Chapter 2

To study the cell cycle progression and morphology switching in *C. albicans* expressing ubiquitin mutations.

2.1. Introduction:

is the most commonly found commensal organism in humans, which can undergo morphogenesis into an opportunistic pathogen. *Candida* species are naturally present in the skin, mouth, digestive tract, and urogenital systems of healthy people (Odds, et al., 1988; latige et al., 2002). They have evolved characteristics that enable them to infect and thrive in various environments while avoiding the host's immune response. Diseases caused by *Candida* infection are termed candidiasis and candidemia. Notably, a bloodstream infection by *C. albicans* is the fourth leading serious condition seen in those who are immunocompromised (Odds, et al., 1988; Talapko et al., 2021; M. runke et al., 2008), *C. albicans* can adopt yeast, pseudohyphal and hyphal forms (Chandra et al., 2001; Nobile et al., 2006). *C. albicans* infections are showing increased resistance to current antifungal medications, an observation that highlights the urgency to discover new treatment targets and develop novel antibiotics. In eukaryotic cells, ubiquitination is crucial in regulating vital functions, which include cell cycling, transcription, translation and coping with stress.

Ubiquitin (Ub) is a small protein, with seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) in its primary structure. The C-terminal glycine of ubiquitin is conjugated to lysine side chains of substrate proteins via sequential actions carried out by three enzymes, in the process of ubiquitination, which requires ATP in the first step. Initially, ubiquitin is activated by an enzyme known as ubiquitin-activating enzyme (E1), which is associated with ATP hydrolysis. This activated ubiquitin is then bound to the enzyme's active site by forming a thioester bond between ubiquitin and E1. Subsequently, ubiquitin is transferred to the second enzyme, ubiquitin-conjugating enzyme (E2), where ubiquitin once again is bound by a thioester linkage. Lastly, the target substrate is identified and bound by the third enzyme, ubiquitin ligase (E3). In the final step, ubiquitin bound to E2 is either directly transferred to the substrate or initially to E3 and subsequently to the substrate. This series of events are repeated between substrate-conjugated ubiquitin and free ubiquitin molecules, leading to the formation of a polyubiquitin chain. The lysine residues of ubiquitin used in polyubiquitin determine the topology of the branches and the resultant signals. For instance, K48 linked polyubiquitin chain typically serves as a signal for the degradation of the substrate by the 26S proteasome.

The conformation of ubiquitin protein and its sequence showcase evolutionary conservation, with only about three alterations in its seventy-six amino acid residues from unicellular

eukaryotes to both animals and plants (Gavilanes et al., 1982; Schelesinge et al., 1975; Wiborg et al., 1985). In earlier studies, to understand the relationship between the conserved primary structure of ubiquitin and the multiple cellular functions in which it serves as a posttranslational modifier, mutants of ubiquitin were generated and examined in *Saccharomyces cerevisiae* (Mishra et al., 2011; Mishra et al., 2009; Prabha et al., 2010). Studies on the effects of ubiquitin mutations like UbEP42 and the four mutations UbS20F, UbA46S, UbL50P, and UbI61T derived from it led to several interesting observations, in *S. cerevisiae*. Interestingly, the mutations UbEP42, UbL50P and UbI61T negatively influenced the growth and various other functions in *S. cerevisiae* (Doshi et al., 2014; Doshi et al., 2017. The rich repository of information available on *S. cerevisiae* positions it as an excellent initial model for hypothesis testing before exploring similar queries in a more complicated yeast such as *C. albicans,* an opportunistic human pathogen (Asleson et al., 2001; Heckmen et al., 2001). Both *C. albicans* and *S. cerevisiae* trace back to a common ancestor, which had existed over 300 million years ago. Notable parallels in the molecular details of cell cycling, mating behavior, metabolism, cell wall formation, and signaling processes have been thoroughly documented for both organisms (Lott et al., 2005). However, CUG codon of *S. cerevisiae* encodes Leucine (Leu), while the same codon encodes Serine (Ser) in *C. albicans* (Mühlhausen et al., 2021). To date little has been reported about ubiquitin regulation and its relation to morphological switching in *C. albicans*. Ubiquitin mutations studied in *S. cerevisiae* have been employed for preliminary study in *C. albicans* CAI4 strain by (Dantuslia et al., 2023) to gain insights into cell cycle dynamics and morphogenesis in *C. albicans,* especially concerning the transition from yeast to hyphal form, an essential step in pathogenesis at the molecular level with compatible vector in fast-growing Strain BWP17. However, the CTG codon nucleotide sequence in the gene was replaced by TTG to have Leucine (Leu) in the 56^{th} position in the protein. The BWP17 strain of *C. albicans* (ura3/ura3 his1Δ/his1Δ arg4Δ/arg4Δ), which is a derivative of CAI4 provided by Prof. Yue Wang from IMCB Singapore, was utilized to study the functional changes produced by ubiquitin mutations in cell cycle progression and morphogenesis.

The BWP17 of *C. albicans* strain lacks virulence. However, it possesses a full complement of genes that express ubiquitin proteins. In *C. albicans*, ubiquitination is vital for pathogenesis (Leach et al., 2011). This organism has two documented ubiquitin genes, UBI3 and UBI4 (Roig

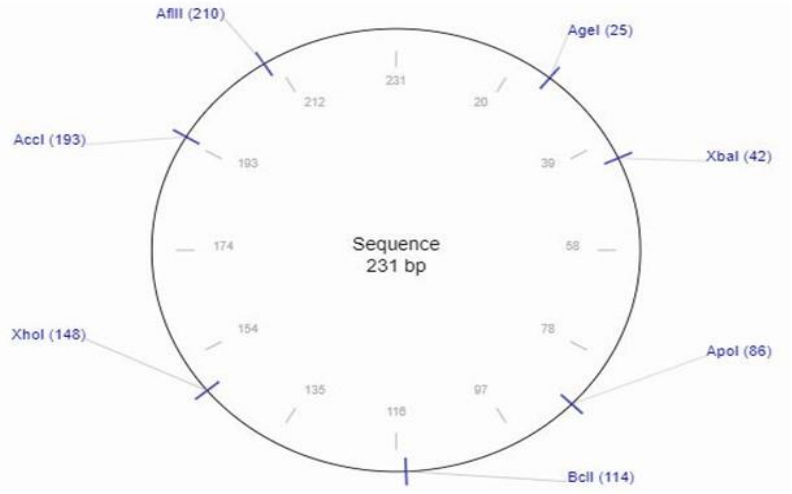
et al., 2001). The UBI3 gene produces a fusion protein of ubiquitin and a ribosomal protein, and it closely resembles the UBI3 gene found in *S. cerevisiae*. On the other hand, the UBI4 gene product is polyubiquitin with three sequential ubiquitin repeats without spacers. Additionally, in *C. albicans* the cyclin B level is modulated by the mitotic transcription factor Fkh2 (Côte et al., 2009). This factor is analogous to the Phd, Sok2, and StuA transcription factors, which influence the activity of secretory aspartyl proteases in *Aspergillus* species (Osiewacz et al., 2002). However, there are no reports on the role of Fkh2 in the regulation of aspartyl protease activity in *C. albicans.* A significant number of genes that typically show increased activity during hyphal growth and pathogenesis were observed to have decreased activity in the fkh2ΔΔ and fkh2 mutants. The aspartyl protease genes *SAP4* and *SAP6* were notably downregulated in the fkh2ΔΔ mutants (Dabas et al., 2008; Greig et al., 2015). Historical research has also shown that individuals with conditions like HIV, cancer and those undergoing radiotherapy for the head and neck cancers tend to be more susceptible to candidiasis. This is possibly due to alterations in the pH of the saliva of these patients.

In this context, it would be interesting to see what would be the effect of ubiquitin mutations on morphology switching, cell surface chitin deposition, β-glucan exposure, secretory proteases associated with pathogenesis, virulence-associated transcription factor levels, and cell cyclins in different forms and stages of the life cycle of *C. albicans*.

2.2. Material and methods

2.2.1. Strains and media

The auxotrophic *C. albicans* strain, BWP17 has been used in the present study (Yang et al., 2018). BWP17 (ura3/ura3 his1Δ/his1Δ arg4Δ/arg4Δ) derivative of CAI4, was a gift from Prof. Yue Wang from IMCB Singapore. It is an avirulent strain of *C. albicans* (Yang et al., 2018). BWP17 was grown in YPD (1% w/v yeast extract, 2 % w/v peptone and 2 % w/v dextrose) supplemented with or without 50 μg/ml uridine for selection as previously described (Lai et al., 2011; Chapa et al., 2005). After transformation by plasmid vector pTET25M-NC (Figure 2.1), the selection of transformants on Synthetic SD minimal media, the integrants were confirmed by

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1    2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17 18
ATG CAG ATA TTC GTC AAG ACT TTG ACC GGT AAA ACC ATA ACT CTA GAA GTT GAA
19   20  21  22  23  24  25  26  27  28 29  30   31  32  33  34  35 36
TCT TCC GAT ACC ATC GAC AAC GTT AAG TCG AAA ATT CAA GAC AAG GAA GGC ATT
37   38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54
CCA CCT GAT CAA CAA AGA TTG ATC TTT GCC GGT AAG CAG CTC GAG GAC GGT AGA
55   56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72
ACG TTG TCT GAT TAC AAC ATT CAG AAG GAG TCT ACC TTA CAT CTT GTC TTA AGA
73  74   75  76
CTA AGA GGT GGT TGA
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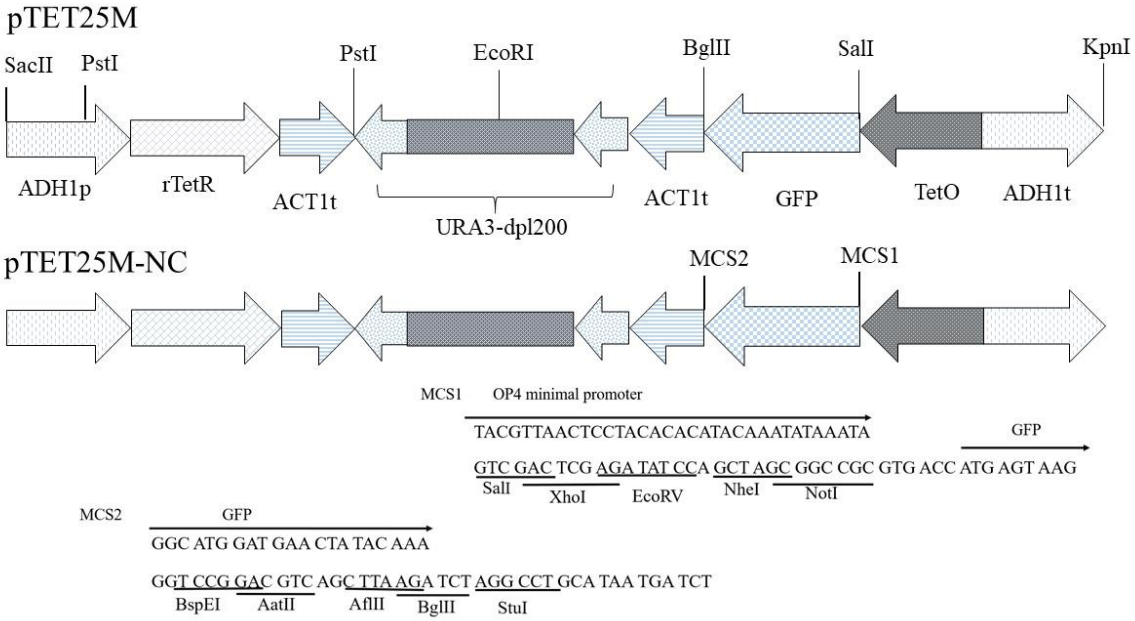


Figure 2.1. Ubiquitin gene mutagenic sequence and Vector map of P-TET25MNC for ubiquitin mutants cloning and screening.

PCR using oligonucleotide primers pTET25-M-NC intF and pTET25M-NC intR listed in Table 1. The *E. coli* strain DH5*α* was used as a host for plasmid DNA construction and routine plasmid maintenance and amplification. The cultures of *E. coli* were grown in Luria-broth or Luria broth supplemented with 50 µg/ml ampicillin. Purification of plasmid was done by using the Thermo Gene-Jet purification Kit, K0502.

2.2.2. Cloning of ubiquitin mutant genes

In *Candida albicans* during evolution, a unique change in genetic code occurred, where the standard codon for leucine CUG encodes serine. In the ubiquitin gene and its mutant forms obtained from *S. cerevisiae* the CTG sequence CUG codon was replaced by TTG sequence UUG codon to keep the leucine as it was in our ubiquitin mutants studied previously (Doshi et al., 2017) . The cloning strategy is mentioned in (figure2.2). Briefly, UbEP42 has the four mutations

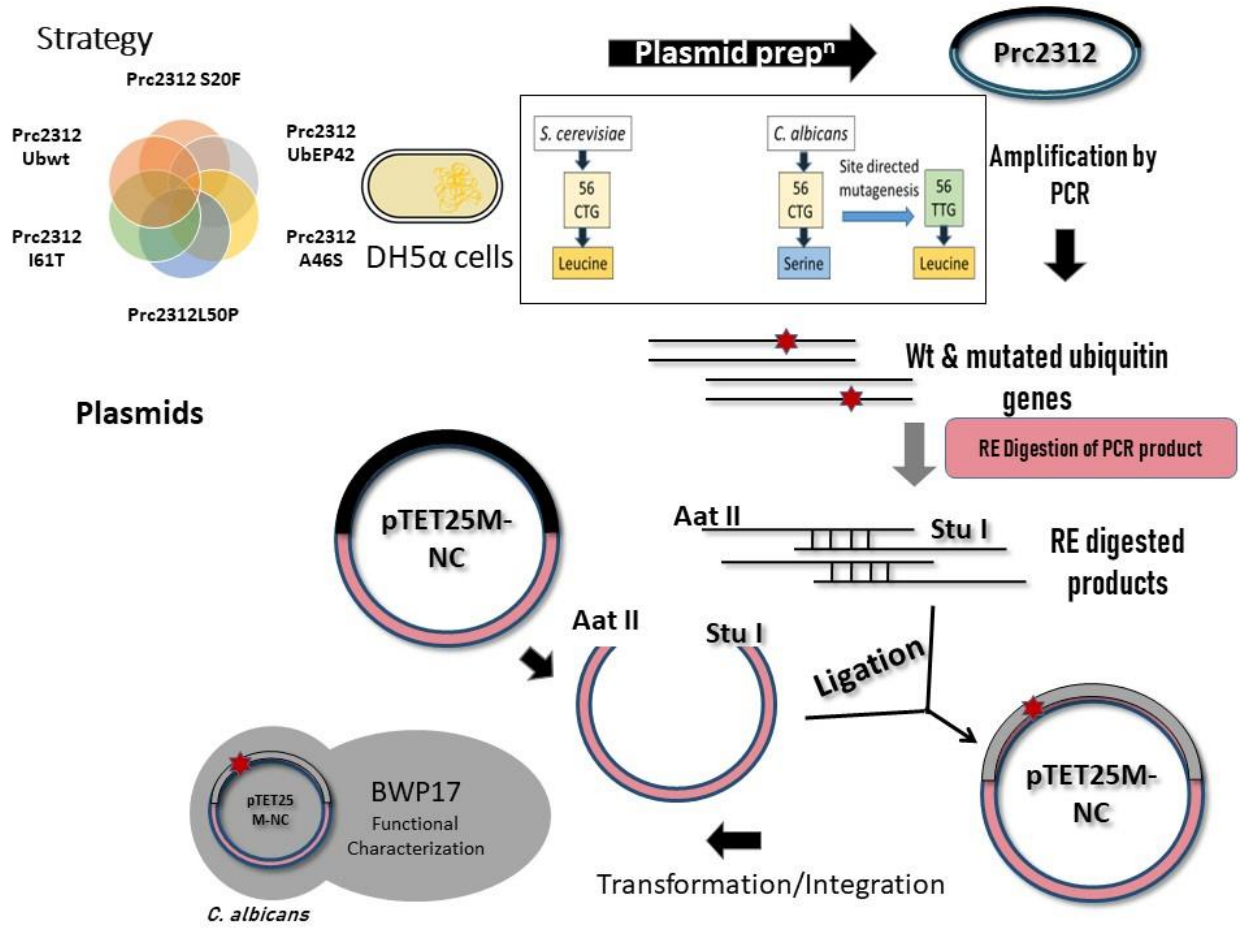


Figure 2.2. Illustration of Cloning Strategy for site-directed mutagenesis of CTG codon in ubiquitin mutants and functional characterization.

mentioned earlier and its isolated single mutations are UbS20F, UbA46S, UbL50P and UbI61T, all five mutant forms of the ubiquitin gene were replaced with TTG sequence (UUG codon) at 56^{th} position in ubiquitin and cloned initially under a constitutive promoter in the pRC2312 vector. Primers (P1, P2, P3, P4) used for UUG codon incorporation are listed in Table 1. At this stage expressing the mutations under an inducible promoter appeared better suited for the purpose. So, for this pRC2312 vectors with inserts of mutant forms of ubiquitin gene were amplified using forward primer FRAatI and REStuI having AatI and StuI restriction sites. Double-digested amplified fragement and pTET25M-NC vector with AatI and StuI restriction enzyme were then ligated under 5' *gfp* tag MCS2 Aat1 & StuI restriction site of pTET25M-NC having Tet- inducible promoter for functional study in *C. albicans*. Genes of interest cloned in the vectors were confirmed by restriction digestion, followed by resolution on 2% agarose gels and sequencing. pRC2312 and pTET25M-NC plasmids were gifted by Dr. Richard Canon from the University of Otago, New Zealand and Dr. Yue Wang from IMCB Singapore respectively. The oligonucleotide primers obtained from Imperial life Science Pvt. Ltd., India, were used for cloning. The DH5α transformants and BWP17 transformants which received the wildtype and mutant forms of ubiquitin gene under the inducible promoter are referred to as UbWT, UbEP42, UbS20F, UbA46S, UbL50P, UbI61T.

Table 1. Primers used in this study.

Cloning Primers	**Discription**	**Sequence (5'->3')**
P1	Forward HindIII	GCTAAGCTTATGCAGATCTTCGTCAAGACG
P2	Reverse BamH1	GATGGATCCTCAACCACCACCTCTTAAGA)
P3	Forward Mutagenic	GAGGACGGTAGAACGTTGTCTGATTACAAC
P4	Reverse Mutagenic	GTTGTAATCAGACAACGTTCTACCGTCCTC
FRAat1	Forward	GACGTCATGCAGATCTTCGTCAAGACT
RevStu1	Reverse	AGGCCTTCAACCACCTCTTAGTCT
pTET25-M-NC intF	Forward	CATGTCAAAGGATTCAAC
pTET25M-NC intR	Reverse	GTATGGTGCCTATCTAAC

2.2.3. Transformation and selection of integrants

Selection of the transformants of *C. albicans* was performed as previously described (Shieh *et al*., 2005). Briefly, the DNA fragment integrents *Sac*II/*Kpn*I cassete (Figure2.1) carrying the Tet-on promoter in pTET25M-NC along with the gene of interest was removed from the plasmid pTET25 MN-C by digestion with *Sac*II and *Kpn*I, purified using the Thermo Gel extraction kit and transformed into *C. albicans* cells by the LiAc-PEG-ssDNA method (Gietz and Woods et al., 2006). The DNA was specifically integrated into the *ADH1* locus of the *C. albicans* genome as a stable integrant and the integration was confirmed by selecting for the Ura^+ prototrophs on minimal medium plates lacking uridine (Berman et al., 2006). The integrants were further confirmed by colony PCR, using the diagnostic primers pTET25-M-NC intF and pTET25M-NC intR (Table 1).

2.2.4. Influence of variants of ubiquitin on different antibiotic stress

An antibiotic complementation study of ubiquitin variants was conducted using plate assays following a standard protocol (J. Hanna et al., 2003; J. Spence et al., 2023). Briefly, BWP17 transformants containing pTET25MN-C plasmids with genes encoding wild type and mutant ubiquitin were cultured at 30°C until reaching an optical density of 0.2. The cultures underwent successive fourfold serial dilutions and were spotted on SD agar plates. Subsequently, cells were induced with 45 μg/ml doxycycline in the presence of antibiotics, including cycloheximide (75 μg/ml), tunicamycin (1 μg/ml), and canavanine (1 μg/ml). Plates were then incubated for 5-6 days at 30°C to evaluate the ability of mutant ubiquitin to complement antibiotic stress by assessing growth.

2.2.5. Confocal microscopy & Fluorescence microscopy for morphological switching analysis

C. albicans BWP17 transformants carrying wild type and mutant ubiquitin genes were cultured until reaching mid-log phase, both with and without serum. Once in mid-log phase, the cultures were treated with the inducer doxycycline (45 μg/ml) and allowed to incubate for 3 hours. Following incubation, the cells were harvested and washed with sterile phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4), pH 8.0) to remove any remaining media components. Subsequently, the cells were fixed in 70% ethanol for 30 minutes and then washed once again with PBS. After this preparation, the suspended cells

were mounted onto a slide for observation under a confocal microscope (Zeiss LSM 700). It's noteworthy that BWP17 cells carrying the wild type ubiquitin gene (UbWT) served as the positive control in this experimental setup.

2.2.6. Cell cycle analysis

To investigate the impact of these mutants on the cell cycle of *Candida albicans* and understand at which phase ubiquitin variants hinder cell cycle progression, fluorescence-activated cell sorting (FACS) analyses were conducted following established protocols (Ramani et al., 1997). In brief, a total culture volume of 100 ml was used, divided into 3 ml aliquots. The cells were pelleted at 6,000 rpm for 5 minutes, and the supernatant was discarded. The cells were then resuspended in 3 ml of dH2O and sonicated to prevent clumping. Fixation of cells was achieved by adding 7 ml of 95% ethanol, followed by incubation at 4°C for 2-16 hours. After centrifugation at 6,000 rpm, the supernatant was discarded, and the cells were resuspended in 5 ml of 50 mM sodium citrate pH 7.4. Subsequently, the cells were again resuspended in 1 ml of 50 mM sodium citrate containing 0.25 mg/ml boiled RNaseA and incubated for 1 hour at 50°C. Proteinase K was then added for degradation of RNA contaminations. The cells were stained with propidium iodide at a final concentration of 16 µg/ml in sodium citrate buffer and incubated at room temperature for 30 minutes. For each FACS assay, the DNA content of 50,000 single cells was monitored using a BD Facs AriaTmLFusion flow cytometer and analyzed with FlowJo software. The experiment was repeated in three independent sets, and the percentage of cells in G0/G1, S, and G2/M phases were plotted for analysis.

2.2.7. Detection of protein levels of Cdc28 by western blot analysis

BWP17 cells carrying plasmids encoding mutant ubiquitin variants and wild type ubiquitin were cultured until reaching the logarithmic growth phase in the presence of 45 µg/ml doxycycline as an inducer. Following this, the cells were harvested and washed with phosphate-buffered saline (PBS), and then subjected to sonication to lyse the cells. The lysate was clarified by centrifugation at high speed. Protein concentration was determined using the Folin-Lowry method, and equal amounts of protein were loaded onto a 15% SDS-PAGE gel. The separated proteins were then transferred onto a nitrocellulose membrane. Subsequently, the membrane was incubated overnight at 4°C with a primary antibody against Cdc28 protein kinase (Santa Cruz, Cat No.: sc-28550). Following primary antibody incubation, the membrane was probed with

Goat anti-Rabbit IgG HRP Conjugate (GeNei, Cat No.: HPO3). The membrane was washed multiple times with PBS-T and PBS. Finally, the blot was developed using an ECL Western Blotting Detection Kit (Amersham Biosciences).

2.2.8. Incorporation of variants in Lys63 and Lys48 linked chain formation by western blot analysis

The transformed ubiquitin genes were induced for overexpression by treating the cells with 45 µg/ml doxycycline for 3 hours after reaching the logarithmic growth phase. Total protein was extracted from the cultured cells following a previously described protocol (Wimalasena et al., 2008). To investigate the formation of K63 and K48 linked polyubiquitin chains, the extraction procedure was followed up to the blocking step. Subsequently, the membrane was probed overnight at 4°C with anti-ubiquitin, Lys63 specific clone HWA4C4 (Merk Millipore, Cat No.: 05-1313, Mouse monoclonal antibody) and K48 linkage specific polyubiquitin antibody (CST, Cat No. 4289), respectively, followed by incubation with HRP-tagged secondary antibody. The membrane was washed six times with PBS-Tween20 solution and then twice with PBS. Finally, the blot was developed using an ECL kit (Amersham).

2.2.9. SEM analysis

C. albicans cells expressing wild type and mutant forms of ubiquitin were treated with 4% formaldehyde in 0.2 M phosphate buffer, pH 7.4 for 2h. at room temperature. Then the cells were washed with PBS and incubated with 8% formaldehyde in 0.2 M phosphate buffer pH 7.4 for 18h. at 5-6 ºC, and again the samples were washed carefully with 0.2 M phosphate buffer pH 7.2. After fixing for 2h. at room temperature with 2% osmium tetroxide, the samples were subjected to dehydration in ethanol gradient in a series of steps: 50% two times for 10 min., 95% two times for 5 min. and 100% twice for one min. Final dehydration of the samples was done with acetone twice for 30 sec. and finally dried by critical point method in liquid CO_2. Samples were then transferred onto SEM stages with carbon discs, and dried in a desiccator for 24 h. in the presence of dry silicone gel and for 2 h. at 180 °C. Afterwards, the stages with the probes were coated with a gold layer using sputtering machine and observed under a scanning electron microscope Hitachi SEM SU1510 Japan.

2.2.10. Calcofluor white staining

Calcofluor white (Sigma-Aldrich) binds to chitin in the fungal cell wall. Equal volumes of 20% aqueous solution of the Calcofluor white fluorochrome and a suspension of BWP17 cells with UbWT and its mutant forms were incubated for 10 min. at room temperature in the dark. Then, 5 µL of the suspension was transferred onto a slide and observed at excitation wavelength 260(nm) under a Nikon Ti-2 confocal fluorescence microscope for chitin deposition and formation of aggregates. The experiment was repeated three times.

2.2.11. Congo red staining

Congo red dye purchased from Sigma-Aldrich was used to visualize β-1,4 glucans of the cell wall. Aqueous solution (2%) of the dye was mixed with the suspension of the different transformants of *C. albicans* cells in a 1:1 ratio and incubated for 5 min. in the dark at room temperature further 5 µL of the suspension was transferred onto a slide and observed at excitation wavelength 543(nm).

2.2.12. Protease Extraction and Precipitation

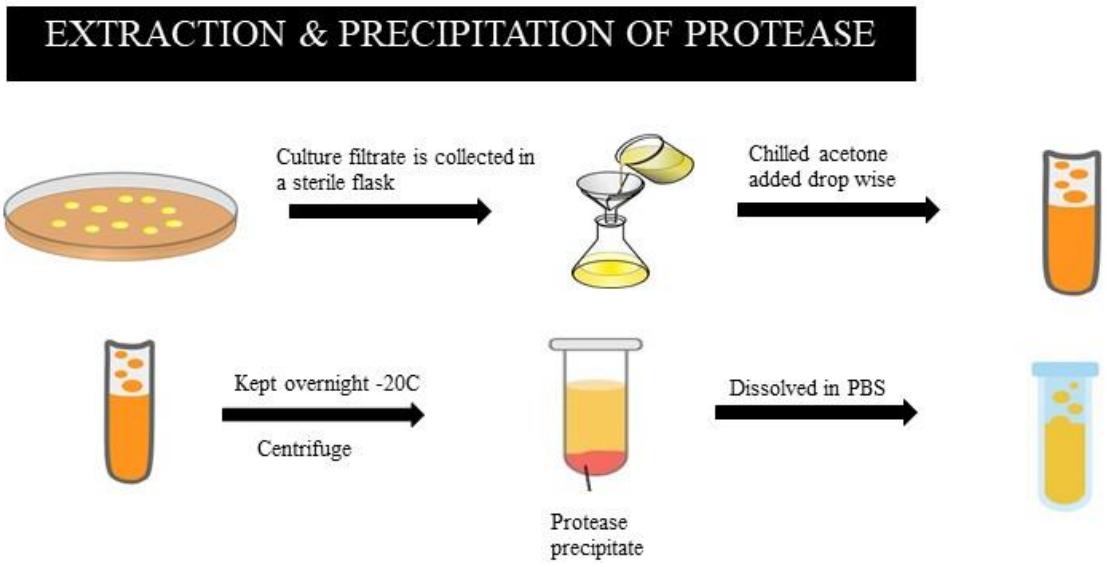


Figure 2.3 Protease extraction and Precipitation method

Procedure Following a 10-day incubation period of fungal growth, the liquid medium containing the fungal byproducts was filtered through sterile Whatman filter paper into a sterile container. The resulting culture filtrate was then subjected to precipitation using chilled acetone. Specifically, 4 ml of chilled acetone was slowly added drop by drop to every 1 ml of fungal culture filtrate. This mixture of culture filtrate and acetone was subsequently stored at a temperature of -26 ºC overnight to facilitate precipitation. The following day, the precipitate was separated from the solution via centrifugation at 5000 RPM for 15 minutes at 4 ºC. The resulting precipitate was then dissolved in PBS, with a ratio of 50 µl of PBS per milliliter of the initial culture filtrate used for precipitation. This dissolved precipitate was utilized for protein estimation through the Folin Lowery method and protease activity on gel through gelatin zymography.

2.2.13. Gelatin Zymography:

Zymography was performed by the method described by Lantz and Ciborowski (1994) using gelatin as a substrate (0.1%) and 12% of polyacrylamide. Twelve percent SDS – polyacrylamide gel was prepared and gelatin was added as a substrate in separating buffer. After electrophoresis, the gel was incubated in 2.5 % Triton –X 100 solution for 1 hour. After incubation, the gel was washed twice with water and again incubated in an incubation buffer containing 50mM Tris, 5 mM CaCl2 and 1 µM ZnCl2. The gel was incubated at 37 °C for 2 hours in an incubation buffer. After incubation, the gel was again washed with water and kept overnight for staining with (0.5 %) Coomassie Brilliant blue R. Next day the stained gel was de-stained to observe the bands of enzyme activity. The bands of substrate degradation were observed as colourless bands against a blue background.

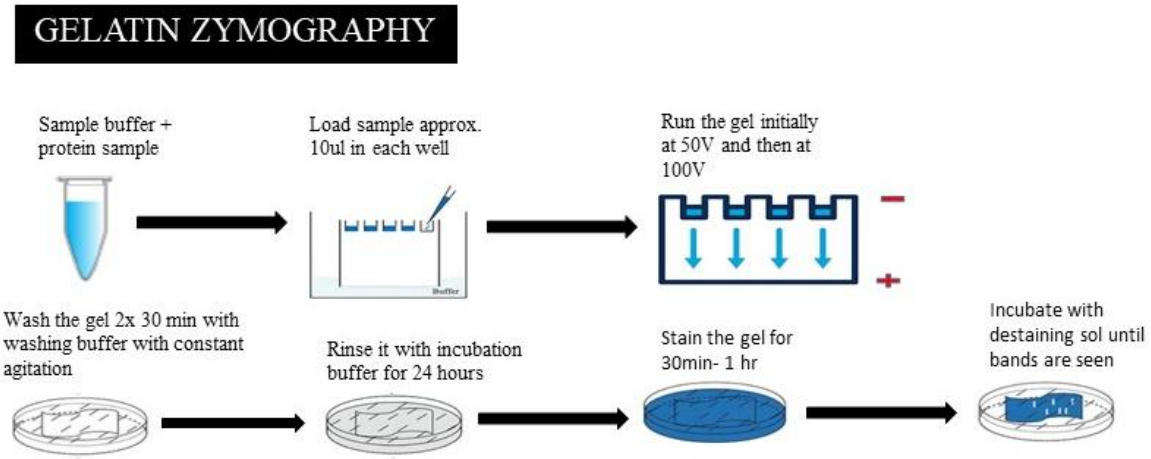


Figure 2.4. Illustration of gelatin zymography method

2.2.14. Statistical analysis

A statistical analysis was performed using GraphPad Prism version 5.01 software for Windows (GraphPad Inc., San Diego, CA). Data are expressed as mean ± standard deviation (SD) of three independent assays. One-way analysis of variance (ANOVA) followed by Bonferroni's test were used for intergroup variation analysis. Statistical significance was established as $p < 0.05$.

2.3. Results

2.3.1. Site-directed mutagenesis of mutant forms of ubiquitin gene & cloning in pRC2312 vector.

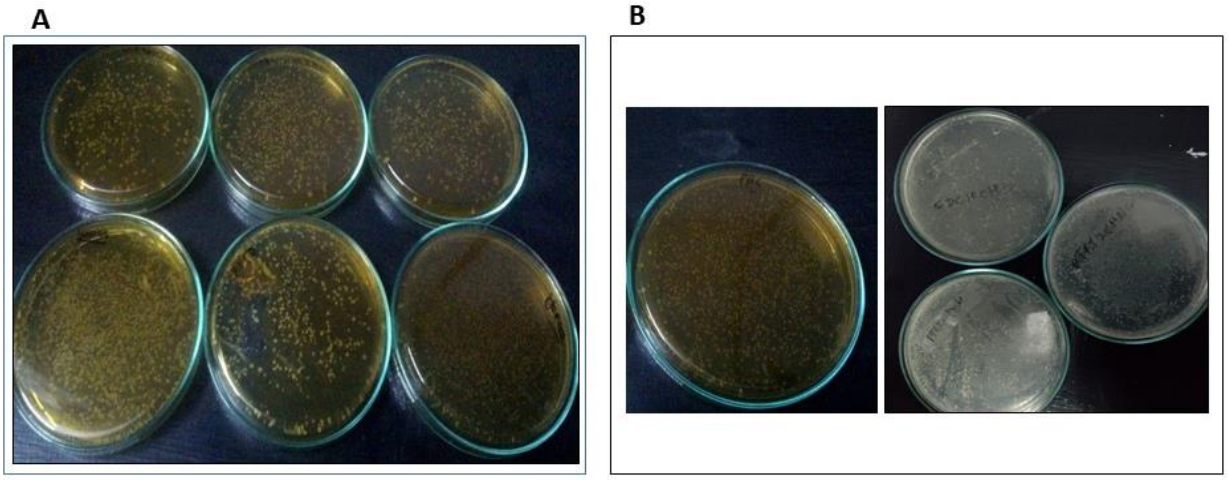


Figure 2.5. Transformation (A)Transformation of pRC2312 vector containing wild type and mutated ubiquitin into DH5*α*. (B) Requested Other inducible Vectors for further study such as PTET25M-NC, PTET25-MCDC10-CH.

2.3.2. Confirmation of pRC2312 vector & Site-directed mutagenesis PCR products confirmation by gel electrophoresis:

pRC2312 plasmid containing *Hind III* and *Bam HI* sites confirmed (Figure 2.6 A). The mutant ubiquitin amplified gene size is 231bp, and the PCR fragments of mutagenic forward and reverse primes shorter than that of non-mutagenic. (Figure 2.6 B).

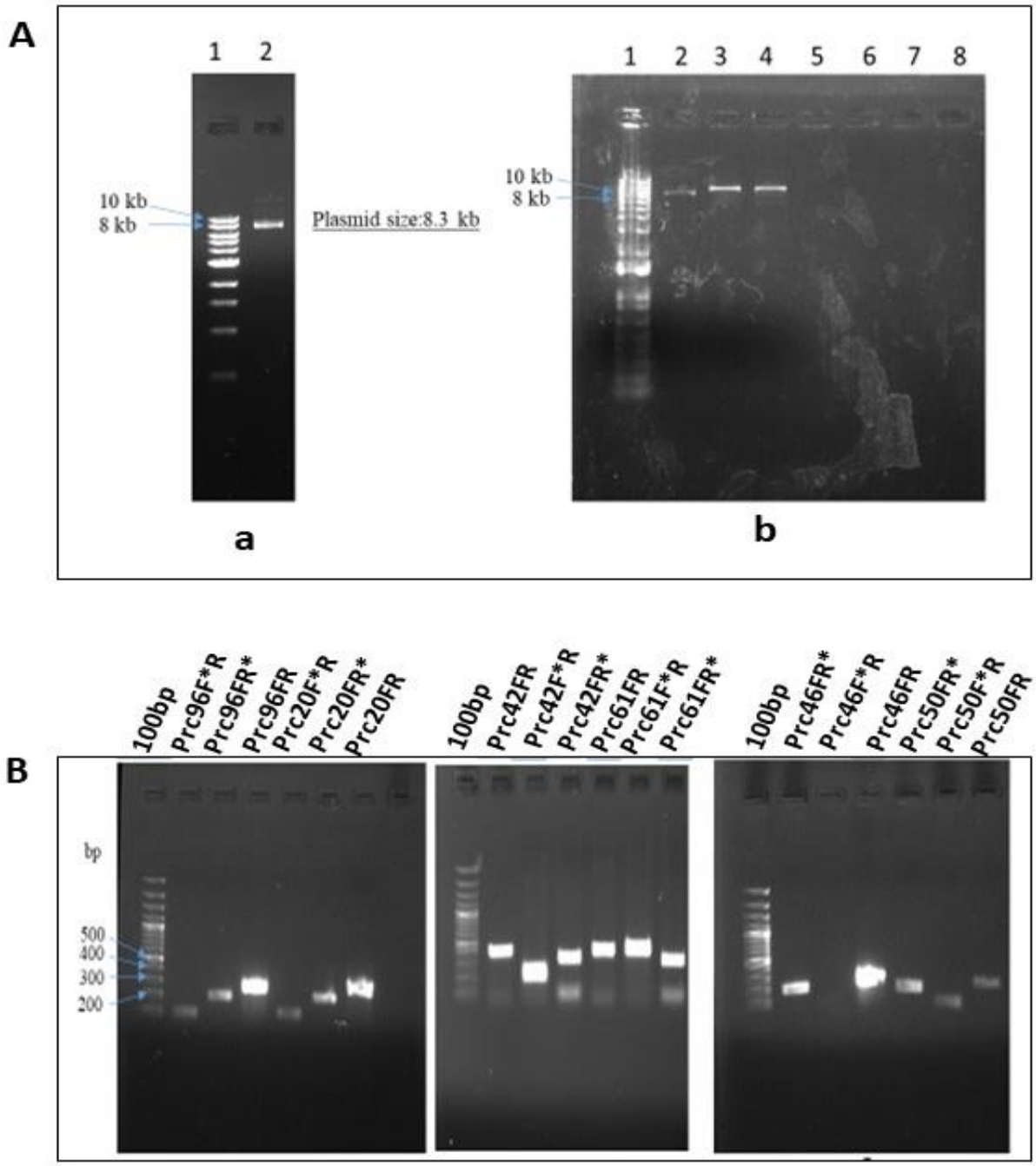


Figure 2.6. Vector Confirmation (A) (a). 1% agarose gel showing 8.3 kb size pRC2312 vector. (b) Restriction digestion of pRC2312 plasmid with *Hind III* and *Bam HI*. The Lanes L1 to L4 contain: L(1):1kb ladder L(2) pRC2312undigested plasmid, L(3) pRC2312 plasmid digested with *HindIII* and L(4) pRC2312 plasmid digested with *BamHI* for cloning purpose. (B) Site-directed mutagenesis PCR products run on 3% Agarose gel. Here, FR is

denoted as = Forward and Reverse non mutagenic primer in combination, F*R= Forward mutagenic& reverse non mutagenic, FR*=Forward non mutagenic and Reverse mutagenic.

2.3.3. Site-Directed mutagenesis CTG to TTG ubiquitin mutants transformed in DH5α cells

UUG codon incorporated in ubiquitin mutants in pRC2312 transformed in DH5α cells grown on Luria Agar plate. Ampicillin(50µg/ml) is used as the selection marker (Figure 2.7 A). further plasmid isolated from transformed cells subjected to restriction digestion with *Hind III* and *Bam HI* to confirmation of ubiquitn mutants inserts. 1% agarose gel showed release of inserts but bands were slightly fant (Figure 2.7 B(a)). later confirmation through pcr amplification of inserts ubiquitn mutants from pRC312(Figure 2.7B(b) final confirmation of TTG sequence (UUG codon) incorporation through sanger DNA sequencing method (2.7 C).

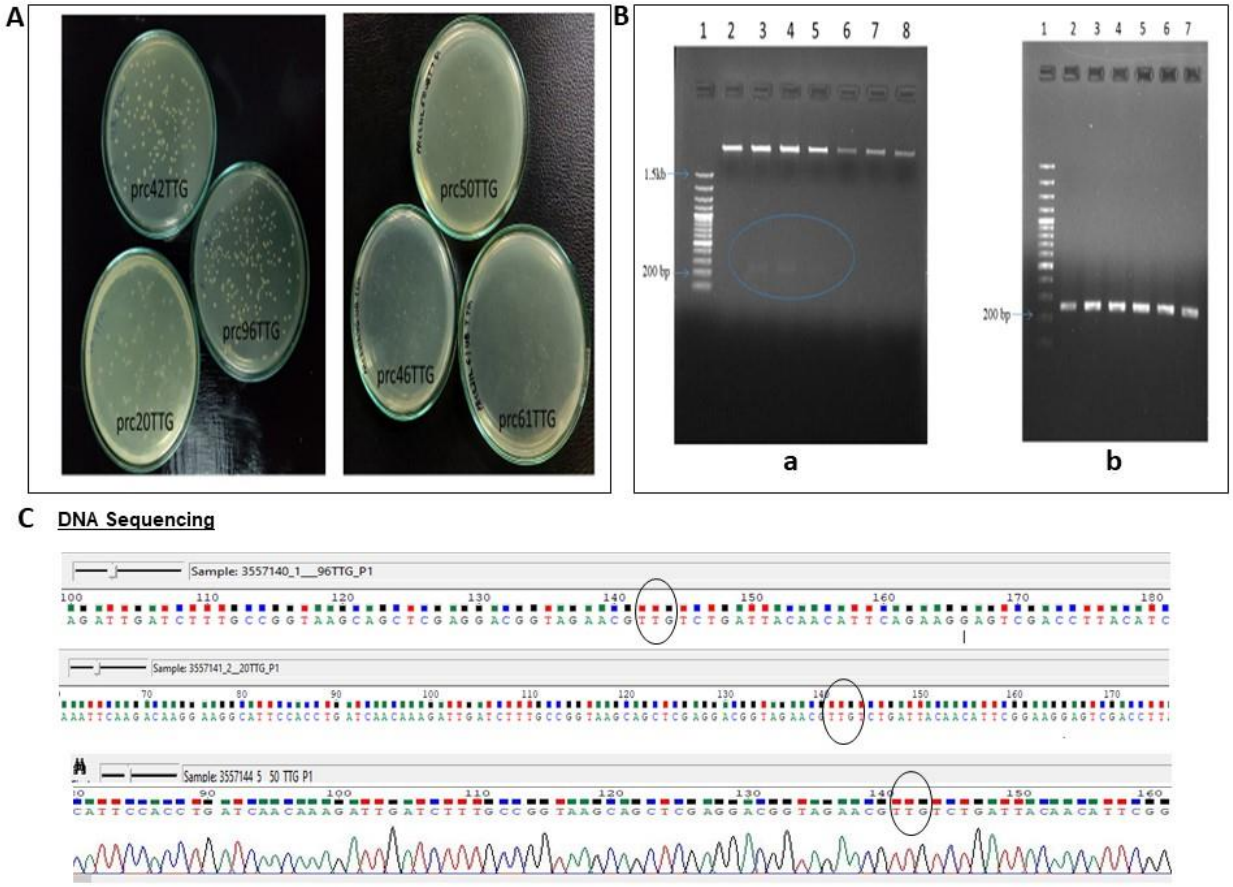


Figure 2.7. Site Directed mutagenesis (A) Transformants of wild type and mutated CTG codon ubiquitin mutants. (B) (a) 1% agarose gel showing results of restriction digestion of pRC2312 plasmid containing ubiquitin mutant genes is double digested with *Hind III* and *Bam HI*. The Lanes 1 contains: 100 bp ladder; Lanes 2 to 8 *Hind III* and *Bam HI* digestion of cloned Prc2312, PrcUbWT TTG, PrcS20F TTG, PrcUbEP42 TTG, PrcA46S TTG, PrcL50P TTG, PrcI61T TTG respectively. (b) 1% agarose gel showing results of PCR amplification for further clarification. (C) Ubiquitin CTG to TTG mutant confirmation by DNA sequencing

2.3.4. Cloning of mutant forms of ubiquitin gene in pTET25M-NC vector and expression in BWP17 strain of C. albicans for functional analysis

The isolated Prc2312 plasmid (Figure 2.8 A), carrying ubiquitin mutant genes pcr amplification successfully analyzed using agarose gel electrophoresis. In the 1% agarose gel, Lane 1 served as a reference with a 100 bp ladder. Lanes 2 to 7 displayed bands representing the respective ubiquitin mutants: UbWT, UbS20F TTG, UbEP42 TTG, UbA46STTG, UbL50P TTG, and UbI61T TTG(Figure 2.8 B). Furthermore, a 2% agarose gel was utilized to analyze PCR products of the ubiquitin mutant genes. In the subsequent analysis, the cloned vector pTET25M-NC was subjected to restriction digestion. Lane 1 of the 1% agarose gel contained the 100 bp ladder, while Lane 2 displayed the undigested vector. Lanes 3 to 8 exhibited the results of AatII and StuI digestion of cloned pTET25M-NC harboring ubiquitin mutants (Figure 2.8 C) given name as UbWT, UbS20F, UbEP42, UbA46S, UbL50P, and UbI61T mutants, respectively. These experiments were conducted following transformation in the BWP 17 strain.

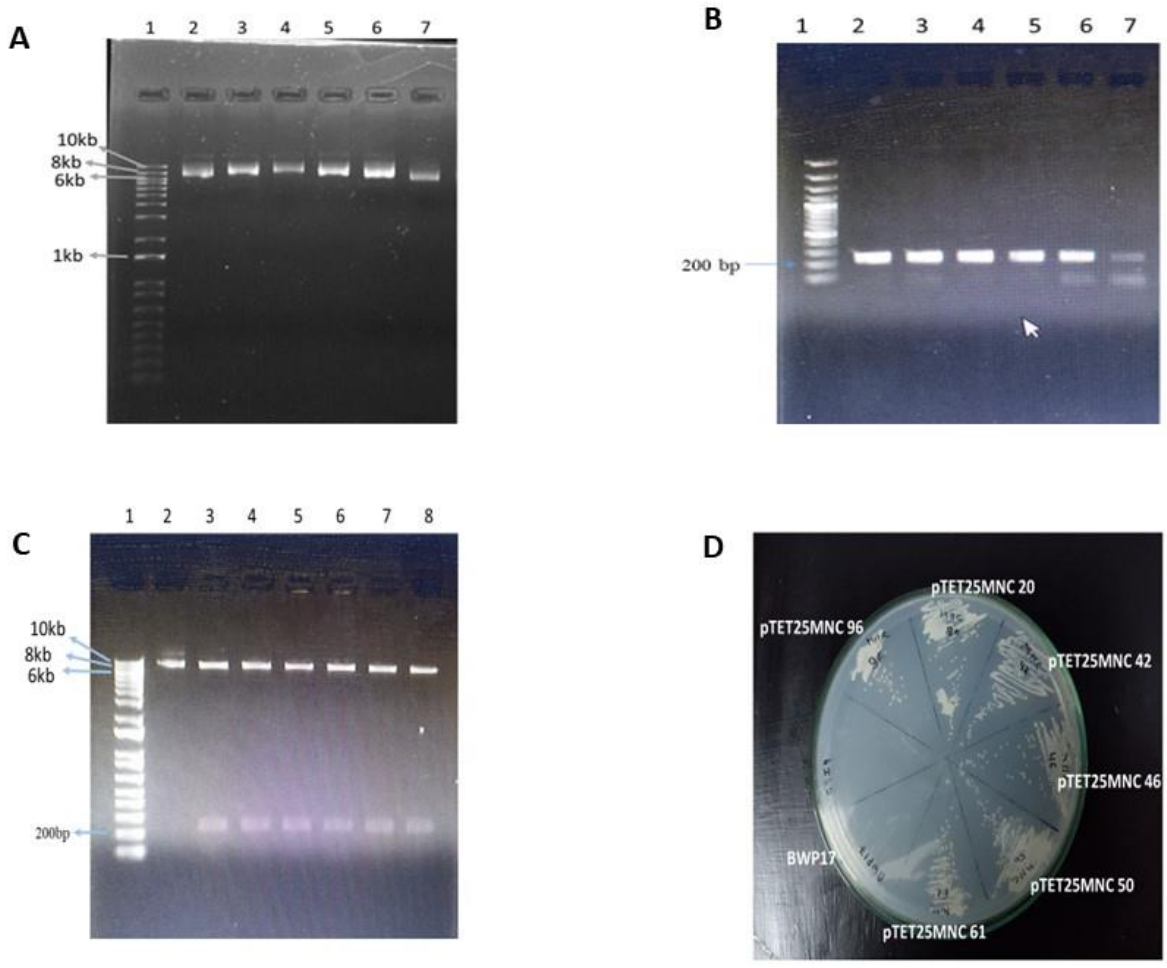


Figure 2.8. Cloning (A) Prc2312 plasmid carrying ubiquitin mutant gene isolated from transformed DH5*α* cells and runned on 1% agarose gel. The Lane1 contain 100 bp ladder, Lane 2 to 7 showing pRC2312 UbWT TTG, UbS20F TTG, UbEP42 TTG, UbA46STTG, UbL50P TTG, UbI61T TTG respectively.(B) 2% agarose gel showing PCR products of ubiquitin mutant genes. (C) 1% agarose gel is showing result of restriction digestion of cloned vector pTET25M-NC with TTG ubiquitin mutant genes. The Lanes L (1):100 bp ladder, Lanes 2 showing undigested vector. Lane 3-8 contains *AatII* and *StuI* digestion of cloned pTET25M-NC showing release of UbWT, UbS20F, UbEP42, UbA46S, UbL50P, UbI61T respectively. (D) Transformation in BWP 17 Strain.

2.3.5. Ubiquitin overexpression dosage-dependent lethality & the survival capacity of the BWP17 strain of *C. albicans*.

The proteins of ubiquitin wild-type UbWT and mutant forms UbS20F, UbEP42, UbA46S, UbL50P, and UbI61T were expressed with GFP tag in the N-terminus under Tet-on promoter in SD minimal broth media containing Doxycycline inducer in the concentration level of 0 µg/ml, 20 µg/ml, and 45 µg/ml of doxycycline. We analyzed the dosage-dependent lethality in the BWP17 strain. UbEP42, UbL50P, and UbI61T exhibit lethality at a lower inducer concentration of 45 µg/ml. However, UbS20F and UbA46S were tolerated with at this concentration and survived (Fig. 2.9 A). Similar results were also found with the streak plate method assay at 45 µg/ml Doxycycline, with growth not observed when the BWP17 cells expressing UbEP42, UbL50P and UbI61T (Fig. 2.9 B).

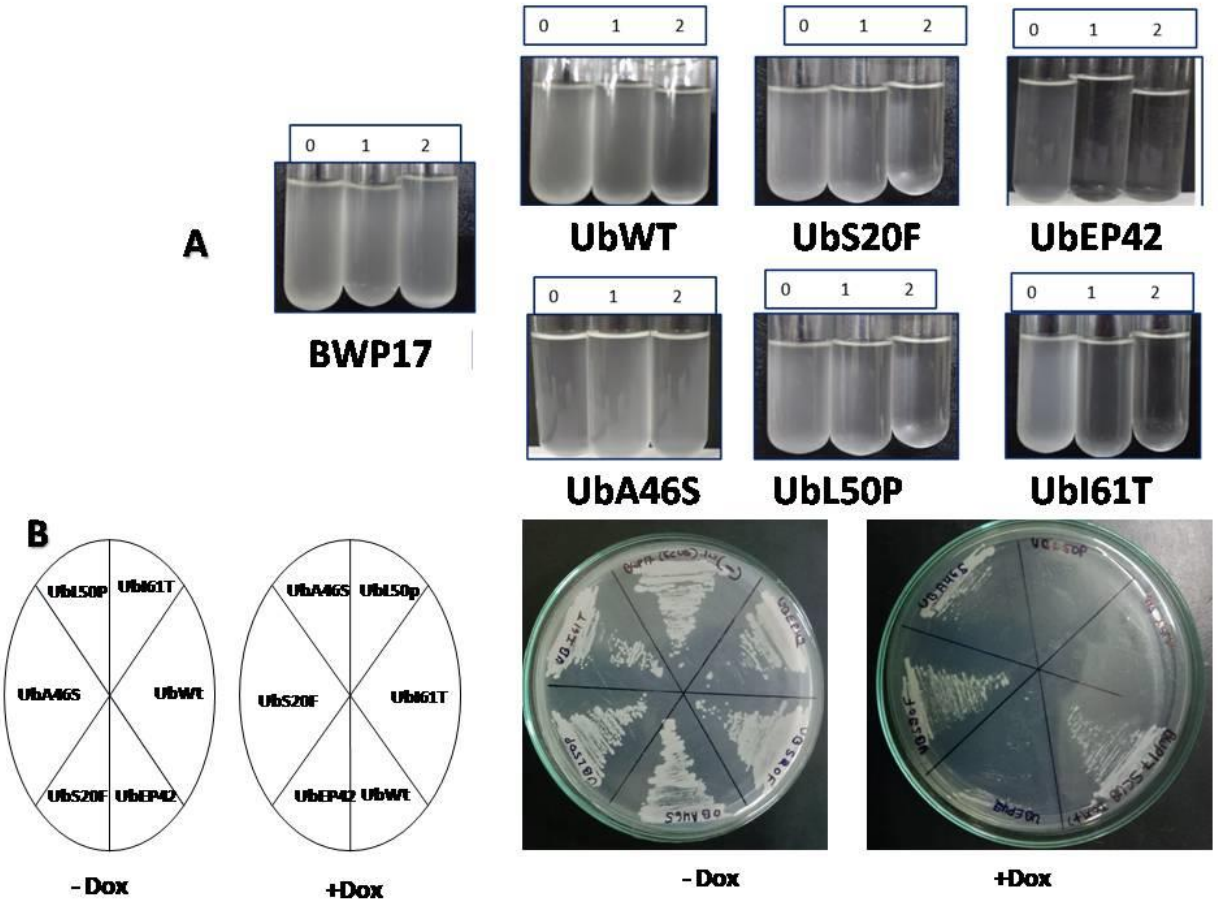


Figure 2.9. (A) Impact of overexpression of UbEP42 and its single mutant derivatives on *C. albicans.* BWP17 transformants with wild type and mutant forms of ubiquitin whose

expression was driven by doxycycline-inducible TET1 promoter were grown in the presence of doxycycline inducer concentration 45ug/ml. BWP17 and it's transformed with the UbWT used as a positive control. (B) Growth was checked on SD minimal media through the streak plate method.

2.3.6. UbEP42, UbL50P, and UbI61T show slow growth phenotype reduced survival and thermotolerance, and impaired heat stress-resisting capacity

At a sublethal concentration of inducer, mutant forms of ubiquitin were overexpressed in C. albicans transformants, and their growth was monitored. Notably, strains expressing UbEP42, UbL50P, and UbI61T exhibited slow growth, with the generation time of these mutants approximately doubling (Figure 2.10 (A)). Conversely, UbS20F and UbA46S showed growth rates similar to wild-type strains, with unaffected generation periods and % viability.

To assess the impact of UbEP42 and its derivatives on the functional efficacy of ubiquitin under heat stress conditions, % survival was determined. BWP17 strain and its transformants were exposed to 30°C for varying durations. While strains expressing UbWT, UbS20F, and UbA46S showed normal growth under optimal temperature conditions, those expressing UbEP42, UbL50P, and UbI61T displayed a decrease in % survival, particularly notable after 10 hours, indicating their inability to withstand heat stress (Figure 2.10 (B)).

Moreover, when subjected to 40°C for varying durations followed by return to 30°C for growth, strains expressing UbEP42, UbL50P, and UbI61T showed reduced survival under heat stress compared to wild type (Figure 2.10 (C)).

Given C. albicans' greater thermotolerance compared to free-living S. cerevisiae, the effect of mutations was further tested by exposing cells grown at 30°C to 55°C for varying durations. However, all transformants expressing both wild type and mutant forms of ubiquitin were unable to survive prolonged exposure to elevated temperatures after one hour at 55°C (Figure 2.10 (D)). These findings emphasize the critical role of ubiquitin mutants in C. albicans' thermotolerance and survival under heat stress conditions.

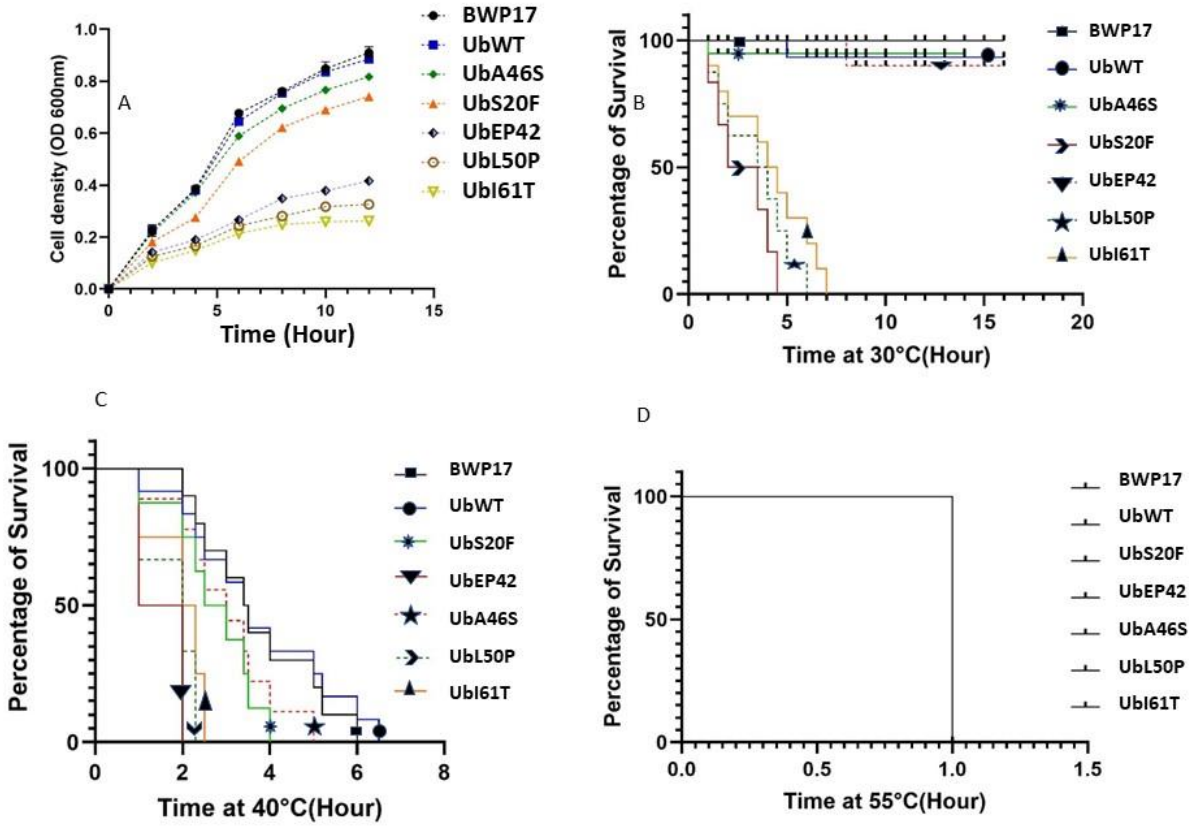


Figure 2.10 The impact of ubiquitin protein mutants on the growth profile and thermotolerance of C. albicans cells. (A) BWP17 cells were genetically modified to express wild type and mutant forms of ubiquitin under the control of the doxycycline-inducible TET1 promoter. These cells were cultivated in the presence of a sublethal concentration of inducer, and their growth profile was monitored by measuring OD at 600nm at 2-hour intervals. Results revealed that strains expressing UbEP42, UbL50P, and UbI61T exhibited slower growth compared to wild type. (B) The transformants were standardized and plated in the presence of the inducer (45 µg/ml doxycycline). Plates were then incubated at 30°C for varying durations (2, 4, 8, 12, and 16 hours), followed by 3-4 days of incubation. % Survival was determined by counting colonies formed. (C) Similarly, the transformants were plated in the presence of the inducer and exposed to 40°C for 2, 4, and 8 hours, followed by return to 30°C and incubation for 3-4 days. Percent % survival was calculated by counting colonies formed. (D) Additionally, BWP17 strain containing mutant ubiquitin

was cultured to log phase and subjected to heat stress at 55°C for 0.5, 1.0, and 1.5 hours. This assay aimed to assess the tolerance of the strains to elevated temperatures.

2.3.7. UbEP42, UbL50P and UbI61T reduce resistance towards antibiotic stress

To investigate how the expression of mutant ubiquitin impacts the survival of C. albicans in the presence of antibiotics, cells of the BWP17 strain and their transformants were cultured on plates containing cycloheximide (75 µg/ml), tunicamycin (1 µg/ml), and canavanine (1 µg/ml) [32]. The results closely resembled the responses observed during heat stress experiments; notably, strains expressing UbEP42, UbL50P, and UbI61T exhibited significantly reduced viability under antibiotic stress conditions. Conversely, among the other two transformants, only UbA46S displayed viability closer to that of the positive controls when exposed to all three antibiotics (Figure 2.11). However, UbS20F having less stability at antibiotic stress levels rather than heat stress. One important thing here we identified is that % survival of control BWP17 varies under different antibiotic stress. Tunicamycin slightly showed a suppressive role in BWP17 survival.

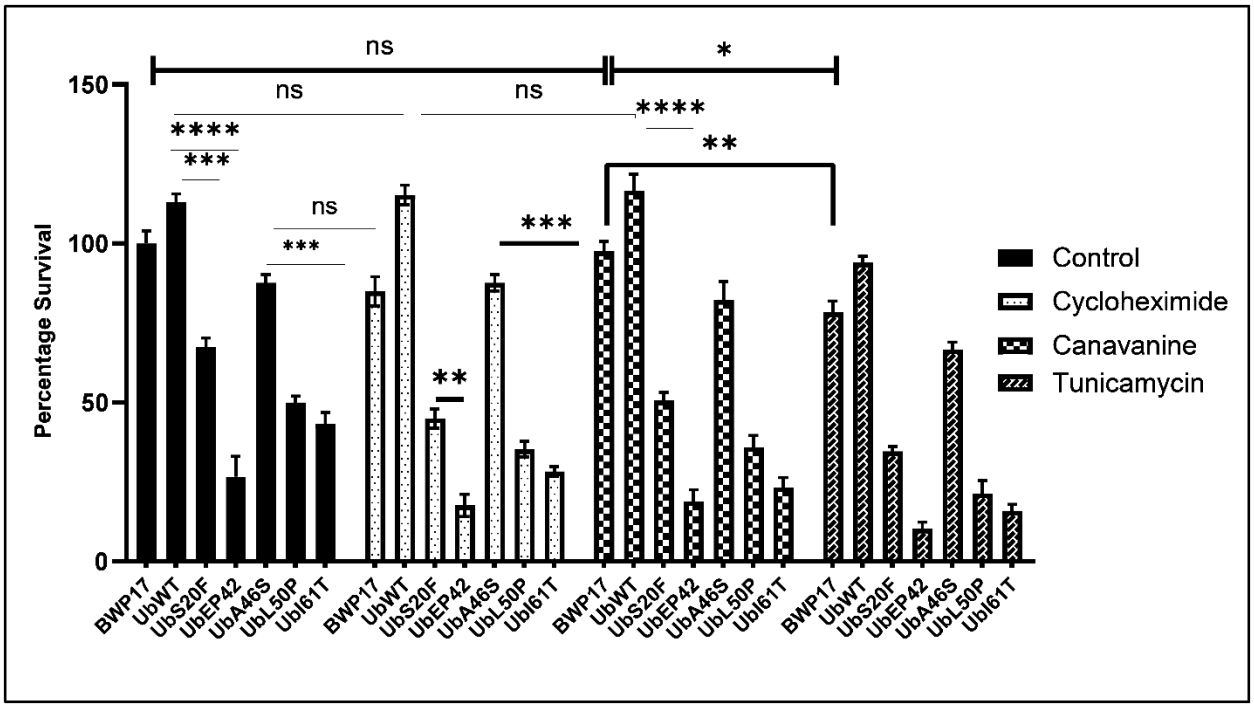


Figure 2.11 Impact of mutant ubiquitin proteins on the survival of C. albicans under antibiotic stress. Overall, the findings indicate that strains expressing UbEP42, UbL50P, and UbI61T were unable to withstand antibiotic stress. The concentrations of antibiotics

utilized in this study were cycloheximide (75 µg/ml), tunicamycin (1 µg/ml), and canavanine (1 µg/ml). Plates were then incubated for 5-6 days at 30°C. Notably, UbS20F showed a slight decrease in viability, while tunicamycin exhibited a potent inhibitory effect on survival. The results are presented as Mean ± SE, with n = 3 replicates. Statistical significance was denoted as P* = <0.001, while NS indicates non-significance.**

2.3.8. Yeast to hyphal transition was affected by mutants of ubiquitin UbEP42, UbL50P and UbI61T confirmed by using N-terminal GFP fusions

C. albicans cells expressing ubiquitin mutations UbEP42, UbL50P and UbI61T no longer change their form from yeast to hyphae, which results in a loss of their infective capability. Confocal analysis results show that the mutation UbEP42 and its segregated single mutations UbL50P and UbI61T hampered yeast to hyphal transition. Even though, the cells showed signs of budding with constriction occurring at the site of septation under serum induced condition, the formation of septa remained incomplete (Fig. 2.12).

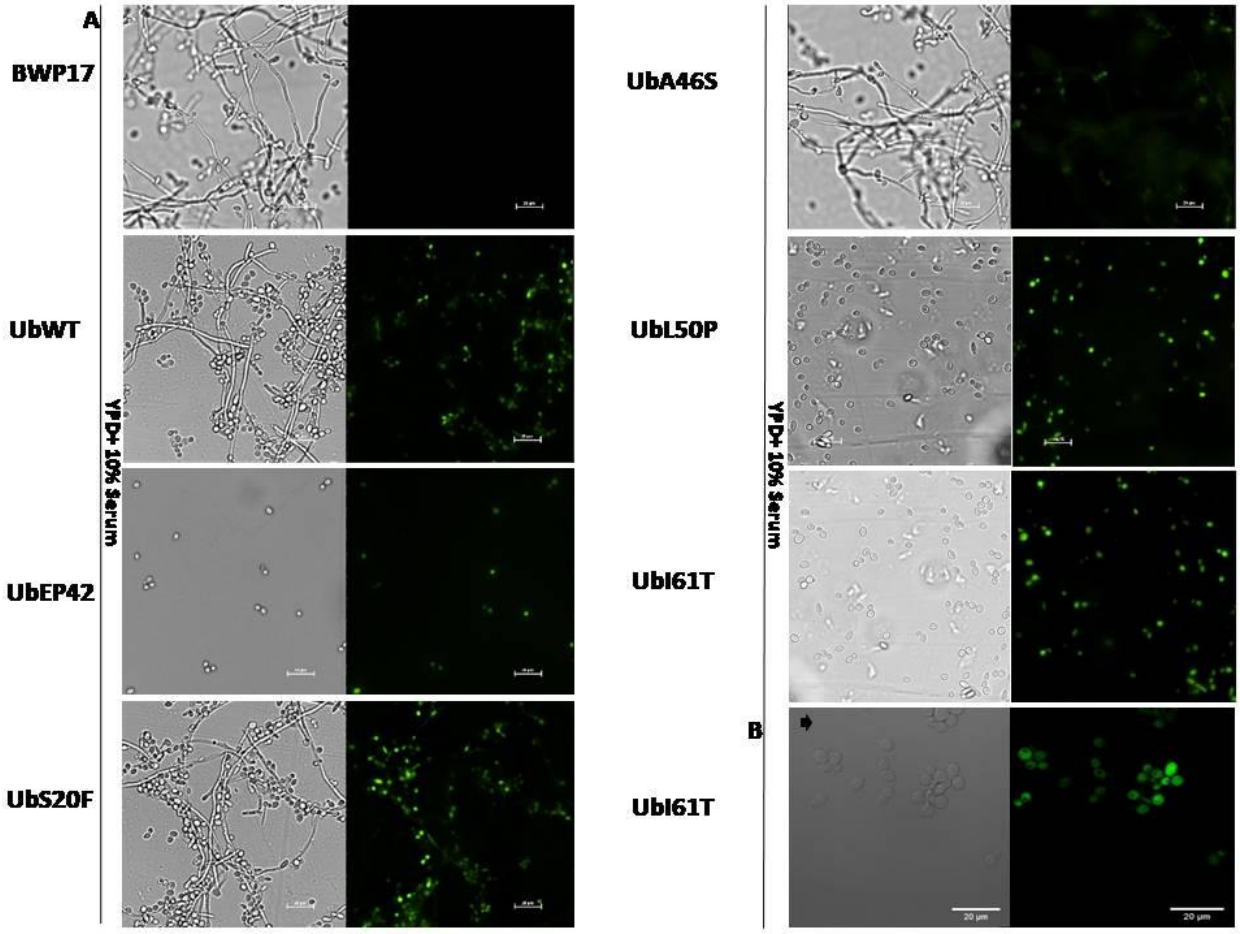


Figure 2.12. Effect of expression of UbWT, UbEP42 derived single mutants of ubiquitin on the yeast to hyphal transition monitored using fluorescence microscopy. (A) BWP17 cells and BWP17 UbWT as a control and the cells expressing wildtype and derived single mutant forms of ubiquitin were observed under GFP filter. Transformants were plated on YPD pates 10% serum condition at 37º C to study filamentous growth. (B) Confocal image of UBI61T resolved at higher magnification for better confirmation of morphology switching.

2.3.9. UbEP42, UbL50P and UbI61T disrupt cell cycle progression in C. albicans

To investigate the impact of ubiquitin mutations on cell cycle progression, log phase cultures of BWP17 cells and their transformants were analyzed using FACS. BWP17 and UbWT cells served as positive controls. The results revealed that UbEP42, UbL50P, and UbI61T cells were arrested in the G0/G1 phase of the cell cycle, leading to delayed entry into the S phase. In contrast, UbS20F and UbA46S were able to progress through the S phase similarly to the positive controls (Figure 2.13). Additionally, we examined cell cycle progression after hyphal induction in the presence of 10% serum. Interestingly, one ubiquitin mutant (designated as H4UbEP42, with "H" denoting hyphal induction and "4" representing the sample number) showed a higher percentage of cells in the G1 phase. However, we did not observe a significant change in the percentage of G1 phase cells in other lethal ubiquitin mutants such as UbL50P and UbI61T compared to the wild type (Figure 2.14). These findings suggest that ubiquitin mutants may interfere with the interaction between yeast cyclins and CDKs complexes, thereby affecting cell cycle progression.

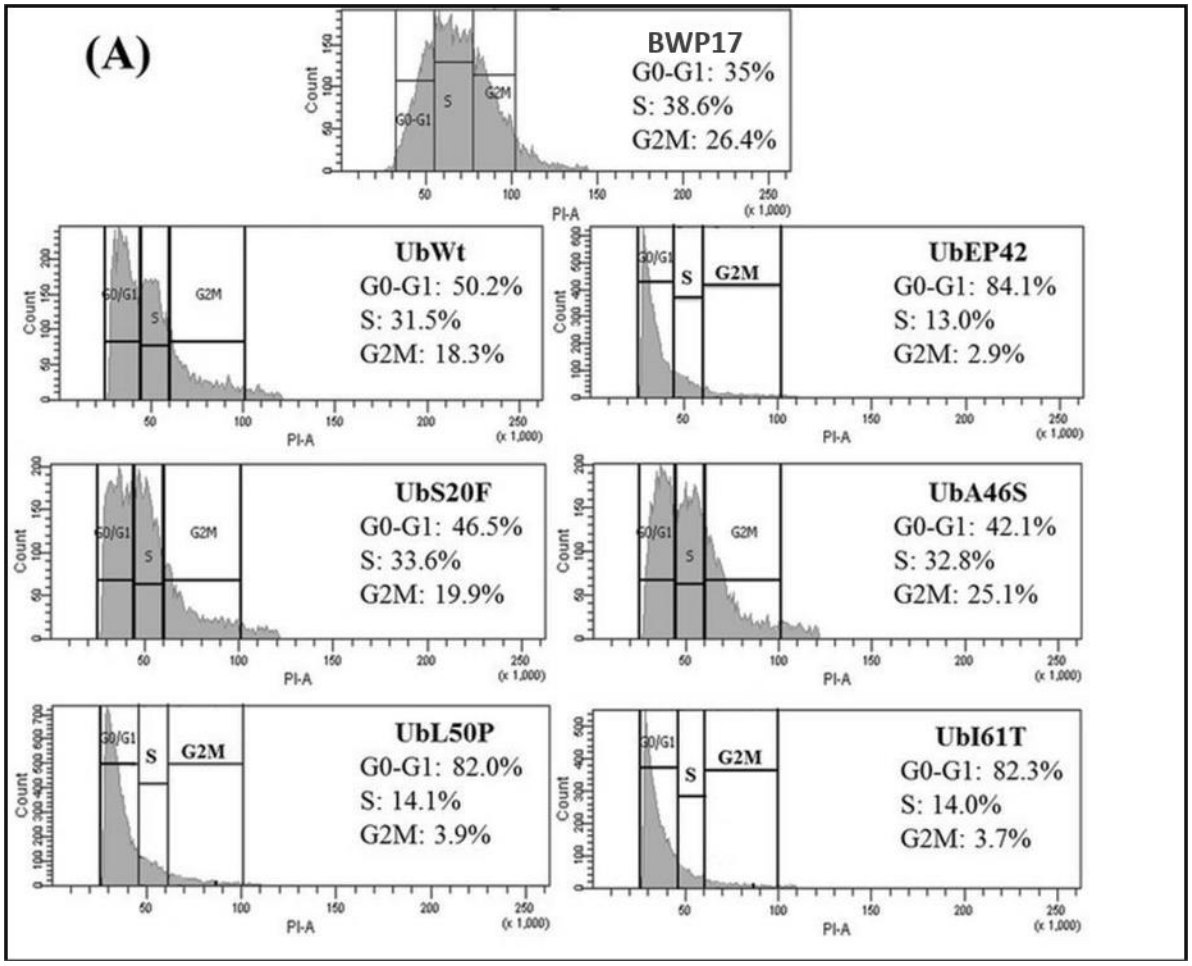


Figure 2.13 Impact of ubiquitin mutant expression on cell cycle progression. (A) FACS analysis was conducted on the BWP17 strain and its transformants. Cells expressing UbEP42, UbL50P, and UbI61T exhibited a delayed G0/G1 phase, while those expressing UbS20F and UbA46S progressed through the G0/G1 phase similar to cells expressing wild type ubiquitin. The results are presented as Mean ± SE, with n = 3 replicates. Statistical significance was denoted as P* = <0.001, while NS indicates non-significance.**

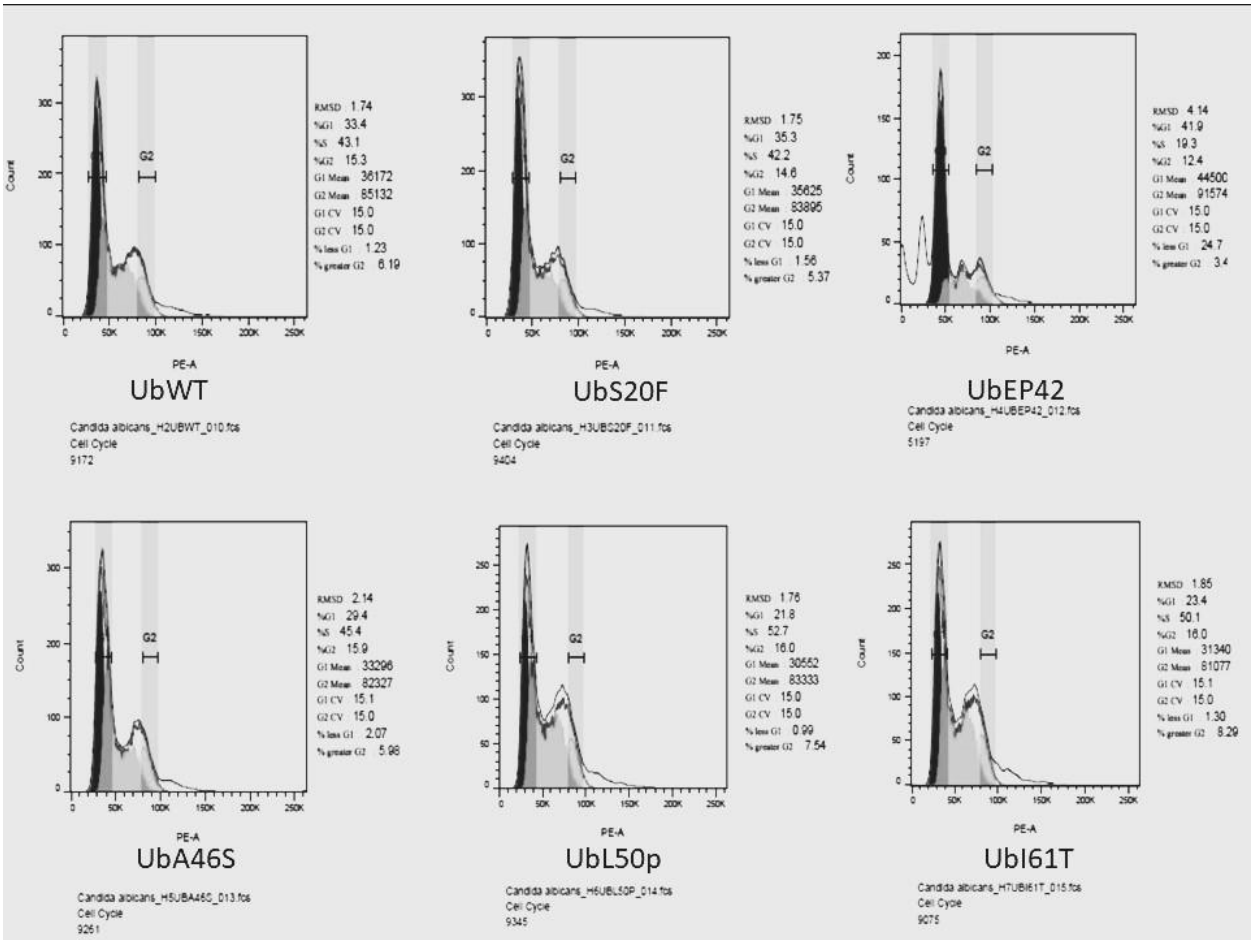


Figure 2.14 Examines the influence of ubiquitin mutant expression on cell cycle progression following hyphal induction. FACS analysis revealed a higher percentage of cells in the G1 phase specifically in BWP17/UbEP42, whereas BWP17/UbL50P and BWP17/UbI61T expressing cells did not exhibit significant changes compared to the wild type. The results are presented as Mean ± SE, with n = 3 replicates. Statistical significance was indicated as P* = <0.001, while NS denotes non-significance.**

2.3.10. Cdc28 protein kinase expression decrease in cells expressing UbEP42, UbL50P and UbI61T

In both S. cerevisiae and C. albicans, there exists a single cyclin-dependent kinase known as Cdc28, which interacts with all cyclins throughout the cell cycle, spanning from the G1 phase to the B phase. Activation of Cdc28 protein kinase by G1-cyclin, Cln3, initiates start-specific transcription, regulates cell size, and facilitates the expression of Cln1 and Cln2, thereby aiding

in the transition of cells from the G1 phase to the S phase (F. Cvrckova et al., 1991). Building upon the findings from the FACS analysis, we conducted western blot analysis to assess the levels of Cdc28 under conditions of ubiquitin mutants. The results revealed a reduction in Cdc28 levels attributed to the overexpression of UbEP42, UbL50P, and UbI61T. Conversely, the expression of UbS20F and UbA46S did not induce any noticeable alteration in the expression pattern of Cdc28 protein kinase compared to the positive controls (Figure 2.15).

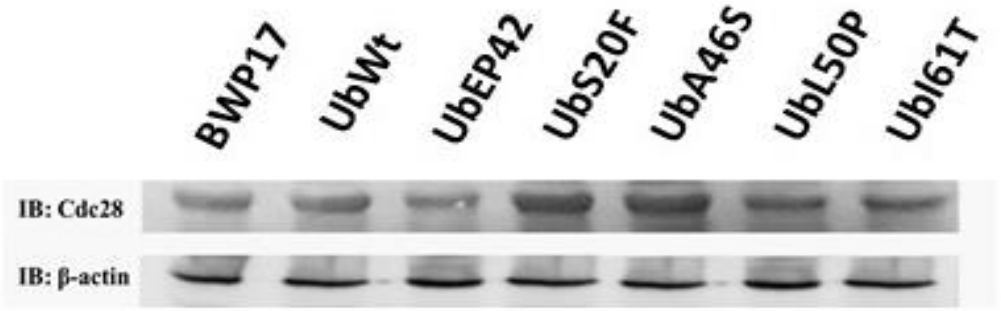


Figure 2.15 Illustrates the levels of Cdc28 protein kinase in BWP17 cells expressing both wild type and mutant forms of ubiquitin. The results indicate a significant reduction in Cdc28 protein kinase levels in cells expressing UbEP42, UbL50P, and UbI61T.

2.3.11. Expression of UbEP42, UbL50P and UbI61T results in impairment of K63 and K48 linked polyubiquitinations

The ubiquitin monomer contains seven lysine residues, all of which play a role in polyubiquitination. The branching pattern of polyubiquitin chains varies depending on the lysine residue involved in isopeptide formation, acting as a signal for regulating diverse functions. For example, polyubiquitin chains with K48 linkage on substrate proteins target them for degradation by proteasomes, while K63-linked polyubiquitin chains serve various purposes including endocytosis, lysosomal degradation, signal transduction, kinase activation, and DNA repair, with the latter being influenced in part by the substrate and its function. Immunoblotting was

performed using anti-K48 and anti-K63 antibodies to investigate the impact of mutant ubiquitins on polyubiquitin chain formation and assess the ability of mutant ubiquitins to participate in K48 and K63 linked polyubiquitination. The results indicated that mutants expressing UbEP42, UbL50P, and UbI61T exhibited impaired polyubiquitination with both K48 and K63 linkages, whereas UbS20F and UbA46S did not significantly affect polyubiquitin chain formation (Figure 2.16).

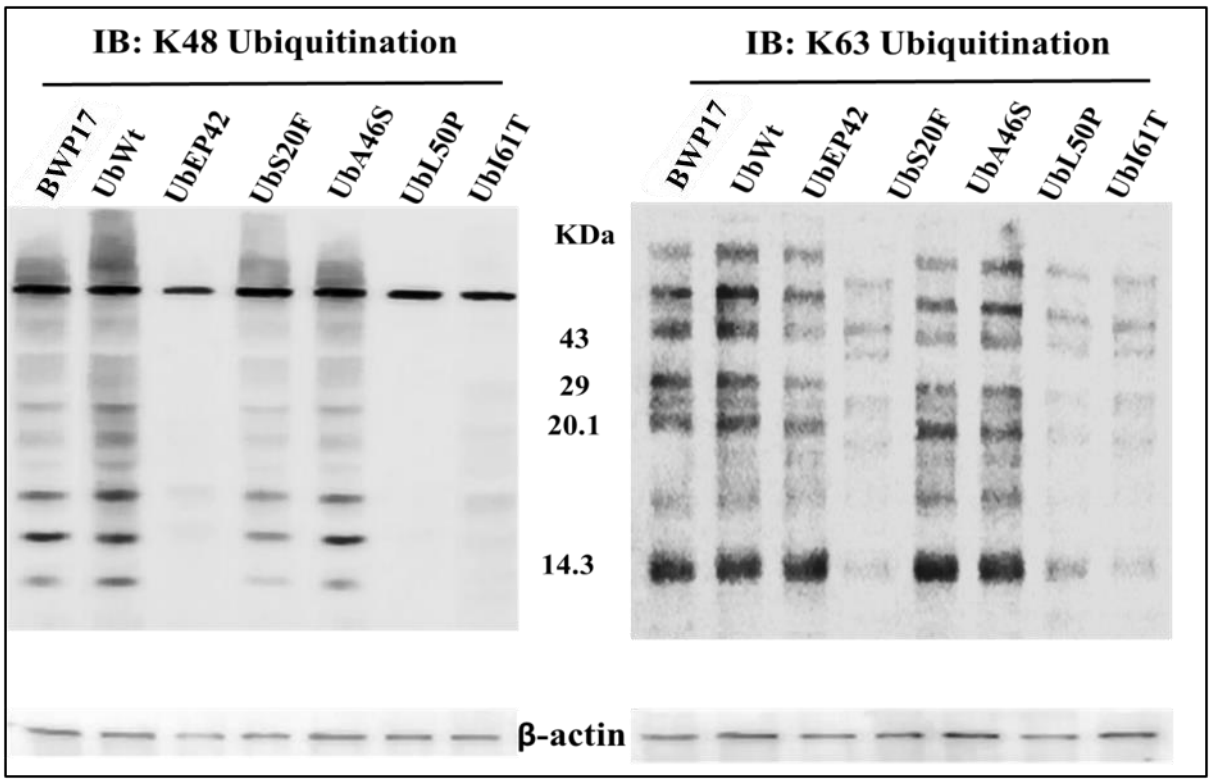


Figure 2.16 Western blot analysis reveals the polyubiquitination profile of K63 and K48 linkages in C. albicans cells expressing mutant forms of ubiquitin. It was observed that UbEP42, UbL50P, and UbI61T mutants exhibited impaired polyubiquitination with both K63 and K48 linkages. Conversely, the polyubiquitination profile of UbS20F and UbA46S mutants did not exhibit significant changes with respect to K63 and K48 linkages.

2.3.12. Cell surface analysis by Scanning Electron Microscope (SEM)

The SEM technique was used to visualize the cell surface of *C. albicans* cells. The SEM images *C. albicans* cells expressing UbWT, UbS20F and UbA46S show the cell surface being smooth

with no deformities However, the cell surface of UbEP42, UbL50P and UbI61T is irregular indicating a disturbance in the structure of the fungal cell wall (Figure 2.17).

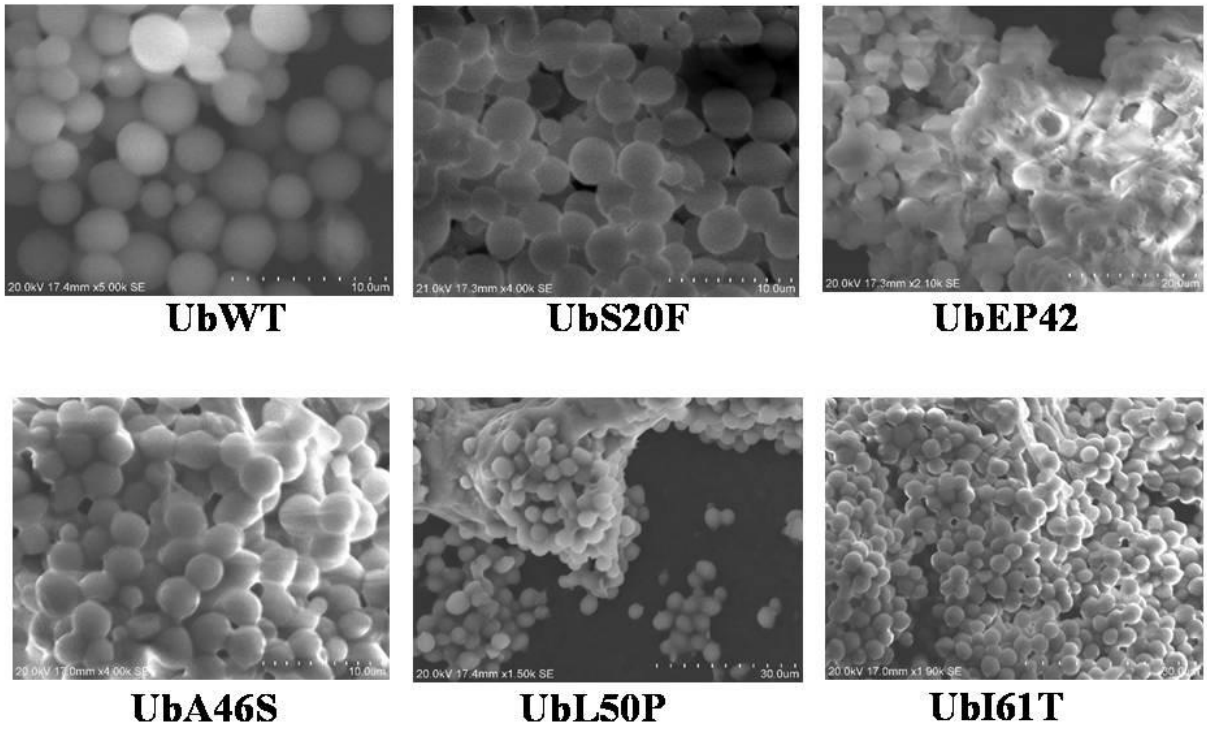


Figure 2.17. SEM image of C. albicans cells under ubiquitin mutant overexpression in the presence of doxycycline inducer concentration 45ug/ml. The minimum scale bar set 10µm and max 30 µm.

2.3.13. Slightly irregular deposition of chitin on C. albicans cell wall under the effect of mutant ubiquitin proteins observed

In *C. albicans*, the protein kinase C (PKC) and HOG MAP kinase cascades and the Ca^{2+}/calcineurin pathway regulates *CHS* gene expression and chitin synthesis in response to cell wall stresses. Coordinated synthesis of chitin also requires the localization of the enzymes to be regulated throughout the cell cycle (Wójcik-Mieszawska et al., 2023). Therefore, investigation of accumulation of chitin on *C. albicans* cells was examined by staining them with Calcofluor white fluorochrome subsequent to the expression of a ubiquitin mutations. Uneven staining on

the cell wall was noticed, suggesting varying thicknesses of the chitin layer on the yeast cells expressing UBEP42, UbL50P, and UbI61T. Additionally, the confocal microscopic images captured of the mutants UBEP42, UbL50P, and UbI61T showed the formation of cell aggregate, while the rest including wild type showed separate cells (Figure 2.18).

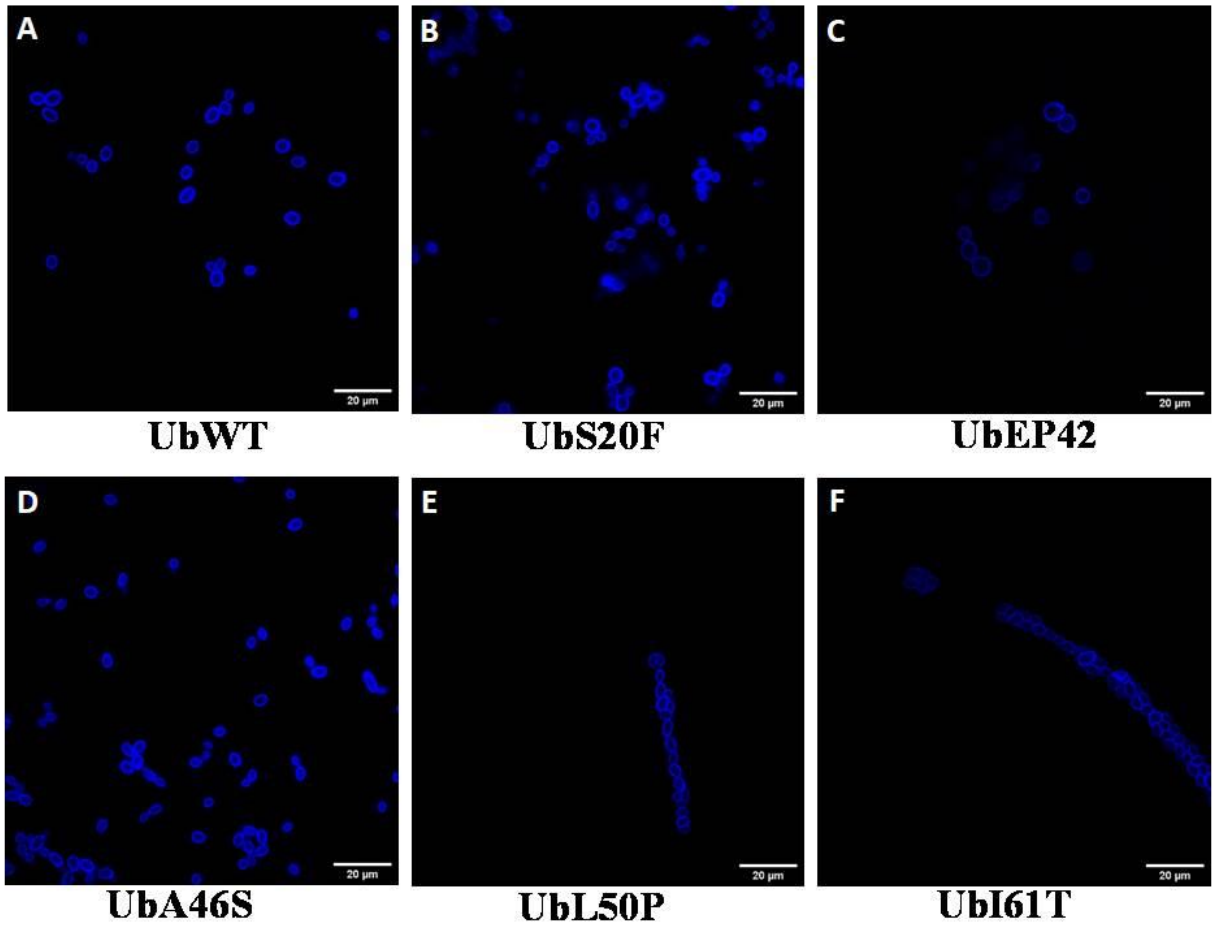


Figure 2.18. Chitin deposition accumulaton and aggregates of C. albicans cells under ubiquitin mutant's overexpression condition (A control B-F ubiquitin mutants) after the staining with Calcofluor white staining. The scale bar represents 20 µm.

2.3.14. Unmasking the effect of mutant ubiquitin proteins over β-glucan exposure on the cell surface of *C. albicans*

The presence of β-glucan on the cell surface of *C. albicans* is influenced by the CEK1-mediated MAPK pathway. It has also been previously reported that Hsp90 and the proteasome impact

morphogenesis via the cAMP-PKA pathway in *C. albicans* (Gong et al., 2017). For analysing whether there is any change in β-glucan exposure on cell surface due to ubiquitin mutations, Congo red fluorochrome staining was done. The stain binds 1,3 β-glucan, causing the fungal cell wall to emit a red fluorescence when viewed under a microscope. Interestingly, it was discovered that yeast cells with UbEP42, UbL50P, and UbI61T mutations displayed a significantly increased β-glucan content in their cell wall, when compared to cells expressing wild type, UbS20F and UbA46S forms of ubiquitin. There are numerous studies on the effect of compounds like the azole derivative drugs in exposing the β-glucan layer. In the present study, β-glucan exposure was observed after overexpression of ubiquitin mutations even when the azole drugs are absent. Further SEM technique was used to visualize the cell wall surface. The *C. albicans* wild type cell wall in the control culture was clearly smooth and uniform. Overexpression of UbEP42 by adding 45 µg/ml doxycycline resulted in a disturbance in the integrity of the fungal cell wall. The extent of β-glucan exposure was less in UbL50P and UbI61T mutations (Figure 2.19).

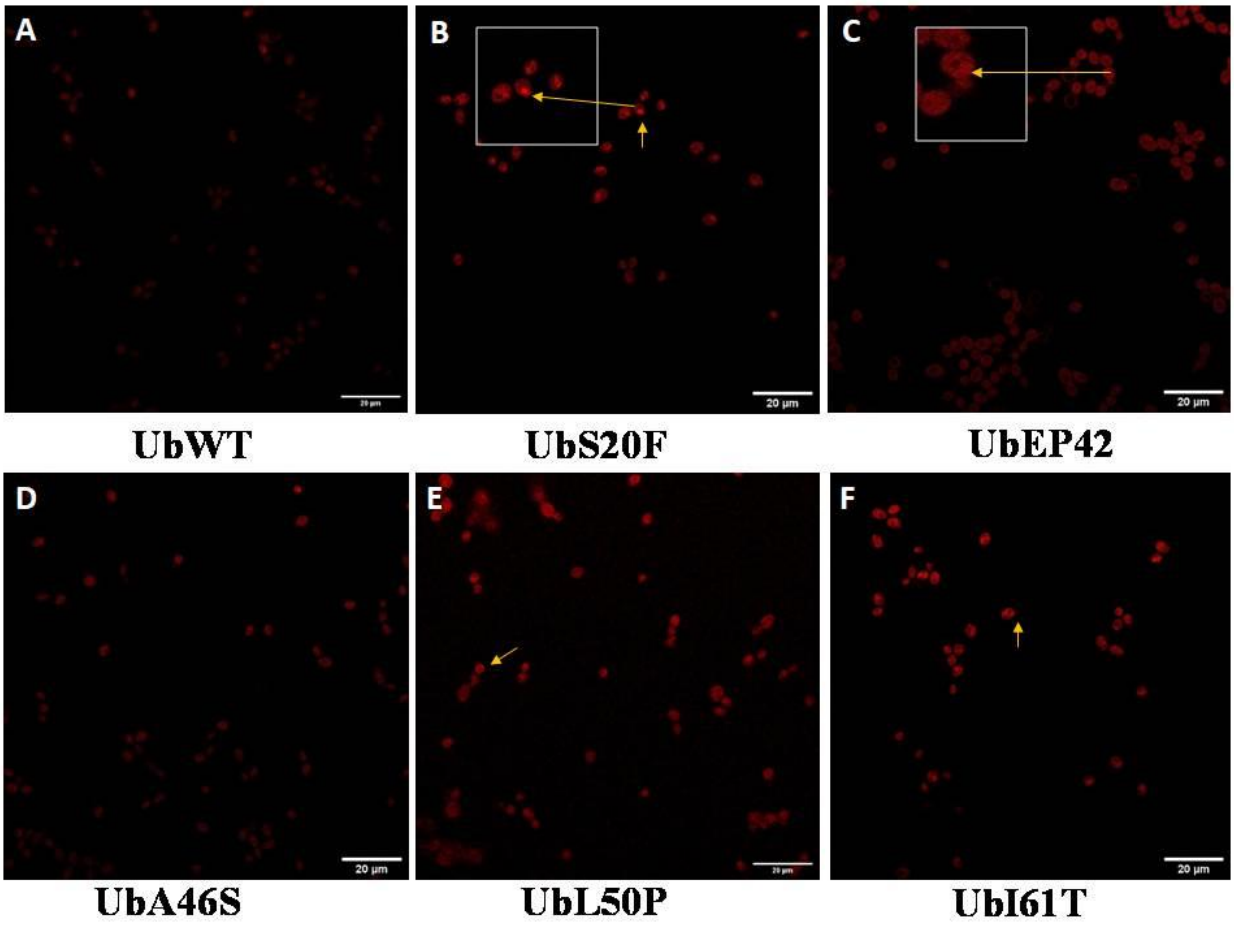
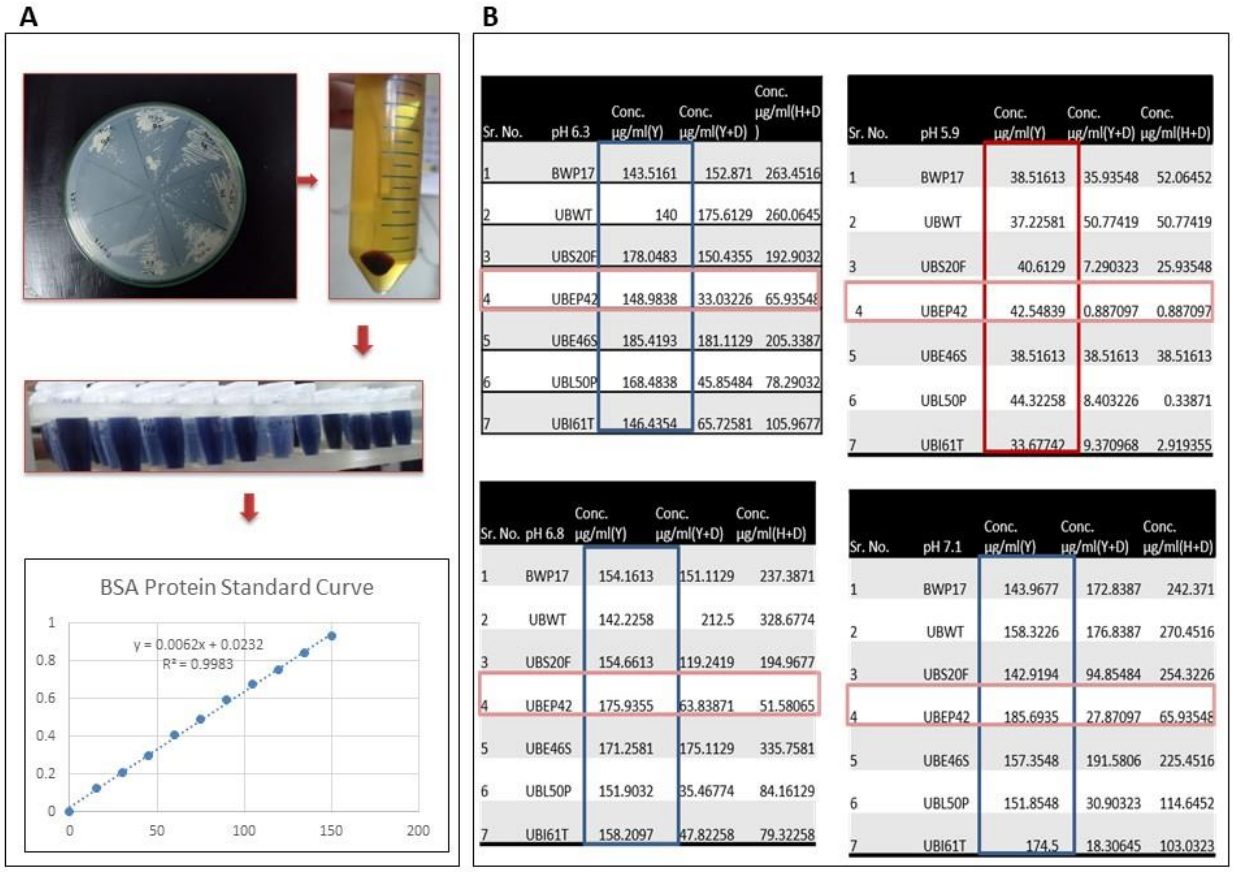


Figure 2.19. C. albicans cell surface β-glucan exposure under ubiquitin mutant overexpression condition after the Congo red staining (A control, (B-F mutants expressing cells). The scale bar represents 20 µm. yellow arrow indicates the higher intensity of dye fluorescence due to binding to β-glucan.

2.3.15. The effect of ubiquitin mutations over secretory aspartyl protease activity at different pH values

To investigate the role of pH of saliva in establishing oral candidiasis infection the present study addressed the regulation of aspartyl protease secretion under different pH conditions, with or without ubiquitin mutations in the background. The regulation of aspartyl protease secretion under four different pH 6.3, 6.8, 7.1and 5.9 conditions was investigated with cells expressing the wild type and mutant forms of ubiquitin. Protease extraction was done through the acetone precipitation method and protein concentration was measured using the Folin-Lowry method (Figure 2.20). Cells expressing wild-type, UbS20F, and UbA46S secreted copious amounts of protease at pH values 6.3, 6.8, and 7.1.

However, cells expressing UbEP42, UbL50P, and UbI61T showed the presence of very low amounts of protease at the same pH values as above. Interestingly, at pH 5.9 cells expressing wild type and all five types of ubiquitin mutations under consideration here were found to secrete extremely low amounts of protease (Figure 2.21).



Sr. No.	pH 6.3	Conc. µg/ml(Y)	Conc. µg/ml(Y+D)	Conc. µg/ml(H+D)
1	BWP17	143.5161	152.871	263.4516
2	UBWT	140	175.6129	260.0645
3	UBS20F	178.0483	150.4355	192.9032
4	UBEP42	148.9838	33.03226	65.93548
5	UBE46S	185.4193	181.1129	205.3387
6	UBL50P	168.4838	45.85484	78.29032
7	UBI61T	146.4354	65.72581	105.9677

Sr. No.	pH 5.9	Conc. µg/ml(Y)	Conc. µg/ml(Y+D)	Conc. µg/ml(H+D)
1	BWP17	38.51613	35.93548	52.06452
2	UBWT	37.22581	50.77419	50.77419
3	UBS20F	40.6129	7.290323	25.93548
4	UBEP42	42.54839	0.887097	0.887097
5	UBE46S	38.51613	38.51613	38.51613
6	UBL50P	44.32258	8.403226	0.33871
7	UBI61T	33.67742	9.370968	2.919355

Sr. No.	pH 6.8	Conc. µg/ml(Y)	Conc. µg/ml(Y+D)	Conc. µg/ml(H+D)
1	BWP17	154.1613	151.1129	237.3871
2	UBWT	142.2258	212.5	328.6774
3	UBS20F	154.6613	119.2419	194.9677
4	UBEP42	175.9355	63.83871	51.58065
5	UBE46S	171.2581	175.1129	335.7581
6	UBL50P	151.9032	35.46774	84.16129
7	UBI61T	158.2097	47.82258	79.32258

Sr. No.	pH 7.1	Conc. µg/ml(Y)	Conc. µg/ml(Y+D)	Conc. µg/ml(H+D)
1	BWP17	143.9677	172.8387	242.371
2	UBWT	158.3226	176.8387	270.4516
3	UBS20F	142.9194	94.85484	254.3226
4	UBEP42	185.6935	27.87097	65.93548
5	UBE46S	157.3548	191.5806	225.4516
6	UBL50P	151.8548	30.90323	114.6452
7	UBI61T	174.5	18.30645	103.0323

Figure 2.20. (A) Extraction of protease visualize and folin lowry method standard curve obtain. (B The secretion of aspartyl protease concentration acetone precipitated samples under different morphology form of *Candida albicans* at different pH under influence of ubiquitin mutants' expression measured by using Folin Lowry method. Results represented in Mean ± SE. (n = 3), (Y= yeast, Y+D= logphase yeast incubated with doxycycline, H+D = After hyphal induction doxycycline inducer added.

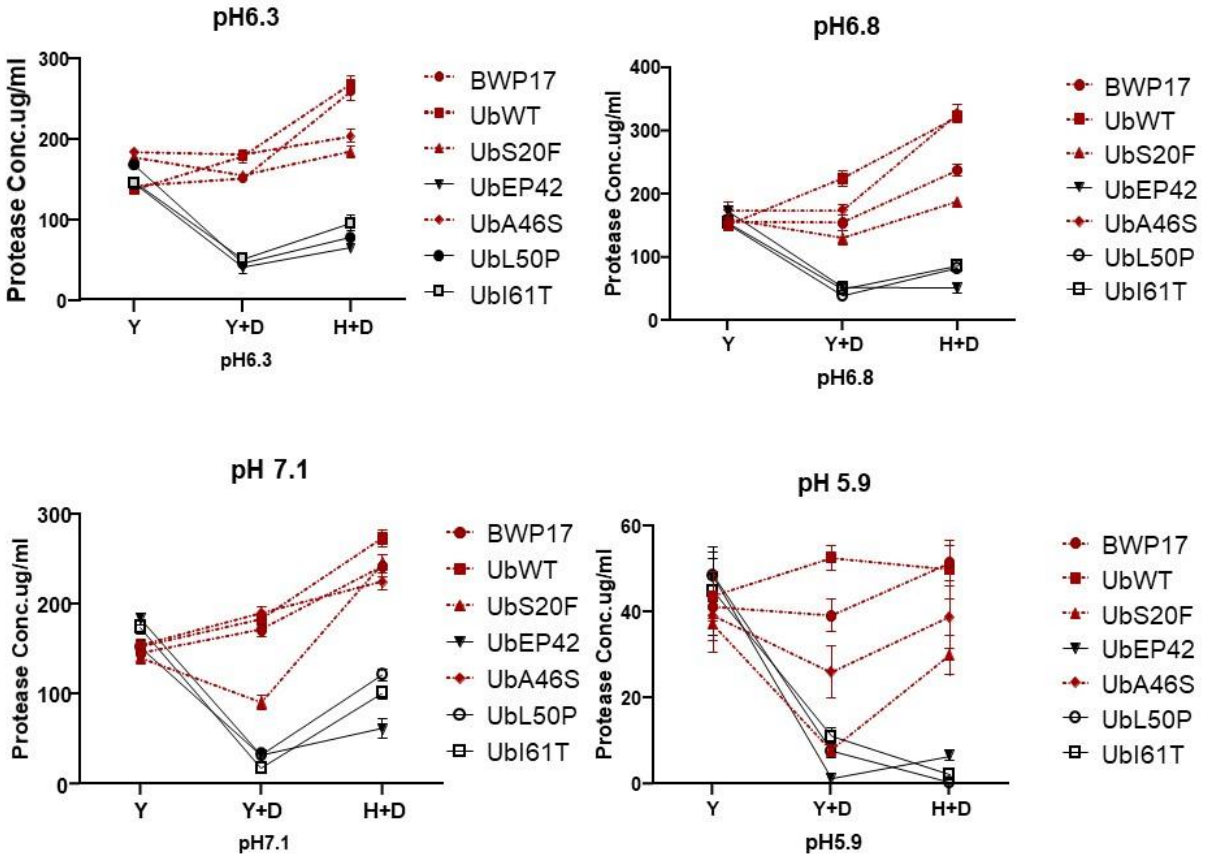


Figure 2.21. The secretion of aspartyl protease concentration acetone precipitated samples represented in chrt form.Under different morphology forms of *Candida albicans* at different pH under the influence of ubiquitin mutants' expression was measured by using the Folin Lowry method. Results represented in Mean ± SE. (n = 3), (Y= yeast, Y+D= logphase yeast incubated with doxycycline, H+D = After hyphal induction doxycycline inducer added.

Further in gelatine zymography Cells expressing UbEP42, UbL50P and UbI61T showed less protease activity compared to UbWT on gelatine substrate (Figure. 2.22). Gelatine zymography was performed at two selective pH values (media pH 6.8 and 5.9) due to low levels of aspartyl protease concentration detected at pH 5.9 by Folin-Lowry method when compared to the control at pH 6.8.

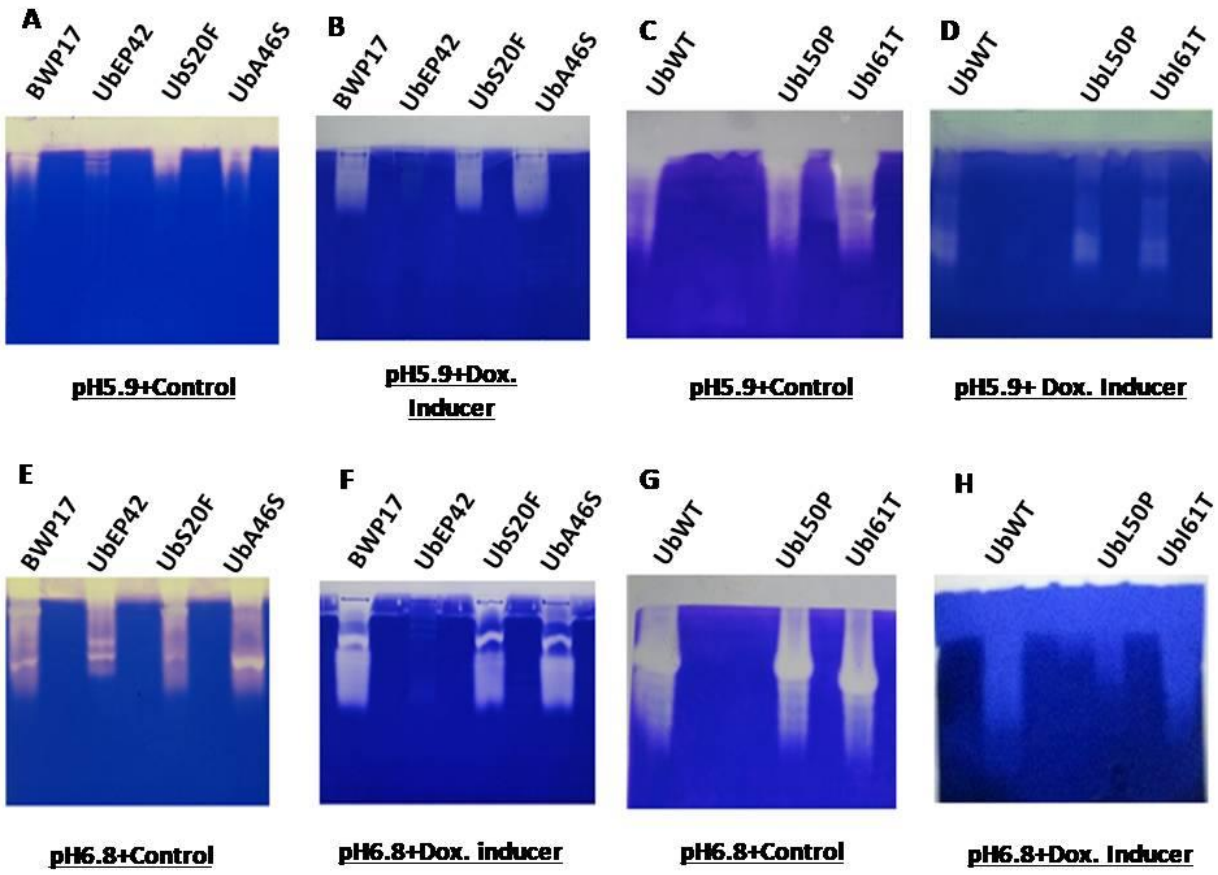


Figure 2.22. 12% Zymography gel using gelatin (0.1%) as a substrate. Results showing the difference in secretory aspartyl protease secretion under pH5.9 and pH6.8 in presence of overexpression of Ubiquitin mutants.

Therefore, to understand the dependence of morphological changes in C. albicans over pH, cultures of *C. albicans* expressing wild type ubiquitin were grown under different pH with YPD medium and 10% bovine calf serum for 6h at 37°C. The cultures of BWP17 strain expressing wild type ubiquitin exhibited more defects in hyphal growth at pH 5.9 and the cells remained in yeast bud form as the morphological switching to hyphal form failed, in spite of growing the cultures in the presence of serum (Figure 2.23).

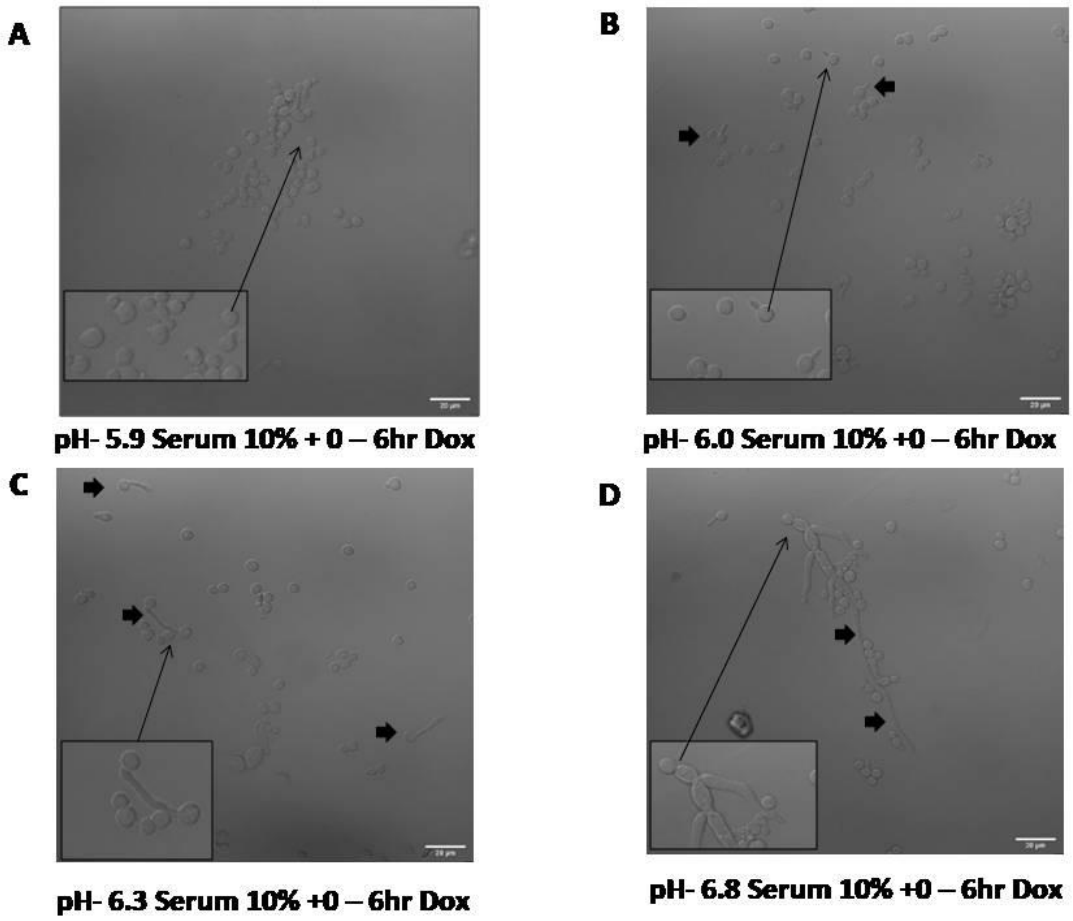


Figure. 2.23: Morphology of cells under different pH of YPD media with 10% bovine calf serum for 0 – 6hr at 37°C exhibited defects in hyphal growth. produced both pseudohyphae and true hyphal filaments. (A, C) Yeast to hyphal transition in presence of 10% serum without inducer conditions in respect to pH5.9, pH6.8. (B, D) Yeast to hyphal transition status in presence of 10% serum with inducer conditions in respect to pH 5.9, 6.8.

While the cells of BWP17 at the other pH in increasing manner (pH6.0,6.3,6.8) conditions grew as both pseudohyphal and true hyphal filaments. Hence, it can be concluded that at acidic pH *C. albicans* find it hard to penetrate host tissues due to low levels of aspartyl protease secretion.

2.4. Discussion

The present study explains at molecular level the earlier findings of decreased viability, failure of morphogeneis, cell cycle arrest, loss of thermotolerance, increased susceptibility to antibiotic

stress in *C. albicans*, under the influence of the ubiquitin mutations UbEP42, UbL50P and UbI61T. Our results suggest that under the influence of mutant forms of ubiquitin - namely UbEP42, UbL50P, and UbI61T *C. albicans* has decreased viability. This decrease in viability could probably be due to the increased exposure of β-glucan, which may subject the cells to immune surveillance. Further, the mutations affected morphogenesis from yeast to hyphal form by degrading the levels of Nrg1(Chapter 4). These lethal mutants hamper G1 to S transition during cell cycle because of: (i) alteration in the levels of Cdc28, (ii) upregulation of G1 cyclins Ccn1 and Cln3 and (iii) decreased expression of the G2 cyclins Clb2 and Clb4, (Chapter 3) which bind to Cdk (Cdc28) leading to suppression of polarized growth. These effects could be due to the impairment of the protein degradation pathway caused by ubiquitin mutants UbEP42, UbL50P and UbI61T. Usually, reduction in Cdc28 levels leads to the formation of hyphae. However, in the case of the mutants UbEP42, UbL50P, and UbI61T, yeast to hyphal transition did not occur. Interestingly, these mutants show elevated expression of the bud-specific gene Nrg1, (Chapter 4) which could be seen as the possible reason for continuing in yeast form. Experimental analysis suggested that pH also plays an important role in yeast-to-hyphal transitions by affecting the Fkh2 transcription factor that is involved in virulence-associated secretory aspartyl protease secretion. C. albicans cultures grown in the presence serum show a ready transformation to hyphal form, which is seen to be associated with the upregulation of Fkh2. On the other hand, ubiquitin mutants suppress the expression of Fkh2 in *C. albicans* grown in serum conditions (Chapter 4). Interestingly it was observed that under ubiquitin mutant conditions of UbEP42, UbL50P and UbI61T the cells contain significantly higher β-glucan in their walls and uneven chitin deposition on the outer surface of the cell wall compared to wild type cells and UBS20F, UbA46S mutants. In conclusion, by probing with ubiquitin mutants the study sheds light on the impact of ubiquitination over intricate molecular mechanisms determining cell morphology, cell cycle progression, protease secretion, and the composition of the yeast cell wall in *Candida albicans*. Further research is needed to investigate cyclins level and virulence-associated transcription factors that's done in chapters 3, and 4 to fully understand the molecular details of these effects by carrying out proteomics analysis to understand the metabolic pathways and their potential implications for fungal virulence and pathogenesis. Further, it is possible to identify and earmark possible targets for developing new strategies to treat the infections caused by *C. albicans.*