ISSN: 0976-0172
Journal of Bioscience And Technology
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DDX3X, a frequently mutated gene in medulloblastoma, encoding a DEAD box family RNA helicase can be expressed successfully as a recombinant protein in bacteria as a MBP-fusion protein.

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1. INTRODUCTION

Medulloblastoma is the most common malignant brain tumor that predominantly occurs in children [1]. Medulloblastomas consists of four core molecular subgroups *viz*. WNT, SHH, Group 3 and Group 4 [2]. The WNT and SHH subgroup tumors are characterized by over-expression of a number of WNT and SHH signaling pathway genes respectively. *DDX3X* gene encoding ATP-dependent RNA helicase is the second most commonly mutated gene in medulloblastoma [3]. It is mutated in about 50% of the WNT subgroup, 11% and 50% of the SHH subgroup medulloblastomas in children and adults respectively [4]. DDX3X is an ATP-dependent RNA helicase that has been reported to be mutated/altered in some other cancers including chronic lymphocytic leukemia, gingivo-buccal oral squamous cell carcinoma from the Indian tobacco chewers and HPV positive head & Neck carcinomas [5,6,7]. Based on the presence of the recurrent mutations in the *DDX3X* gene in various cancers, *DDX3X* is now recognized as a gene having pathogenic role in cancer. However, the precise role of *DDX3X* in pathogenesis of these cancers is not understood.

DDX3X belongs to a highly conserved subfamily of the DEAD (Asp-Glu-Ala-Asp) box containing RNA helicases, abundantly expressed in all tissues and has been found to play multiple roles in RNA metabolism including transcription, splicing, translation and mRNA transport [8]. Homozygous deletions of *DDX3X* gene in oral cancer and down-regulation of its expression in hepatocellular carcinoma suggest *DDX3X* to function as a tumor suppressor gene. DDX3X has been reported to bring about transcriptional upregulation of cell cycle inhibitor p21 [9]. On the other hand, *DDX3X* has also been shown to act as an oncogene by upregulating Snail transcription factor and repressing E-cadherin expression to bring about epithelial mesenchymal transition of MCF7 breast cancer cells [10,11].

DDX3X is a multifunctional protein with its N-terminal domain required and sufficient for some of its transcriptional regulator activity that is independent of its ATPase and helicase activity [12]. On the other hand, its ATP-dependent helicase activity has been reported be necessary for its role as a regulator of translation [13]. Mutations in *DDX3X* gene identified in WNT and SHH medulloblastomas are missense mutations located in two helicase domains of the protein. It is therefore necessary to check if these mutations disrupt helicase activity of the protein. For this purpose, we expressed human DDX3X (661 amino acid long) encoding cDNA in bacterial system. Full length DDX3X cDNA expressed well in bacterial system but expressed only in insoluble fraction. The present study describes various modifications to the expression protocol made in order to get the DDX3X expression in soluble active form.

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2. METHODOLOGY

2.1. Cloning of human DDX3X cDNA in bacterial expression vectors

Human DDX3X cDNA was a kind gift from Dr. Laura Madrigal-Estebas, Trinity College, Dublin. DDX3X cDNA was cloned in the multiple cloning site of pET28a vector (Novagen, USA), a bacterial expression vector using BamHI and, XhoI restriction sites at the 5' and 3' end respectively. pET28a is Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible vector which contains N-terminal 6X-His tag that can be used for purification on Nickel column**.**

DDX3X cDNA was also cloned in pMAL-c5E vector (New England Biolabs, Ipswich, MA, USA) which contains N-terminal MBP (Maltose binding protein) tag. MBP tag is believed to act as a chaperone helping proper folding and thereby solubilisation of recombinant protein. DDX3X cDNA was cloned in the multiple cloning sites using BamHI site at the 5' end and EcoRI site at the 3' end. The DDX3X constructs were confirmed for the presence of DDX3X insert in the correct orientation by restriction enzyme digestion and by Sanger sequencing.

2.2. Expression of recombinant human DDX3X protein in bacteria

E. coli strain BL21 competent cells were transformed with the pET28a-DDX3X construct and the transformed colonies were selected in the presence of Kanamycin. Expression of recombinant DDX3X protein was studied by varying the incubation temperature and varying concentration of IPTG inducer as well as by addition of various detergents, chemical chaperones like Sorbitol and other additives like mannitol each at 100 mM concentration and 2% glycol to increase the solubility. DDX3X expression in various E.coli strains like pLysS, Rosetta-Gami strains was also carried out to control inducible expression levels as well as provide chaperones for proper folding.

2.3. SDS-PAGE analysis of the expressed protein

Bacterial pellet was extracted for soluble protein fraction in Lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 2mM beta-mercaptoethanol and 5% glycerol). The insoluble bacterial pellet fraction was extracted in 1X SDS-PAGE sample buffer (60 mM Tris-HCl, 2% SDS, 10% Glycerol). Soluble and insoluble fractions were analysed by electrophoresis on a 10% SDS-PAGE.

2.4. Purification of the recombinant protein by affinity chromatography.

E. coli BL21 (DE3) pLysS, Rosetta-Gami strain competent cells were transformed with the pMAL-c5E-DDX3X construct and grown overnight in 50 ml starter culture containing specific antibiotic. Large scale cultures were prepared by inoculating 10 mL of starter culture per liter of fresh LB medium containing specific antibiotic. The cultures were shaken at 200 rpm at 37^0C and induced for protein expression with IPTG once O.D. reached 0.6. Induced cells were harvested by centrifugation for 15 min at 6000 rpm. The cells were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Trition X-100, 2 mM BME, 5% glycerol and 1X EDTA-free protease inhibitor cocktail from Roche, Basel, Switzerland) and lysed using sonication. The soluble fraction was isolated after centrifugation at 15000 rpm for 45 min and pellet was resuspended in 1X Laemmli buffer (60 mM Tris-HCl, 2% SDS, 10% Glycerol) and loaded as insoluble fraction for SDS-PAGE analysis.

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Amylose resin was washed with autoclaved milli-Q water and equilibrated with lysis buffer. Soluble fraction was bound to equilibrated amylose resin for 1hr at 4^0C followed by three washes with wash buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% Glycerol 0.1% Triton X-100, 2 mM BME and 1 mM Maltose) and eluted with elution buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM dTT, 10% Glycerol and 10 mM Maltose). Maltose was removed from purified protein by dialysis using Amicon Ultra centrifugal filter (Millipore) in dialysis buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM dTT and 10% Glycerol). Freshly dialysed protein was used for doing RNA helicase assay.

2.5. Helicase assay for determination of helicase activity of the recombinant protein

DDX3X can unwind only a hybrid double stranded RNA/ single stranded RNA substrate. Therefore, a partial RNA duplex was prepared by annealing a 18 mer RNA oligonucleotide (5'-CCCAAGAACCCAAGGAAC-3') labeled with 6-Carboxyfluorescein (6-FAM) fluorescent dye, having complementary sequence to a 36 RNA oligonucleotide (5'-ACCAGCUUUGUUCCUUGGGUUCUUGGGAGCAGCAGG-3') for the helicase assay. Annealed RNA oligonucleotides at a final concentration of 20 nM were incubated in the assay buffer (50 mM Tris-HCl [pH 7.5], 1 mM DTT, 0.2 mg/ml BSA, 1 mM ATP, 10 mM $MgCl_2$, 5 units of RNase inhibitor, 5% glycerol) with various concentrations (0.2 to 0.8 μM) of the MBP-fusion protein (as eluted protein or as bound to amylase resin) at 37 °C for 40 min. The reaction was stopped by addition of 5X RNA loading buffer (100 mM Tris-HCl [pH 7.5], 20 mM EDTA, 0.5% SDS, 0.1% Bromophenol Blue, 50% Glycerol) and loaded onto a 8% Native Polyacrylamide gel. The electrophoresis was carried out in 1X Tris-Borate-EDTA / 0.1% SDS buffer.

3. RESULTS AND DISCUSSION:

Human DDX3X cDNA (1989 bp, 662 amino acid long) was cloned in an inducible bacterial expression vector pET28a (Novagen, Merck Millipore, USA). This vector contains T7lac promoter that enables expression of the gene of interest in an IPTG-inducible manner and adds N-terminal His tag to the protein of interest. For cloning DDX3X cDNA a forward primer was designed that contained the first 21 nucleotides starting with initiation codon ATG and a TEV protease cleavage site upstream the ATG codon so as to enable release of DDX3X without the Histidine tag

Abundant DDX3 protein expression was seen in pET28a-DDX3X E. coli BL21 cells grown at 37° C as well as at lower temperature of 18 °C at concentration of IPTG ranging from 0.1 mM to 0.4 mM. However the expression was obtained only in the insoluble fraction (Fig. 1). Since the expression levels of DDX3X did not change appreciably even upon incubation at lower temperature or at low IPTG concentration, the E. coli cells expressing pET28a-DDX3X construct were grown at 18°C until the Optical Density (O.D.) of the culture reached 0.5 at 600 nm and then IPTG induction was done at 0.3 mM concentration for increasing time intervals. Figure 2 shows detectable DDX3X expression upon IPTG induction for 3 h that increases gradually with increasing incubation time of 6 h and 9 h in the whole cell lysates. However, significant DDX3X expression is not seen at any of these time intervals in soluble fraction. Thus, even after controlling DDX3X expression to a minimum detectable level by controlling temperature and time of induction, DDX3X expression is still obtained only in the insoluble fraction.

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Next, increasing concentrations of different detergents *viz*. Triton X-100, NP-40 and Sarkozyl were used for increasing solubility of DDX3X. However, only in the presence of 0.3% to 1% Sarkosyl, the DDX3X protein was obtained in the soluble fraction (Fig. 3). Even 0.1% Sarkosyl was not found to be sufficient to solubilize DDX3X protein. The presence of sarkosyl does not allow efficient binding of the His-tagged protein to the Nickel column. Therefore, other additives like sucrose, glycol, mannitol, mannose, sorbitol were also added to the extraction buffer to increase solubility of the protein. However, none of these additives could get DDX3X recombinant protein in the soluble fraction (Fig. 4). We then tried using the E. coli strain Rosetta-gami 2(DE3) pLysS strain for recombinant DDX3X expression. This E. coli strain allows for disulfide bond formation of eukaryotic proteins. The pLysS expresses T7 lysozyme that suppresses basal expression of T7 RNA polymerase thus eliminating expression of recombinant protein before induction with IPTG. However, even in this strain DDX3X protein expression was not found in the soluble fraction (Fig. 5).

Since all attempts to obtain expression of recombinant His-tagged DDX3X protein in soluble fraction failed, human DDX3X cDNA was then cloned in pMAL-c5E vector that expresses the gene of interest as a fusion downstream *malE* gene encoding Maltose binding protein (MBP). The fusion protein can be purified by amylose affinity chromatography and the protein of interest can be cleaved of MBP using specific protease enterokinase. The MBP is known to enhance solubility of eukaryotic proteins it is fused to and the vector used expresses fusion protein in cytoplasm. The MBP-DDX3X fusion protein (MW = 120 kDa) was found to be expressed at all the three temperatures tested 18 °C , 24 °C and 37 °C in soluble fraction (Fig. 6) . The fusion protein therefore was purified using amylose affinity column. Elution fractions of amylose affinity column showed the presence of more than 90% pure 120 KDa MBP-DDX3X fusion protein (Fig. 7).

Helicase activity of MBP-DDX3X fusion protein was assessed by incubating with a 18 mer RNA oligonucleotide annealed to a 36 mer RNA oligonucleotide having 18 nucleotide long complementary sequence in a hybridization buffer at 37 C for 40 min. The after the incubation the reaction mixture was separated on a native acrylamide gel by electrophoresis in the presence of 0.1% SDS to inhibit secondary structure formation. The 18 mer oligonucleotide was labeled using fluorescent dye 6-FAM so that its separation from the 18 mer/36 mer hybrid RNA can be traced using UV transilluminator. Figure 8 shows PAGE analysis of the helicase assay reaction products indicating the position of 18 mer oligonucleotide and that of the hybrid. Incubation of the hybrid with the bead bound MBP-DDX3X protein shows migration of the hybrid at intermediate position between that of hybrid and free 18 mer oligonucleotide indicating helicase activity of the fusion DDX3X protein.

Recently a truncated DDX3X (AA 135-582) was expressed successfully in bacterial system that was assessed for its ATPase activity [14]. Some of the medulloblastoma associated DDX3X mutants were found to be impaired in their ATPase activity. However, not all mutants could be expressed successfully even as truncated version and the helicase activity could not be assessed for either wild type (135-582 AA construct) or mutant protein. The MBP-DDX3X fusion protein does show helicase activity at least partially and hence can be used to study the effect of mutations on the helicase activity of DDX3X.

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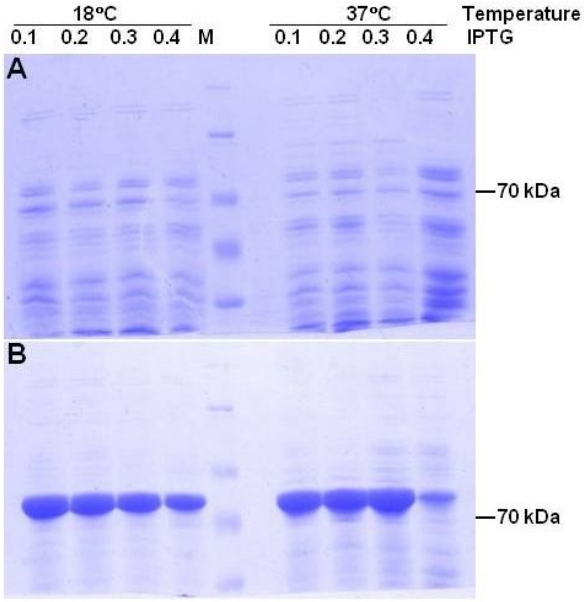

Figure 1. SDS-PAGE analysis of soluble (A) and insoluble pellet (B) fractions of E. coli cells expressing pET28a-DDX3X construct and induced at the indicated concentration ranging from 0.1 to 0.4 mM of IPTG at 18°C or 37 °C.

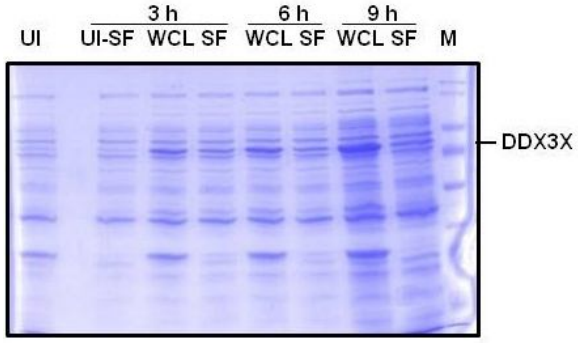

Figure 2. SDS-PAGE analysis of uninduced soluble (UI-SF) and induced soluble (SF) fractions as well whole cell lysates (WCL) of E. coli. BL21 cells carrying the pET28a-DDX3X construct. The cells were induced with 0.3 mM IPTG for the indicated time intervals.

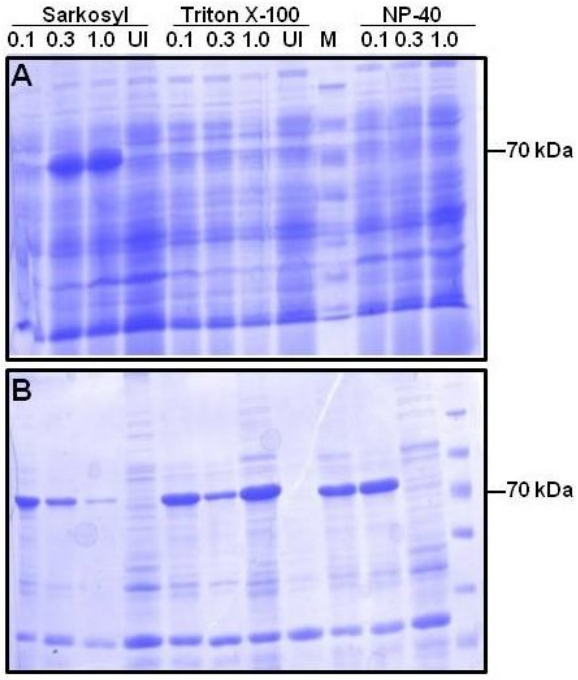

Figure 3. Expression of the recombinant DDX3X protein in fraction solubilized (A) with the indicated detergent at 0.1%, 0.3% and 1.0% concentration and the correspondin g insoluble fraction (B). UI indicated un-induced lysate in 1% concentration of the indicated detergent. M indicates molecular weight marker.

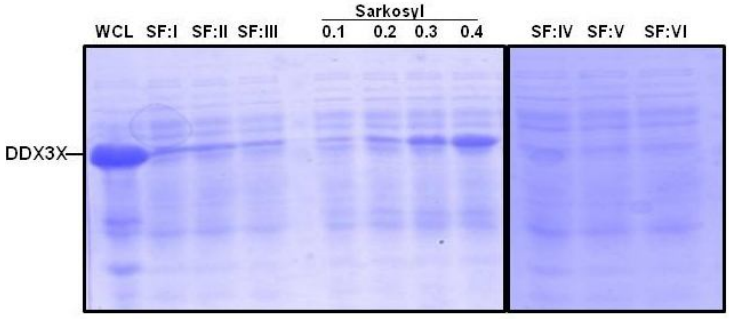

Figure 4: SDS-PAGE analysis of the expression of recombinant DDX3X protein in WCL: whole cell lysate; SF: Soluble Fractions in buffers (I, II, III, IV, V, VI) with various additives and those solublized in the presence of 0.1% to 0.4% Sarkosyl.
SF:I 10% Glycerol + 2% Glycol + 100 mM Sucrose; SF:II 10% Glycerol + 2% Glycol + 100 mM Mannose; SF:III 10% Glycerol + 2% Glycol + 100 mM Mannitol; SF:IV 100 mM Sucrose + 100 mM Sorbitol + 100 mM Mannitol; SF:V 100 mM Sucrose + 100 mM Sorbitol + 2% PEG 6000; SF: VI 100 mM Sucrose + 100 mM Sorbitol + 100mM Mannose

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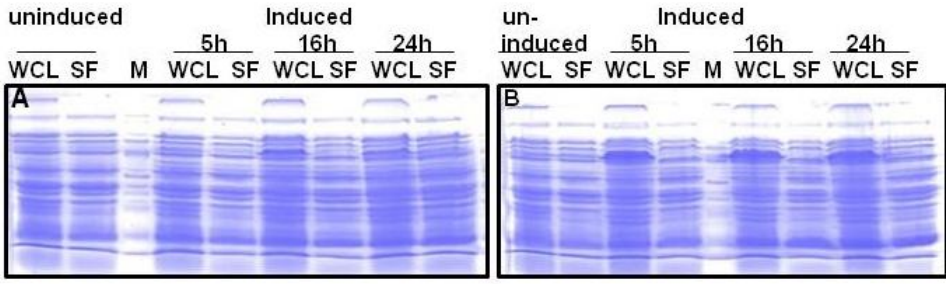

Figure 5. SDS-PAGE analysis of pET28a-DDX3X expressed in E. coli Rosetta gami pLys cells. The cells were treated with 0.3 mM IPTG for 5h, 16 h or 24 h at 18 C (A) or 24 C and the whole cell lysate and soluble fractions were analyzed by SDS-PAGE.

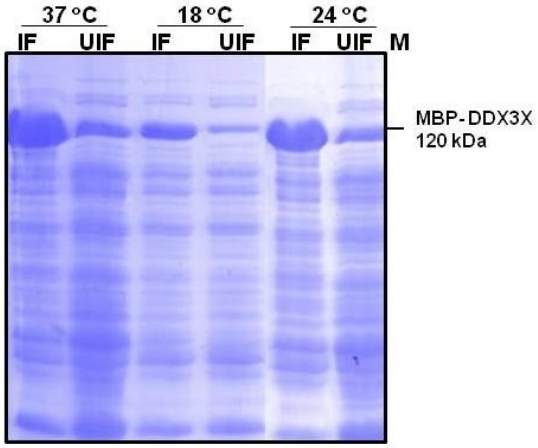

Figure 6. SDS-PAGE analysis of the recombinant MBP-DDX3X fusion protein expressed in E. coli. BL21 grown at the indicated temperature in soluble uninduced fraction (UIF) or in fraction induced (IF) with 0.3 mM IPTG.

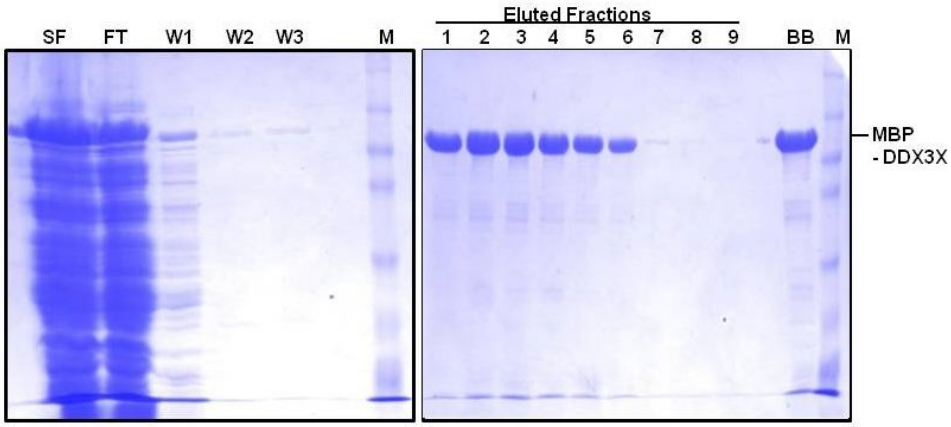

Figure 7. SDS-PAGE analysis of total soluble fraction (SF) of E. coli. Protein extract expressing recombinant MBP-DDX3X fusion protein and that separated on amylose affinity column. FT: Flow through fraction from the column; W1, W2, W3: 1st, 2nd and 3rd wash given to the column and eluted fractions 1 to 9 and BB: Column beads bound fraction; M: Molecular weight marker

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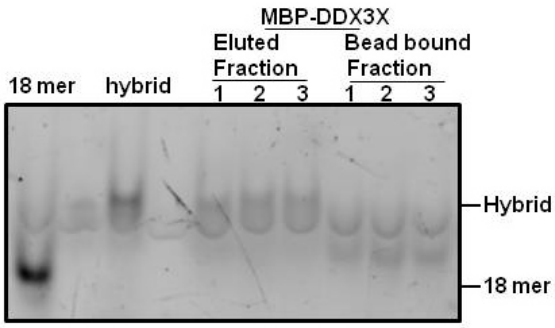

Figure 8. Helicase assay reaction products separated by native PAGE electrophoresis. The 18 mer/ 36mer RNA hybrid incubated with bead bound MBP-DDX3X fusion protein at increasing concentrations from 1 to 3 shows product at position intermediate between that of the hybrid and the 18 mer RNA oligonucleotide.

4. CONCLUSIONS

Full length human DDX3X protein is expressed well in Bacterial system. However, it is expressed in insoluble fraction and no detergent (Triton X-100, NP-40) or additive like Mannitol, Sorbitol can solubilize the recombinant protein other than the ionic surfactant Sarkosyl. Sarkosyl however, does not allow efficient binding of the protein to affinity columns. Therefore, the human DDX3X protein was expressed as a MBP fusion protein that expressed well in the soluble fraction and could be purified as a fusion protein. The resin bound MBP-DDX3X fusion protein showed partial helicase activity that can be used to study effect of cancer associated DDX3X mutations on its helicase activity.

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Acknowledgements:

We thank Dr. Ashok Varma, Dr. Rajan Choudhary, ACTREC for valuable suggestions. This work was supported from a grant from Department of Biotechnology.