



## LIST OF FIGURES

Figure No.	Title	Page No.
<b>Chapter 1</b>	<b>GENERAL INTRODUCTION</b>	
<b>Figure 1.1.</b>	General structural formula of $\alpha$ -amino acid	2
<b>Figure 1.2.</b>	Resonance interaction of peptide bond	5
<b>Figure 1.3.</b>	The right handed $\alpha$ -helix	6
<b>Figure 1.4.</b>	The hydrogen bonding pattern of several polypeptide helices	6
<b>Figure 1.5.</b>	$\beta$ -pleated sheets: parallel and anti-parallel	7
<b>Figure 1.6.</b>	Folding funnel representing various state of energy levels	9
<b>Figure 1.7.</b>	The amino acid sequences of yeast, plant and human ubiquitins. The residues which were changed have been highlighted	13
<b>Figure 1.8.</b>	Ubiquitin structure	14
<b>Figure 1.9.</b>	Ubiquitin gene family	19
<b>Figure 1.10.</b>	The isopeptide linkage	20
<b>Figure 1.11.</b>	Polyubiquitin chains through different ubiquitin lysine residues. (Cecile M Pickart and David Fushman, 2004)	21
<b>Figure 1.12.</b>	Positions of lysine residue in ubiquitin.	22
<b>Figure 1.13.</b>	Ubiquitin proteasome system (UPS)	23
<b>Figure 1.14.</b>	The 26S proteasome System. (Dieter H. Wolf and Wolfgang Hilt, 2004)	24
<b>Figure 1.15.</b>	Pathogenesis of ubiquitin system related diseases	28
<b>Figure 1.16.</b>	Position of Glu64, Ser65 and Gln2 in the $\beta$ -bulge at C-terminal	30
<b>Figure 1.17.</b>	Schematic representation of proposed structural work.	31
<b>Figure 1.18.</b>	Schematic representation of proposed work for functional complementation.	32
<b>Figure 1.19.</b>	Schematic representation for screening of error prone PCR mutants and structure function relationships of ubiquitin.	33

<b>Chapter 2</b>	<b>CONSTRUCTION, EXPRESSION, PURIFICATION OF UbE64G AND ITS STRUCTURAL ANALYSIS</b>	
<b>Figure 2.1.</b>	Percentage frequencies of amino acids in the first position of $\beta$ -bulge.(Chan et al., 1993)	<b>35</b>
<b>Figure 2.2.</b>	Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)	<b>36</b>
<b>Figure 2.3.</b>	<i>E.coli</i> expression vector pRB463.4. The plasmid carries Yeast-wild type ubiquitin gene (YUbWt) under tac promoter	<b>37</b>
<b>Figure 2.4.</b>	<i>E.coli</i> expression vector pKK223-3. The plasmid carries Yeast ubiquitin gene with F45W mutation (YUb-F45W) under tac promoter. (Platt et al., 2003)	<b>38</b>
<b>Figure 2.5.</b>	Sequence of synthetic yeast ubiquitin gene with mutation for UbF45W.	<b>39</b>
<b>Figure 2.6.</b>	Ubiquitin expression and purification gel profile. Lane 1. Shows the protein molecular weight marker. Lane 2. Shows protein profile of JM109 cells transformed by pRB463.4 (YUbWt) induced with IPTG. Lane 3. Shows supernatant of cell lysate after lysozyme treatment and sonication. Lane 4. Shows 0.2% polyethyleneimine (PEI) treated supernatant. Lane 5. Shows heat treated supernatant. Lane 6. Shows protein precipitated by 85% ammonium sulphate, loaded after dialysis and Lane 7. Shows the pure ubiquitin obtained after Sephadex G-50 column.	<b>42</b>
<b>Figure 2.7.</b>	Restriction digestion patterns of (a) pKK223-3-UbF45W and (b) pKK223-3-UbE64G. Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3-UbF45W (a) and pKK223-3-UbE64G (b). Lane 3. Contains <i>Eco</i> RI digest. <i>Eco</i> RI has a unique restriction site. Lane 4. Contains <i>Sal</i> I digest. <i>Sal</i> I has two sites in (a) pKK223-3-UbF45W and releases a 728bp fragment and (b) the site is lost in pKK223-3-UbE64G after subcloning of UbE64G resulting in no fragment release. Lane 5. Contains <i>Xho</i> I digest and gives a single band of 4791bp fragment with pKK223-3-UbF45W (a) and also for (b) pKK223-3-UbE64G, which is the actual size of the vector. and Lane 6. Contains <i>Hind</i> III digest. <i>Hind</i> III also has a unique restriction site. Lane 7. Shows the vector fragmentation with <i>Eco</i> RI and <i>Pvu</i> II releasing 2848bp and 1943bp fragment. Lane 8. Contains <i>Pvu</i> II digest. <i>Pvu</i> II also has a unique restriction site.	<b>43</b>

<b>Figure 2.8.</b>	Electrophoretogram of UbE64G gene in pKK223-3 vector. The marked region confirms the E64G substitution.	<b>43</b>
<b>Figure 2.9.</b>	Far uv CD spectra of three forms of ubiquitin UbWt, UbF45W and UbE64G at various pH buffers 2.2, 5, 7.4 and 10.	<b>44</b>
<b>Figure 2.10.</b>	Near uv CD spectra for UbF45W and UbE64G at various pH buffers 2.2, 5, 7.4 and 10.	<b>45</b>
<b>Figure 2.11.</b>	Fluorescence emission spectra of UbWt, UbF45W and UbE64G recorded after exciting the protein at (a) 280nm and (b) 295nm	<b>46</b>
<b>Figure 2.12.</b>	Fluorescence emission spectra of extrinsic fluorophores ANS, Bis-ANS and Pyrene are shown in panels (a), (b) and (c) respectively. The emission spectra of UbWt, UbF45W and UbE64G are shown in each panel.	<b>48</b>
<b>Figure 2.13.</b>	Fluorescence resonance energy transfer spectra of UbF45W and UbE64G recorded in absence and presence of ANS.	<b>48</b>
<b>Figure 2.14.</b>	CD spectra showing thermal denaturation of UbF45W and UbE64G at 25°C and 90°C.	<b>49</b>
<b>Figure 2.15.</b>	Intrinsic fluorescence emission spectra for guanidine hydrochloride denaturation of (a) UbF45W and (b) UbE64G and (c) denaturation curves of UbE64G and UbF45W.	<b>51</b>
<b>Figure 2.16.</b>	Guanidine hydrochloride denaturation curves of UbE64G and UbF45W. (a) Far uv CD and (b) Near uv CD spectra of UbF45W and UbE64G recorded in 0M guanidine hydrochloride and 4M guanidine hydrochloride.	<b>52</b>
<b>Chapter 3</b>	<b>CONSTRUCTION, EXPRESSION, PURIFICATION OF Ubs65D AND ITS STRUCTURAL ANALYSIS</b>	
<b>Figure 3.1.</b>	Percentage frequencies of amino acids in the second position of $\beta$ -bulge. (Chan et al., 1993)	<b>54</b>
<b>Figure 3.2.</b>	Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)	<b>55</b>
<b>Figure 3.3.</b>	Strategy adapted for recombinogenic PCR.	<b>56</b>
<b>Figure 3.4.</b>	Vector back bone pBluescript II KS/SK(+) and sequence of multiple cloning site	<b>57</b>

<b>Figure 3.5.</b>	Restriction digestion patterns of (a) pKK223-3/UbF45W and (b) pKK223-3/ UbS65D. Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3/UbF45W (a) and pKK223-3/UbS65D (b). Lane 3. Contains <i>Eco</i> RI digest. <i>Eco</i> RI has a unique restriction site. Lane 4. Contains <i>Sal</i> I digest. <i>Sal</i> I has two sites in the vector backbone one in the ubiquitin gene and other out side the gene (a) and releases a 728bp fragment and the site is lost after subcloning of UbS65D in (b) resulting in no fragment release. Lane 5. Contains <i>Xho</i> I digest and gives a single band of 4791bp fragment with pKK223-3/UbF45W (a) and also for (b) pKK223-3/UbS65D, which is the actual size of the vector. <i>Xho</i> I site is present near the <i>Sal</i> I site. Lane 6. Contains <i>Sal</i> I- <i>Xho</i> I double digest which releases 684bp. Lane 7. Contains <i>Hind</i> III digest which is also a unique restriction site in the vector. UbS65D gene was cloned in <i>Eco</i> RI and <i>Hind</i> III.	<b>59</b>
<b>Figure 3.6.</b>	Electrophoretogram of UbS65D gene in pKK223-3 vector. The marked region confirms the UbS65D substitution.	<b>60</b>
<b>Figure 3.7.</b>	Far uv CD spectra of the two variants of ubiquitin UbF45W and UbS65D at various pH buffers 2.2, 5, 7.4 and 10.	<b>61</b>
<b>Figure 3.8.</b>	Near uv CD spectra of UbF45W and UbS65D at various pH buffers 2.2, 5, 7.4 and 10.	<b>61</b>
<b>Figure 3.9.</b>	Fluorescence emission spectra of UbF45W and UbS65D recorded after exciting the proteins at 280nm (a) and 295nm (b)	<b>62</b>
<b>Figure 3.10.</b>	Fluorescence emission spectra showing the fluorescence of extrinsic fluorophores ANS and Bis- ANS bound to the two variants of ubiquitin UbF45W and UbS65D shown in panels (a) and (b) respectively.	<b>63</b>
<b>Figure 3.11.</b>	Spectra showing fluorescence resonance energy transfer between tryptophan of ubiquitin UbF45W and UbS65D in absence and presence of ANS.	<b>64</b>
<b>Figure 3.12.</b>	CD spectra showing thermal denaturation of UbF45W and UbS65D.	<b>64</b>
<b>Figure 3.13.</b>	Intrinsic fluorescence spectra for guanidine hydrochloride denaturation of (a) UbF45W and (b) UbS65D. (c) Guanidine hydrochloride denaturation curves of UbF45W and UbS65D.	<b>66</b>

<b>Chapter 4</b>	<b>CONSTRUCTION, EXPRESSION, PURIFICATION OF UbQ2N AND ITS STRUCTURAL ANALYSIS</b>	
<b>Figure 4.1.</b>	Percentage frequencies of amino acids in the X-position of $\beta$ -bulge. (Chan et al., 1993)	<b>68</b>
<b>Figure 4.2.</b>	Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)	<b>68</b>
<b>Figure 4.3.</b>	Restriction digestion patterns of (a) pKK223-3-UbF45W and (b) pKK223-3-UbQ2N. Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3-UbF45W (a) and pKK223-3-UbQ2N (b). Lane 3. Contains <i>Eco</i> RI digest. <i>Eco</i> RI has a unique restriction site. Lane 4. Contains <i>Bgl</i> II digest and gives a single band of 4791bp fragment with (a) pKK223-3-UbF45W and (b) the site is lost after cloning of UbQ2N resulting in no digestion of vector backbone. Lane 5. Contains <i>Sal</i> I digest. <i>Sal</i> I has two sites in (a) and releases a 728bp fragment. Lane 6. Contains <i>Hind</i> III digest. <i>Hind</i> III also has a unique restriction site. <i>Eco</i> RI and <i>Hind</i> III sites were used for cloning UbQ2N gene in pKK223-3.	<b>71</b>
<b>Figure 4.4.</b>	Electrophoretogram of UbQ2N gene in pKK223-3 vector. The marked region confirms the UbQ2N substitution.	<b>72</b>
<b>Figure 4.5.</b>	Far uv CD spectra of the two variants of ubiquitin UbF45W and UbQ2N at various pH buffers 2.2, 5, 7.4 and 10.	<b>73</b>
<b>Figure 4.6.</b>	Near uv CD spectra of UbF45W and UbQ2N at various pH values 2.2, 5, 7.4 and 10.	<b>73</b>
<b>Figure 4.7.</b>	Fluorescence emission spectra of UbF45W and UbQ2N recorded after exciting the protein at 280nm (a) and 295nm (b)	<b>74</b>
<b>Figure 4.8.</b>	Fluorescence emission spectra of extrinsic fluorophores (a) ANS and (b) Bis- ANS, bound to UbF45W and UbQ2N.	<b>75</b>
<b>Figure 4.9.</b>	Fluorescence resonance energy transfer spectra between tryptophan of UbF45W and UbQ2N in absence and presence of ANS.	<b>76</b>
<b>Figure 4.10.</b>	CD spectra showing thermal denaturation of UbF45W and Q2N.	<b>76</b>

<b>Figure 4.11.</b>	Intrinsic fluorescence spectra for guanidine hydrochloride denaturation of (a) UbF45W and (b) UbQ2N. (c) Guanidine hydrochloride denaturation curves of UbF45W and UbQ2N.	<b>78</b>
<b>Chapter 5</b>	<b>FUNCTIONAL ASSESSMENT OF THREE SITE DIRECTED MUTANTS OF UBIQUITIN IN <i>Saccharomyces cerevisiae</i></b>	
<b>Figure 5.1</b>	Ubiquitin Proteasome System (UPS) and its various biological roles.	<b>80</b>
<b>Figure 5.2</b>	Changing amino acid residue of $\beta$ -galactosidase at ubiquitin $\beta$ -gal.	<b>82</b>
<b>Figure 5.3.</b>	Yeast expression vector pUB175-UbWt. The plasmid pUB175-UbWt carries wild type synthetic yeast ubiquitin gene under CUP1 promoter induced by 10-20 $\mu$ M copper sulphate. The plasmid when transformed into <i>E.coli</i> , DH5 $\alpha$ strain was maintained under a selection pressure of 50 $\mu$ g-100 $\mu$ g/ml of ampicillin. The backbone of the pUB175-UbWt was similar to YEp96 except for URA3 fragment cloned into <i>Bam</i> H I site. TRP1 and URA3 are the selection markers and 2 $\mu$ fragment maintains YEp 96 at 50 copies/cell.	<b>86</b>
<b>Figure 5.4.</b>	Schematic representation of YEp96 and pUB23 yeast double transformants to study the N-end rule as a degradation signal.	<b>88</b>
<b>Figure 5.5.</b>	Schematic representation of functional complementation studies.	<b>89</b>
<b>Figure 5.6.</b>	PCR amplification of ubiquitin gene. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. are the reagent blanks. Lane 3. and lane 5. Contain 364bp extension form vector pKK-223-UbF45W and pKK-223-UbE64G respectively using primer combination of FORWARD PRIMER: 5'GC GCCGACATCATAACGGTTCTGGC'3 and REVERSE PRIMER: 5'TCCGGTACCCGCTCAACCACCTCTTAG'3.	<b>91</b>

<p><b>Figure 5.7.</b></p>	<p>Restriction analysis of pUB175-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contain 234bp extension from pUB175-UbWt and pUB175-UbF45W respectively using primer combination of FORWARD PRIMER: 5'ATGCAGATCTTCGTCAAGA CGTTAACCGG'3 and REVERSE PRIMER: 5'TCCGG TACCCGCTCAACCAC CTCTTAG'3. Lane 3. and Lane 5. PCR products digested by <i>Sau96</i> I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in pUB175 the gene develops a new restriction site for <i>Sau96</i> I, which cuts the gene into two fragments of 100bp and 134bp.</p>	<p>92</p>
<p><b>Figure 5.8.</b></p>	<p>Restriction pattern analysis of (a) pUB175-UbF45W and (b) pUB175-UbE64G. Lane 1. Contains <math>\lambda</math> <i>Hind</i> III Digest marker. Lane 2. Contains undigested plasmid (a) pUB175-UbF45W and (b) pUB175-UbE64G. Lane 3. There are three <i>EcoR</i> I sites in the vector and hence <i>EcoR</i> I digest shows three fragments of 628bp, 2818bp, 3733bp fragments. Lane 4. Contains <i>Bgl</i> II digest. Lane 5. Contains <i>Sal</i> I digest with a unique restriction site, giving a single band of 7220bp fragment with pUB175-UbF45W (a), and is lost after subcloning of gene with UbE64G (b). Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites were used for cloning.</p>	<p>93</p>
<p><b>Figure 5.9.</b></p>	<p>Restriction digestion pattern of YEp96-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contains 234bp extension from YEp96-UbWt and YEp96-UbF45W respectively using primer combination of FORWARD PRIMER: 5'ATGCAGATCTTCGTCAAGACGT TAAC CGG'3 and REVERSE PRIMER: 5'TCCGGTACCCGC TCAACCACCTCTTAG'3. Lane 3. and Lane 5. PCR products digested by <i>Sau96</i> I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in YEp96 the gene develops a new restriction site for gene <i>Sau96</i> I which cuts the gene into two fragments of 100bp and 134bp</p>	<p>93</p>

<b>Figure 5.10.</b>	Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbE64G. Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbE64G. Lane 3 There are three <i>Hind</i> III sites in the vector and hence the <i>Hind</i> III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4 contains <i>Bgl</i> II digest. Lane 5. Contains <i>Sal</i> I digest with a unique restriction site, gives a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of the gene with UbE64G mutation. Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites were used for subcloning. Lane 7. There are three <i>Eco</i> R I sites in the vector, and therefore the <i>Eco</i> R I digest shows three fragments of 628bp, 2818bp and 2733bp.	<b>94</b>
<b>Figure 5.11.</b>	Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbS65D. Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbS65D. Lane 3. There are three <i>Hind</i> III sites in the vector and hence the <i>Hind</i> III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4. Contains <i>Bgl</i> II digest. Lane 5. Contains <i>Sal</i> I digest with a unique restriction site giving a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of UbS65D gene. Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites were used for subcloning. Lane 7. There are three <i>Eco</i> R I sites in the vector so the digest contains three fragments of 628bp, 2818bp and 2733bp.	<b>95</b>
<b>Figure 5.12.</b>	Restriction digestion pattern of pBSK KS/SK(+). Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid of pBSK KS/SK(+). Lane 3., Lane 4., Lane 5., Lane 6., and Lane 7. Contains <i>Eco</i> RV digest, <i>Sal</i> I digest, <i>Bgl</i> II digest, <i>Afl</i> II digest, <i>Bam</i> HI digest and <i>Kpn</i> I digest respectively. Restriction sites of <i>Eco</i> RV, <i>Sal</i> I, <i>Bam</i> HI and <i>Kpn</i> I are unique to the vector and gives a single band of 2961bp on digestion. <i>Bgl</i> II and <i>Afl</i> II site are absent in the pBSK KS/SK(+).	<b>96</b>

<b>Figure 5.13.</b>	Restriction digestion pattern of pBSK-KS/SK(+)-Ub(Wt.ORF) and pBSK KS/SK(+)-Ub(Q2N.ORF). Lane 1. Contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. contains undigested plasmid (a) pBSK-KS/SK(+)-Ub(Wt.ORF) and (b) pBSK KS/SK(+)-Ub(Q2N.ORF). Lane 3. and Lane 4. Contatins <i>Bam</i> HI and <i>Eco</i> RI respectively digesting the vector to give a single band of 3639 bps. Lane 5. Contains <i>Bgl</i> II digest with a unique restriction site, giving a single band of 3639bp fragment with (a) pBSK KS/SK(+)-Ub(Q2N.ORF) and (b) and is lost after subcloning of UbQ2N gene in <i>Eco</i> RI- <i>Kpn</i> I site. Lane 6. Contains <i>Kpn</i> I digest. Lane 7. Contains <i>Bam</i> HI- <i>Kpn</i> I double digest, which releases 678bp ubiquitin ORF along with CUP1 promoter leaving 2961bp backbone.	97
<b>Figure 5.14.</b>	Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbQ2N. Lane 1. contains $\lambda$ <i>Hind</i> III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbQ2N. Lane 3. There are three <i>Hind</i> III sites in the vector and the <i>Hind</i> III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4. contains <i>Bam</i> HI digest. Lane 5. contains <i>Bgl</i> II digest with a unique restriction site, giving a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of gene with UbS65D mutation. Lane 6. contains <i>Kpn</i> I digest. Lane 7. Contains <i>Bam</i> HI- <i>Kpn</i> I double digest, which releases 678bp ubiquitin ORF along with CUP1 promoter leaving 5501bp backbone. <i>Bam</i> HI and <i>Kpn</i> I sites were used for subcloning.	97
<b>Figure 5.15.</b>	Growth curves of <i>Saccharomyces cerevisiae</i> (SUB60 and SUB62) and SUB 60 transformant under (a) uninduced and (b) induced conditions.	98
<b>Figure 5.16.</b>	UV-C Complementation of <i>Saccharomyces cerevisiae</i> (SUB60 and SUB62) and SUB 60 transformant under (a) uninduced and (b) induced conditions.	99
<b>Figure 5.17.</b>	Heat stress complementation of <i>Saccharomyces cerevisiae</i> (SUB60 and SUB62) and SUB 60 transformants under (a) uninduced and (b) induced conditions.	100

<b>Figure 5.18.</b>	Antibiotic stress complementation of <i>Saccharomyces cerevisiae</i> (SUB60). Cycloheximide resistance of SUB62, SUB60 cells transformed with UbWt and UbF45W gene. The SUB60 and SUB60 transformed with UbE64G, UbS65D and Q2N fail to grow in presence of cycloheximide. Three fold serial dilutions (2), (3) and (4) spotted on YPD plates containing 100µM of copper and 4µg/ml of cycloheximide.	<b>101</b>
<b>Figure 5.19.</b>	Effect of UbE64G, UbS65D and UbQ2N mutations on the half-life of proteins in Ubi4 background. SUB60, SUB62, SUB60 transformed with YEp96-UbWt (60/Wt), YEp96-UbF45W (60/45), YEp96-UbE64G (60/64), YEp96-UbS65D (60/65) and YEp96-UbQ2N (60/2) expressing the wild type ubiquitin UbWt, UbF45W and three mutants of ubiquitin, namely UbE64G, UbS65D and UbQ2N respectively. These transformant were also cotransformed with pUb23 expressing Ub-β-galactosidase fusion with (I) Met (M) and (II) Pro (P) as the N-terminal residues. SUB60 and SUB62 were also cells were transformed by plasmid pUb23 expressing Ub-β-galactosidase fusion with (I) Met (M) (60/M, 62/M) and (II) Pro (P) (60/M, 62/M) as the N-terminal residues. SUB60 and SUB62 were used as controls.	<b>102</b>
<b>Figure 5.20.</b>	Restriction digestion pattern of pUB221-c-myc-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contains 234bp extension from pUB221/c-myc-UbWt and pUB221/c-myc-UbF45W respectively using primer combination of FORWARD PRIMER: 5'ATGCAG ATCTTCGTCAAGACGTTAACCGG '3 and REVERSE PRIMER: 5'TCCGGTACCCGCTCAACCACCTCTTA G'3. Lane 3. and Lane 5. Digested PCR products of <i>Sau96</i> I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in pUB221 the gene develops a new restriction site to the gene <i>Sau96</i> I which digests the gene into two fragments of 100bp and 134bp.	<b>103</b>

<b>Figure 5.21.</b>	Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbE64G. Lane 1. Contains $\lambda$ Hind III Digest marker. Lane 2. Contains undigested plasmid of (a) YEp96-UbWt and (b) pUB221-c-myc-UbE64G. Lane 3. There are three <i>EcoR</i> I sites in the vector so which contains <i>EcoR</i> I digest shows three fragments of 694bp, 2818bp and 3733bp fragments. Lane 4. Contains <i>Bgl</i> II digest. Lane 5. contains <i>Sal</i> I digest is a unique restriction sites and give a single band of 7286bp fragment with (a) pUB221/c-myc-UbWt, which is the actual size of the vector and is lost after subcloning of c-myc-UbE64G gene (b). Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites used for subcloning the gene.	<b>104</b>
<b>Figure 5.22.</b>	Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbS65D. Lane 1. Contains $\lambda$ Hind III digest as marker. Lane 2. Contains undigested plasmid (a) pUB221-c-myc-UbWt and (b) pUB221-c-myc-UbS65D. Lane 3. There are three <i>EcoR</i> I sites in the vector and hence <i>EcoR</i> I digest contains three fragments of 694bp, 2818bp and 3733bp. Lane 4. Contains <i>Bgl</i> II digest. Lane 5. Contains <i>Sal</i> I digest is a unique restriction site, giving a single band of 7286bp fragment with (a) pUB221/c-myc-UbWt and (b) is lost after subcloning of c-myc-UbS65D. Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites were used for subcloning the gene.	<b>104</b>
<b>Figure. 5.23.</b>	Western blot showing ubiquitination profile. Lane 1. Rain bow molecular weight marker. Lane 2. SUB60 as negative Control. Lane 3. SUB60-UbWt and Lane 4. SUB60-UbF45W as positive control and Lane 5. SUB60-UbE64G Lane 6. SUB60S65D	<b>105</b>
<b>Chapter 6</b>	<b>IN VITRO EVOLUTION OF UBIQUITIN</b>	
<b>Figure 6.1</b>	YEp96-UbWt vector map	<b>111</b>
<b>Figure 6.2</b>	Strategies for generation of error prone mutants and their screening.	<b>113</b>

<b>Figure 6.3.</b>	YEp 96-UbWt digestion pattern. Lane 1. Contains $\lambda$ <i>Hind</i> III digest marker. Lane 2. Contains YEp96-UbWt undigested plasmid. Lane 3. <i>Hind</i> III digest of the vector with three <i>Hind</i> III sites showing three fragments of 646bp, 2099bp and 3434bp. Lanes 4., Lane 5. and Lane 6. Show <i>Bam</i> HI digest, <i>Bgl</i> II digest and <i>Kpn</i> I digest respectively. <i>Kpn</i> I and <i>Bgl</i> II sites were used for cloning error prone amplicons. All these restriction sites are unique and give a single band of 6179bp fragment, which is the actual size of the vector. In lane 7, <i>Bam</i> HI, <i>Kpn</i> I double digest shows release of 678bp ubiquitin ORF along with CUP1 promoter and 5501bp backbone.	<b>115</b>
<b>Figure 6.4.</b>	PCR amplicons under different error prone conditions. Lane 1. Contains 100 bp ladder. Lane 2. Contains amplicons of gene under standard conditions. Lanes 3., Lane 4., Lane5., Lane 6. and Lane 7. Contain amplicons of the gene under error prone conditions 10mM MgCl <sub>2</sub> , 0.5mM MnCl <sub>2</sub> , 1mM dATP, 1mM dGTP and 1mM dATP/dGTP respectively.	<b>116</b>
<b>Figure 6.5.</b>	Screening of mutants by heat complementation in SUB60 yeast cells	<b>117</b>
<b>Figure 6.6.</b>	Failure of complementation of stress hypersensitive phenotype by mutated ubiquitin gene in yeast cell transformants. Mutagenesis under different error prone conditions (A) MnCl <sub>2</sub> , (B) MgCl <sub>2</sub> and dGTP, (C) dATP.	<b>118</b>
<b>Figure 6.7.</b>	$\lambda$ <i>Hind</i> III Digest (DNA Marker) Lane 1. YEp96-UbWt in lane 2. and plasmids of all the mutants (colony No. 6, 9, 11, 26, 30, 34, 37, 38, 40, 42, 64 and 68) were loaded and resolved in lane 3 to 14 respectively on 1% agarose.	<b>118</b>
<b>Figure 6.8.</b>	The 100 bp ladder (DNA Marker) in lane 1, PCR of YEp96-UbWt in lane 2 and PCR products of the ubiquitin gene of mutants (Colony No. 6, 9, 11, 26, 30, 34, 37, 38, 40, 42, 64 and 68) were in lane 3 to 14 respectively on 2 % agarose.	<b>119</b>

<b>Figure 6.9.</b>	Culture tubes with UBI4 mutant of <i>S.cerevisiae</i> (SUB60) (I, II and III) with 0 $\mu$ M, 100 $\mu$ M and 200 $\mu$ M CuSO <sub>4</sub> , transformed with plasmid carrying YEp96-UbWt [II] and YEp96-EP42Ub [III] the lethal form of ubiquitin gene under CUP1 promoter, which is induced by Cu <sup>2+</sup> at 30°C (permissive temperature). <i>S.cerevisiae</i> (SUB62) (IV) with 0 $\mu$ M, 100 $\mu$ M and 200 $\mu$ M CuSO <sub>4</sub> transformed with plasmid carrying YEp96-EP42Ub. <i>S.cerevisiae</i> (SUB60-EP42) [V] the lethal form of ubiquitin gene under CUP1 promoter with 0 $\mu$ M, 100 $\mu$ M and 200 $\mu$ M CuSO <sub>4</sub> cotransformed with YEp96-UbWt, which is induced by Cu <sup>2+</sup> at 30°C (permissive temperature).	<b>120</b>
<b>Figure 6.10.</b>	Electrophoretogram of UbEP42 gene in YEp96-UbEP42 vector. Start and end arrow indicated the start and end of the gene.	<b>120</b>
<b>Figure 6.11.</b>	Percentage survival of SUB60, SUB62, SUB60/UbWt, SUB60/UbF45W and SUB60/ UbEP42 yeast cells at differential time of incubation 0, 4, 8, 12 and 16 hours of incubation.	<b>122</b>
<b>Figure 6.12.</b>	Cycloheximide resistance of SUB62, SUB60 cells transformed with UbWt and UbF45W gene. The SUB60 and SUB60 transformed with UbEP42 fail to grow in presence of cycloheximide. Three fold serial dilutions (2), (3) and (4) spotted on YPD plates containing 100 $\mu$ M of copper and 4 $\mu$ g/ml of cycloheximide.	<b>122</b>
<b>Figure 6.13.</b>	Effect of UbEP42 on the half-life of proteins in Ub14 background. SUB60, SUB62, SUB60 transformed by plasmids YEp96-UbWt, YEp96-UbF45W and YEp96-UbEP42 expressing the three forms of ubiquitin, namely wildtype UbWt, UbF45W and UbEP42. These cells were also transformed by pUb23 expressing Ub- $\beta$ -galactosidase fusion with (I) Met(M) and (II) Pro(P) as the N-terminal residues. SUB60 and SUB62 were also cells were co-transformed by plasmid pUb23 expressing Ub- $\beta$ -galactosidase fusion with (I) Met(M) and (II) Pro(P) as the N-terminal residues. SUB60 and SUB62 were used as controls.	<b>124</b>

<b>Figure 6.14.</b>	Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbEP42). Lane 1. Contains $\lambda$ <i>Hind</i> III Digest marker. Lane 2. Contains undigested plasmid (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbEP42). Lane 3. There are three <i>EcoR</i> I sites in the vector and so <i>EcoR</i> I digest shows three fragments of 694bp, 2818bp and 3733bp. Lane 4. contains <i>Bgl</i> II digest. Lane 5. Contains (a) <i>Xho</i> I digest is a unique restriction sites and give a single band of 7286bp fragment with pUB221/c-myc-UbWt and (b) is lost after subcloning of c-myc-UbEP42 gene Lane 6. Contains <i>Kpn</i> I digest. <i>Bgl</i> II and <i>Kpn</i> I sites were used for subcloning of the gene.	<b>125</b>
<b>Figure 6.15.</b>	Western blot showing ubiquitination profile. Lane 1. Rain bow molecular weight marker. Lane 2. SUB60 as negative Control. Lane 3. SUB60/UbWt and Lane 4. SUB60/UbF45W as positive control and Lane 5. SUB60/UbEP42.	<b>126</b>
<b>Figure 6.16.</b>	Restriction digestion pattern (a) pKK223-3/UbWt and (b) pKK223-3/UbEP42. Lane 1. Contains $\lambda$ <i>Hind</i> III digest marker. Lane 2. Contains (a) undigested plasmid of pKK223-3/UbWt and (b) pKK223-3/UbEP42. Lane 3. Contains <i>EcoR</i> I digest with a unique restriction site. Lane 4. (a) Contains <i>Xho</i> I digest and gives a single band with pKK223-3/UbWt and (b) the site is lost after subcloning of UbEP42 gene. Lane 5. Contains <i>Sal</i> I digest which has two sites and hence releases a 700bp fragment. Lane 6. Contains <i>Hind</i> III digest with a unique restriction site. <i>Eco</i> RI and <i>Hind</i> III sites were used for subcloning of the gene.	<b>126</b>
<b>Figure 6.17.</b>	UbEP42 expression gel profile. Lane 1. Shows the protein molecular weight marker. Lane 2. and Lane 3. Show protein profile of JM109 cells without and with IPTG induction respectively. Lane 4. and Lane 5. Show protein profile of JM109/ pKK223-3-UbEP42 transformed cells without and with IPTG induction respectively.	<b>127</b>
<b>Figure 6.18.</b>	UbEP42 expression and purification gel profile.Lane 1. Shows the protein molecular weight marker. Lane 2. Shows protein profile of JM109/ pKK223-3-UbEP42 transformed cells with IPTG induction. Lanes 3., Lanes 4. and Lanes 5. Show cell lysate, 0.2% PEI treated supernatant and heat treated supernatant respectively.	<b>128</b>
<b>Figure 6.19.</b>	Shows the position of four mutations S20F, A46S, L50P and I61T accompanied with the secondary structural features of ubiquitin.	<b>129</b>