, so these proteins themselves share interestingly little sequence similarity, subunit composition, and oligomerization states. All SSB proteins consist of at least DNA-binding oligonucleotide/oligosaccharide binding (OB) fold, which is made up of minimally of a five stranded □-sheet arranged as a □-barrel covered on by single □-helix. The OB fold binds with ssDNA and helps in oligomerization (in case of those SSBs that works as oligomers). The overall organization of OB fold differs between bacteria, eukaryotes, and archaea (6).

SSB proteins from eubacteria shows two common structural features. First is the use of oligonucleotide/oligosaccharide-binding (OB) domains to bind ssDNA through an electrostatic interaction with the phosphodiester backbone as well as base stacking interactions with nucleotide bases (7, 8). Second is the oligomerization of SSB that brings together four DNA-binding OB fold in the active form of protein (8-12). *E. coli* SSB is known to function as tetramer and each monomer provides a single OB fold and this was considered as prototypical SSB protein for long times (13-15). Rare exception to this in eubacterial system is SSB from *Deinococcus-*

thermus genera, which is known to have two OB fold per monomer and functions as homodimer (16-18). Non-eubacterial SSBs such as bacteriophage and viral SSBs are found to function as monomers (T4 gp32) (10) and dimers (T7 gene 2.5) (10, 19), whereas eukaryotic replication protein A (RPA) acts as heterotrimer (20). Eubacterial SSBs bind to ssDNA in an extremely cooperative manner that give rise to clustering of SSBs and form protein filament on long ssDNA (21-23).

1.4 Role of SSB protein in recruitment of genome maintenance complex:

Eubacterial SSBs have been found to bind several different proteins. In most of the cases C-terminal region of SSB (SSB-Ct) plays an important role in complex formation, correlates that SSB-Ct is recognized by different proteins by a conserved mechanism to bind SSB. C-terminus of SSB is considered as amphipathic sequence element as it has been found that the C-terminal region is richer in acidic amino acid residues (in *E. coli*, Asp residue is present in high amount, and is very well conserved in others) (24) as well as conserved hydrophobic tripeptide is present at the extreme C-terminus. Mutations in the SSB-Ct have deleterious effect on *E. coli* cell survival. One well-observed mutation (ssb113, penultimate Pro to Ser) has given rise to a temperature sensitive variant of SSB (25, 26). This temperature sensitive variant can bind DNA but cannot support DNA replication at non-permissive temperature (26) and it is hypersensitive to DNA damage even under permissive conditions (27-31). In most of the cases, where mutations were carried out within the C-terminal sequences, give proteins that is found to interact improperly with few of SSB’s binding partners (32). These studies have appreciably brighten the significance to cellular genome maintenance pathways of the interactions of SSB protein with many other proteins. SSB plays important role in

genome integrity.

List of some proteins which are found to have interaction with SSB

SSB-interacting Proteins	**Whether require SSB-Ct**	**Involved in**
DNA polymerase III, chi	Yes	DNA Replication
Topoisomerase III	Yes	
Primase	Unknown	
RecQ	Yes	Recombination
RecO	Yes	
RecG	Yes	
RecJ	Unknown	
PriA	Yes	Replication Restart
PriB	Unknown	
Exonuclease I	Yes	DNA Repair
Uracil DNA glycosylase	Yes	
DNA polymerase II	Yes	
DNA polymerase V	Yes	
Exonuclease IX	Unknown	Unknown
vRNA polymerase	Unknown	Transcription

The main objectives of the study are:

1. Cloning, expression and purification of GST-AlkB,
2. Cloning, expression and purification of His-SSB,
3. Cloning, expression and purification of GST-ABH3,
4. Cloning, expression and purification of GST-ABH2.
5. Interaction study of SSB and AlkB *in-vitro*.
6. Functional Assay of purified GST-AlkB etc.

Chapter 2

Materials and Methods

2.1 General Techniques

2.1.1 Agarose gel electrophoresis:

Horizontal agarose gel (0.6-1.2%) electrophoresis were routinely performed for separation of DNA fragments of various lengths (between 100bp-10kb). Appropriate amount of agarose (SeaKem®LE, LONZA, Cat. no. 50005) was dissolved in 1X TAE buffer (40mM Tris-acetate; 1mM EDTA pH 8.0) by heating in microwave oven. After cooling, ethidium bromide of concentration 1µg/ml was added into the gel solution and then poured into a mould and a comb was inserted to generate wells. After 30-45 minutes, when the gel got solidified completely, gel was mounted into electrophoresis chamber containing 1X TAE (40mM Tris-acetate; 1mM EDTA pH 8.0) and comb was removed gently. DNA samples and size marker were mixed with appropriate volume of 6X DNA loading dye (6x stock: 0.25% bromophenol blue; 0.25 xylene cyanol FF; 30% v/v glycerol (Thermo scientific, Cat. No. R0611). Electrophoresis were generally carried out at 60-120 volt till the xylene cyanol dye migrated to distance of half of the gel. DNA bands were visualized in gel documentation system from SynGene (Model no: Chemi XR5, S.No DR4V2/2355). DNA ladder of 1kb (Cat no: SM0311 Thermo scientific) was used for SSB, AlkB, ABH3, ABH2 and linearized plasmid DNA for fragment size determination.

2.1.2 Preparation of competent *E. coli* cells:

Competent *E. coli* cells of DH5α and BL21(DE3)pLysS were prepared as described by Alexander by using MnCl2 and CaCl2. 20µl of *E. coli* strains (DH5α and BL21(DE3)pLysS) were taken from frozen glycerol stock and inoculated in 3ml of LB medium containing no antibiotics and incubated for overnight in a shaking incubator at 200 rpm. 1ml of this overnight grown culture was inoculated in a pre-warmed 100 ml of LB medium (1% tryptone, 0.5% yeast extract) prepared in 250ml Erlenmeyer flask. Inoculated culture was grown for about 4h at 30□C at 200 rpm in shaking incubator and the culture growth was monitored by measuring OD**600** spectrophotometer every 20 minutes. When culture had reached OD600 of ~0.35 it was taken out and incubated on

ice for 1hr. Cells were harvested in two sets by centrifugation at 4000 rpm for 15 min at 4□C. The supernatant was discarded and the cell pellets were resuspended in 12ml of acid salt buffer-A (ASB-A, sodium acetate- 40mM, CaCl2- 100mM, MnCl2- 70mM, pH 5.5) and incubated on ice for 1 hour. Then solution from both the falcon tube were combined in one falcon tube and the ASB treated cells were then pelleted by centrifugation carried out at 3500 rpm for 15 min at 4□C and resuspended in 2ml of ASB-B (Sodium acetate- 40mM, CaCl2- 100mM, MnCl2- 70mM, 15% glycerol, pH 5.5) and were stored in Eppendorf tubes (aliquots of 50□l) in deep freezer at -86 □C for future use.

2.1.3 Purification of DNA from agarose gels (gel extraction):

DNA was purified from agarose gels using GeneJET gel extraction kit (Fermentas Life Sciences, cat. No. K0691) following the instructions recommended by the manufacturer.

2.1.4 Restriction digests and Ligation:

All restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Ligation reactions were performed using T4-DNA ligase (New England Biolabs), used as recommended by the manufacturer. Whole ligation reactions were used for transformation into *E. coli* (DH5α).

2.1.5 Plasmid DNA Isolation from mini bacterial culture:

A single colony was picked out from the plate containing colonies of DH5α transformed cells and inoculated in 2ml of LB medium containing ampicillin. The culture was incubated in a shaking incubator at 37°C, 200 rpm for overnight. Entire culture was used for isolation of plasmid DNA using Plasmid Mini-Prep kit (Fermentas Life Sciences, cat. No. K0502) following the instructions recommended by the manufacturer.

2.1.6 Transformation of competent *E. coli* cells:

(I) ***Transformation of competent DH5a cells*****:** DH5α competent *E. coli* cells were prepared as described above. 5µl of a ligation mixture was added to 25µl of competent DH5α cells and incubated on ice for 30 minutes. After that heat shock was given at 42°C for 30 seconds, cells were again briefly incubated on ice for 5 minutes and transformed competent cells were plated on LB- agar plates containing 100µg/ml ampicillin. Plates were incubated at 37°C overnight.

(II) ***Transformation of competent BL21(DE3)pLysS cells*****:** BL21(DE3)pLysS competent *E. coli* cells were prepared as described above. 1µl of a plasmid DNA was added to 20µl of competent cells and the mixture was incubated on ice for 5 minutes. After a 42°C heat shock for 30 seconds, the cells were briefly incubated on ice for 5

minutes. After this incubation, entire transformation mixture was plated on LB-agar plates containing 100µg/ml ampicillin. Plates were incubated at 37°C overnight for growth of cells.

2.1.7 SDS-PAGE:

Recombinant proteins were analyzed through SDS-PAGE. SDS-PAGE was carried out using discontinuous buffer system as described by Laemmli using Bio-Rad gel electrophoresis apparatus (Cat. no: 165-8001). Throughout this study 1.5mm thick 10% and 12% polyacrylamide (30:0.8 acrylamide to bisacrylamide ratio) gel containing 0.1% SDS was used for electrophoretic separation of proteins. The protein samples were mixed with half volume of 3X sample buffer (2.4ml 1M Tris-HCl, pH 6.5; 3ml 20% SDS, 3ml 100% Glycerol, 1.6ml β-mercaptoethanol, 0.006g Bromophenol blue) and heated at 100°C for 10 minutes and were loaded into the wells of precast polyacrylamide gel. Electrophoresis was performed at a constant voltage range of 100-140V by diluting 10x buffer (Tris base 30.3g, Glycine 144g, SDS 10g in 1000ml). After the electrophoresis, the gel was stained with coomassie brilliant blue (0.4% w/v Coomassie blue R250, 30% v/v Methanol, 10 % Acetic acid). Spectra multicolor broad range protein ladder was used to confirm the size of protein. Composition of 10% and 12% SDS-PAGE gel is given below:

SDS-PAGE Gel Composition

Resolving Gel			Stacking Gel	
Components	**10%**	**12%**	**Components**	**4%**
30% Acrylamide bisacrylamide	1.65ml	2.0ml	30% Acrylamide bisacrylamide	0.65 ml
Tris-HCl, pH 8.8, 1.5M	1.25ml	1.25ml	Tris-HCl, pH 6.8, 0.5M	1.25 ml
H_2O	2.05ml	1.70ml	H_2O	3.05 ml
SDS (10%)	50.0µl	50.0µl	SDS (10%)	50µl
APS	45.0µl	45.0µl	APS	25 µl
TEMED	20.0µl	20.0µl	TEMED	10.0µl

2.1.8 Media and Solutions:

De-ionized water was used for all buffers solutions and media.

Media	Composition
LB media	1%Tryptone w/v, 0.5% w/v Yeast Extract
TB media	1.2% Tryptone w/v, 2.4% Yeast Extract, 0.4% v/v glycerol

2.1.9 Other solutions:

i) **Isopropyl β-D-1-thiogalactopyranoside (1M):** IPTG 4.7g, H_2O 20ml.

ii) **20% SDS**: SDS (20g), H_2O (80ml). Make final volume 100 ml with stirring.

iii) **20% Ammonium per Sulfate:** APS (0.2g), H_2O (0.8 ml).

2.1.10 General buffers used in the study:

Buffers	Composition
TE (Tris-EDTA) 50X	1mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0. A 10X stock solution was routinely used to prepare 1X TE.
SDS-PAGE Resolving gel buffer	1.5 M Tris-HCl, (for Resolving Gel) ,pH 8.8
SDS -PAGE Stacking gel buffer	0.5 M Tris-HCl (for stacking gel), pH 6.5
10X SDS-PAGE gel running buffer	Tris base 30.3g, Glycine 144g, SDS 10g (add last) , Make final volume to 1000ml.
1X Gradient gel running buffer	25mM Tris Base, 192mM Glycine, 0.1 % (w/v) SDS, methanol 20%.
3X SDS-PAGE loading dye (10ml)	1M Tris-Cl, pH 6.5 (2.4 ml), 20% SDS (3 ml), Glycerol (100%) (3 ml), β-mercaptoethanol (1.6 ml), Bromophenol blue (0.006g).
SDS-PAGE staining solution.	Coomassie blue R250-0.4% w/v, Methanol-30% v/v, Acetic acid 10% v/v
SDS-PAGE de-staining solution	Methanol- 30 % v/v, Acetic acid 10 % v/v.

2.2.1 Cloning of GST-ABH3:

2.2.1.1 PCR amplification of ABH3:

The HeLa (S3) cDNA obtained from Clontech Laboratories was used as template to amplify the ABH3 gene. The PCR was carried out using Phusion High-Fidelity DNA Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers ABH3-BamHI-Sen (5'CCTGGGATCC ATGGAGGAAA AAAGACGGCG AGCC3') and ABH3-EcoRI-Anti (5'CTTCGAATTC TCACCAGGGT GCCCCTCGAG GGTC3') were synthesized by Imperial Life Sciences, Haryana, India. Full-length ABH3 gene was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98℃ for 30 seconds: followed by 98℃ for 30 seconds: 57℃ for 30 seconds: 72℃ for 1 min: final extension 72℃ for 10 min: and 4℃ on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1 kb DNA ladder (NEB)

was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.2.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 4- 4.0 µl, BamHI-1.0 U, EcoRI-1.0 U, incubated at 37˚C for 4 h. The entire digested product was analyzed by 0.8 % agarose gel at 70 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.2.1.3 Restriction digestion of vector:

For cloning of ABH3, pGEX-6P-1 (GE Healthcare), a vector with N-terminal GSTtag was used. Vector digestion was carried out with plasmid DNA-17.0 µl, 10X NEB buffer 4- 2.0 µl, BamHI-0.5 U, EcoRI-0.5 U and incubated at 37℃ for 4 h. The digested product was separated in 0.8 % agarose gel, at 70 V for 30 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.2.1.4 Ligation of BamHI, EcoRI digested ABH3 and pGEX-6P-1:

Ligation of BamHI, EcoRI digested ABH3 and pGEX-6P-1 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: BamHI, EcoRI cut ABH3 (insert) 7.0 µl, BamHI, EcoRI cut pGEX-6P-1 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16℃ for 15 h.

2.2.1.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5 µl of the ligation mix was added to 25 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42℃ for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37℃ for 12 h. Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37℃ / 200 rpm / 12 h. Alkaline lysis method was followed to isolate plasmid from the grown culture.

Cells were pelleted at 8000 rpm / 5 min / 4℃. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4℃. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20℃ for 40 min. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4℃. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4℃. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min. Later the positive clones were regrown and isolated using GeneJET plasmid purification kit (Thermo Scientific: Cat. #K0502).

2.2.1.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. Two restriction sites were chosen, one from the vector backbone (BamHI) and one from the gene of interest (NdeI). In a ligated vector the position of BamHI and NdeI are 945 and 1640 nucleotide respectively and the presence of correct insert would result in release of an insert of size 695 nucleotide. The reaction conditions were as follows: Plasmid DNA: 4.0 µl, BamHI and NdeI- 0.4 U, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.2.2 Expression of recombinant ABH3:

2.2.2.1Transformation of pGEX-ABH3 into BL21(DE3)pLysS

The positive clones, after restriction analysis, were selected for transformation into bacterial expression system, BL21 (pLysS). 1.0 µl of plasmid DNA was added to 25 µl of BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5 min. Heat shock was given at 42℃ for 30 sec. After incubation on ice for 5 min., entire transformation mix was plated on LB-Amp agar plates. The plates were then incubated at 37℃ for 13 h.

2.2.2.2 Small-scale expression analysis of recombinant GST-ABH3

After the transformation, 5-10 colonies were inoculated into 5 ml of LB-Amp broth. Cells were allowed to grow till log phase (approximately 3 h) at 37℃, 200 rpm. 2 ml of the culture was aliquoted as uninduced sample and the remaining 3 ml was induced with

1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30℃ for 4 h. After the induction, 1 ml of uninduced and induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 μl of Protein Extraction Buffer (Tris, pH 8.0 - 50 mM; NaCl - 250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 40 μl of resuspended cells were mixed with 20 μl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100℃ for 10 min. 15 μl of the uninduced and 10 μl of the induced samples were loaded into 10 % SDS-PAGE, run at 140 V for 70 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.2.3 Purification of recombinant ABH3

2.2.3.1 Large scale culture of recombinant ABH3

The positive clone(s) for expression was inoculated into 100 ml LB-Amp broth and grown overnight at 37℃ / 200 rpm. The preinoculum was then diluted into 1 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37℃ / 200 rpm / 3 h using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30℃ / 200 rpm / 4 h. Cells were then pelleted at 8000 rpm / 15 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80℃ refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.2.3.2 Purification of recombinant ABH3 protein by Glutathione-affinity chromatography Method:

1 L cell pellet was resuspended in 20 ml of extraction buffer (Tris, pH 7.4 – 50 mM; NaCl 550 mM; Triton X100 0.05 %; DTT – 2 mM) by vortex. GST fusion proteins are susceptible to proteolytic cleavage. To avoid this, proteolytic inhibitors are usually added to the extraction buffer. One protease inhibitor cocktail tablet, EDTA free (Roche, Cat. No. 11873580001) was dissolved in 50 ml extraction buffer. Total extract was then subjected to sonication on ice for 15 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4℃ using centrifuge (Thermo

Scientific, Model No. SORVALL Legend XTR). Glutathione sepharose 4 fast flow beads (GE Life Sciences, Cat. No.: 17-5132-01), equilibrated with the extraction buffer, were added to the soluble fraction. Binding was carried out by gentle rotation at 10 rpm / 4℃ for 3 h using an orbital rotator (Tarson, Cat. No. 3090). After binding unbound fraction was removed by centrifugation at 500 g / 4℃ / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed 4 times with wash buffer (Tris, pH 7.4 – 50mM; NaCl 1 M; Triton X100 0.05 %; DTT – 2 mM). The bound protein was then eluted using elution buffer (Tris, pH 8.0 – 50 mM; NaCl 100 mM DTT – 2 mM, reduced glutathione, Sigma Cat. No. G4251 – 10 mM, NaOH – 0.01 N).Usually binding of the GST-tagged protein is optimal at neutral pH (PBS or Tris, pH 7.5). The excess reduced glutathione (10 mM) is added which displaces the immobilized GST-fusion protein from the beads. Reduced glutathione when added to the elution buffer decreases the pH of the elution buffer. To neutralize this reduced pH, 10 N NaOH is added. Pure protein was collected in 4 different fractions as elute – 1, 2, 3 and 4. The protein elutes along with soluble fraction, unbound and wash fractions were analyzed by in 10 % SDS-PAGE at 140 V / 60 min. Pre-stained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cut-off 3500 Da (Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80℃.

2.3.1 Cloning of GST-ABH2:

2.3.1.1 PCR amplification of ABH2:

The HeLa (S3) cDNA obtained from Clontech was used as template to amplify the ABH2 gene. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers ABH2- EcoRI-Sen (5’TCGAGAATTC ATGGACAGAT TCCTGGTGAA AGGGG3’) and ABH2-SalI-NheI-Anti (5’CAGCGCTAGC GTCGACTTAT TTTTTAGTAA GCAAAATTTT ACG3’) were synthesized by Imperial Life Sciences, Haryana, India. Full-length ABH2 gene was

amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98℃ for 30 seconds: followed by 98℃ for 30 seconds: 57℃ for 30 seconds: 72℃ for 1 min: final extension 72℃ for 10 min: and 4℃ on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1 kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.3.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 4- 4.0 µl, EcoRI-1.0 U, SalI-1.0 U, incubated at 37℃ for 5 h. The entire digested product was analyzed by 0.7 % agarose gel at 90 V for 25 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.7 % agarose gel run at 120 V for 15 min.

2.3.1.3 Restriction digestion of vector:

For cloning of ABH3, pGEX-6P-1 (GE Healthcare), a vector with N-terminal GST-tag was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 4- 2.0 µl, EcoRI-1.0 U, SalI-1.0 U and incubated at 37℃ for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.3.1.4 Ligation of EcoRI, SalI digested ABH2 and pGEX-6P-1:

Ligation of EcoRI, SalI digested ABH2 and pGEX-6P-1 was carried out using T4- DNA ligase (NEB: M0202). The composition of the ligation was as follows: EcoRI, SalI cut ABH2 (insert) 7.0 µl, EcoRI, SalI cut pGEX-6P-1 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16℃ for 20 h.

2.3.1.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. Entire ligation mix (10.0 µl) was added to 50 µl DH5α competent cells and incubated on ice for

30 min. The incubation was followed by heat shock at 42℃ for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 μg/ml). The plates were incubated at 37℃ for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37℃ / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4℃. Pellet was resuspended in 250 μl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 μl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 μl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4℃. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20℃ for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4℃. Plasmid DNA pellet was washed with 250 μl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4℃. Pellet was air dried and resuspended in 50 μl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min.

2.3.1.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. Two restriction sites were chosen, one from the vector backbone (EcoRI) and one from the gene of interest (KpnI). In a ligated vector the position of EcoRI and KpnI are 954 and 1664 nucleotide respectively and the presence of correct insert would result in release of an insert of size 710 nucleotide. The reaction conditions were as follows: Plasmid DNA: 10.0 μl, EcoRI and KpnI- 0.5 U, in a total volume of 15 μl. The reaction was incubated at 37℃ for 5 h 30 min and 15 μl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.3.2 Expression of recombinant ABH2:

2.3.2.1 Transformation of pGEX-ABH2 into BL21(DE3)pLysS

The positive clones, after restriction analysis, were selected for transformation into bacterial expression system, BL21 (pLysS). 1.0 μl of plasmid DNA was added to 25 μl of BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5 min. Heat shock was given at 42℃ for 30 sec. After incubation on ice for 5 min., entire

transformation mix was plated on LB-Amp agar plates. The plates were then incubated at 37℃ for 14 h.

2.3.2.2 Small-scale expression analysis of recombinant GST-ABH2

After the transformation, 5-10 colonies were inoculated into 6 ml of LB-Amp broth. Cells were allowed to grow till log phase (approximately 3 h) at 37℃, 200 rpm. 2 ml of the culture was aliquoted as uninduced sample and the remaining 4 ml was induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30℃ for 4 h. After the induction, 1 ml of uninduced and 800 µl of induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 µl of Protein Extraction Buffer (Tris, pH 8.0 - 50 mM; NaCl - 250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 20 µl of resuspended cells were mixed with 10 µl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100℃ for 10 min. 15 µl of the uninduced and 10 µl of the induced samples were loaded into 10 % SDS-PAGE, run at 140 V for 60 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.3.3 Purification of recombinant ABH2

2.3.3.1 Large scale culture of recombinant ABH2

The positive clone(s) for expression was inoculated into 200 ml LB-Amp broth and grown overnight at 37℃ / 200 rpm. The preinoculum was then diluted into 2 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37℃ / 200 rpm / 3 h using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30℃ / 200 rpm / 5 h 30 min. Cells were then pelleted at 8000 rpm / 15 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80℃ refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.3.3.2 Purification of recombinant ABH2 protein by Glutathione-affinity chromatography Method:

2 L cell pellet was resuspended in 25 ml of extraction buffer (Tris, pH 7.4 – 50 mM; NaCl 550 mM; Triton X100 0.05 %; DTT – 2 mM) by vortex. GST fusion proteins are

susceptible to proteolytic cleavage. To avoid this, proteolytic inhibitors are usually added to the extraction buffer. One protease inhibitor cocktail tablet, EDTA free (Roche, Cat. No. 11873580001) was dissolved in 50 ml extraction buffer. Total extract was then subjected to sonication on ice for 20 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Glutathione sepharose 4 fast flow beads (GE Life Sciences, Cat. No.: 17-5132-01), equilibrated with the extraction buffer, were added to the soluble fraction. Binding was carried out by gentle rotation at 10 rpm / 4℃ for 3 h using an orbital rotator (Tarson, Cat. No. 3090). After binding unbound fraction was removed by centrifugation at 500 g / 4℃ / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed 4 times with wash buffer (Tris, pH 7.4 – 50mM; NaCl 1 M; Triton X100 0.05 %; DTT – 2 mM). The bound protein was then eluted using elution buffer (Tris, pH 8.0 – 50 mM; NaCl 100 mM DTT – 2 mM, reduced glutathione, Sigma Cat. No. G4251 – 10 mM, NaOH – 0.01 N). Usually binding of the GST-tagged protein is optimal at neutral pH (PBS or Tris, pH 7.5). The excess reduced glutathione (10 mM) is added which displaces the immobilized GST-fusion protein from the beads. Reduced glutathione when added to the elution buffer decreases the pH of the elution buffer. To neutralize this reduced pH, 10 N NaOH is added. Pure protein was collected in 4 different fractions as elute – 1, 2, 3 and 4. The protein elutes along with soluble fraction, unbound and wash fractions were analyzed by in 10 % SDS-PAGE at 140 V / 60 min. Pre-stained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cut-off 3500 Da (Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80℃.

2.4.1 Cloning of His-SSB

2.4.1.1 PCR amplification of SSB:

The genomic DNA isolated from *E. coli* K-12 strain was used as template to amplify the SSB gene fragment. The PCR was carried out using Phusion High- Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers SSB-BamHI-Sen (5'CCTGGGATCC ATGGCCAGCA GAGGCGTAAA CAAGG3') and SSB-XhoI-Anti (5'GCCGCTCGAG TCAGAACGGA ATGTCATCAT CAAAGTCC3') were synthesized by Imperial Life Sciences, Haryana, India. SSB gene was amplified using the specified primers. The PCR reaction had dNTP- 200 μM, primers-1.0 μM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98℃ for 30 seconds: followed by 98℃ for30 seconds: 56℃ for 30 seconds: 72℃ for 1 min: final extension 72℃ for 10 min: and 4℃ on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.4.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA - 44 μl, 10X NEB buffer 4- 5.0 μl, BamHI-0.5 U, XhoI-0.5 U, incubated overnight at 37℃. The entire digested product was analyzed by 0.8 % agarose gel at 80 V for 20 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.4.1.3 Restriction digestion of vector:

For cloning of SSB gene, pRSET-A (GE Healthcare), a vector with N-terminal polyhistidine tag was used. Vector digestion was carried out with plasmid DNA- 17.0 μl, 10X NEB buffer 4- 2.0 μl, BamHI-0.5 U, XhoI-0.5 U and incubated overnight at 37℃. The digested product was separated in 0.8 % agarose gel at 80 V for 20 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel

extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.4.1.4 Ligation of BamHI, XhoI digested SSB and pRSET-A:

Ligation of BamHI, XhoI digested SSB and pRSET-A was carried out using T4- DNA ligase (NEB: M0202). The composition of the ligation was as follows: BamHI, XhoI cut SSB (insert) 7.0 µl, BamHI, XhoI cut pRSET-A (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 17 h.

2.4.1.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. Entire 10.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42℃ for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37℃ for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚C / 200 rpm / 13 h. The colonies obtained were screened using GeneJET plasmid purification kit (Thermo Scientific, Cat.no: #K0502) as per instruction. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min.

2.4.1.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. One restriction site (PvuII) was chosen that is present in both the vector backbone and the gene of interest. In a ligated vector the position of PvuII are 457 and 758 nucleotide and the presence of correct insert would result in release of an insert of size 302 nucleotide. The reaction conditions were as follows: Plasmid DNA: 5.0 µl, PvuII- 1.0 U, in a total volume of 10 µl. The reaction was incubated at 37℃ for 4 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.4.2 Expression of recombinant SSB:

2.4.2.1 Transformation of pRSETA-SSB into BL21(DE3)pLysS

The positive clones, after restriction analysis, were selected for transformation into bacterial expression system, BL21(pLysS). 1.0 µl of plasmid DNA was added to 50 µl of BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5

min. Heat shock was given at 42℃ for 30 sec. After incubation on ice for 5 min., entire transformation mix was plated on LB-Amp agar plates. The plates were then incubated at 37℃ for 12 h.

2.4.2.2 Small-scale expression analysis of recombinant His-SSB

After the transformation, 5-10 colonies were inoculated into 5 ml of LB-Amp broth. Cells were allowed to grow till log phase (approximately 3 h) at 37℃, 200 rpm. 2 ml of the culture was aliquoted as uninduced sample and the remaining 3 ml was induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30℃ for 4 h. After the induction, 1 ml of uninduced and 750 µl of induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 µl of Protein Extraction Buffer (Tris, pH 8.0 - 50 mM; NaCl - 250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 40 µl of resuspended cells were mixed with 20 µl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100℃ for 10 min. 15 µl of the uninduced and 10 µl of the induced samples were loaded into 10 % SDS-PAGE, run at 140 V for 50 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.4.3 Purification of recombinant SSB

2.4.3.1 Large scale culture of recombinant SSB

The positive clone(s) for expression was inoculated into 100 ml LB-Amp broth and grown overnight at 37℃ / 200 rpm. The preinoculum was then diluted into 1 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37℃ / 200 rpm / 3 h using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30℃ / 200 rpm / 4 h 30 min. Cells were then pelleted at 8000 rpm / 15 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80℃ refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.4.3.2 Purification of recombinant SSB protein by Ni-NTA affinity

The cell pellet was resuspended in 20 ml of extraction buffer (Tris, pH 8.0 – 50 mM; NaCl 300 mM; Triton X100 0.1 %; Imidazole – 1 mM) by vortex. Total extract was then

subjected to sonication on ice for 15 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Clear supernatant was taken for Ni-NTA (Qiagen, Cat. No. 30210) bead binding. Before addition of the beads to supernatant, beads were equilibrated with the extraction buffer. Binding was carried out by gentle rotation at 10 rpm / 4℃ for 4 h. After binding unbound fraction was removed by centrifugation at 1000 g / 4℃ / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed 3 times with wash buffer (Tris, pH 8.0 – 50mM; NaCl 350 mM; Triton X100 0.1 %; Imidazole – 5 mM). Washing step was repeated 3 times with 15 ml wash buffer. The bound protein was eluted using elution buffer (Tris – 10 mM; NaCl 100 mM; Imidazole – 250 mM). Protein was collected in 4 different fractions as elute – 1, 2, 3 and 4. The protein elutes along with soluble, insoluble, unbound and wash fractions were loaded and checked in 10 % SDS-PAGE at 140 V / 50 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cut-off 3500 Da (Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80℃.

2.5.1 Cloning of His-SSB ΔC8

2.5.1.1 PCR amplification of SSB ΔC8:

The genomic DNA isolated from *E. coli* K-12 strain was used as template to amplify the SSB gene fragment. The PCR was carried out using Phusion High- Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers SSB-BamHI-Sen (5'CCTGGGATCC ATGGCCAGCA GAGGCGTAAA CAAGG3') and SSB-dC8-XhoI-Anti (5'GCCGCTCGAG TCACATCGGC GGCTCGTTAG ACG3') were synthesized by Imperial Life Sciences, Haryana, India. SSB-CTD delta8 gene fragment was amplified using the specified primers. The PCR reaction had dNTP-200 µM,

primers-1.0 μM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98℃ for 30 seconds: followed by 98℃ for30 seconds: 56℃ for 30 seconds: 72℃ for 1 min: final extension 72℃ for 10 min: and 4℃ on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 1kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.5.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 μl, 10X NEB buffer 4- 4.0 μl, BamHI-1.0 U, XhoI-1.0 U, incubated at 37℃ for 4 h. The entire digested product was analyzed by 0.8 % agarose gel at 80 V for 20 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.5.1.3 Restriction digestion of vector:

For cloning of SSB gene fragment (ΔC8), pRSET-A (GE Healthcare), a vector with N-terminal polyhistidine tag was used. Vector digestion was carried out with plasmid DNA-17.0 μl, 10X NEB buffer 4- 2.0 μl, BamHI-0.5 U, XhoI-0.5 U and incubated at 37˚C for 12 h. The digested product was separated in 0.8 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.5.1.4 Ligation of BamHI, XhoI digested SSB ΔC8 and pRSET-A:

Ligation of BamHI, XhoI digested SSB ΔC8 and pRSET-A was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: BamHI, XhoI cut SSB (insert) 7.0 μl, BamHI, XhoI cut pRSET-A (vector) - 1.0 μl in a 10.0 μl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.5.1.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 μl of the ligation mix was added to 50 μl DH5α competent cells and incubated on ice for 30

min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚C / 200 rpm / 10 h. The colonies obtained were screened using GeneJET plasmid purification kit (Thermo Scientific, Cat.no: #K0502) as per instruction. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min.

2.5.1.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. One restriction site (PvuII) was chosen that is present in both the vector backbone and the gene of interest. In a ligated vector the position of PvuII are 457 and 734 nucleotide and the presence of correct insert would result in release of an insert of size 278 nucleotide. The reaction conditions were as follows: Plasmid DNA: 6.0 µl, PvuII- 0.5 U, in a total volume of 10 µl. The reaction was incubated at 37˚C for 4 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.5.2 Expression of recombinant SSB:

2.5.2.1 Transformation of pRSETA-SSB (ΔC8) into BL21(DE3)pLysS

The positive clones, after restriction analysis, were selected for transformation into bacterial expression system, BL21(pLysS). 1.0 µl of plasmid DNA was added to 25 µl of BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5 min. Heat shock was given at 42℃ for 30 sec. After incubation on ice for 5 min., entire transformation mix was plated on LB-Amp agar plates. The plates were then incubated at 37℃ for 12 h.

2.5.2.2 Small-scale expression analysis of recombinant His-SSB

After the transformation, 5-10 colonies were inoculated into 5 ml of LB-Amp broth. Cells were allowed to grow till log phase (approximately 3 h) at 37℃, 200 rpm. 1.5 ml of the culture was aliquoted as uninduced sample and the remaining 3.5 ml was induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30℃ for 4 h. After the induction, 1.5 ml of each uninduced and induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 µl of Protein

Extraction Buffer (Tris, pH 8.0 – 50 mM; NaCl -250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 40 µl of resuspended cells were mixed with 20 µl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100℃ for 10 min. 25 µl of the uninduced and 15 µl of the induced samples were loaded into 10 % SDS-PAGE, run at 140 V for 50 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.5.3 Purification of recombinant SSB ΔC8

2.5.3.1 Large scale culture of recombinant SSB ΔC8

The positive clone(s) for expression was inoculated into 100 ml LB-Amp broth and grown overnight at 37℃ / 200 rpm. The preinoculum was then diluted into 1 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37℃ / 200 rpm / 2 h 30 min using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30℃ / 200 rpm / 4 h 45 min. Cells were then pelleted at 8000 rpm / 15 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80℃ refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.5.3.2 Purification of recombinant SSB ΔC8 protein by Ni-NTA affinity

The cell pellet was resuspended in 20 ml of extraction buffer (Tris, pH 8.0 – 50 mM; NaCl 300 mM; Triton X100 0.1 %; Imidazole – 1 mM) by vortex. Total extract was then subjected to sonication on ice for 15 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Clear supernatant was taken for Ni-NTA (Qiagen, Cat. No. 30210) bead binding. Before addition of the beads to supernatant, beads were equilibrated with the extraction buffer. Binding was carried out by gentle rotation at 10 rpm / 18-20℃ for 4 h. After binding unbound fraction was removed by centrifugation at 1000 g / 4℃ / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed with washbuffer (Tris, pH 8.0 – 50mM; NaCl 350 mM; Triton X100 0.1 %;

Imidazole – 5 mM). Washing step was repeated 3 times with 15 ml wash buffer. The bound protein was eluted using elution buffer (Tris – 10 mM; NaCl 100 mM; Imidazole – 250 mM). Protein was collected in 4 different fractions as elute – 1, 2, 3 and 4. The protein elutes along with soluble, insoluble, unbound and wash fractions were loaded and checked in 10 % SDS-PAGE at 140 V / 50 min. Pre-stained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cutoff 3500 Da (Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80℃.

2.6.1 Cloning of GST-AlkB:

Cloning of AlkB was carried out in two steps: first AlkB was cloned into pBlueScript II (SK+) vector using BamHI and HindIII restriction sites. From pBS-AlkB, AlkB was sub-cloned into pGEX-6P-1 vector by digesting pBS-AlkB with BamHI and SalI restriction sites.

2.6.1.1 PCR amplification of AlkB:

The HeLa (S3) cDNA obtained from Clontech Laboratories was used as template to amplify the ABH3 gene. The PCR was carried out using Phusion High-Fidelity DNA Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers F-EcAlkB-BamHI (5'AAAAGGATCC ATGTTGGATC TGTTTGCCGA TGCTGAACCG TGGC3') and R-EcAlkB-HindIII (5'ATTCAAGCTT TTATTCTTTT TTACCTGCCT GACGGAATGT CAGG3') were synthesized by Imperial Life Sciences, Haryana, India. Full-length ALKB gene was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98℃ for 30 seconds: followed by 98℃ for 30 seconds: 56℃ for 30 seconds: 72℃ for 1 min: final extension 72℃ for 10 min: and 4℃ on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA)

at 120 V for 15 min. Standard 1 kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.6.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA- 34 µl, 10X NEB buffer 4- 4.0 µl, BamHI-1.0 U, HindIII-1.0 U, incubated at 37˚C for 4 h. The entire digested product was analyzed by 0.8 % agarose gel at 70 V for 25 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.6.1.3 Restriction digestion of pBS vector:

pBlueScript plasmid, a vector withmultiple cloning site (MCS) located within LacZ gene, was used for the cloning of AlkB. Insertion of gene in the MCS disrupts the LacZ gene. This results in formation of white colonies when selected on X-gal plate. Plasmid digestion reaction was as follows: pBlueScript (SK+) 7.0 µl, BamHI 1.0 U, HindIII 1.0 U in a 10.0 µl reaction. Incubation was carried out for 5 h at 37℃. The digested product was separated on 0.8 % agarose gel and the linearized plasmid DNA was excised from the gel using scalpel. DNA was extracted using GeneJET gel extraction kit according to the manufacturer's instructions. The DNA was eluted using molecular grade water.

2.6.1.4 Ligation of BamHI and HindIII digested AlkB and pBS

Ligation was carried out using T4-DNA ligase. The reaction composition was as follows: BamHI and HindIII digested AlkB – 7.0 µl and BamHI and HindIII digested pBS - 1.0 µl in a 10.0 µl reaction. The reaction was incubated at 16℃ for 12 h.

2.6.1.5 Transformation and X-gal selection

The ligation mix was transformed into DH5α competent cells according to the protocol as described in the section 1.5. After transformation, entire transformation mix was plated on LB-Amp plates containing X-gal and IPTG. Preparation of X-gal plates: X-gal, chemically known as 5-Bromo-4-chloro-3- indolyl β-D-galactopyranoside was purchased from sigma (Cat. No. B4252). It was dissolved in Dimethyl sulfoxide (Vetec, D2658) to a final concentration of 20 mg/ml. 1 M stock of Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758) was prepared in autoclaved water. 200 µl of X-gal and 50 µl of IPTG were mixed and spreaded uniformly on solidified LB-Amp agar plates and allowed to dry.

After drying the transformation mix was plated on this plates. The plates were then incubated at 37℃ for 16 h.

2.6.1.6 Colony screening of pBS-AlkB

Colonies appeared after 16 h of incubation. The plate was then shifted to 4℃ for completion of reaction between X-gal and IPTG. This was confirmed by appearance of some blue color colonies on the plate. Usually non-transformants appear in blue color whereas transformed colonies appear in white color even after incubation. The white color colonies were selected and incubated into 2 ml of LB-Amp broth and the miniprep for the plasmid was carried out as per the protocol described in section 2.2.1.5.

2.6.1.7 Restriction analysis of pBS-AlkB

After the miniprep, clones showing slow mobility on agarose gel were selected for confirmation by restriction analysis. Two restriction sites were selected from the plasmid map – BamHI (1346 bp) and SalI (674 bp). The reaction conditions were as follows: Plasmid DNA 4.0 µl, BamHI 0.5 U and SalI 0.5 U in a 10.0 µl reaction. Incubation was carried out at 37℃ for 4 h. the reaction was analyzed on 0.8 % agarose gel run at 120 V for 20 min.

2.6.1.8 Restriction digestion of pBS-AlkB and pGEX-6P-1

pBS-AlkB positive clone was enriched in DH5α and plasmid was isolated by GeneJET plasmid purification kit. AlkB insert with flanking restriction sites, BamHI and SalI, was prepared by digesting pBS-AlkB with BamHI and SalI. Reaction conditions were as follows: pBS-AlkB – 20.0 µl, BamHI – 1.0 U, SalI – 1.0 U. Incubation was overnight at 37℃. the reaction was then loaded in a 0.8 % agarose gel and the 672 bp fragment was gel eluted using GeneJET Gel extraction kit. DNA was eluted using molecular grade water.

2.6.1.9 Restriction digestion of pGEX-6P-1

For sub-cloning of AlkB, pGEX-6P-1 (GE Healthcare), a vector with N-terminal GST-tag was used. Vector digestion was carried out with plasmid DNA-17.0 µl, 10X NEB buffer 4- 2.0 µl, BamHI-0.5 U, HindIII-0.5 U and incubated at 37℃ for 4 h. The digested product was separated in 0.8 % agarose gel, at 70 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.6.1.10 Ligation of BamHI, SalI digested AlkB and pGEX-6P-1:

Ligation of BamHI, EcoRI digested ABH3 and pGEX-6P-1 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: BamHI, EcoRI cut AlkB (insert) 7.0 μl, BamHI, EcoRI cut pGEX-6P-1 (vector) - 1.0 μl in a 10.0 μl reaction. The ligation reaction was incubated at 16℃ for 12 h.

2.6.1.11 Transformation of ligation and colony screening

The ligation mixture after incubation was transformed into DH5α competent cell. 5 μl of the ligation mix was added to 50 μl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42℃ for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 μg/ml). The plates were incubated at 37℃ for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37℃ / 200 rpm / 12 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4℃. Pellet was resuspended in 250 μl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 μl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 μl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4℃. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20℃ for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4℃. Plasmid DNA pellet was washed with 250 μl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4℃. Pellet was air dried and resuspended in 50 μl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min. Later the positive clones were regrown and isolated using GeneJET plasmid purification kit (Thermo Scientific: Cat. #K0502) as per the instruction.

2.6.1.12 Confirmation of the clones:

Transformed colonies were screened for the mobility shift (slower mobility) on a 0.8 % agarose gel.

2.6.2 Expression analysis of recombinant GST-AlkB

2.6.2.1 Transformation of pGEX-AlkB into BL21(DE3)pLysS

The positive clones, after confirmation, were selected for transformation into bacterial expression system, BL21 (pLysS). 1.0 μl of plasmid DNA was added to 50 μl of

BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5 min. Heat shock was given at 42℃ for 30 sec. After incubation on ice for 5 min., entire transformation mix was plated on LB-Amp agar plates. The plates were then incubated at 37℃ for 12 h.

2.6.2.2 Small-scale expression analysis of recombinant GST-AlkB

After the transformation, 5-10 colonies were inoculated into 4 ml of LB-Amp broth. Cells were allowed to grow till log phase (approximately 3 h) at 37℃, 200 rpm. 1 ml of the culture was aliquoted as uninduced sample and the remaining 3 ml was induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30℃ for 4 h. After the induction, 1 ml of uninduced and induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 µl of Protein Extraction Buffer (Tris, pH 8.0 - 50 mM; NaCl - 250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 40 µl of resuspended cells were mixed with 20 µl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100℃ for 10 min. 15 µl of the uninduced and 10 µl of the induced samples were loaded into 12 % SDS-PAGE, run at 140 V for 70 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.6.3 Purification of recombinant AlkB

2.6.3.1 Large scale culture of recombinant AlkB

The positive clone(s) for expression was inoculated into 270 ml LB-Amp broth and grown overnight at 37℃ / 200 rpm. The preinoculum was then diluted into 2.7 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37℃ / 200 rpm / 3 h using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30℃ / 200 rpm / 4 h. Cells were then pelleted at 8000 rpm / 15 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80℃ refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.6.3.2 Purification of recombinant AlkB protein by Glutathione-affinity chromatography Method:

3 L cell pellet was divided into two falcon and each was resuspended in 20 ml of extraction buffer (Tris, pH 7.4 – 50 mM; NaCl 550 mM; Triton X100 0.05 %; DTT – 2 mM) by vortex. GST fusion proteins are susceptible to proteolytic cleavage. To avoid this, proteolytic inhibitors are usually added to the extraction buffer. One protease inhibitor cocktail tablet, EDTA free (Roche, Cat. No. 11873580001) was dissolved in 50 ml extraction buffer. Total extract was then subjected to sonication on ice for 15 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4℃ using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Soluble fractions were collected in one falcon tube. Glutathione sepharose 4 fast flow beads (GE Life Sciences, Cat. No.: 17-5132-01), equilibrated with the extraction buffer, were added to the soluble fraction. Binding was carried out by gentle rotation at 10 rpm / 4℃ for 2 h using an orbital rotator (Tarson, Cat. No. 3090). After binding unbound fraction was removed by centrifugation at 500 g / 4℃ / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed 3 times with wash buffer (Tris, pH 7.4 – 50mM; NaCl 1 M; Triton X100 0.05 %; DTT – 2 mM). The bound protein was then eluted using elution buffer (Tris, pH 8.0 – 50 mM; NaCl 150 mM; DTT – 2 mM, reduced glutathione, Sigma Cat. No. G4251 – 10 mM, NaOH – 0.01 N). Usually binding of the GST-tagged protein is optimal at neutral pH (PBS or Tris, pH 7.5). The excess reduced glutathione (10 mM) is added which displaces the immobilized GST-fusion protein from the beads. Reduced glutathione when added to the elution buffer decreases the pH of the elution buffer. To neutralize this reduced pH, 10 N NaOH is added. Pure protein was collected in 4 different fractions as elute – 1, 2, 3 and 4. The protein elutes along with total extract, soluble, insoluble, unbound and wash fractions were analyzed by in 10 % SDS-PAGE at 140 V / 60 min. Pre-stained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cut-off 3500 Da

(Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80℃.

(Cloning for Yeast Two Hybrid Assay)

2.7 Cloning of AlkB N-terminal 75

2.7.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBNcoI- Sen (5'AAAACCATGG AGATGTTGGA TCTGTTTGCC GATGC3') and AlkB75-SalI-Anti (5'TTTTGTCGAC ACCTTGCCGA TGGGTCGTCC AGC3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB 75) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TEbuffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.7.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.7.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 3.1- 2.0 µl, NcoI-1.0 U, XhoI-1.0 U and incubated at 37˚

C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.7.4 Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2:

Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB75 (insert) 7.0 µl, NcoI, XhoI cut pACT2 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.7.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚ C for 12 h. Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.7.6 Confirmation of the clones:

Plasmids containing insert were initially selectedbased on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and

orientation of the insert by two different restriction analysis. In both the restriction analysis, single restriction site was chosen, which is present in vector at two different positions, one before the multiple cloning site (MCS) and one after the MCS. In a ligated vector the positions of BglII are 3037 and 3335 nucleotide whereas the positions of HindIII are 2618 and 3578 nucleotide and the presence of correct insert would result in release of an insert of size 299 and 961 nucleotide respectively. The reaction conditions were as follows: Plasmid DNA: 10.0 µl, 0.5 U of both the enzymes BglII and HindIII (separate reactions) were used, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.8 Cloning of AlkB Middle 75

2.8.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBd75- NcoI-BamHI-Sen (5'AAAACCATGG GATCCTATCT CTATTCGCCC ATTGATCCGC3') and AlkB150-SalI-Anti (5'TTTTGTCGAC TAAGCCCAGA GAAACAGAAA CAATTGG3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB Mid75) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.8.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using

GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.8.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 3.1- 2.0 µl, NcoI-1.0 U, XhoI-1.0 U and incubated at 37˚ C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.8.4 Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2:

Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB Mid75 (insert) 7.0 µl, NcoI, XhoI cut pACT2 (vector)- 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.8.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚ C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated

by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.8.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by two different restriction analysis. In both the restriction analysis, single restriction site was chosen, which is present in vector at two different positions, one before the multiple cloning site (MCS) and one after the MCS. In a ligated vector the positions of BglII are 3037 and 3338 nucleotide whereas the positions of HindIII are 2618 and 3581 nucleotide and the presence of correct insert would result in release of an insert of size 302 and 964 nucleotide respectively. The reaction conditions were as follows: Plasmid DNA: 10.0 µl, 0.5 U of both the enzymes BglII and HindIII (separate reactions) were used, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.9 Cloning of AlkB C-terminal (Δ150)

2.9.1 PCR amplification of AlkB C-terminal (Δ150) fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBd150- NcoI-BamHI-Sen (5’AAAACCATGG GATCCCCCGC GATTTTTCAA TTTGGCGGCC3’) and AlkB-SalI-Anti (5’TTTTGTCGAC TTATTCTTTT TTACCTGCCT GACGG3’) were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB delta150) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.9.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.9.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 3.1- 2.0 µl, NcoI-1.0 U, XhoI-1.0 U and incubated at 37˚C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.9.4 Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2:

Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB delta150 (insert) 7.0 µl, NcoI, XhoI cut pACT2 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.9.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚C / 200 rpm / 10 h. Alkaline lysis method was

followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.9.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by two different restriction analysis. In both the restriction analysis, single restriction site was chosen, which is present in vector at two different positions, one before the multiple cloning site (MCS) and one after the MCS. In a ligated vector the positions of BglII are 3037 and 3314 nucleotide whereas the positions of HindIII are 2618 and 3557 nucleotide and the presence of correct insert would result in release of an insert of size 278 and 940 nucleotide respectively. The reaction conditions were as follows: Plasmid DNA: 10.0 µl, 0.5 U of both the enzymes BglII and HindIII (separate reactions) were used, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.10 Cloning of AlkB N-terminal 150

2.10.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBNcoI- Sen (5'AAAACCATGG AGATGTTGGA TCTGTTTGCC GATGC3') and AlkB150-SalI-Anti (5'TTTTGTCGAC TAAGCCCAGA GAAACAGAAA CAATTGG3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB 150) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0

μM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.10.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 μl, 10X NEB buffer 3.1- 4.0 μl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.10.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA-16.0 μl, 10X NEB buffer 3.1- 2.0 μl, NcoI-1.0 U, XhoI-1.0 U and incubated at 37˚C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.10.4 Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2:

Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB 150 (insert) 7.0 μl, NcoI, XhoI cut pACT2 (vector) - 1.0 μl in a 10.0 μl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.10.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB- agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚ C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚ C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.10.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by two different restriction analysis. In both the restriction analysis, single restriction site was chosen, which is present in vector at two different positions, one before the multiple cloning site (MCS) and one after the MCS. In a ligated vector the positions of BglII are 3037 and 3560 nucleotide whereas the positions of HindIII are 2618 and 3803 nucleotide and the presence of correct insert would result in release of an insert of size 524 and 1186 nucleotide respectively. The reaction conditions were as follows: Plasmid DNA: 10.0 µl, 0.5 U of both the enzymes BglII and HindIII (separate reactions) were used, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.11 Cloning of AlkB Δ75

2.11.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBd75- NcoI-BamHI-Sen (5'AAAACCATGG GATCCTATCT CTATTCGCCC ATTGATCCGC3') and AlkB-SalI-Anti (5'TTTTGTCGAC TTATTCTTTT TTACCTGCCT GACGG3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB delta75) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.11.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.11.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 3.1- 2.0 µl, NcoI-1.0 U, XhoI-1.0 U and incubated at 37˚C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using

GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.11.4 Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2:

Ligation of NcoI, SalI digested AlkB fragment and NcoI, XhoI digested pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB delta75 (insert) 7.0 µl, NcoI, XhoI cut pACT2 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.11.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚ C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚ C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.11.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by two different restriction analysis. In both the restriction analysis, single restriction site was chosen, which is present in vector at two different positions, one before the multiple cloning site (MCS) and one after the MCS. In a ligated

vector the positions of BglII are 3037 and 3539 nucleotide whereas the positions of HindIII are 2618 and 3782 nucleotide and the presence of correct insert would result in release of an insert of size 503 and 1165 nucleotide respectively. The reaction conditions were as follows: Plasmid DNA: 10.0 µl, 0.5 U of both the enzymes BglII and HindIII (separate reactions) were used, in a total volume of 10 µl. The reaction was incubated at 37˚C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.12 Cloning of AlkB N-terminal 75 (in pGBKT7 Vector)

2.12.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBNcoI- Sen (5'AAAACCATGG AGATGTTGGA TCTGTTTGCC GATGC3') and AlkB75-SalI-Anti (5'TTTTGTCGAC ACCTTGCCGA TGGGTCGTCC AGC3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB 75) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TEbuffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.12.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.12.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pGBKT7 (Clontech Laboratories), a vector with GAL4 binding domain (BD) was used. Vector digestion was carried out with plasmid DNA-25.0 µl, 10X NEB buffer 3.1- 3.0 µl, NcoI-1.0 U, SalI-1.0 U and incubated at 37℃ for 4 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.12.4 Ligation of NcoI, SalI digested AlkB fragment and pGBKT7 vector:

Ligation of NcoI, SalI digested AlkB fragment and pGBKT7 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB75 (insert) 7.0 µl, NcoI, XhoI cut pGBKT7 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.12.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Kanamycin (final conc. 25 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Kan broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚ C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚ C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.12.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. Two restriction sites were chosen, one from the vector backbone (XhoI) and one from the gene of interest (XmaI). In a ligated vector the position of XhoI and XmaI are 979 and 1447 nucleotide respectively and the presence of correct insert would result in release of an insert of size 468 nucleotide. The reaction conditions were as follows: Plasmid DNA: 8.0 µl, XhoI and XmaI- 0.5 U, in a total volume of 10 µl. The reaction was incubated at 37˚C for 4 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.13 Cloning of AlkB Middle 75 (in pGBKT7 vector)

2.13.1 PCR amplification of AlkB fragments:

The previous AlkB clone (pET-28-a-AlkB) was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High-Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkBd75- NcoI-BamHI-Sen (5'AAAACCATGG GATCCTATCT CTATTCGCCC ATTGATCCGC3') and AlkB150-SalI-Anti (5'TTTTGTCGAC TAAGCCCAGA GAAACAGAAA CAATTGG3') were synthesized by Imperial Life Sciences, Haryana, India. Truncated AlkB gene (AlkB Mid75) was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for 10 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 1.2 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 25 min. Standard 100 bp DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.13.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 3.1- 4.0 µl, NcoI-1.0 U, SalI-1.0 U, incubated at 37˚C for 5 h. The entire digested product was analyzed by 1.2 % agarose gel at 90 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using

GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 1.2 % agarose gel run at 120 V for 20 min.

2.13.3 Restriction digestion of vector:

For cloning of truncated AlkB fragment, pGBKT7 (Clontech Laboratories), a vector with GAL4 binding domain (BD) was used. Vector digestion was carried out with plasmid DNA-16.0 µl, 10X NEB buffer 3.1- 2.0 µl, NcoI-1.0 U, SalI-1.0 U and incubated at 37˚C for 5 h. The digested product was separated in 0.7 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.7 % agarose gel.

2.13.4 Ligation of NcoI, SalI digested AlkB fragment and pGBKT7:

Ligation of NcoI, SalI digested AlkB fragment and pGBKT7 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB Mid75 (insert) 7.0 µl, NcoI, SalI cut pGBKT7 (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.13.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB- agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Kanamycin (final conc. 25 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Kan broth and incubated at 37˚ C / 200 rpm / 10 h. Alkaline lysis method was followed to isolate plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4˚ C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4˚ C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20˚C for 1 h. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4˚C. Plasmid DNA pellet was washed with

250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4˚C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.6 % agarose gel, run at 120 V for 20 min.

2.13.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. Two restriction sites were chosen, both from the vector backbone (XhoI and SalI). In a ligated vector the position of XhoI and SalI are 979 and 1519 nucleotide respectively and the presence of correct insert would result in release of an insert of size 540 nucleotide. The reaction conditions were as follows: Plasmid DNA: 8.0 µl, XhoI and SalI- 0.5 U, in a total volume of 10 µl. The reaction was incubated at 37˚C for 4 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.14 Cloning of SSB in pACT2

2.14.1 PCR amplification of SSB:

The genomic DNA isolated from *E. coli* K-12 strain was used as template to amplify the SSB gene fragment. The PCR was carried out using Phusion High- Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers SSB-NcoI-Sen (5'AAAACCATGG AGATGGCCAG CAGAGGCGTA AAC3') and SSB-XhoI-Anti (5'GCCGCTCGAG TCAGAACGGA ATGTCATCAT CAAAGTCC3') were synthesized by Imperial Life Sciences, Haryana, India. SSB gene was amplified using the specified primers. The PCR reaction had dNTP- 200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for30 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TEbuffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1kb DNAladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.14.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA- 43 µl, 10X NEB buffer 4- 5.0 µl, NcoI-1.0 U, XhoI-1.0 U, incubated overnight at 37℃.

The entire digested product was analyzed by 0.8 % agarose gel at 80 V for 20 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.14.3 Restriction digestion of vector:

For cloning of SSB gene, pACT2 (Clontech Laboratories), a vector with GAL4 activation domain (AD) was used. Vector digestion was carried out with plasmid DNA- 17.0 μl, 10X NEB buffer 4- 2.0 μl, NcoI-0.5 U, XhoI-0.5 U and incubated at 37˚C for 12 h. The digested product was separated in 0.8 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.14.4 Ligation of BamHI, XhoI digested SSB and pACT2:

Ligation of NcoI, XhoI digested SSB and pACT2 was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, XhoI cut SSB (insert) 7.0 μl, NcoI, XhoI cut pACT2 (vector) - 1.0 μl in a 10.0 μl reaction. The ligation reaction was incubated at 16˚C for 12 h.

2.14.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. Entire 10.0 μl of the ligation mix was added to 50 μl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚ C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Ampicillin (final conc. 100 μg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Amp broth and incubated at 37˚ C / 200 rpm / 10 h. The colonies obtained were screened using GeneJET plasmid purification kit (Thermo Scientific, Cat.no: #K0502) as per instruction. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min.

2.14.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. One restriction site (BglII) was chosen that is present in the vector backbone twice. In a ligated vector the position of BglII are 3036 and 3645 nucleotide and the presence of correct insert would result in release of an insert of size 609 nucleotide. The reaction conditions were as follows: Plasmid DNA: 8.0 μl, NcoI and XhoI - 0.5 U each, in a total reaction volume of 10 μl. The reaction was incubated overnight at 37℃ and 10 μl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.15 Cloning of AlkB in pGBKT7

2.15.1 PCR amplification of AlkB:

The genomic DNA isolated from *E. coli* K-12 strain was used as template to amplify the AlkB gene fragment. The PCR was carried out using Phusion High- Fidelity Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers AlkB-NcoI-Sen (5'AAAACCATGG AGATGTTGGA TCTGTTTGCC GATGC3') and AlkB-SalI-Anti (5'TTTTGTCGAC TTATTCTTTT TTACCTGCCT GACGG3') were synthesized by Imperial Life Sciences, Haryana, India. AlkB gene was amplified using the specified primers. The PCR reaction had dNTP-200 μM, primers-1.0 μM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98˚C for 30 seconds: followed by 98˚C for30 seconds: 56˚C for 30 seconds: 72˚C for 1 min: final extension 72˚C for 10 min: and 4˚C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.15.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the sequential restriction digestion were as follows: Cleaned-up PCR DNA-43 μl, 10X NEB buffer 3.1- 5.0 μl, NcoI-1.0 U, SalI- 1.0 U and incubated at 37℃ for 5 h. The entire digested product was analyzed by 0.8 % agarose gel at 80 V for 20 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested product was

extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.15.3 Restriction digestion of vector:

For cloning of AlkB gene, pGBKT7 (Clontech Laboratories), a vector with GAL4 binding domain (BD) was used. Vector digestion was carried out with plasmid DNA- 26.0 µl, 10X NEB buffer 4- 3.0 µl, NcoI-1.0 U and incubated overnight at 37℃. The digested product was cleaned up and re-digestion was carried out with plasmid DNA- 25.0 µl, NcoI-1.0 µl, SalI -1.0 µl in a 30.0 µl of reaction volume and incubated at 37℃ for 5 h. The digested product was separated in 0.8 % agarose gel, at 90 V for 25 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.15.4 Ligation of NcoI, SalI digested AlkB and pGBKT7:

Ligation of NcoI, SalI digested AlkB and pGBKT7 was carried out using T4- DNA ligase (NEB: M0202). The composition of the ligation was as follows: NcoI, SalI cut AlkB (insert) 7.0 µl, NcoI, SalI cut pGBKT7 (vector)- 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16˚C for 14 h.

2.15.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. Entire 5.0 µl of the ligation mix was added to 50 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42˚ C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Kanamycin (final conc. 25 µg/ml). The plates were incubated at 37˚C for 12 h.

Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Kan broth and incubated at 37℃ / 200 rpm / 10 h. The colonies obtained were screened using GeneJET plasmid purification kit (Thermo Scientific, Cat.no: #K0502) as per instruction. The plasmid DNA thus obtained was analyzed on 0.7 % agarose gel, run at 120 V for 20 min.

2.15.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and

orientation of the insert by restriction analysis. Two restriction sites (NcoI and SalI) were chosen that are present in the vector backbone. In a ligated vector the position of NcoI and SalI are 1288 and 1945 nucleotide and the presence of correct insert would result in release of an insert of size 657 nucleotide. The reaction conditions were as follows: Plasmid DNA: 4.0 µl, NcoI- 0.5 U and SalI- 0.5 U, in a total volume of 10 µl. The reaction was incubated at 37˚C for 4 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.16 Interaction study of SSB and AlkB by *in-vitro* pull-down assay

Pull-down assays with Ni–NTA beads (QIAGEN) were performed using purified His-SSB. GST-AlkB (concentration 5µM) was mixed with His-SSB (concentration 200µM) and incubated at 4°C for 2h in 100µl of interaction buffer (IB: 50mM Tris–HCl, pH 7.4; 100mM NaCl; 5% glycerol; 1mM dithiothreitol (DTT) and 0.005% Tween-20). 50µl of Ni-NTA beads slurry equilibrated with equal amount (50µl) of interaction buffer, a total volume of 100µl was added to each reaction and incubated at 18-20°C on rotor for 1 hr. The beads were separated and washed three times with 0.5ml of interaction buffer supplemented with 5mM imidazole, and bound proteins were eluted in 2ml of interaction buffer supplemented with 250mM imidazole. Elution was followed by 30 min. incubation at 18°C. Eluted proteins were analyzed by 10% SDS-PAGE followed by coomassie staining.

Chapter 3

Figures

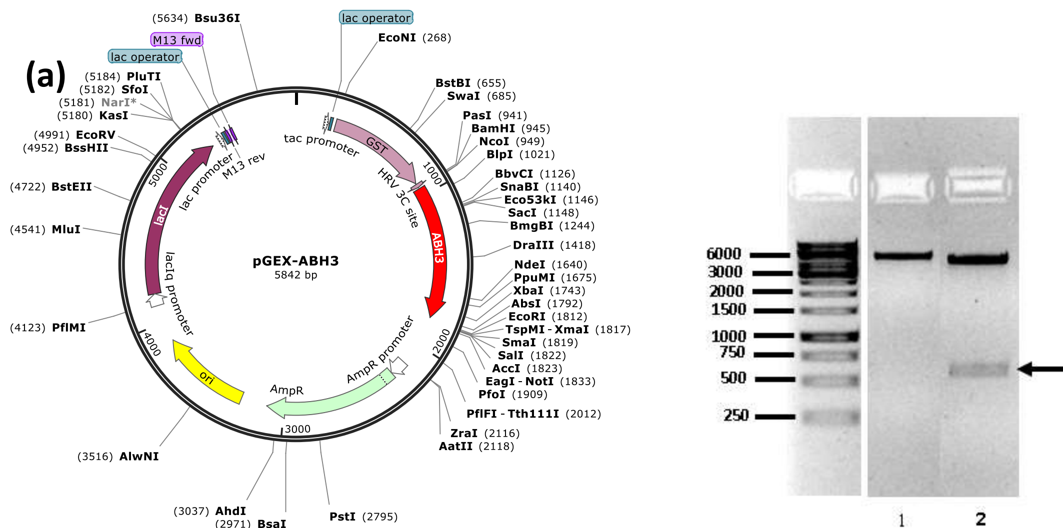

Figure 3.1: Construction of pGEX-ABH3

(a) Plasmid map of pGEX-ABH3 generated using SnapGene software. Unique restriction sites are indicated in bold.

(b) The pGEX-ABH3 plasmid obtained from the plasmid miniprep was confirmed for the presence of ABH3 insert by restriction analysis. pGEX-ABH3 plasmid was digested with BamHI (945) and NdeI (1640) (Lane 2). 1kb ladder was used to compare the released insert. The Gel was viewed under Syngene Gel Doc system. The presence of ABH3 insert in correct orientation was confirmed by release of 695 bp insert.

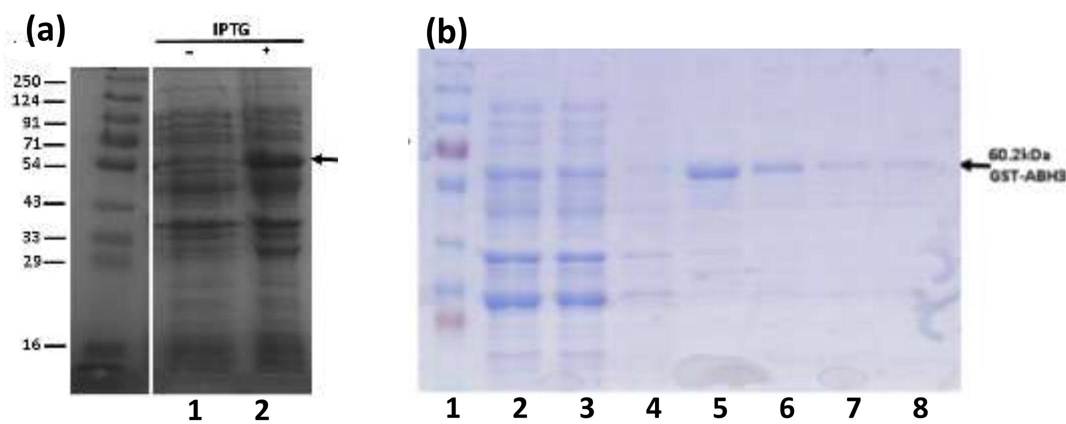

Figure 3.2: Expression and purification of recombinant GST-ABH3

(a) pGEX-ABH3 was transformed into BL21(DE3)pLysS and was analyzed for expression of GST-ABH3(60.2kDa) by induction, using 1 mM IPTG. The expression level of the induced cell extract (lane 2) was compared with the uninduced cell extract (lane 1), analyzed in 10% SDS-PAGE.

(b) Recombinant GST-ABH3 was purified from 1 L culture by one-step glutathione-affinity chromatography. Soluble fraction (lane 2) was loaded along with unbound (lane 3) and wash fraction (lane 4). The purified GST-ABH3 (lane 5-8) was collected as different fractions (elute 1, 2, 3 and 4). All the fractions were loaded in 10% SDS-PAGE along with Pre-stained Protein Marker (lane 1) and the bands were visualized by Coomassie staining.

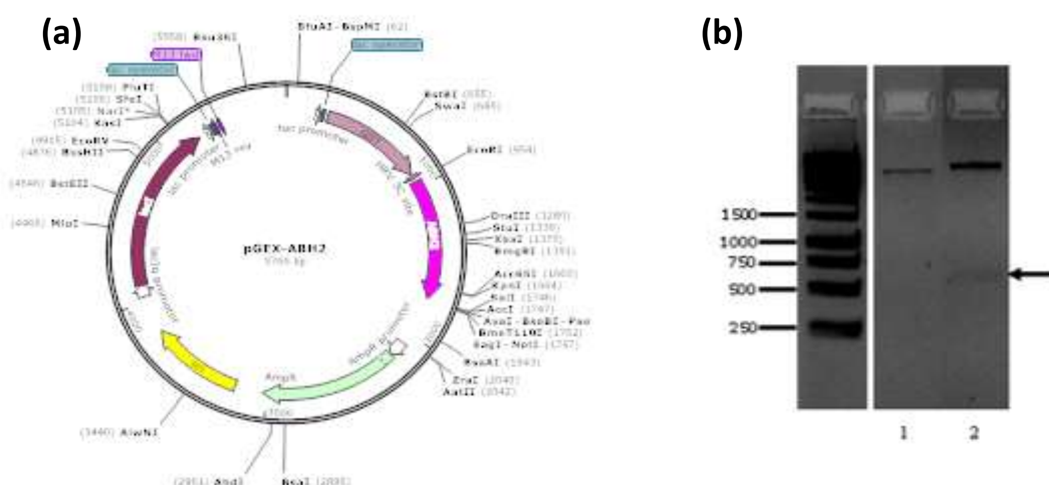

Figure 3.3: Construction of pGEX-ABH2

(a) Plasmid map of pGEX-ABH2 generated using SnapGene software. Unique restriction sites are indicated in bold.

(b) The pGEX-ABH2 plasmid obtained from the plasmid miniprep was confirmed for the presence of ABH2 insert by restriction analysis. pGEX-ABH2 plasmid was digested with EcoRI (954) and KpnI (1664) (Lane 2). 1kb ladder was used to compare the released insert. The Gel was viewed under Syngene Gel Doc system. The presence of ABH2 insert in correct orientation was confirmed by release of 710 bp insert.

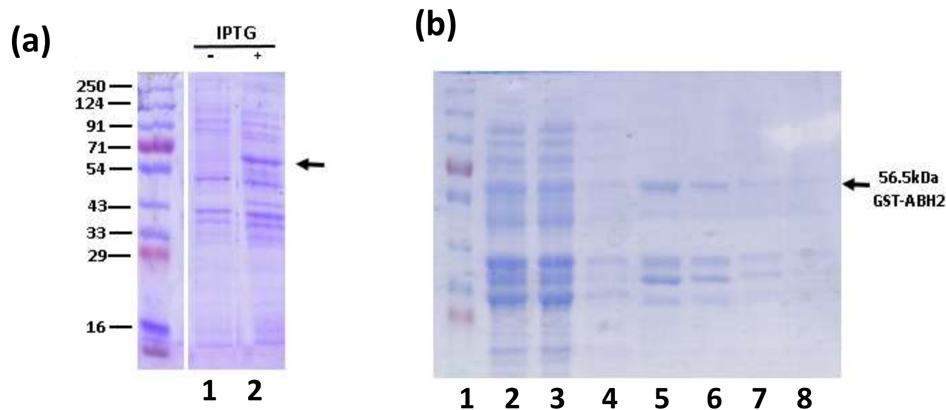

Figure 3.4: Expression and purification of recombinant GST-ABH2

(a) pGEX-ABH2 was transformed into BL21(DE3)pLysS and was analyzed for expression of GST-ABH2(56.5kDa) by induction, using 1 mM IPTG. The expression level of the induced cell extract (lane 2) was compared with the uninduced cell extract (lane 1), analyzed in 10% SDS-PAGE.

(b) Recombinant GST-ABH2 was purified from 1 L culture by one-step glutathione-affinity chromatography. Soluble fraction (lane 2) was loaded along with unbound (lane 3) and wash fraction (lane 4). The purified GST-ABH2 (lane 5-8) was collected as different fractions (elute 1, 2, 3 and 4). All the fractions were loaded in 10% SDS-PAGE along with Pre-stained Protein Marker (lane 1) and the bands were visualized by Coomassie staining.

Figure 3.5: Construction of pRSETA-SSB

(a) Plasmid map of pRSETA-SSB generated using SnapGene software. Unique restriction sites are indicated in bold.

(b) The pRSETA-SSB plasmid obtained from the plasmid miniprep was confirmed for the presence of SSB insert by restriction analysis. pRSETA-SSB plasmid was digested with PvuII (457 and 758) (Lane 5). 1kb ladder (lane 1) was loaded along with pRSETA (lane 2), PvuII cut pRSETA (lane 3), pRSETA-SSB (lane 4) to compare the released insert. The Gel was viewed under Syngene Gel Doc system. The presence of SSB insert in correct orientation was confirmed by release of 302 bp insert.

Figure 3.6: Expression and purification of recombinant His-SSB

(a) pRSETA-SSB was transformed into BL21(DE3)pLysS and was analyzed for expression of His-SSBH2(23.1kDa) by induction, using 1 mM IPTG. The expression level of the induced cell extract (lane 3) was compared with the uninduced cell extract (lane 2), analyzed in 10% SDS-PAGE.

(b) Recombinant His-SSB was purified from 1 L culture by one-step Ni-NTA-affinity chromatography. Bacterial lysate (lane 1) was loaded along with Soluble (lane 2), insoluble (lane 3), unbound (lane 4) and wash fraction (lane 5). The purified His-SSB (lane 5 and lane 7-9) was collected as different fractions (elute 1, 2, 3 and 4). All the fractions were loaded in 10% SDS-PAGE along with Pre-stained Protein Marker (lane 6) and the bands were visualized by Coomassie staining.

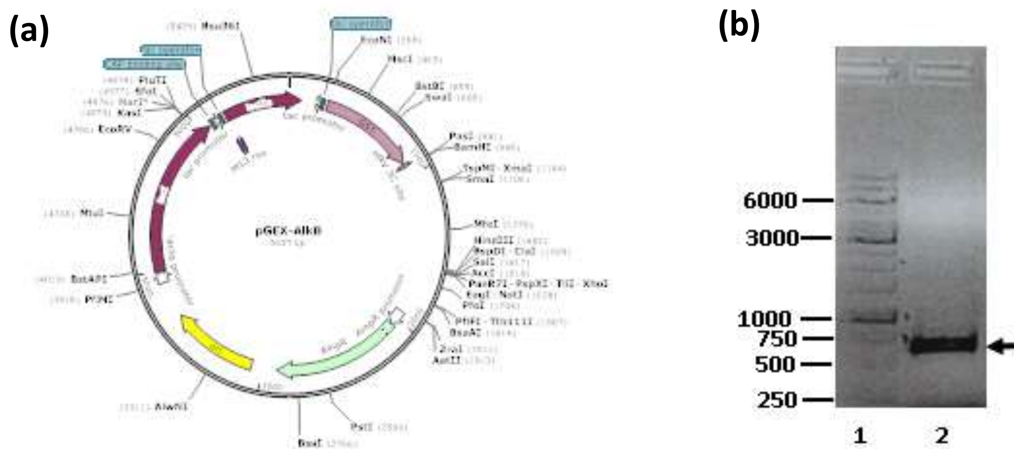

Figure 3.7: Construction of pGEX-AlkB

(a) Plasmid map of pGEX-AlkB generated using SnapGene software. Unique restriction sites are indicated in bold.

(b) The pGEX-AlkB plasmid obtained from the plasmid miniprep was confirmed for the presence of AlkB insert by PCR amplification. AlkB gene was PCR amplified with pBlueScript-AlkB template (Lane 2). 1kb ladder was used to compare the size of PCR amplified product. The Gel was viewed under Syngene Gel Doc system.

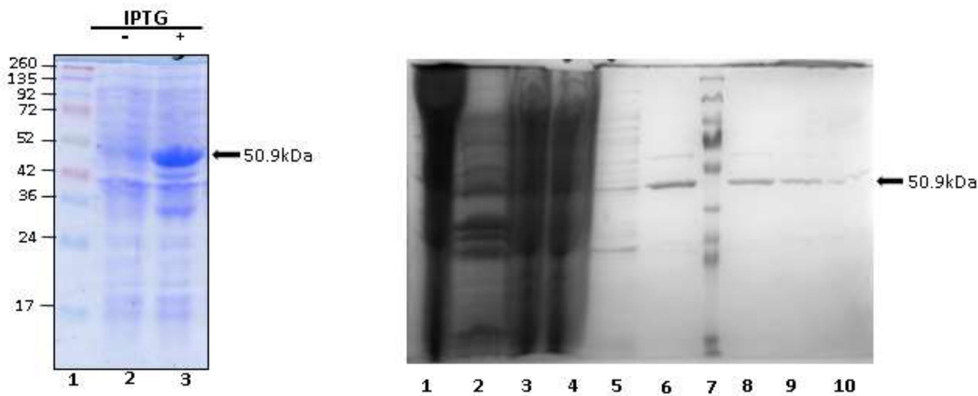

Figure 3.8: Expression and purification of recombinant GST-AlkB

(a) pGEX-AlkB was transformed into BL21(DE3)pLysS and was analyzed for expression of GST-AlkB(50.9kDa) by induction, using 1 mM IPTG. The expression level of the induced cell extract (lane 2) was compared with the uninduced cell extract (lane 1), analyzed in 10% SDS-PAGE.

(b) Recombinant GST-AlkB was purified from 3 L culture by one-step glutathione-affinity chromatography. Bacterial lysate (lane 1) was loaded with insoluble (lane 2), soluble (lane 3), unbound (lane 4) and wash fraction (lane 5). The purified GST-AlkB (lane 6 & 8-10) was collected as different fractions (elute 1, 2, 3 and 4). All the fractions were loaded in 10% SDS-PAGE along with Pre-stained Protein Marker (lane 1) and the bands were visualized by Coomassie staining.

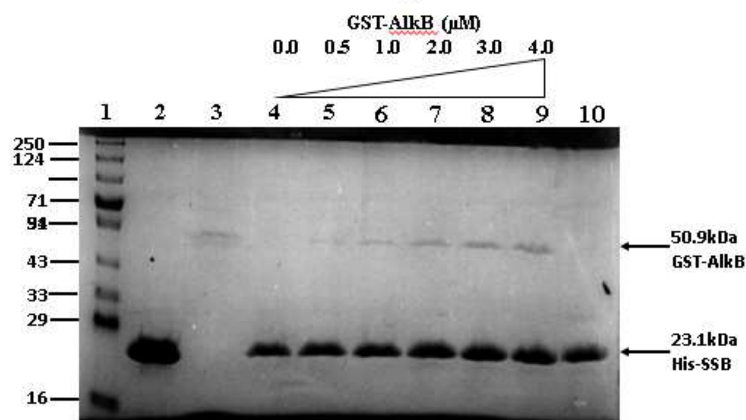

Figure 3.9: Interaction study of AlkB & SSB by *in-vitro* pull-down assay: Ni-NTA bound His-SSB and purified GST-AlkB were used for interaction study. Increasing volume of purified GST-AlkB was used for the interaction. Lane 1, Protein molecular weight marker; Lane 2, purified His-SSB (10μl) control; Lane 3, GST-AlkB (30μl) control; Lane 4-9, 4μM of His-SSB in combination with increasing concentration of GST-AlkB(0μM, 0.5μM, 1μM, 2μM, 3μM, 4μM). It was checked with only GST (lane 10) as a negative control.

Gururaj Shivange

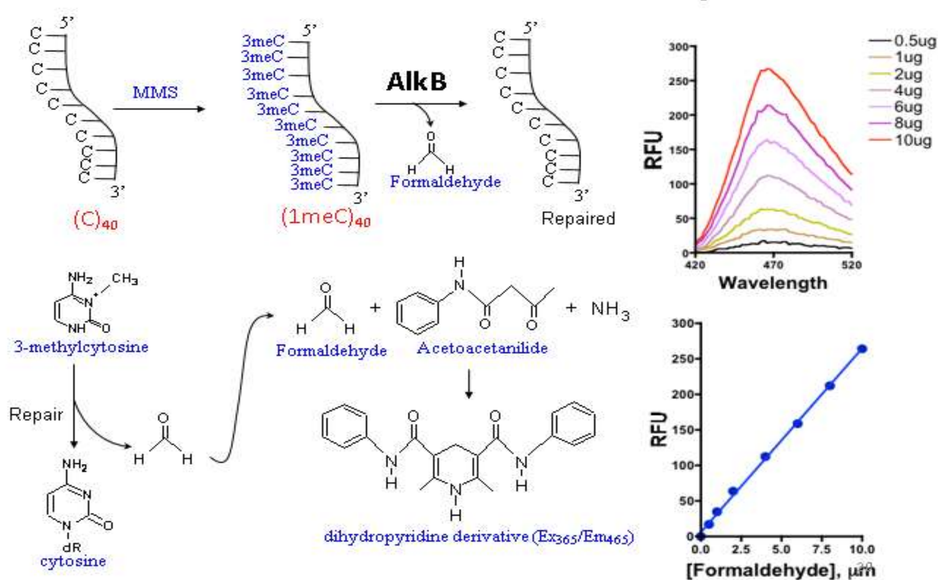

Figure 3.10: Experimental set up to detect AlkB activity: 40mer oligo were used for the experiment. 40mer oligo were treated with MMS for the duration of 14-15 h, then repair assay was performed by adding AlkB in the reaction and incubated for an hour at 37 ℃. The repair activity was checked by determining the amount of formaldehyde released by converting this into dihydropyridine derivative which has excitation wavelength of 365 nm and emission wavelength of 465 nm.

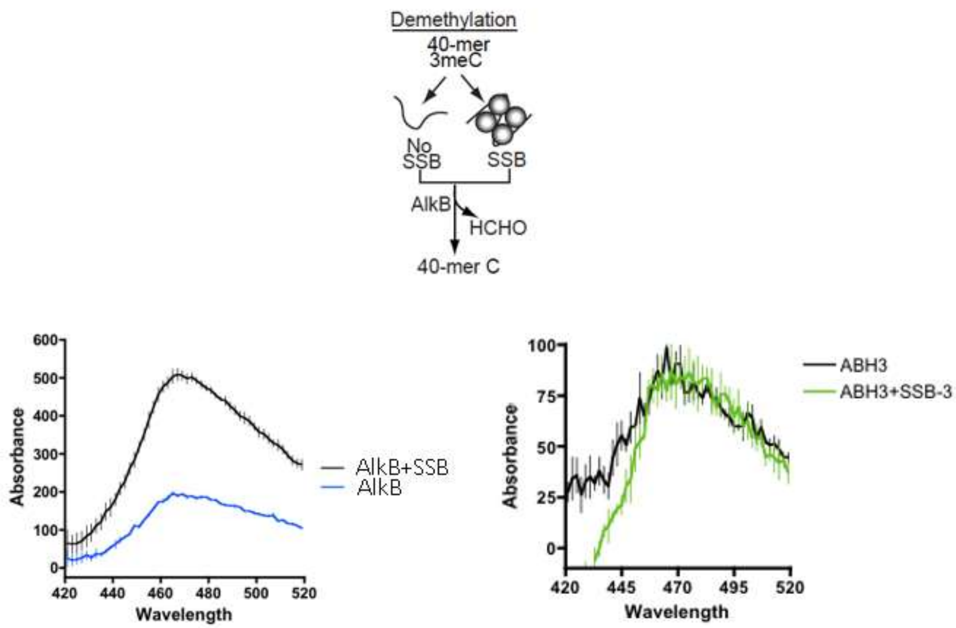

Figure 3.11: Effect of SSB on AlkB and ABH3 activity: 40mer oligo used were damaged and repair activity was performed in the presence and absence of SSB with the AlkB and ABH3 in separate reactions. In the presence of SSB the activity of AlkB and ABH3 activity was checked by determining the amount of formaldehyde released.

Chapter 4

Result & Discussion

In this research, I studied the effect of single-stranded DNA-binding protein on the activity of *Escherichia coli* AlkB. AlkB is a direct repair protein and found involved in the direct repair of 1-methyl adenine and 3-methyl cytosine. These two gene from the *E. coli* K12 strain were used for the cloning purpose. For His-tagged SSB protein, pRSET-A vector and for GST-tagged AlkB protein, pGEX-6P-1 vector were used. They were cloned successfully and the recombinant protein were purified as mentioned in the chapter 2. The interaction study was carried out by *in-vitro* pull-down assay and they are found to have interaction. The constant volume of His-SSB were used for the interaction study with the increasing concentration of GST-AlkB. The fractions were eluted with 250 mM imidazole. The appearance of both the band on elution shows that AlkB interacts with the SSB protein and with increase in the concentration of GST-AlkB, the intensity of the band also get increased. This clears the interaction more strongly. The same interaction was also checked with only GST to be sure that the interaction is not with the GST protein but with the AlkB and it was found as we expected.

AlkB homologs ABH3 and ABH2 were also cloned in pGEX-6P-1 to study the comparative analysis of effect of SSB on these proteins. Both the cloning were done successfully and purified the recombinant GST-ABH3 and GST-ABH2, but unfortunately the later one got degraded. To study the effect of SSB protein on the activity of AlkB and ABH3, 40mer adenine and 40mer cytosine were used. They were damaged with MMS (methylmethane sulfonate) and repair assay was carried out for 1 h with either of the purified protein (GST-AlkB and GST-ABH3). These reactions were used in both the combination with and without SSB. As the reaction result we got, one can easily understand that in the presence of SSB, the activity of AlkB gets increased while SSB has no effect on ABH3 activity. This repair activity was determined by the detection of amount of formaldehyde released during repair activity. The amount of formaldehyde released are then reacted with acetoacitanilide and ammonium acetate to get dihydropyridine derivative, which has excitation wavelength of 365 nm and emission wavelength of 465 nm. The absorption profile

at 465 nm suggests the effect of SSB on the activity of AlkB and it showed a significant difference in the absorption profile in the presence and absence of SSB. The same reaction with ABH3 did not give significant difference in the absorption profile, so I can be concluded that SSB protein has no effect or negligible effect on ABH3 protein.

The same interaction study is being carried out *in-vivo* by yeast two hybrid assay and we are hopeful that this will bring some important findings that will help in describing interaction of these two protein.

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