Studies on Yeast Model of Amyotrophic Lateral Sclerosis and Alzheimer's Disease

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Declaration

I declare that this written submission represents my ideas in my own words, and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.

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Approval Sheet

This thesis entitled **"Studies on Yeast Model of Amyotrophic Lateral Sclerosis and Alzheimer's Disease"** by S. Kiruthika is approved for the degree of Master of Technology from IIT Hyderabad.

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Abstract

Amyotrophic lateral Sclerosis (ALS) is a fatal neurodegenerative disease which affects the motor neurons. TDP-43 was identified as the pathogenic protein in ALS. The cause of the sporadic ALS is unknown but, there are some links with occupations involving heavy labour, exposure to heavy metals, or a history of traumatic injury. TDP-43 has been expressed in yeast to act as a disease model for ALS. This model exhibits some of the pathological features associated with ALS in humans, like cytoplasmic aggregation of the protein and cytotoxicity. In this work, yeast model of ALS was studied using fluorescence microscopy and spot assay to check for toxic effect of TDP43-YFP expression on the growth of cells. When basal level of protein expression is maintained, diffused fluorescence is observed which implies the protein is in monomeric state. When the protein is overexpressed, the TDP43-YFP fusion proteins tend to aggregate and form fluorescent foci. The TDP43-YFP when expressed in yeast forms toxic aggregates which hinders the growth of cells. Effect of zinc was also checked on the aggregation of TDP43-YFP. The data shows that zinc promotes the aggregation of TDP43-YFP in yeast. Alzheimer's disease (AD) is a neurodegenerative disease and it is the most common cause of dementia. When Amyloid Precursor Protein (APP) is cleaved into Aβ peptide, the peptide tends to aggregate and form amyloid deposits. The hallmark of AD is the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain. These amyloid plaques contain the aggregated Aβ peptide. Aβ42 fused to the functional domain of the Sup35, when expressed in yeast acts as a disease model for AD. This model has been used for the screening of potential anti-oligomer drug. Prions can propagate as different strains due to different aggregated conformation of the same protein. It has been observed that Arctic and Swedish mutations in Aβ lead to two prion-like different strains of Aβ. It is being proposed here that different variants of Aβ prion, even from wild-type Aβ sequence, can be obtained in yeast as well, which can act as a disease model for AD. Furthermore, preliminary data show that expression of Aβ42 in yeast causes mitochondrial loss.

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Chapter 1

1. Introduction

1.1. Amyloids and Prions

Proper folding of a protein is essential for proper functioning of the protein. When the protein is present in a non-native structure the protein is said to be misfolded, the residues exposed on the surface might interact with the cellular components causing cellular toxicity. Misfolding also causes reduction in the number of native protein available to perform its normal function. Protein misfolding is mostly prevented by molecular chaperones and degradation process; but, sometimes the protein escapes this process and tends to misfold [1].

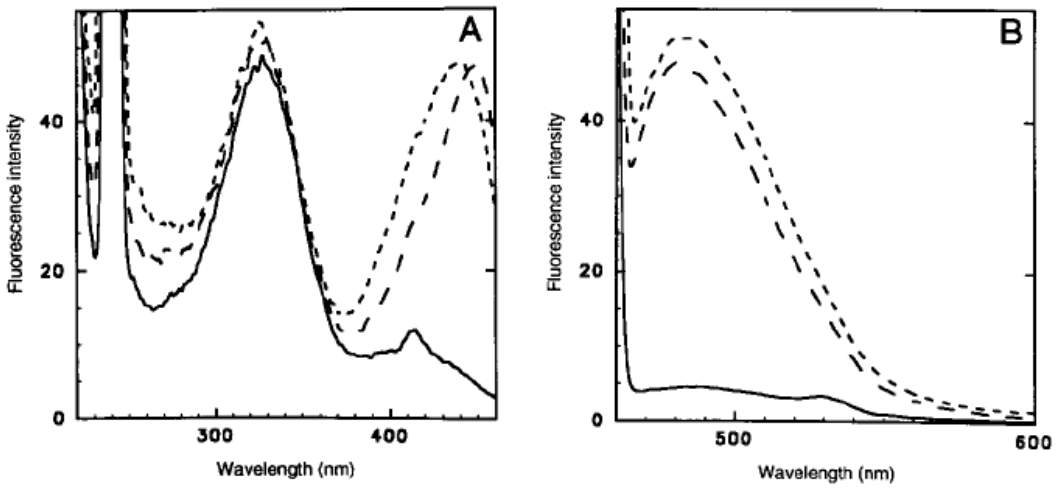


Figure 1: Fluorescence spectra of ThT in the absence (solid line) of peptide, and in the presence of preaggregated β (1-28) (short dashed line), or β (1-40) peptide (long dashed line) A) Excitation spectra B) Emission spectra [2]

Misfolding of a protein in general, causes formation of highly organized and thread-like aggregates called amyloid fibrils. Amyloids have cross-β structure where the peptide molecules form β-strands perpendicular to the axis of the fibril [3]. These fibrils bind specifically to Thioflavin T (ThT) dye causing increase in the fluorescence [2] and Congo red (CR) dye causing green birefringence when viewed under cross-polarized light [4].

When these amyloid fibrils are infectious in nature, they are called prions. The word prion is derived from protein and infectious. Prions are self seeding, have high stability and are resistant to protease [1].

Prions can propagate as different strains due to different conformation of the protein within the protein aggregates. These strains have different pathogenic properties. [5-7]

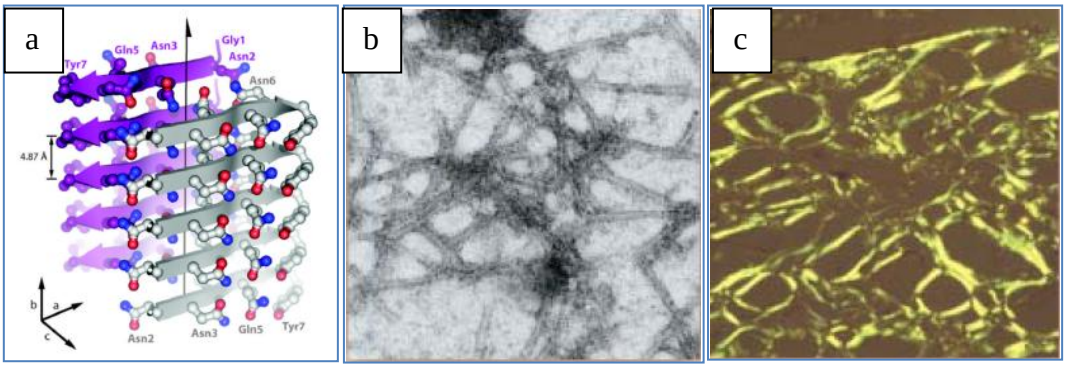


Figure 2: Structural features of Amyloid

a) Cross-beta structure present in amyloids [8]
b) Transmission electron microscopy (x100,000) image of amyloid fibrils with a diameter of 7.5 to 10 nm [9]
c) Apple-green birefringence of amyloid fibrils upon binding of Congo red under polarized light microscopy. [9]

Misfolding of a protein is the main cause of major neurodegenerative diseases like Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS) and the Frontal Temporal Dementias [10].

Table 1: List of examples of neurodegenerative protein misfolding diseases [1, 10, 11]

Disease	**Amyloid Protein/Peptide**
Alzheimer's Disease	Aβ(40-42)
Transmissible Spongiform Encephalopathies	Prion Protein (PrP)
Parkinson's Disease	α-Synuclein
Amyotrophic Lateral Sclerosis	TDP-43
Huntington's Disease	Huntingtin with PolyQ Expansion

1.2. Yeast: Model for Prion and Amyloid diseases.

The yeast *Saccharomyces cerevisiae* is an ideal *in vivo* model to study human disease mechanisms [12] due to conserved basic cellular mechanisms, rapid growth on defined media and easy mutant isolation [13].Yeast provides a model system for studying mechanisms of protein misfolding and aggregation, which is also applicable to human diseases [14].

Yeast has been used as a model system to study the disease mechanism of various neurodegenerative diseases like Alzheimer's [15], Parkinson's [16], and Huntington's [17] by expression of the pathological protein. TDP-43 has been expressed in yeast to act as a disease model of ALS [18].

Sup35 protein is involved in the recognition of stop codons in yeast. Sup35 N-terminal domain [1-123] is unstructured and is responsible for prion formation, the highly charged, middle (M) domain [124-254] promotes the non-prion state. The C-terminal folded domain [255-685] is responsible for its translation termination activity [19].

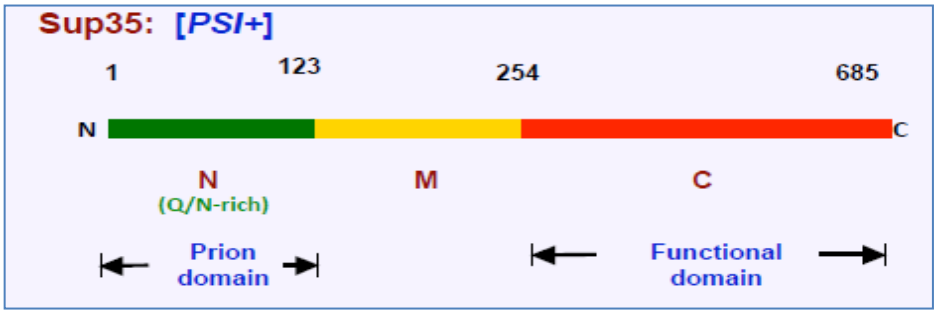


Figure.3: Domain architecture of Yeast Sup35 Protein [20].

When the Sup35 is converted from its soluble non-prion state [*psi*–] to its aggregated prion state [*PSI*+], termination activity of the protein is reduced, causing the read-through of stop codons [21].

[*psi*-] cells in *ade1–14* background having a premature stop codon, will form red colonies on rich growth medium (YPD) due to functional C-terminal region of Sup35MC which would perform the translation termination and due to the accumulation of a metabolic intermediate of the adenine biosynthesis pathway.

If the protein is insoluble then it will give white colonies indicative of prion formation leading to loss of function of C-terminal of Sup35MC and formation of full length Ade1 protein (functional) [22,23].

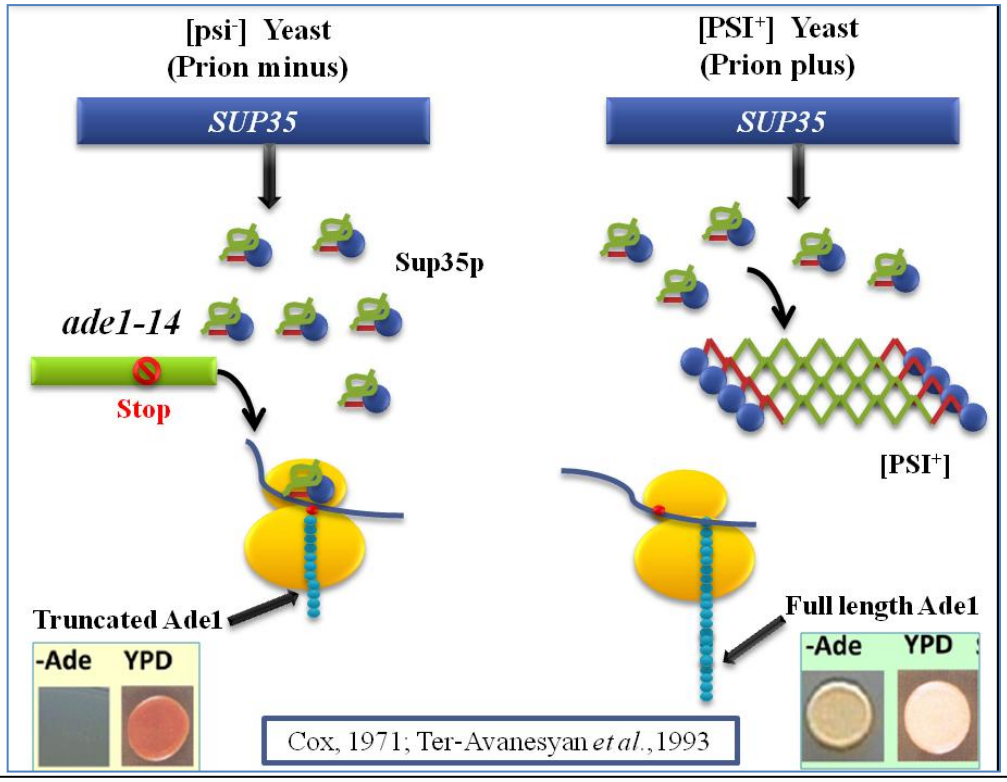


Figure 4: The [*PSI*+] yeast prion. [21]
Sup35 is soluble in [*psi*-] state. In *ade1–14* background, cells will form red colonies on YPD due to functional C-terminal region of Sup35MC which would perform the translation termination. When Sup35 is converted to its aggregated prion state [*PSI*+], termination activity is reduced, causing the read-through of stop codons.

A previous study done by Sondheimer and Lindquist, shows that the Prion domains are modular and transferable. Fusion will not affect the function of Sup35MC [24].

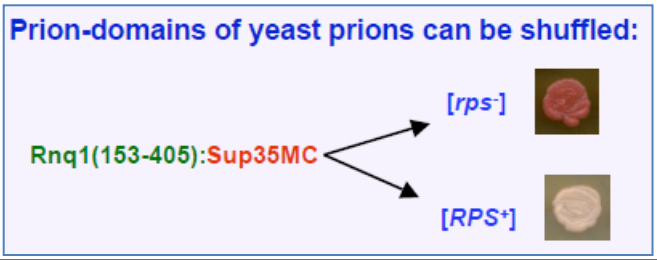


Figure 5: The [*RPS*+] and [*rps*-] prion in yeast. Prion domain of Rnq1 when fused with Sup35 mimics [*PSI*+] prion. When the fusion protein is soluble the cells appear red on YPD and when the fusion protein is aggregated it forms white colonies on YPD. [24].

Prion Domain of Pathogenic protein can be fused with Sup35MC and the fusion protein can be expressed in yeast to study the mechanism of the disease. The Sup 35 can act as a reporter to study the aggregation of the protein.

1.3. Prion strains

Prion variants are called prion strains. Prion variants are present in mammalian as well as yeast prions. Scrapie is a prion disease in sheep, which has two variants: drowsy and scratchy [25].

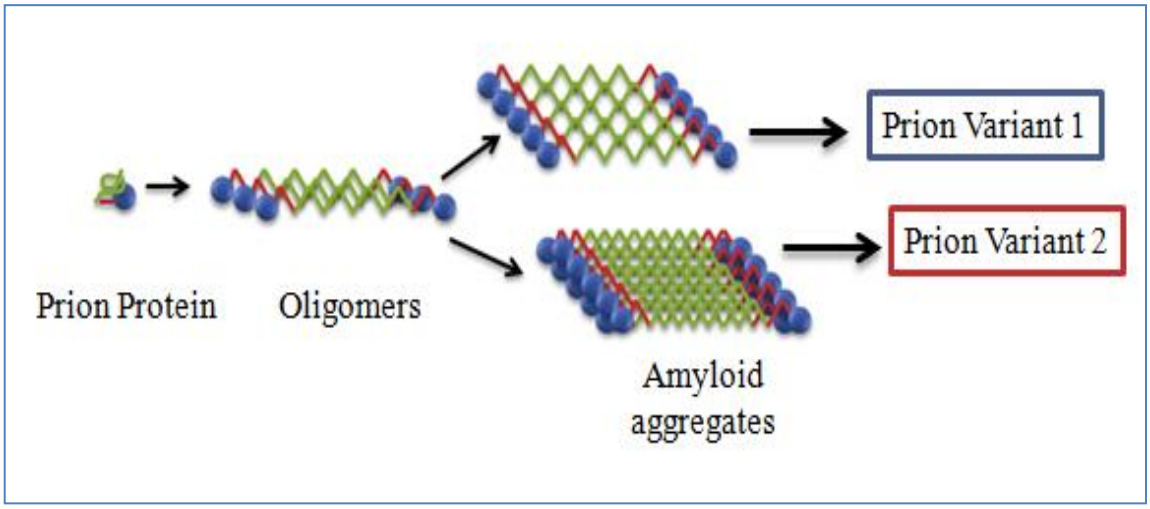


Figure 6: Prions can exhibit self propagating variants/strains

The variants of yeast prion [*PSI*+] depend on the level of soluble Sup35 present in the yeast cell. The [*PSI*+] prion exhibits three strains: strong [*PSI*+] (low level of soluble Sup35), weak [*PSI*+] and [*psi*–], which appear white, pink and red respectively on YPD.

Figure 7: The [*PSI*+] yeast prion exhibits three variants [26]: Strong [*PSI*+] (White), Weak [*PSI*+] (Pink) and [*psi*–] (red).

The strong [*PSI*+] can grown well on –Ade media, weak [*PSI*+] grows poorly on –Ade and [*psi*–] cannot grow on –Ade media [26]. They also exhibit faithful propagation of the prion variant. Strong [*PSI*+] being dominant over weak [*PSI*+] and [*psi*–] [27].

1.4. Amyotrophic Lateral Sclerosis

Amyotrophic lateral Sclerosis (ALS) is a fatal neurodegenerative disease which affects the motor neurons. Upper motor neurons (brain) send the signals to the lower motor neurons (spinal cord) which then sends the message to the voluntary muscles. ALS causes the degeneration of both upper and lower motor neurons, which causes muscular atrophy and muscle twitching. ALS is characterized by progressive weakness and death within few years [11]. The major symptoms include difficulty in speaking, chewing and swallowing; stumbling while walking and eventually patients lose the ability to breathe.

Prevalence of the disease is 5 individuals per 100,000 and the risk increases after the age of 60. Most of the ALS cases are sporadic and only 10% are associated with familial mutations [11]. The cause of the sporadic ALS is largely unknown but, there are some links with occupations involving heavy labour, exposure to environmental toxicants, or a history of traumatic injury [28]. There is no known cure for ALS yet; but, the disease progression can be slowed down by an anti-glutamate drug called Rilutek which blocks the release of glutamate from the nerve cells. Rilutek prolongs median survival by two to three months in ALS patients [29].

TDP-43 was identified as the pathogenic protein in ALS, using specific monoclonal antibodies [30]. Trans Active Response (TAR) DNA-binding protein 43 (TDP-43) is a ubiquitously expressed 414 amino-acid nuclear protein, encoded by *TARDBP* gene on chromosome 1 [31]. Under certain conditions, the protein mislocalises to the cytoplasm and forms toxic inclusions; this depletes the nuclear pool of soluble TDP-43. The pathological TDP-43 is hyperphosphorylated, ubiquitinated and cleaved to generate C-terminal fragments of the protein [32].

Mutations in the *TARDBP* gene are mostly located in the C-terminal glycine rich region [33]. TDP-43 aggregates are non-amyloid like and mutation of the protein in the C-terminal region increases its aggregation and cytotoxicity [34]. These aggregates are heat and proteinase resistant; transmissible in nature and are able to seed the aggregation of the soluble protein. Hence, these aggregates have prion like properties [31].

TDP-43 was originally identified as a repressor of HIV-1 which binds to the TAR DNA element of HIV [31]. Functions of TDP-43 include transcriptional repression [35], translational repression [34] and splicing of the CFTR pre-mRNA [36].

Figure 8: Domain architecture of TDP-43. [32]

TDP-43 is a homodimer with four RNA recognition motifs (RRMs) and a glycine rich C-terminal. RRMs have two highly conserved hexameric and octameric segments called as ribonucleoprotein 2 (RNP2) and ribonucleoprotein 1 (RNP1) respectively. The conserved RNP segments in TDP-43 are involved in binding to TAR DNA sequences and RNA sequences with UG-repeats and the C-terminal is involved in the splicing [32].

TDP-43 fused with green fluorescent protein (GFP) has been expressed in yeast to act as a disease model for ALS. This model exhibits some of the pathological features associated with ALS in humans, like nuclear localisation and cytoplasmic aggregation of the protein. And it has been proposed that the toxicity is caused by the loss of function of TDP43 protein due to aggregation rather than due to accumulation of the protein. [36]

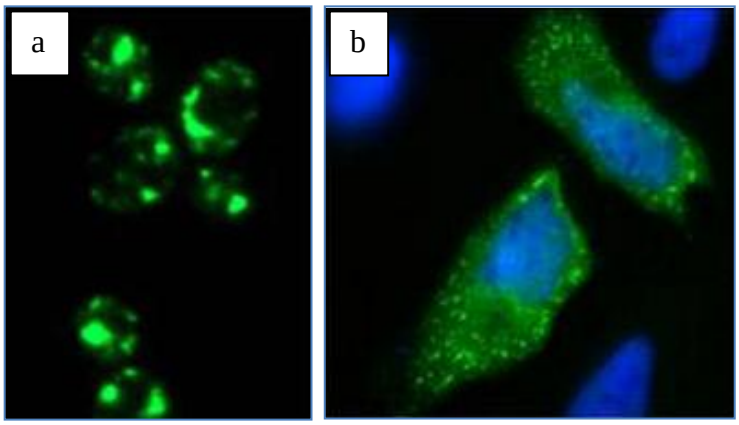


Figure 9: Aggregates of TDP-43

a) Yeast cells showing TDP43-GFP aggregation. [36]
b) HeLa cells showing cytoplamic aggregation of TDP-43 (green) and the nucleus is shown in blue. [37]

When basal level of protein expression is maintained, diffused fluorescence is observed under fluorescence microscope which implies the protein is in monomeric state. When the protein is overexpressed, the TDP43-GFP fusion proteins tend to aggregate and form fluorescent foci.

1.5. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease and it is the most common cause of dementia. AD is characterized by memory impairment (delayed recall), amnestic mild cognitive impairment and Alzheimer's dementia. Dementia causes decline in cognition, function and behaviour. [38] In 2005, 24.2 million people were affected by dementia worldwide and 70% of these patients had AD. In of 60 year-olds population of North America and Western Europe, the prevalence and incidence of dementia is the highest. [39]. AD is classified as early onset (onset before 65) and late onset (after 65 years) based on the age of onset. Late onset AD affects more than 95% of the affected individuals. [38] The hallmark of AD is the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain. [40]. These amyloid plaques contain the aggregated Aβ peptide [41].

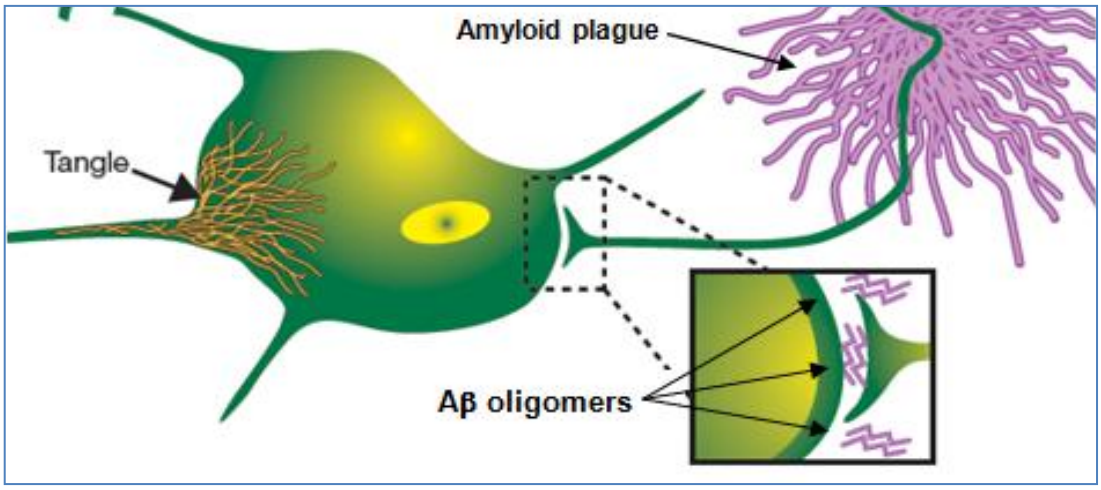


Figure 10: Hallmarks of AD: Amyloid plaque and neurofibrillary tangles [42]

The Amyloid Precursor Protein is a transmembrane protein. Function of this protein is not clear but, when it is cleaved into Aβ peptide, the peptide tends to aggregate and form amyloid deposits. [43]

Figure 11: Amyloid Precursor Protein (APP) showing the N-Terminal signal peptide, a serine protease inhibitor domain of the Kunitz type (KPI) and amyloid β-peptide (Aβ) including the transmembrane domain (TM) [43]

The Amyloid Precursor Protein (APP) is cleaved by the enzyme β-secretase (BACE). Then the γ-secretase enzymes act on the APP to generate Aβ peptide of different lengths. The γ cleavage site is variable which decides the length of the peptide and determines the ability of the peptide to aggregate. For eg. Aβ-42 is more prone to aggregation than Aβ-38. [43]

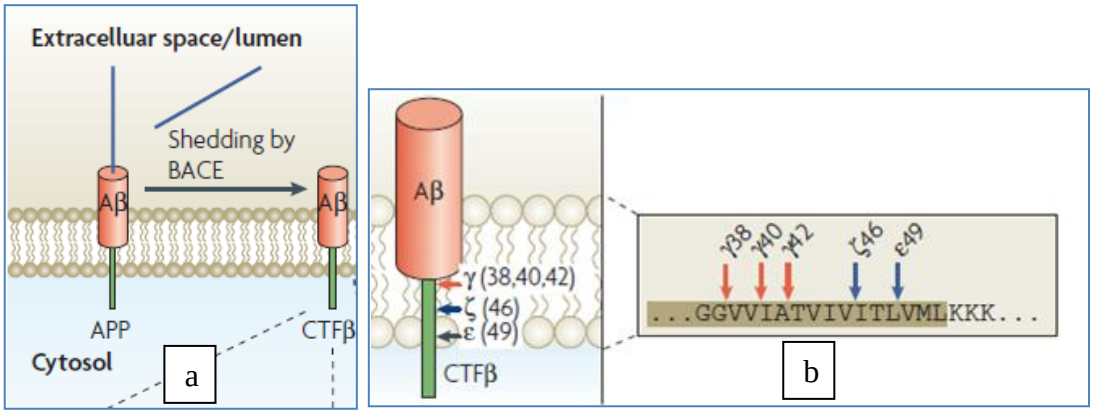


Figure 12: Generation of Aβ peptide [43]

a) The amyloid precursor protein (APP) is first cleaved by β-secretase (BACE)
b) Sites where γ-secretase enzymes can act, to release the Aβ peptide.

Aβ42 fused to the functional domain of the Sup35, when expressed in yeast produces Aβ42-Sup35MC fusion proteins, which in the [*PSI*+] *ade1–14* strain, will give rise to rise to red colonies on rich growth medium (YPD) due to functional C-terminal region of Sup35MC and will not survive on SD-Ade media as the Ade protein produced is truncated. If the protein is insoluble then it will give white colonies indicative of prion formation leading to loss of function of C-terminal of Sup35MC and formation of full length Ade protein and will survive on SD-Ade. Aβ42-Sup35MC fusion proteins when expressed in yeast were able to form sodium dodecyl sulfate (SDS)-stable low-n oligomers. Yeast growth in SD-Ade was used to assay for the formation of oligomers, and the compound which is able to restore the activity of the functional domain of Sup35 can act as a potential anti-oligomer drug. [44].

Prions can propagate as different strains due to different conformation of the protein aggregates and it has been observed that at least two different strains of Aβ prions i.e. Arctic and Swedish strains exist leading to different manifestation of the disease [45]. It is being proposed that different strains of Aβ prions can be obtained in the yeast model as well, which can act as a better disease model for AD.

Chapter 2

2. Materials and Methods

2.1. Materials

All the culture media components, Zinc chloride, Copper chloride and DMSO, were purchased from HiMedia. Yeast strain INV*Sc*1 was purchased from Invitrogen. Qiagen mini-prep kit was purchased from Qiagen. Ammonium Sulphate was purchased from SRL. Ampicillin Sodium salt, galactose, glycine, histidine, L- Leucine, L- Tryptophan, Lithium acetate, uracil and raffinose were purchased from Sigma. Yeast strains and plasmids were kind gifts from Dr. Susan Liebman.

2.2. Methods used for studying TDP-43

2.2.1. Preparation of Media

Bacterial culture media

Luria Bertani (LB) + Ampicilin media was used for reviving and maintaining the bacterial transformants containing pRS416Gal TDP43 WT YFP and pRS416 plasmids. These plasmids have ampicilin as antibiotic selection marker. LB media contains Tryptone (1%), yeast extract (0.5%) and sodium chloride (1%). The pH was adjusted to 7 using sodium hydroxide and for the preparation of solid media 1.5% agar was used. Media was autoclaved for 20 minutes at 121 °C. The media was cooled to 60°C and 1 ml from 1mg/ml ampicilin stock, was added to the media.

Yeast culture media

Standard yeast media YPD [46] was used for reviving yeast strains from -80°C. YPD is a non selective rich media which contains 1% Yeast extract, 2% Peptone and 2% Dextrose. For preparation of solid media 2% agar was added. The media was autoclaved for 20 minutes at 121 °C.

For maintaining the plasmid and for the selection of plasmid containing yeast transformants, synthetic “dropout” media was used which contains all the nutrients except for uracil. The synthetic defined media used were SD-Ura (2% Dextrose) and SRaf-Ura (1% Raffinose) containing Yeast Nitrogen Base (YNB),

Ammonium Sulfate (AS) and all the amino acids except uracil. For preparation of solid media 2% agar was added. Media was autoclaved for 18 minutes at 115 C. Yeast strains were grown at 30°C and stored at 4°C.

While checking the effect of the metal ions and the small molecule, these compounds were added along with the SRaf (1%) +0.1%Gal-Ura media. Galactose solution, salt solution and 3-Aminophenol solutions were filter sterilized before addition to the media.

2.2.2. Plasmid Isolation and Yeast Transformation

Plasmid pRS416Gal TDP43 WT YFP is a low copy number Yeast Centromeric Plasmid (YCP) under GAL promoter. This plasmid when transformed in INVSc1 yeast expresses TDP-43-YFP fusion proteins under GAL promoter. The expression of the protein can be induced by galactose and the level of expression can be controlled by changing the concentration of galactose.

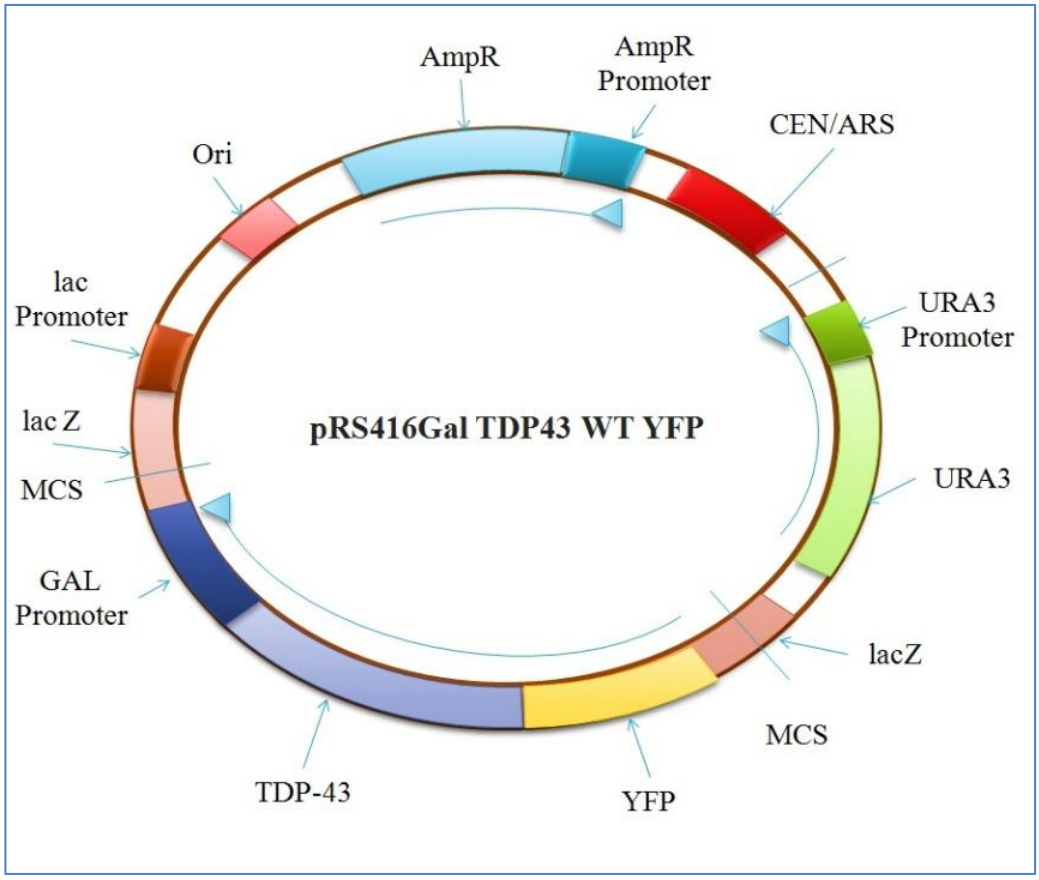


Figure 13: Plasmid map of pRS416Gal TDP43 WT YFP

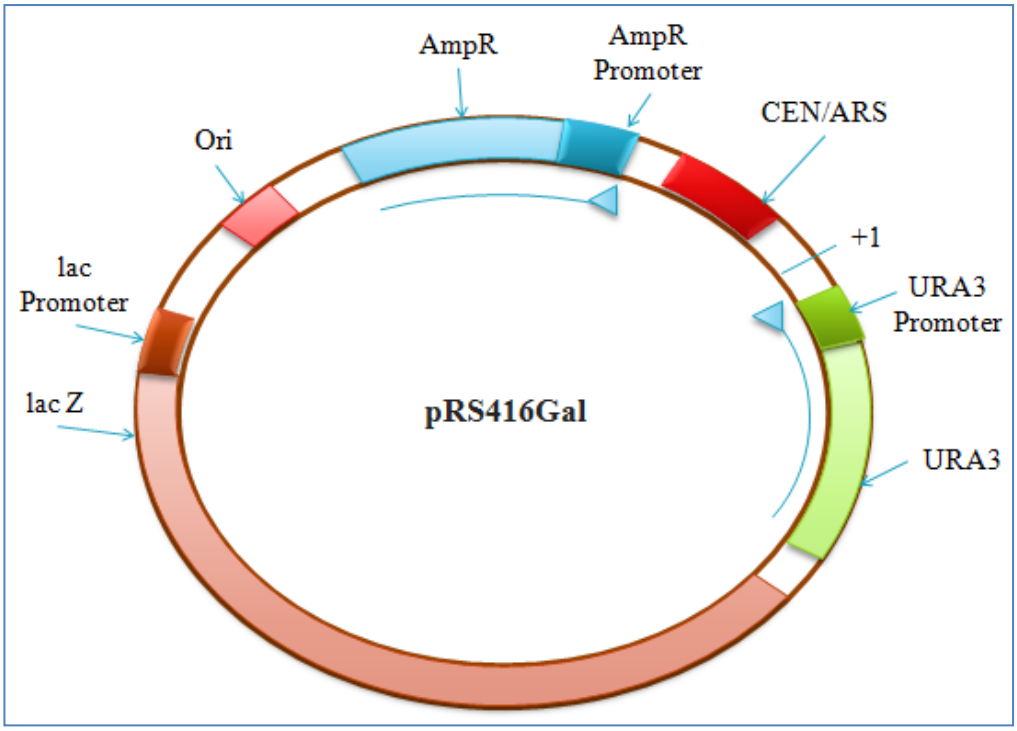


Figure 14: Plasmid map of pRS416

Plasmid Isolation: Mini prep

Plasmid pRS416Gal TDP43 WT YFP and pRS416 (control plasmid) were isolated using Qiagen mini-prep kit. Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA), Buffer P2 (200 mM NaOH, 1% SDS), Buffer N3 (4.2M Gu-HCl, 0.9M potassium acetate, pH 4.8), Buffer PB (5M Gu-HCl, 30% isopropanol and Buffer PE (10 mM Tris-HCl, pH 7.5, 80% ethanol)

1. The bacterial transformants were grown overnight in LB+Amp broth in rotary shaker at 200 rpm at 37°C.
2. Cells were pelleted by centrifuging at 6000 rpm for 5 mins at 4°C.
3. The supernatant was discarded and the cells were resuspended in 250µl of P1 (containing RNase A) buffer.
4. 250 µl P2 buffer was added and mixed thoroughly till the solution turned clear blue.
5. 350 µl of N3 buffer and was mixed thoroughly by inverting the tube until the solution becomes clear and colorless.
6. Centrifuged for 10 min at 13000 rpm.

7. Supernatant from previous step (6) was applied to the QIA prep spin column by pipetting and centrifuged at 13000 rpm for 2 mins, and the flow through was discarded
8. 500 µl PB buffer was added and allowed to stand for 1 min then centrifuged at 10000 rpm for 2 mins and the flow through was discarded.
9. 700 µl PE buffer was added and was centrifuged at 10000 rpm for 2 mins and the flow through was discarded.
10. Residual ethanol in the column was allowed to dry and the column was centrifuged for 2 mins at 10000 rpm.
11. The QIA prep column was placed in a clean eppendorf and 50µl of autoclaved water was added to the center of the column.
12. This was allowed to stand for 5mins and centrifuged at 14000 rpm for 2 mins and the concentration was found using nanodrop. And the plasmid was stored at -20°C

Yeast Transformation: PEG/Lithium acetate-DMSO method

The yeast strain INVSc1**:** *MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52* was revived on Yeast extract Peptone Dextrose (YPD) plate. Both the TDP43-YFP and Control plasmids were transformed into INVSc1 using PEG/Lithium acetate-DMSO method. The transformants were selected on synthetic complete dextrose media lacking uracil (SD-Ura plate) as the plasmids contain URA3 as selectable marker.

1. A single colony of Inv.Sc.1 was inoculated in 5 ml YPD broth and incubated overnight in rotary shaker at 200 rpm at 30°C.
2. 2 ml of overnight pre-inoculum was added to 60 ml YPD broth and was incubated till the O.D reaches 1.0 (~5 hours) in rotary shaker at 200 rpm at 30°C.
3. The cells were pelleted by centrifuging at 10000 rpm for 5 mins and washed with 1 ml water and 1 ml 1X TE-LiAc at 10000 rpm for 2 mins.
4. The cells were resuspended in 250 µl 1X TE-LiAc and 5µl of plasmid (TDP43-YFP and Control plasmid) and 5 µl carrier DNA was added. The carrier DNA was boiled for 10 min at 95°C and was placed on ice immediately before adding.

5. 300 µl of sterile 1X PEG-LiAc was added and incubated at 30°C for 60 mins with occasional shaking.
6. 40 µl DMSO was added and mixed thoroughly then heat shock at 42°C for 15 mins.
7. The cells were pelleted at 10000 rpm for 5 mins and resuspended in 100 µl autoclaved water.
8. The cells were spread on SD-Ura plate and allowed to grow for 3-5 days.

2.2.3. Spot Assay to study the toxicity of TDP-43 expression in yeast cells.

1. The INVSc1+TDP43-YFP transformants from the SD-Ura plate were picked from the plate and inoculated in 10 ml of SD-Ura media
2. The cells were grown to an optical density of 0.6 $OD_{600,}$ at 30°C in a rotary shaker at 200 rpm; till they reach log phase.
3. One to five fold serial dilutions were performed using 100 µl of the culture.
4. 5 µl of the culture was spotted and allowed to dry.
5. Triplicates of each dilution were spotted on each plate: YPD, SD+12, SD-Ura, SRaf-Ura, SRaf-Ura + 2% Gal and SRaf-Ura + 0.1% Gal.
6. Incubated at 30°C, for 48 hours.

2.2.4. Spot Assay to study the effect of 3-Aminophenol on toxicity of TDP-43 expression in yeast cells.

1. The INVSc1+TDP43-YFP transformants from the SD-Ura plate were picked from the plate and inoculated in 10 ml of SD-Ura media
2. The cells were grown to an optical density of 0.6 $OD_{600,}$ at 30°C in a rotary shaker at 200 rpm; till they reach log phase.
3. One to five fold serial dilutions were performed using 100 µl of the culture.
4. 5 µl of the culture was spotted and allowed to dry.
5. Triplicates of each dilution were spotted on each plate: SD+12, SD-Ura, SRaf-Ura + 2% Gal, SRaf-Ura + 0.1% Gal, SRaf-Ura + 0.2% Gal and SRaf-Ura + 0.2% Gal + 3mM 3-Aminophenol.
6. Incubated at 30°C, for 48 hours.

2.2.5. Selection of Carbon source to study the effect of zinc/copper [47]

1. Inv Sc1 + TDP43-YFP cells were grown in a rotary shaker at 200 rpm and 30°C, for 24 hours in 5 ml of SD-Ura.
2. 100 µl of this culture was inoculated in 5ml of:
    1) SRaf–Ura
    2) SRaf–Ura + 10mM Zinc chloride
    3) SRaf–Ura + 5mM Copper sulphate
    4) SD–Ura
    5) SD–Ura + 10mM Zinc chloride
    6) SD–Ura + 5mM Copper sulphate
3. The culture was allowed to grow overnight in a rotary shaker at 200 rpm and 30°C; 2 ml of the culture was centrifuged and washed with 1 ml of autoclaved water to remove traces of media
4. The pellet was resuspended in 200 µl of water.
5. 100 µl of this culture was serially diluted in 900 µl of water up to 10000 dilutions.
6. 5 µl was spotted on SD-Ura plate and YPD.
7. The growth was checked after 48 hours.

2.2.6. Effect of 1% Galactose and 10mM zinc chloride / 5mM copper sulphate

1. Inv Sc1+TDP43-YFP cells were grown in 10 ml of SRaf-Ura for 24 hours in a rotary shaker at 200 rpm and 30°C.
2. The absorbance was measured at 600 nm after 24 hours taking SRaf-Ura media as blank.
3. 200 µl of this culture was inoculated in 5ml of:
    1. SRaf – Ura
    2. SRaf + 1% Galactose.
    3. SRaf + 1% Galactose + 10mM Zinc chloride
    4. SRaf + 1% Galactose + 5mM Copper sulphate
4. The experiment was performed in triplicates.
5. The culture was allowed to grow overnight in a rotary shaker at 200 rpm and 30°C; and the absorbance was measured at 600nm.

6. 2ml of the culture was centrifuged and washed with 1ml of water to remove traces of media
7. The pellet was resuspended in 200 µl of water.
8. 100 µl of this culture was serially diluted in 900 µl of water up to 1000 dilutions.
9. 5 µl was spotted on SD-Ura plate.
10. Growth was checked after 3 days.

2.2.7. Optimization of zinc concentration

1. Inv Sc1 + control plasmid was grown overnight in 5 ml of SD-Ura in a rotary shaker at 200 rpm and 30°C.
2. 100 µl of this culture was inoculated in 5 ml of:
    1) SD–Ura
    2) SD+ 0.1mM Zinc chloride-Ura
    3) SD+ 0.5mM Zinc chloride-Ura
    4) SD+ 1mM Zinc chloride-Ura
    5) SD+ 2mM Zinc chloride-Ura
    6) SD+ 4mM Zinc chloride-Ura
    7) SD+ 6mM Zinc chloride-Ura
    8) SD+ 8mM Zinc chloride-Ura
3. The culture was allowed to grow overnight in a rotary shaker at 200 rpm and 30°C. 5 ml of the culture was centrifuged and washed with 1 ml autoclaved water to remove traces of media.
4. The pellet was resuspended in 200 µl autoclaved water. 100 µl of this culture was serially diluted in 900 µl of autoclaved water up to 1000 dilutions.
5. 5 µl was spotted on SD-Ura and SD+12 plates. The growth was checked after 48 hours at 30°C.

2.2.8. Optimization of copper concentration

1. Inv Sc1 + control plasmid cells were grown overnight in 5 ml of SRaf-Ura in a rotary shaker at 200 rpm and 30°C.
2. 100 µl of this culture was inoculated in 5 ml of:

1. SRaf–Ura
2. SRaf-Ura + 10 μM Copper sulphate
3. SRaf-Ura + 50 μM Copper sulphate
4. SRaf-Ura + 100 μM Copper sulphate

3. The culture was allowed to grow overnight in a rotary shaker at 200 rpm and 30°C. 3 ml of the culture was centrifuged and washed with 1 ml autoclaved water to remove traces of media.
4. The pellet was resuspended in 200 μl autoclaved water and the cells were normalized. 100 μl of this culture was serially diluted in 900 μl of autoclaved water up to 1000 dilutions.
5. 5 μl was spotted as duplicates on SD-Ura. The growth was checked after 48 hours at 30°C.

2.2.9. Optimisation of Galactose concentration

1. Inv Sc1 + control plasmid, Inv Sc1 + TDP43-YFP, Yeast A (TDP43-YFP) were grown overnight in 5 ml of SRaf-Ura at 30°C.
2. The absorbance of the culture was measured and the OD was normalized to 0.36 OD.
3. 100 μl of this culture serially diluted in 900 μl autoclaved water up to 1000 dilutions.
4. 5 μl was spotted as duplicates on plates:
    1) SRaf(1%)-Ura + 0.0001% Gal
    2) SRaf(1%)-Ura + 0.001% Gal
    3) SRaf(1%)-Ura + 0.01% Gal
    4) SRaf(1%)-Ura + 0.1% Gal
    5) SRaf(1%)-Ura + 1% Gal
    6) SRaf(1%)-Ura + 2% Gal
5. Growth was checked after 48 hours of incubation at 30°C.

2.2.10. Fluorescence Microscopy to check for different levels of expression of TDP43-YFP

1. Cells in the log phase from the different plates [SRaf(1%)-Ura + 0.0001% Gal, SRaf(1%)-Ura+0.001% Gal, SRaf(1%)-Ura+0.01% Gal, SRaf (1%)-

Ura+0.1% Gal, SRaf(1%)-Ura + 1% Gal, SRaf(1%)-Ura + 2% Gal] were suspended in 10μl of autoclaved water on a micro slide and were covered with a cover slip.

2. The cells were first observed under 40 x magnifications.
3. Then a drop of immersion oil was placed on the cover slip and the cells were observed under oil immersion 63x and 100x magnification.

2.2.11. Effect of 0.1% and 1% Galactose and 3mM Zinc chloride

1. Inv Sc1 + control plasmid and Inv Sc1 + TDP43-YFP was grown overnight in 5ml of SRaf-Ura.
2. 100 μl of culture was inoculated in 5μl of:
    1. SRaf–Ura
    2. SRaf+ 0.1% Gal –Ura
    3. SRaf+ 0.1% Gal + 3mM Zinc chloride-Ura
    4. SRaf+ 1% Gal –Ura
    5. SRaf+ 1% Gal + 3mM Zinc chloride-Ura
3. The culture was allowed to grow overnight and 2 ml of the culture was centrifuged and washed with 1 ml of autoclaved water to remove traces of media.
4. The pellet was resuspended in 200 μl of water. 100 μl of this culture was serially diluted in 900 μl of water up to 1000 dilutions.
5. 5 μl was spotted on two SD-Ura plates. The growth was checked after 48 hours.

2.3. Methods used for studying Aβ42-Sup35MC fusion

The yeast strain 74D-694 (*MATa ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200*) [48] where the genomic *SUP35* (*sup35*Δ::*LEU2*) [49] and *ERG6* of the ergosterol biosynthetic pathway was deleted [50] carrying the plasmid p1364 (pRS313, *CEN, Ura3, CUP1::* Aβ-*MRF*) [51] was used as a disease model of Alzheimer's disease.

2.3.1. Preparation of Media

YPD was used [43] for reviving yeast strains from -80°C. YPD is a non selective rich media which contains 1% Yeast extract, 2% Peptone and 2% Dextrose. For preparation of solid media 2% agar was added. YPG contains

2% Glycerol instead of 2% dextrose and it forbids the growth of respiratory defective petite cells which cannot survive on non fermentable carbon sources and allows the growth of healthy cells [52]. The media was autoclaved for 20 minutes at 121 °C. YPD was cooled to 60°C and 50 µl from 10mM Copper sulfate stock solution, was added to 10 ml media to make YPD+50 µM Copper sulphate ($CuSO_4$) broth. Aβ42-Sup35MC is under *CUP1* promoter which can be induced with 50 µM $CuSO_4$. Yeast strains were grown at 30°C and stored at 4°C.

2.3.2. Toxicity of Aβ42-Sup35MC expression in yeast

1. The yeast strain was revived from -80°C on a YPD plate and was subcultured on YPD plates.
2. The cells were streaked on YPG plates and were incubated for 5 days at 30°C for the selection of healthy cells.
3. Cells from the YPG plate were inoculated in 10 ml YPD broth and in 10 ml YPD+50µM $CuSO_4$ broth. And the cells were grown till 0.6 OD_{600} in a rotary shaker at 200 rpm, at 30°C.
4. One to five fold serial dilutions were performed using 100 µl of the culture.
5. 5µl of the culture was spotted in triplicates on YPD and YPG plates and was incubated at 30°C, for 2 days (YPD) and 5 days (YPG).

2.3.3. Search for Aβ42-Sup35MC amyloid variants

1. Cells from YPD were streaked on YPG plates and were incubated for 5 days at 30°C for the selection of healthy cells.
2. Cells from the YPG plate were inoculated in 10 ml YPD broth and in 10 ml YPD+50µM $CuSO_4$ broth. And the cells were grown till 0.6 OD_{600} in a rotary shaker at 200 rpm, at 30°C.
3. One to five fold serial dilutions were performed using 100 µl of the culture.
4. 100 µl of the culture was spread on YPD plates in triplicates and was incubated at 30°C, for 2 days (YPD) and 5 days (YPG).

Chapter 3

3. Results and discussion

3.1. Toxicity of TDP-43 expression in yeast cells.

Spot assay was performed to study the toxicity caused by aggregation of TDP-43 in yeast cells.

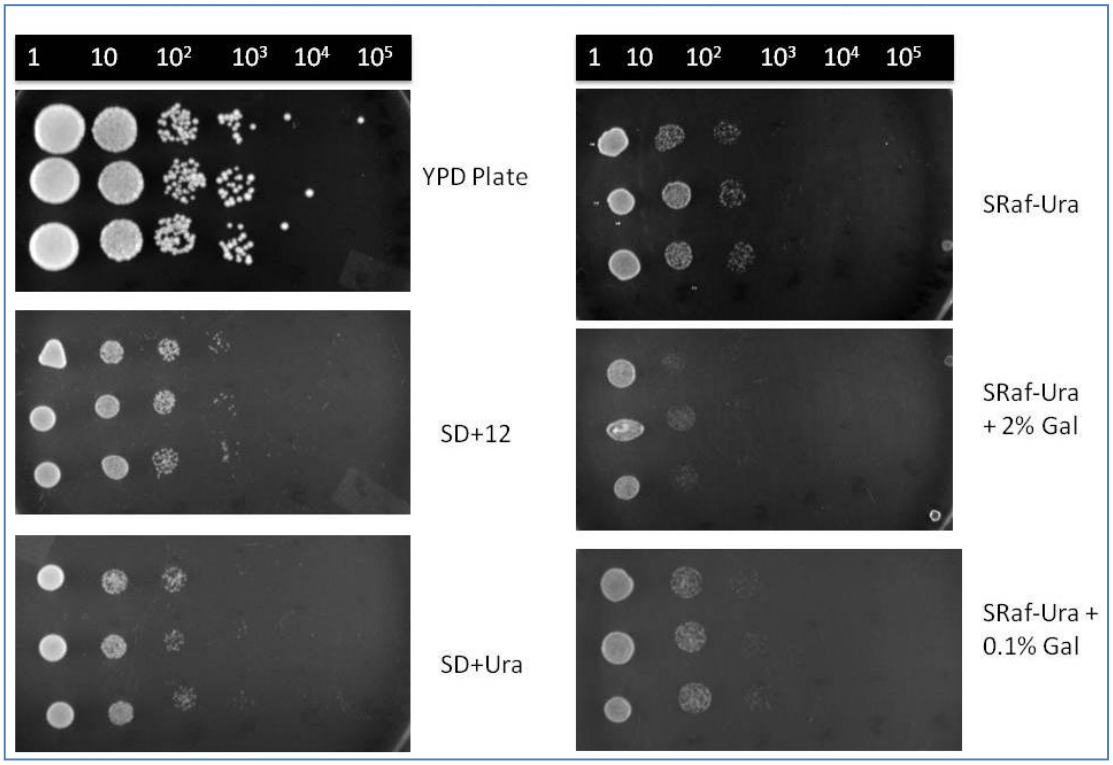


Figure 15: Spot Assay to study the toxicity of TDP-43 expression in yeast cells.

The YPD serves as a rich media which supports the growth of transformed and as well as non-transformed yeast cells. Growth on synthetic complete media (SD+12) is restricted compared to rich media. Synthetic media lacking uracil (SD-Ura) selects for transformed colonies having the plasmid with a URA3 selection marker. To overcome the problem of glucose repression in the yeast cells, raffinose was used as the carbon source in SRaf-Ura plate. The protein was overexpressed by inducing the promoter with 2% Galactose. When the protein was expressed by inducing the promoter with 0.1% galactose, the toxicity was reduced compared to the 2% galactose sample. These results show that expression of TDP-43 in yeast cells, results in the inhibition of growth.

3.2. Spot Assay to study the effect of 3-Aminophenol on toxicity of TDP-43 expression in yeast cells.

The fusion protein when overexpressed by inducing with 2% Galactose causes high toxicity. And when the protein was expressed by inducing the promoter with 0.1% and 0.2% galactose, the toxicity was reduced compared to the 2% galactose sample. 3-Aminophenol when used with 0.2% Galactose induction causes high toxicity which is comparable to 2% Galactose sample. These results show that 3-Aminophenol is increasing the toxicity caused by expression of TDP-43 in yeast cells, results in the inhibition of growth.

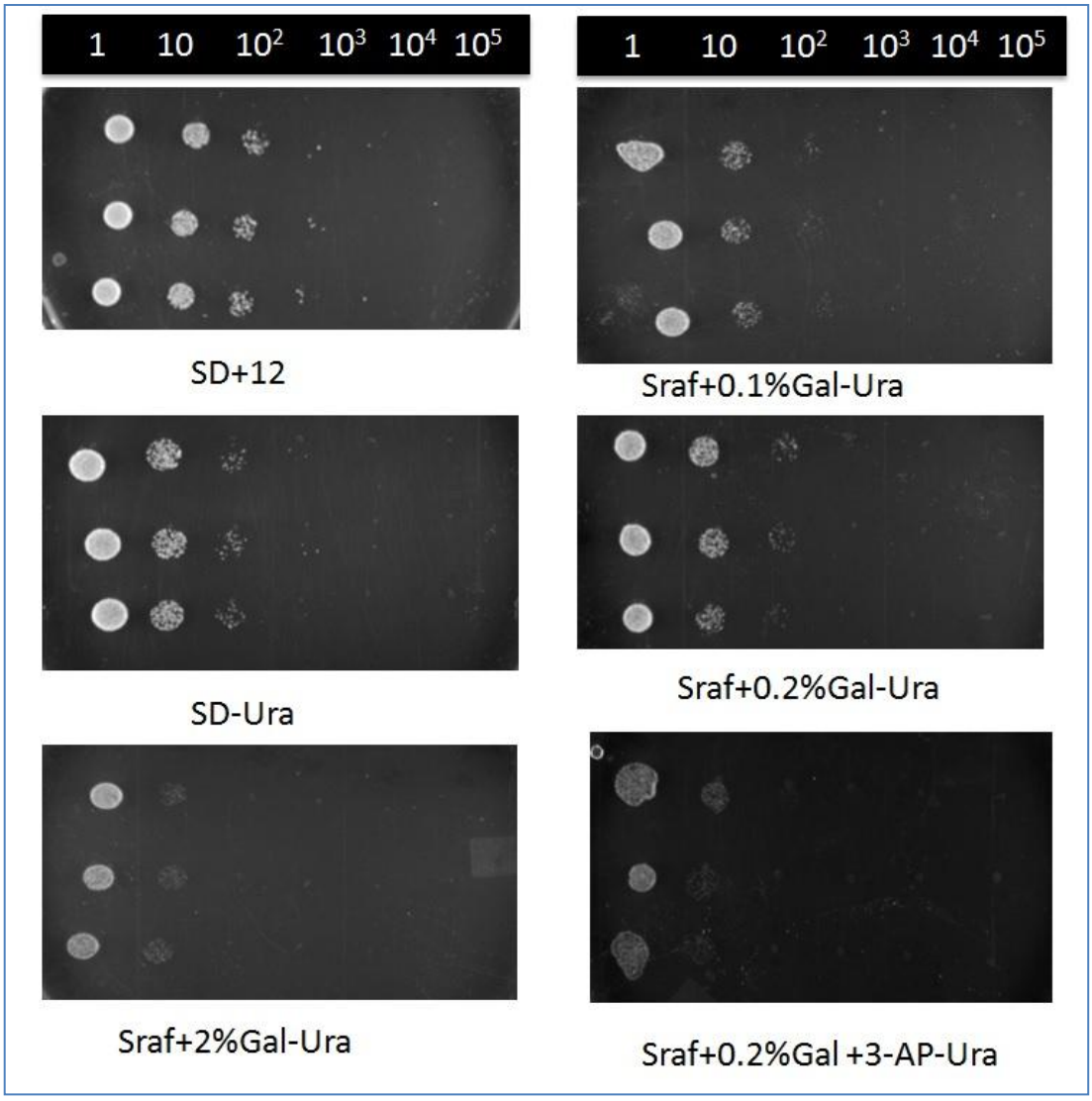


Figure 16: Spot Assay to study the effect of 3-Aminophenol on toxicity of TDP43-YFP expression in yeast cells.

3.3. Selection of Carbon source

The cell grown in SD-Ura broth face the problem of glucose repression in the yeast cells and when raffinose was used as the carbon source, the cell growth is better. When the cells are exposed to 10 mM Zinc chloride or 5 mM Copper sulphate [47], the cell growth is further hindered in the SD-Ura sample owing to the toxic effects of zinc/copper and glucose repression.

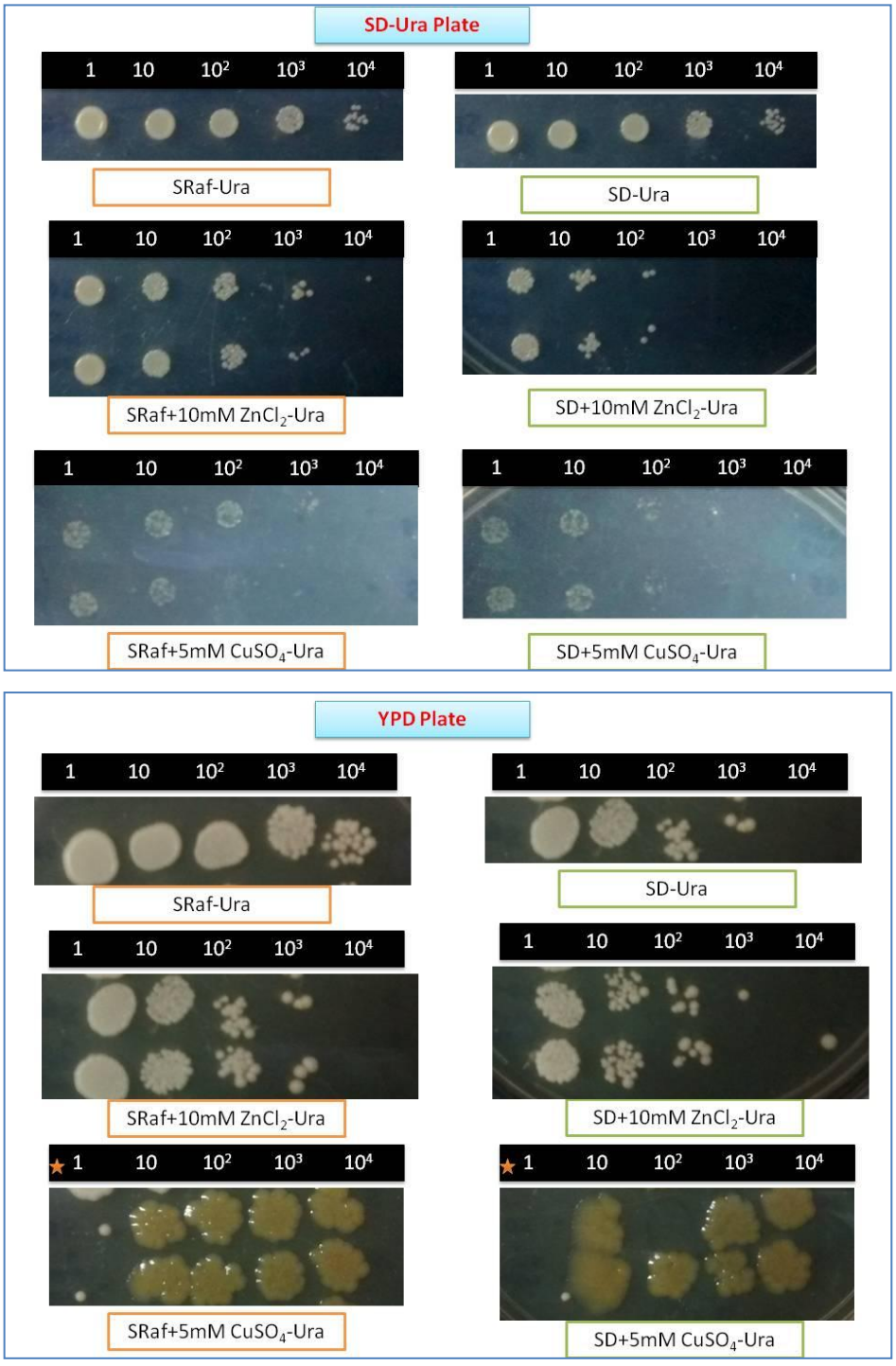


Figure 17: Selection of Carbon source

3.4. Effect of 1% Galactose and 10mM zinc chloride /5mM copper sulphate

1% Galactose induction causes high expression of TDP43-YFP fusion protein and the concentration of zinc chloride and copper sulphate used was determined from a previous work [47]. High expression of TDP43-YFP along with Zinc chloride is causing toxicity compared to 1% galactose sample. Copper sulphate in mill molar concentration is causing very high toxicity compared to 1% Galactose sample.

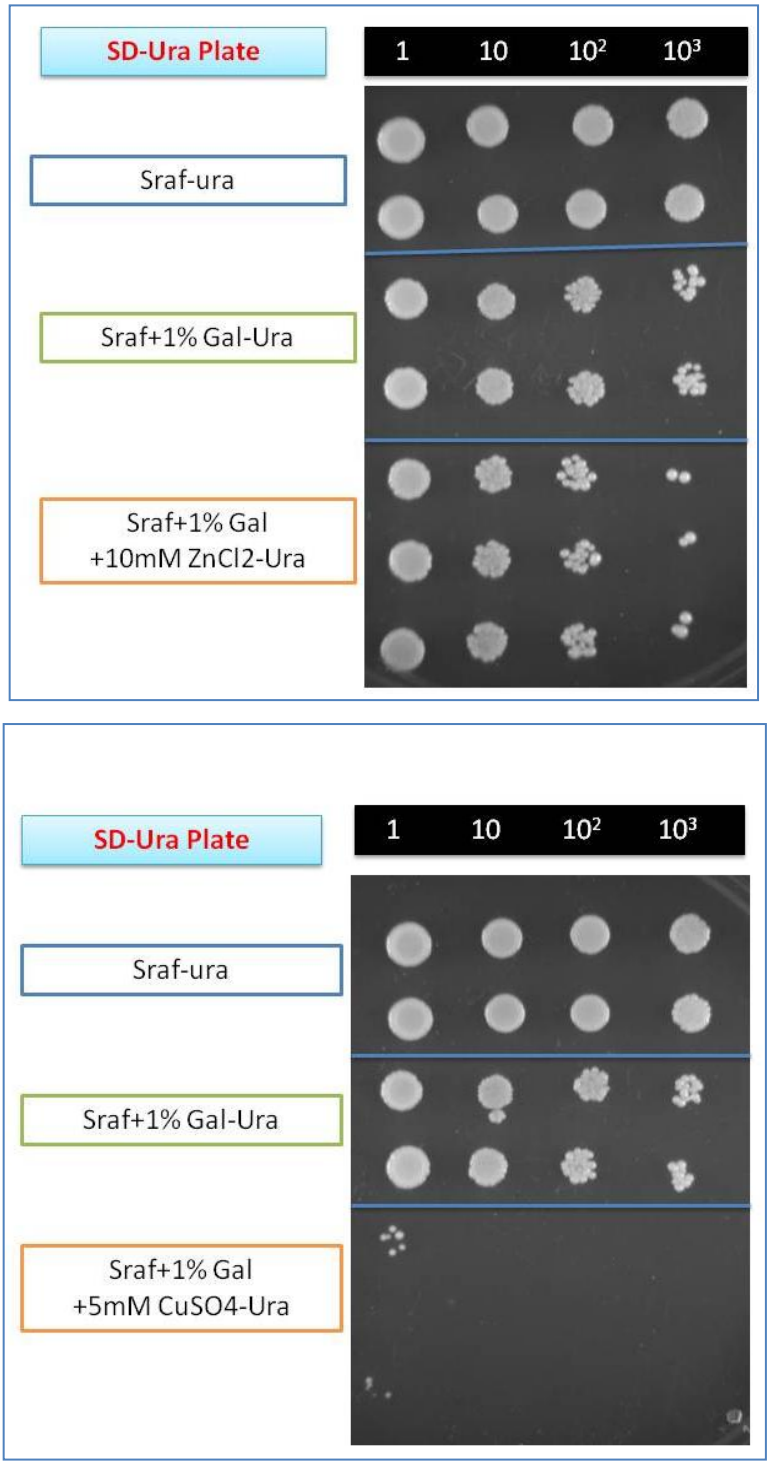


Figure 18: Effect of zinc/copper along with high expression TDP43-YFP.

3.5. Optimization of Zinc Concentration

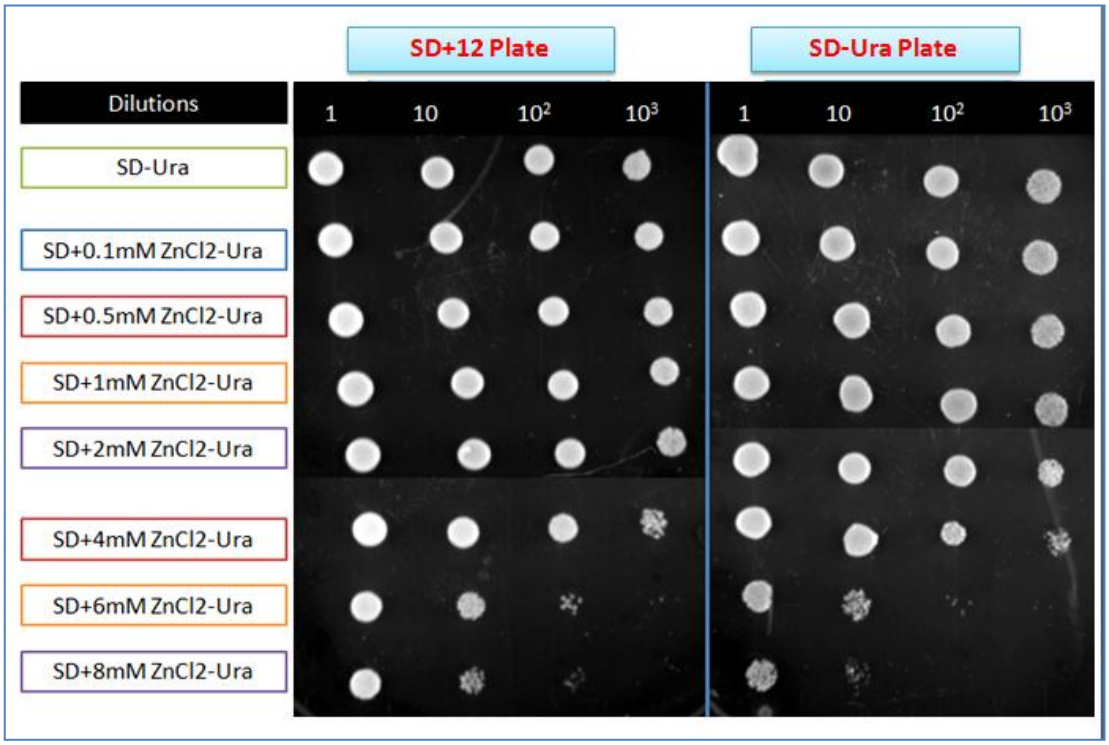


Figure 19: Optimization of Zinc Concentration

To find the optimum concentration of zinc, different concentrations of were checked The optimum concentration of zinc was found to be between 2 mM and 4 mM i.e. 3 mM. If the concentration of Zinc is more than 3 mM, zinc itself is toxic to the cells as the growth is hindered and the effect of expression of TDP43-YFP cannot be studied.

3.6. Optimization of Galactose concentration

TDP43-YFP is under Gal promoter and when the promoter is induced with a high concentration of galactose the protein is overexpressed and the protein tends to misfold. The misfolding leads to aggregation of the protein. If the protein aggregates are not amyloid they are degraded by the cell. If the aggregates are amyloid then they seed the normal soluble protein as well. This causes the depletion of normal protein which is soluble. And this causes growth inhibition in cells.

The optimal concentration of galactose where a balance of aggregation and toxicity was observed was 0.1% Galactose.

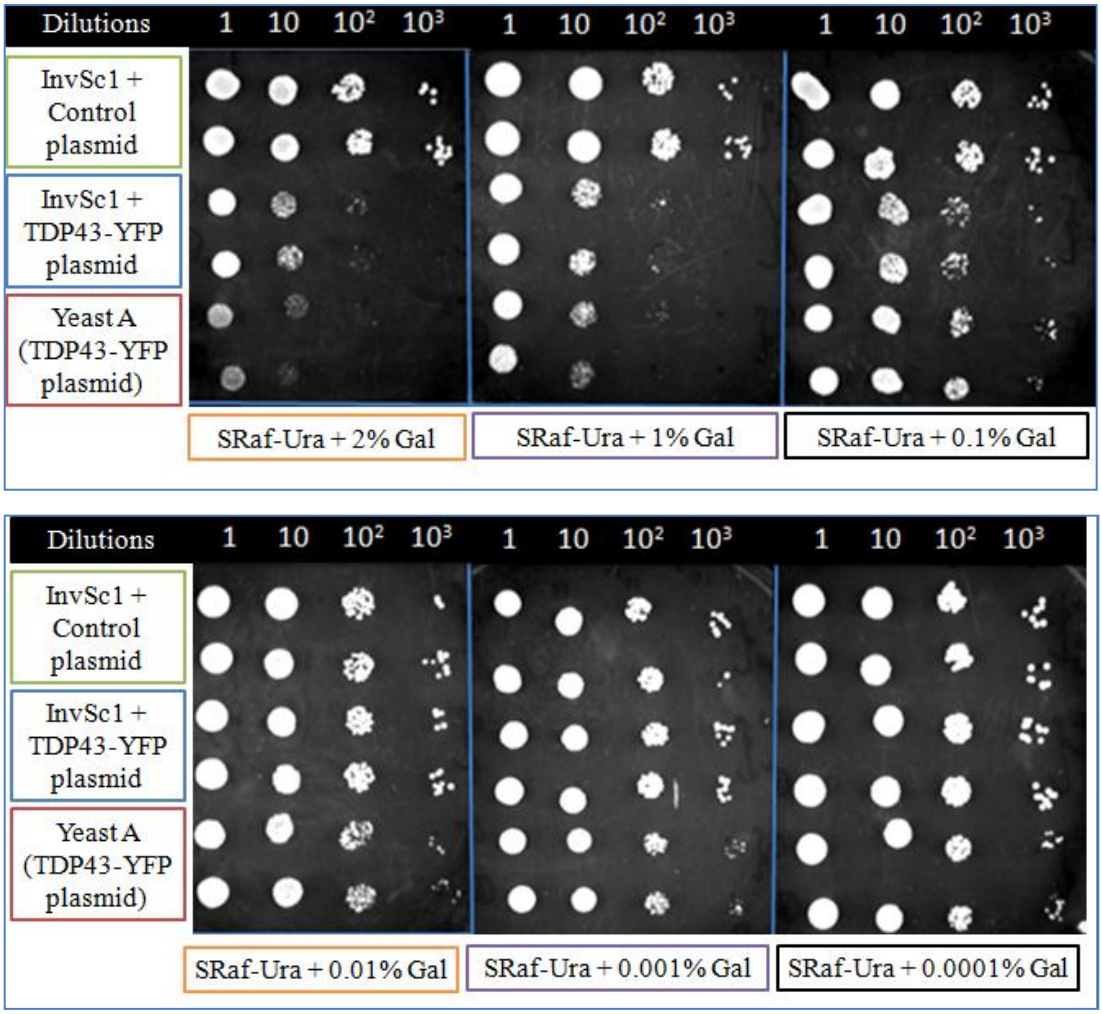


Figure 20: Optimization of Galactose concentration

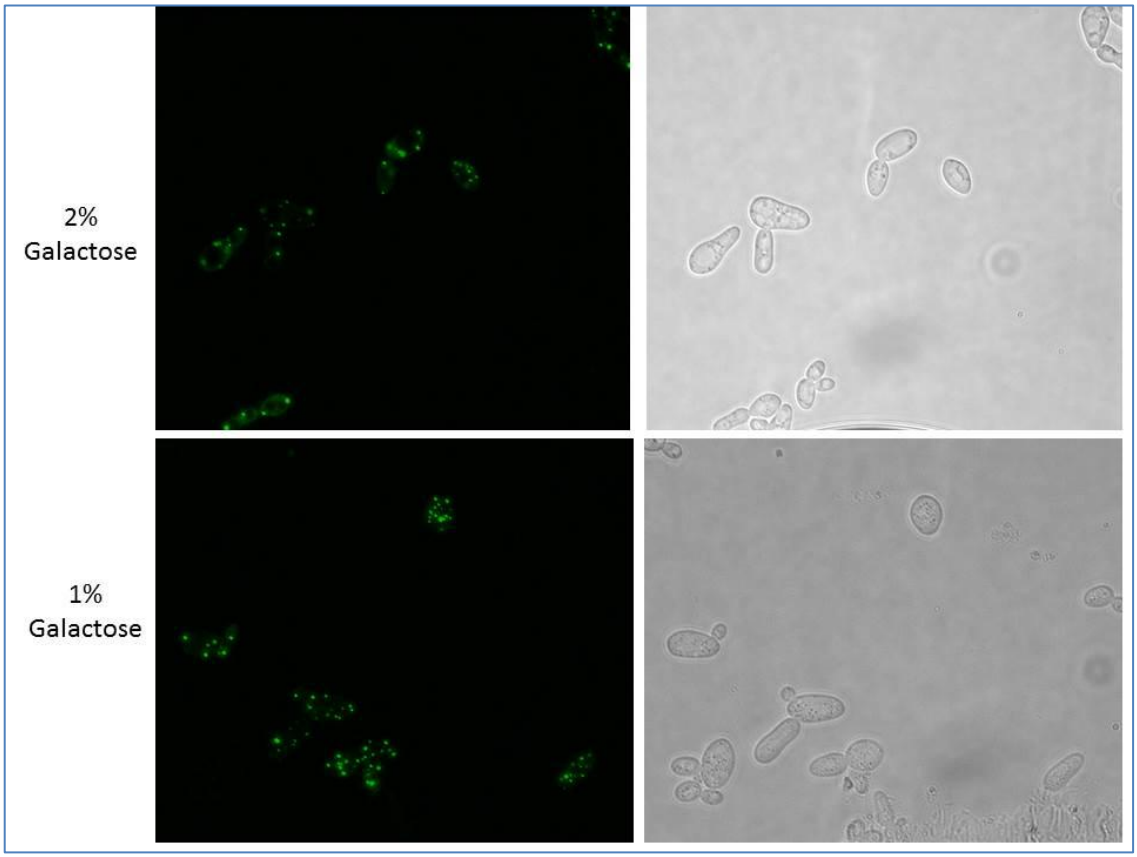
3.7. Fluorescence microscopy to check for different levels of expression of TDP43-YFP

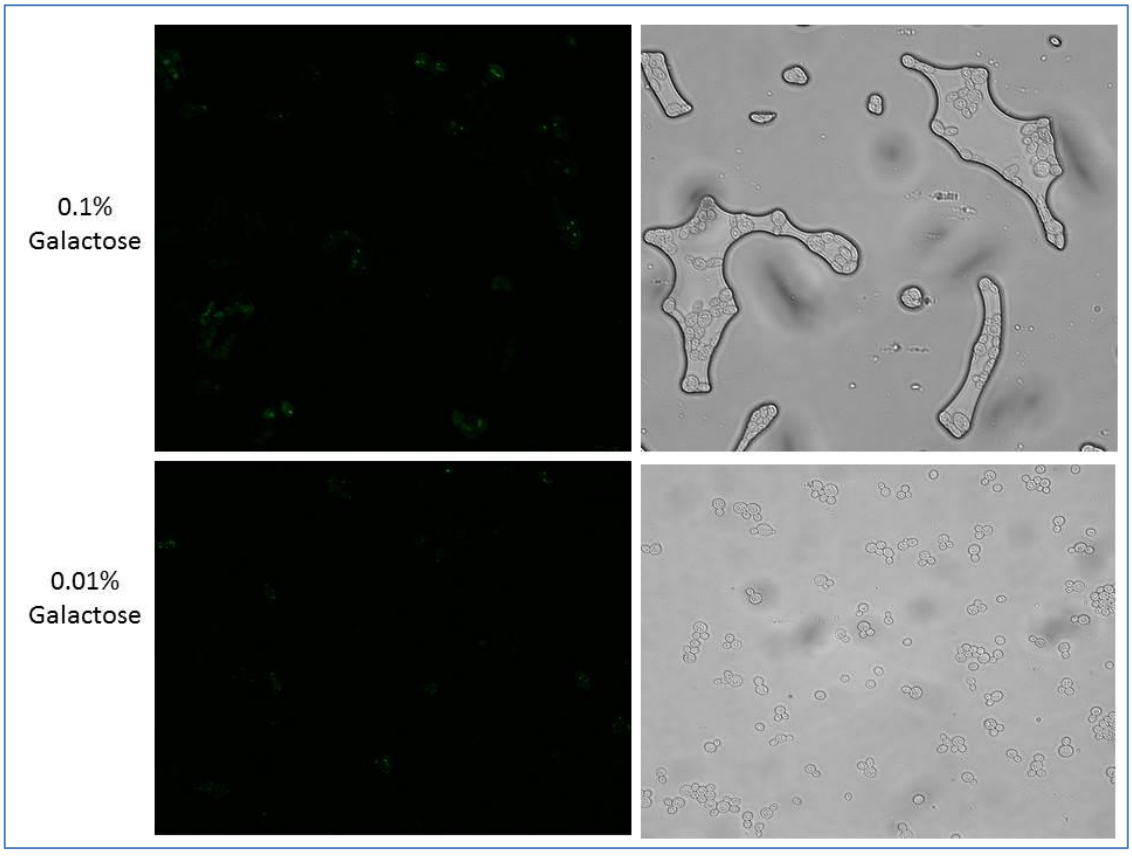
Greyscale images were obtained from the experiments and the image was processed by ImageJ software developed at the National Institutes of Health.

To check the time required for formation of fluorescent foci, the cells were incubated for different intervals of time 24 hours, 48 hours and 72 hours. After 24 hours of over-expression, the cells tend to lose the plasmid owing to the toxicity caused by TDP-43. To check effect of different levels of expression of TDP43-YFP in yeast different concentration of galactose 0.0001% 0.001%, 0.01%, 0.1%, 1% and 2% concentrations were tested and the fluorescence was checked.

2% and 1% sample showed fluorescent dots due to the aggregation of TDP43-YFP. 0.1% and 0.01% Galactose were found to be the optimum concentration for the maintenance of TDP43-YFP foci.

0.001% and 0.0001% are showing diffused fluorescence which shows basal level of protein expression





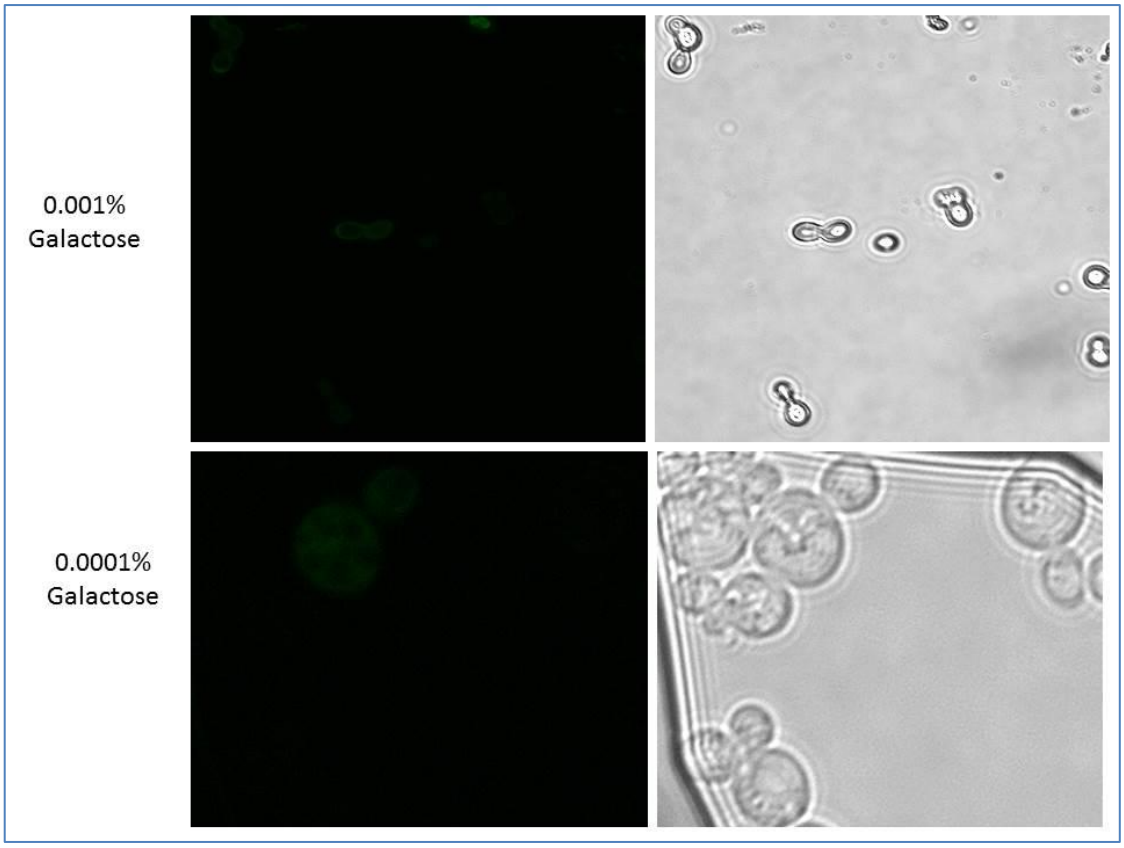


Figure 21: Fluorescent and DIC images indicating different level of TDP43-YFP

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3.8. Effect of 0.1% Galactose and 3mM zinc chloride.

The concentration of galactose and zinc as determined by previous experiments was used. 0.1% galactose was used to maintain the balance between aggregation and toxicity caused by the protein.

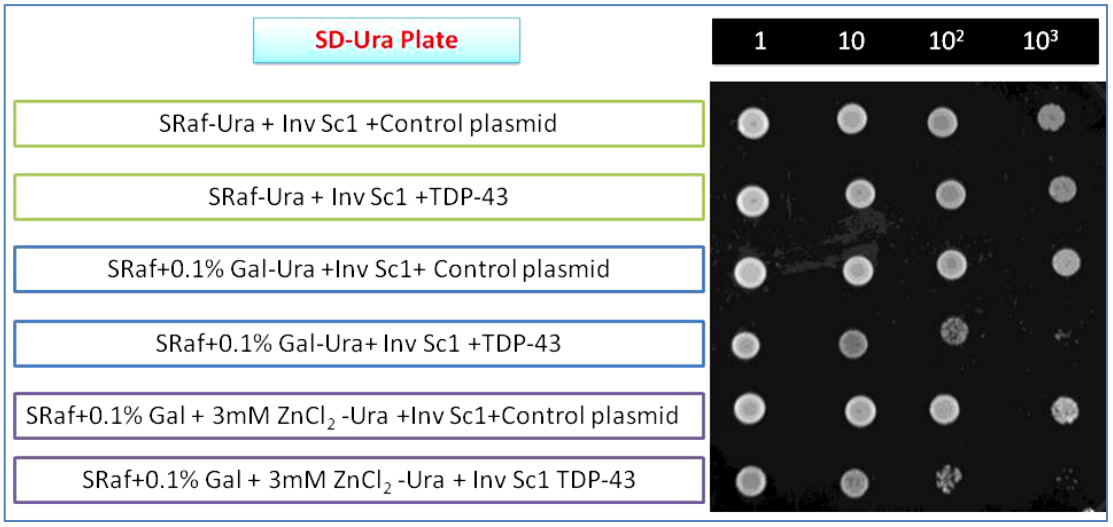

Figure 22: Effect of 0.1% Galactose and 3mM $ZnCl_2$

3.9. Effect of 1% Galactose and 3mM zinc chloride.

The concentration of galactose and zinc as determined by previous experiments was used. 1% galactose was used for high level of expression of the protein. Toxicity caused by the protein increased in presence of zinc

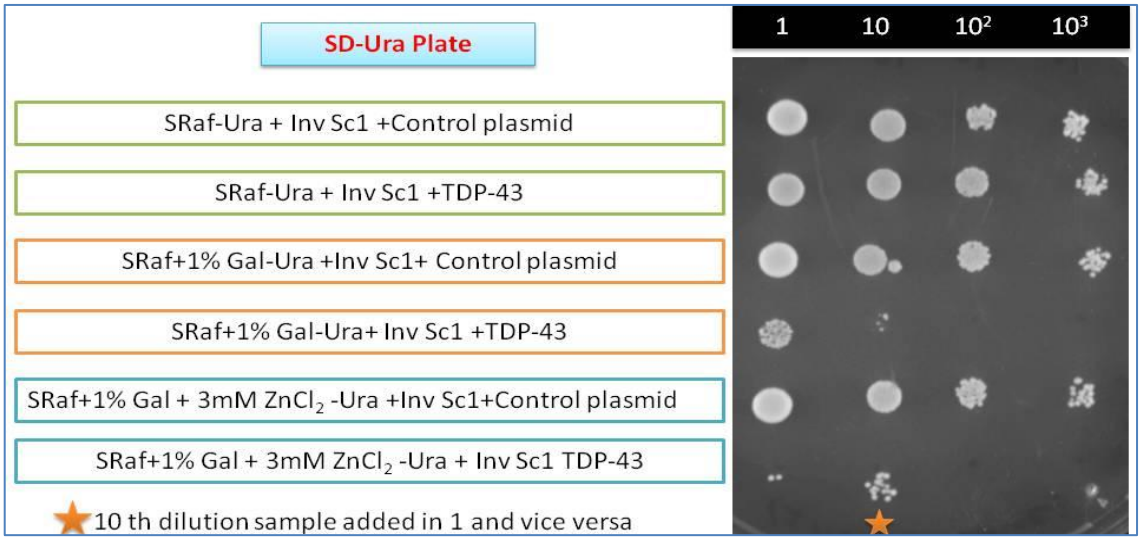

Figure 23: Effect of 1% Galactose and 3mM $ZnCl_2$

3.10. Concentration optimization of copper sulphate

Results obtained from the previous experiments suggest that mill molar concentration of copper is toxic to the cells. The micro molar concentrations are not toxic to the yeast cells. Hence, concentrations in the range of 0.1 mM to 5 mM needs to be checked.

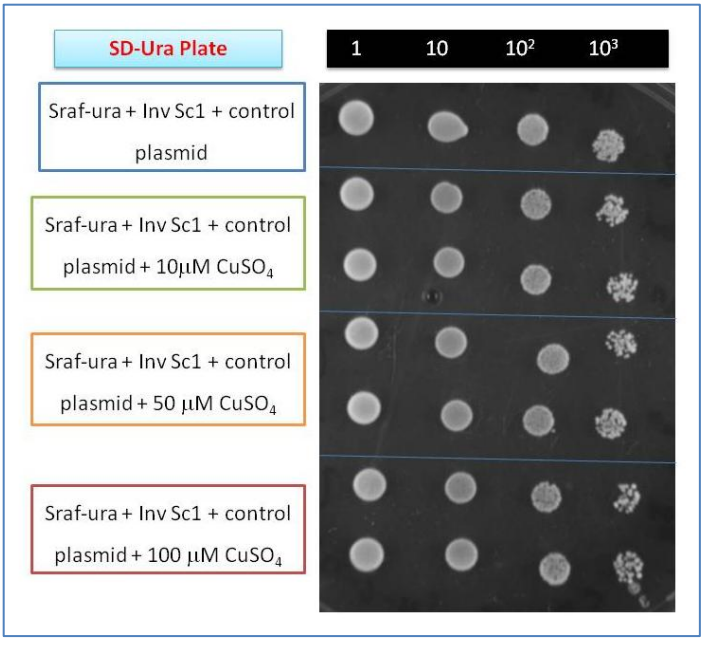

Figure 24: Optimization of copper concentration

3.11. Toxicity of Aβ42-Sup35MC expression in yeast

Aβ42-Sup35MC was expressed by inducing the CUP promoter with 50 μM copper sulphate. Growth of cells expressing Aβ42-Sup35MC (YPD+50 μM copper sulphate) is lesser than the cells not expressing Aβ42-Sup35MC (YPD).

YPG contains 2% Glycerol instead of 2% dextrose and it forbids the growth of respiratory defective petite cells which cannot survive on non fermentable carbon sources and allows the growth of healthy cells [52].

The cells expressing Aβ42-Sup35MC seem to have mitochondrial dysfunction as the cell growth is inhibited on YPG plate as compared to the cells not expressing Aβ42-Sup35MC.

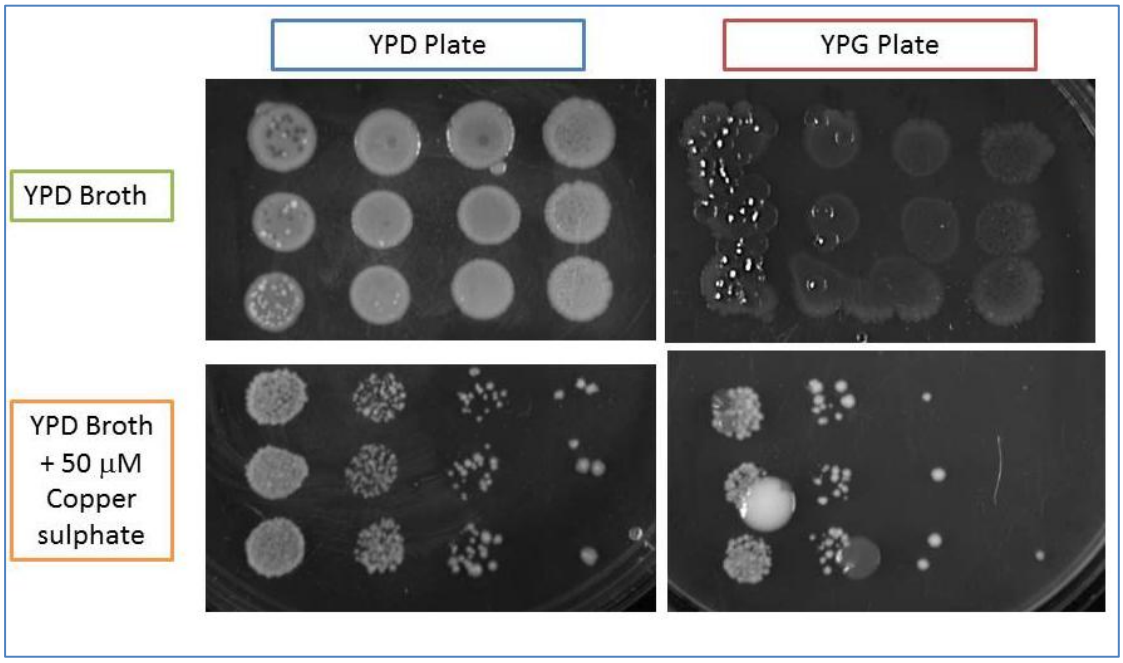

Figure 25: Growth of cells expressing Aβ42-Sup35MC on YPD and YPG

3.12. Search for Aβ42-Sup35MC amyloid variants

Aβ42 fused to the functional domain of the Sup35, when expressed in yeast produces Aβ42-Sup35MC fusion proteins, which in the [*PSI*+] *ade1–14* strain, will give rise to rise to red colonies on rich growth medium (YPD) due to functional C-terminal region of Sup35MC. If the protein is insoluble then it will give white colonies indicative of prion formation leading to loss of function of C-terminal of Sup35MC. It has been observed that at least two different strains of Aβ prions i.e. Arctic and Swedish strains exist [45]. It is

being proposed that different strains of Aβ can be obtained in the yeast model, which can act as a better disease model for AD.

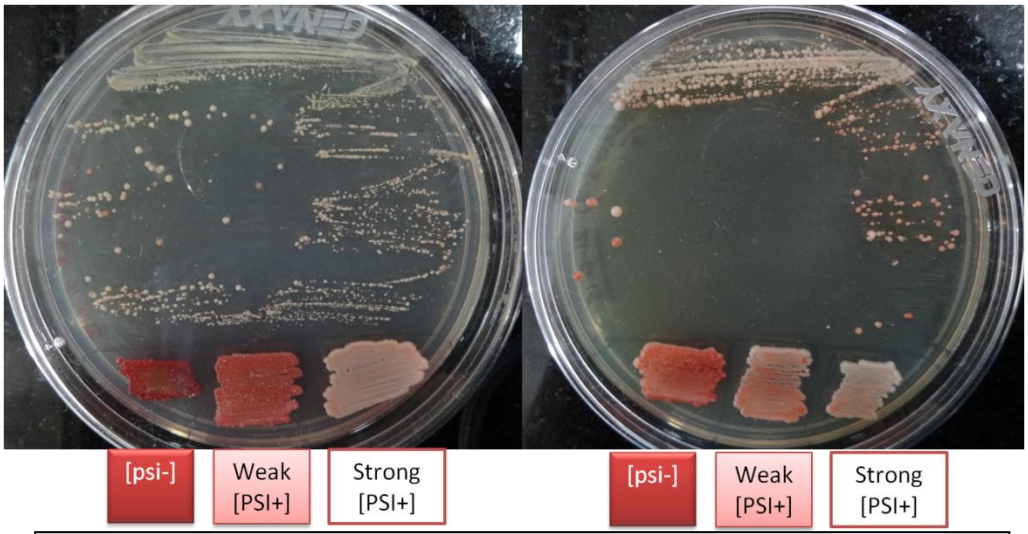

Figure 26: Different coloured strains (White, pink and red) of Aβ42-Sup35MC fusion protein in yeast cells.

Cells on YPD not expressing Aβ42-Sup35MC fusion protein show 7.14% (5/70) petite cells. And cells expressing Aβ42-Sup35MC fusion protein show 13.12% (29/221) petites.

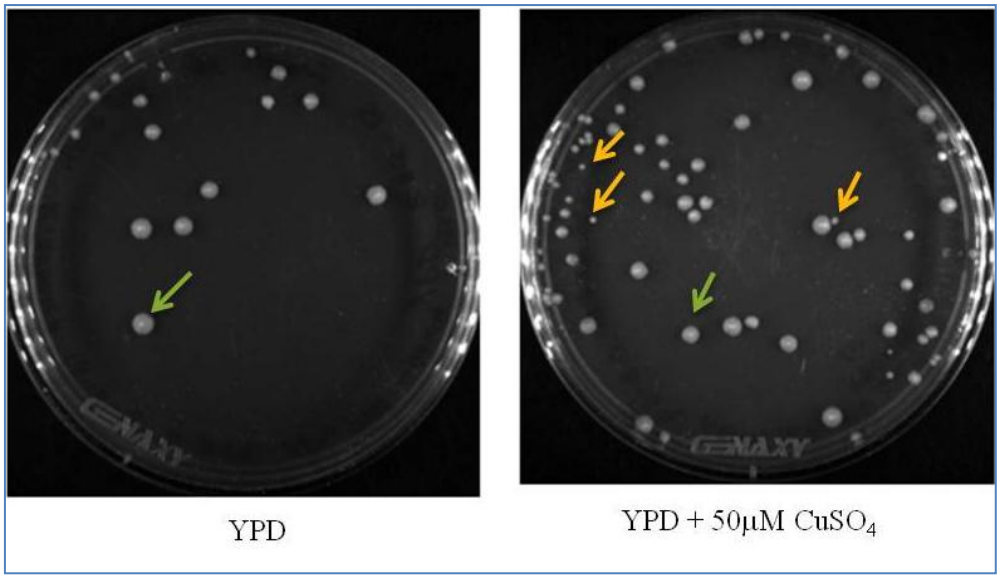

Figure 27: Showing petite cells are more in Aβ42-Sup35MC expressing cells induced with 50 μM copper sulphate, than the non expressing cells. Healthy colony is indicated by green arrow and petite colony indicated by yellow arrow.

Chapter 4

4. Conclusion

In this work, yeast model of ALS was studied using fluorescence microscopy and spot assay to check for toxic effect of TDP43-YFP expression on the growth of cells. When basal level of protein expression is maintained, diffused fluorescence is observed which implies the protein is in monomeric state. When the protein is overexpressed, the TDP43-YFP fusion proteins tend to aggregate and form fluorescent foci. The TDP43-YFP when expressed in yeast forms toxic aggregates which hinders the growth of cells. Effect of zinc was also checked on the aggregation of TDP43-YFP. The data shows that zinc promotes the aggregation of TDP43-YFP in yeast.

Aβ42 fused to the functional domain of the Sup35, when expressed in yeast acts as a disease model for AD. This model has been used for the screening of potential anti-oligomer drug. Prions can propagate as different strains due to different conformation of the protein aggregates and it has been observed that at least two different strains of Aβ prions i.e. Arctic and Swedish strains exist. It is being proposed that different strains of Aβ prions can be obtained in yeast model as well, which can act as a better disease model for AD. Preliminary data shows that expression of Aβ42 in yeast causes mitochondrial dysfunction.

Chapter 5

5. References

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