Study on the effect of Stress conditions on Alzheimer's disease using yeast Aβ42-MRF oligomerisation model

N. Aarthi

BO14MTECH11005

A Dissertation Submitted to

Indian Institute of Technology Hyderabad

In Partial Fulfillment of the Requirements for

The Degree of Master of Technology

Department of Biotechnology

June, 2016

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.

N.Aarthi

BO14MTECH11005

Approval Sheet

This thesis entitled **"Study on the effect of Stress conditions on Alzheimer's disease using yeast Aβ42-MRF Oligomerisation model"** by N.Aarthi is approved for the degree of Master of Technology from IIT Hyderabad.

Basant Kumar Patel

Dr. Basant Kumar Patel
Thesis Advisor
Department of Biotechnology
IIT Hyderabad

N.K. Raghavendra

Dr. NK Raghavendra
Department of Biotechnology
IIT Hyderabad

Dr. Anamika Bhargava
Department of Biotechnology
IIT Hyderabad

Dr. Parag Pawar
Department of Chemical Engineering
IIT Hyderabad

Acknowledgements

I take this opportunity to express my deep sense of gratitude and indebtedness to my supervisor Dr. Basant Kumar Patel for his indispensable guidance and encouragement throughout the period of the M.Tech thesis work. I also acknowledge my sincere regards to my thesis committee members: Dr. Anamika Bhargava, Dr. Rajakumara Eerappa, Dr. NK Raghavendra, Dr. Jyothsnendu Giri and Dr. Parag Pawar for their thoughtful and valuable comments, constructive criticism and tireless review of the work.

I would like to add a special note of thanks to my senior lab mates, S. Vishwanath, Archana Prasad, Neetu Sharma, Aman and my colleague Vidhya Bharathi for their kind help and support whenever it was required.

I am highly grateful to my family for selflessly extending their ceaseless help and moral support at all times.

Abstract

Alzheimer's disease (AD) is a severe neurodegenerative disorder characterized by an extracellular deposition of amyloid plaques, and an intraneuronal accumulation of neurofibrillary tangles in the brain of affected individuals. A 42 amino acid long Aβ42 peptide generated by proteolytic processing of the APP protein is a major component of the amyloid plaques, in which it is mainly represented in the form of detergent-insoluble amyloid fibers. Previously, the Aβ42 fibers have been considered to be the major pathogenic agents of AD. Thus Small molecules that prevent the formation of Aβ42 aggregates that lead to the formation of large plaques had previously been of interest. Recently, this hypothesis has been challenged by findings suggesting that fibrillar aggregates may represent inert dead-end products of the Aβ42 aggregation pathway. Considerable evidence now suggests that the primary neurotoxic effects are associated with soluble SDS-stable assemblies of Aβ42, such as 56 kDa Aβ42 dodecamers, or even smaller, low-n (dimers, trimers, and tetramers) oligomers of Aβ42, which seem to appear during the early stages of Aβ42 assembly, and could give rise to larger oligomers. Thus, the focus of putative therapeutic interventions have shifted towards unravelling compounds that inhibit the earliest stages of Aβ42 oligomerization.

In yet another finding, the frequency of [PSI^{+}] induction, the prion of Sup35 (Yeast Translation Termination Factor) increased with stress. This stress related prion induction can be extrapolated to AD using Aβ42-MRF oligomerisation model that mimics [PSI^{+}].

Contents

Chapter 1

Introduction

1.1 Amyloids and Prion diseases

Proper folding of proteins is essential for proper functioning of proteins. A broad range of human diseases arises from the failure of a specific peptide or protein to adopt, or remain in, its native functional conformational state. These pathological conditions are generally referred to as "*protein misfolding diseases*". They include pathological states in which an impairment in the folding efficiency of a given protein results in a reduction in the quantity of the protein that is available to play its normal role. This reduction can arise as the result of one of several post translational processes, such as an increased probability of degradation via the quality control system of the endoplasmic reticulum, or the improper trafficking of a protein [1].

The largest group of misfolding diseases, however, is associated with the conversion of specific peptides or proteins from their soluble functional states ultimately into highly organized fibrillar aggregates. These structures are referred as "amyloid fibrils" (Fig.1). 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]. Amyloids have cross-β structure where the peptide molecules form β-strands perpendicular to the axis of the fibril [2]. These fibrils bind specifically to Thioflavin T (ThT) dye causing increase in the fluorescence [3] and Congo red (CR) dye causing green birefringence when viewed under cross-polarized light [4].

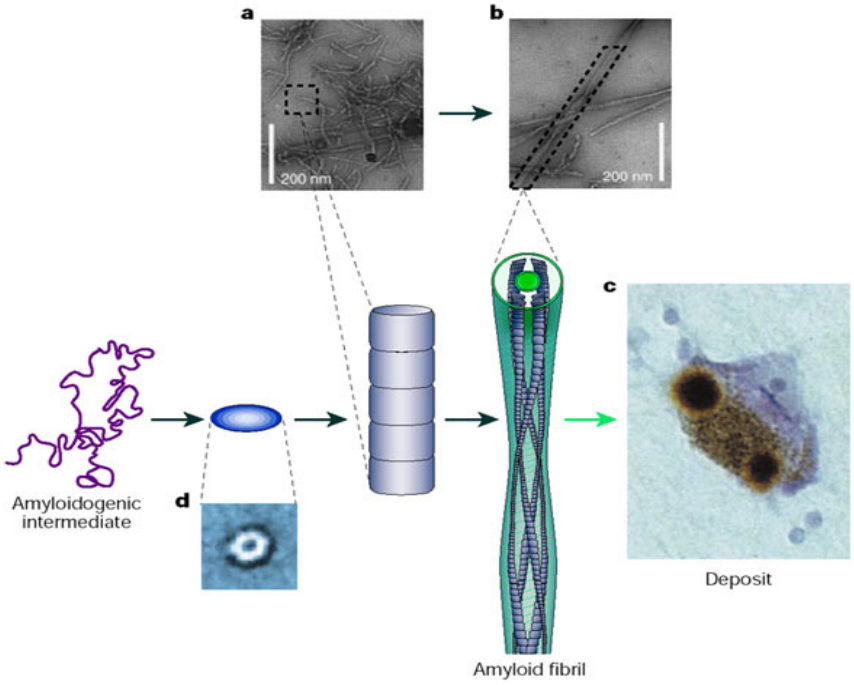

Fig 1: Formation of amyloid fibrils [5]

a. Electron microscopy image of Protofibrils
b. Electron microscopy image of mature fibrils
c. Fibrils accumulate as plaques or lewy bodies
d. Early aggregates in Amorphous structure

Misfolding of a protein is the main cause of major neurodegenerative diseases like Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis etc [5].

Table 1: List of examples of neurodegenerative protein misfolding diseases [6]

Disease	Genetic causes	Function
Alzheimer's disease	APP	Gives rise to Aβ, the primary component of senile plaques
Parkinson's disease	PS1 and PS2	A component of γ-secretase, which cleaves APP to yield Aβ
Parkinson's disease	α-Synuclein	The primary component of Lewy bodies
Parkinson's disease	Parkin	A ubiquitin E3 ligase
Parkinson's disease	DJ-1	Protects the cell against oxidant-induced cell death
Parkinson's disease	PINK1	A kinase localized to mitochondria. Function unknown. Seems to protect against cell death
Parkinson's disease	LRRK2	A kinase. Function unknown
Parkinson's disease	HTRA2	A serine protease in the mitochondrial intermembrane space. Degrades denatured proteins within mitochondria. Degrades inhibitor of apoptosis proteins and promotes apoptosis if released into the cytosol
Amyotrophic lateral sclerosis	SOD1	Converts superoxide to hydrogen peroxide. Disease-causing mutations seem to confer a toxic gain of function
Huntington's disease	Huntingtin	Function unknown. Disease-associated mutations produce expanded polyglutamine repeats

1.2 YEAST: Model for Prion and Amyloid diseases

The yeast *Saccharomyces cerevisiae* is an ideal *in vivo* model to study human disease mechanism due to conserved basic cellular mechanisms, rapid growth on defined media and easy mutant isolation [7]. Yeast provides a model system for studying

mechanisms of protein misfolding and aggregation, which is also applicable to human diseases [8].

Yeast has been used as a model system to study the disease mechanism of various neurodegenerative diseases like Alzheimer's [9], Parkinson's [10], and Huntington's [11] by expression of the pathological protein.

Sup35 protein is involved in the recognition of stop codons in yeast. Sup35 N-terminal domain [1-123] is unstructured (Fig.2) 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 [12].

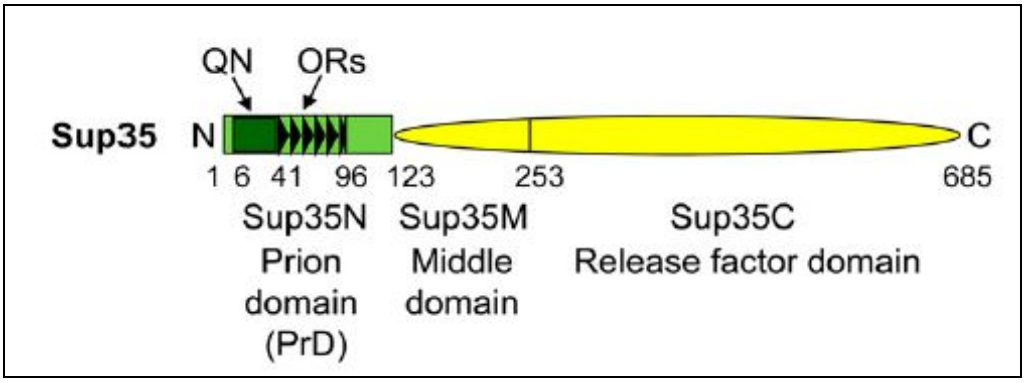

Fig 2: Domain architecture of Yeast Sup35 Protein [13].

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 [13]. [*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. Sup35 tagged with GFP is diffuse in [*psi-*] cells but forms aggregates in [*PSI+*] cells. When total cell lysates (T) are separated into supernatant (S) and pellet (P) fractions and Western blots made of boiled SDS acrylamide gel separations are developed with Sup35 antibody,Sup35 is found largely in the supernatant in [*psi-*] lysates but mostly in the pellet in [*PSI+*] lysates. When lysates are separated on agarose gels, following room temperature incubation with 2% SDS, Sup35 runs as a monomer in [*psi-*] cells but mostly as oligomers in the other case [13].

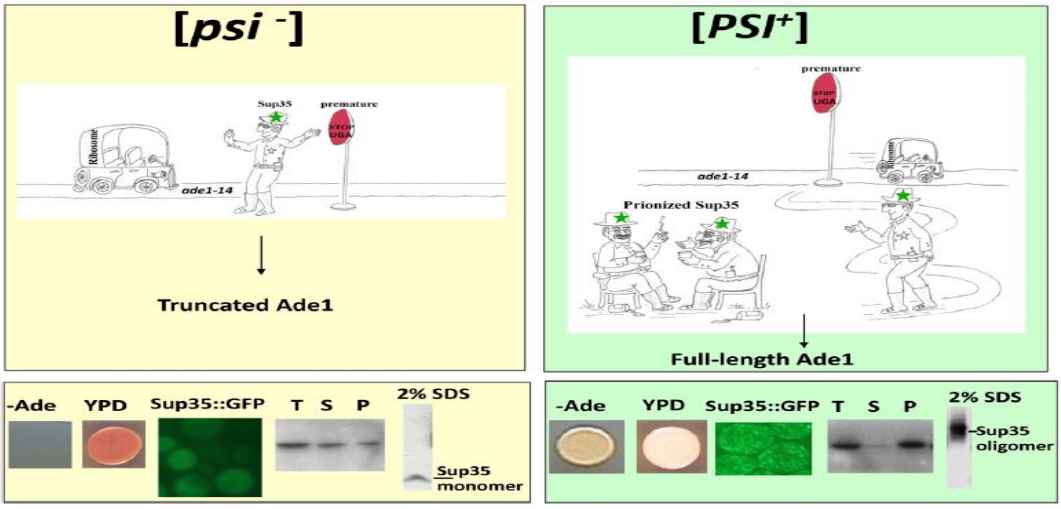

Fig 3: [*PSI+*] Phenotypes [13]

1.3 Prion Variants

Different heritable states of prions are called as prion variants (Fig: 4). Different [PSI+] variants were associated with inherently different ratios of aggregated vs. non-aggregated Sup35 protein [13].

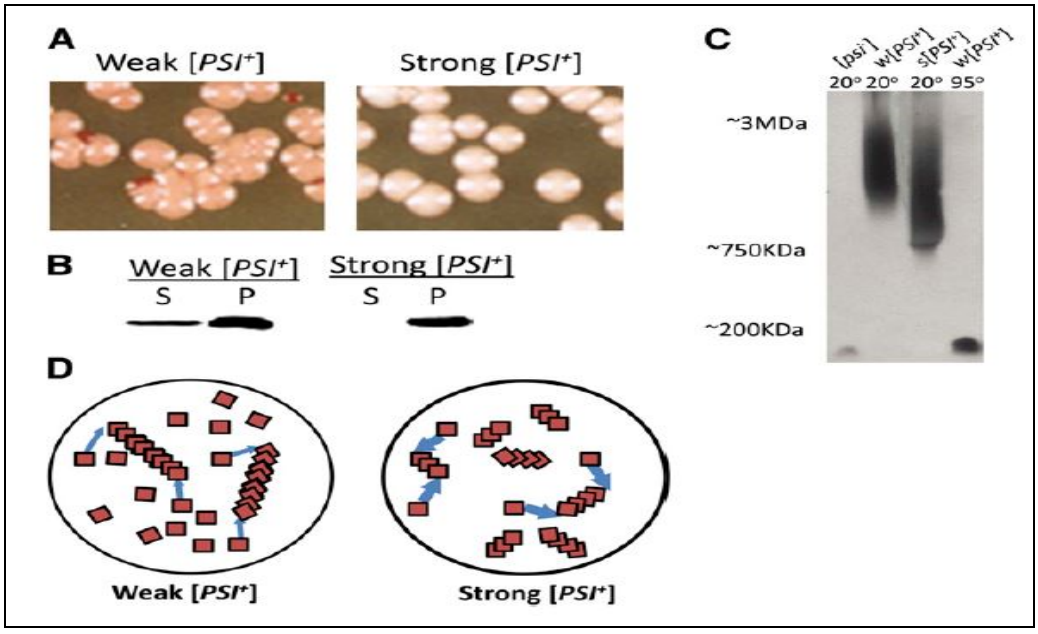

Fig 4: Differences between weak and strong [PSI+] prion variants [13].

A. Colony colour difference (red vs pink)
B. Levels of soluble Sup35 in weak vs. strong [PSI+] strains
C. Size comparison of SDS resistant Sup35 prion polymers
D. Cartoon of weak vs. strong [PSI+]

1.4 Alzheimer's disease (AD)

Alzheimer's disease (AD) is a severe neurodegenerative disorder characterized by an extracellular deposition of amyloid plaques, and an intraneuronal accumulation of neurofibrillary tangles in the brain of affected individuals [14].

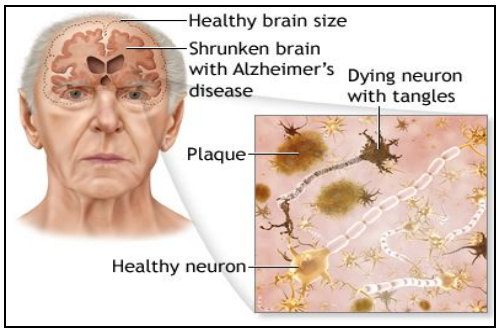

Fig 5: Hallmark of AD [15]

A 42 amino acid long Aβ42 peptide generated by proteolytic processing of the APP (Amyloid Precursor Protein) protein is a major component of the amyloid plaques, in which it is mainly represented in the form of detergent-insoluble amyloid fibers.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 [14].

1.5 Stress conditions and AD

Prion switching in response to environmental stress was studied which concluded that prion induction increased by as much as 60-fold when cells were exposed to various stressful conditions, such as oxidative stress (H2O2) or high salt concentrations [16].

Since the frequency of [PSI^{+}] induction, the prion form of Sup35 (Yeast Translation Termination Factor) increased with stress. This stress related prion induction can be extrapolated to AD using yeast Aβ42-MRF oligomerisation model that mimics [PSI^{+}] [16].

1.6 Aβ42-MRF Oligomerisation model

Several aggregated forms of the amyloid β peptide (Aβ), which are generated by proteolytic processing of the amyloid precursor protein (APP), in normal brains and cerebrospinal fluid (CSF) are believed to have a crucial role in the development of Alzheimer's disease (AD). Although extracellular amyloid plaques and neurofibrillary tangles formed by insoluble fibrils in brains are hallmarks of AD, recent findings suggest that smaller non-fibrillar oligomeric forms of the Aβ peptide are a more likely cause of AD.

Indeed, studies in mice as well as mammalian cell culture showed that detergent-stable Aβ oligomers are potent neurotoxins. Thus the Prevention of formation of Aβ42 fibers that lead to plaque deposits was therapeutic area of interest previously. Recently focus shifted to earlier stages of Aβ42 oligomerisation [17].

Yeast Aβ42-MRF model provides a tool to screen for agents that target early stages of Aβ42 oligomerisation. It is an Aβ42 fusion to the essential functional domain (MRF) of the translational release factor, Sup35 provides an Aβ42 aggregation-specific probe tied to a functional Sup35 readout [17].

Chapter 2

Materials and Methods

2.1 Yeast strain

The yeast strain L3149 containing a genomic deletion of *SUP35* (*sup35Δ*::*LEU2*) was used. The strain was maintained under the copper-inducible *CUP1* promoter p1364-based *URA3* plasmid encoding Aβ42-MRF.

2.2 Yeast culture media

Standard yeast media YPD 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 mins 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).

2.3 Yeast cultivation

A single colony of yeast cells were spotted on agar plates, and incubated at 30°C for 3 days (complex medium). The desirable colour saturation on complex medium was achieved by incubating the plates at 4°C for 3 more days.

Expression of the Aβ42MRF constructs driven by the copper-inducible *CUP1* promoter was stimulated by the addition of 50 μM $CuSO_4$ to all media. Low concentration of $CuSO_4$ used was 1 μM.

2.4 Prion induction study with stress conditions

Stress conditions used were 2mM H_2O_2, 10mM H_2O_2, 12mM Dithiothreitol (DTT), 1M Ammonium Acetate (NH_4Ac). 1 ml of YPD broth was inoculated with Aβ42-MRF L3149 strain with the stress conditions applied and grown till 0.6 OD_{600} in rotary shaker at 200 rpm at 30°C. One to five fold serial dilutions were performed using 100 μl of the culture. 5 μl of the culture was spotted in duplicates on YPD and YPD + 1 μM $CuSO_4$ plates and was incubated at 30°C, for 2 days YPD.

Chapter 3

Results and Discussion

3.1 Yeast Growth

Usually, when Sup35 protein, which is a translation termination factor, is over expressed it leads to prion conversion to [PSI+] that partially inactivates Sup35 protein thereby allowing read-through of the premature stop codon in ade1-14 allele. This leads to functional ADE1 protein formation that allows for growth on media lacking adenine and also, the red intermediate pigment does not accumulate hence the [PSI+] yeast is white on YPD-agar. Thus, this red to white conversion in ade1-14 allele containing yeast strain (e.g. 74-D-694) upon over-expression of Sup35 protein is scored as de novo [PSI+] prion formation. This applies to scoring of Aβ42 amyloid aggregation in strain containing Sup35 fusion protein.

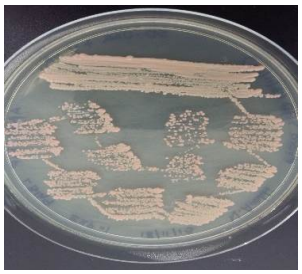

Fig 6: Aβ42-MRF L3149 on SD –URA +1/4 YPD

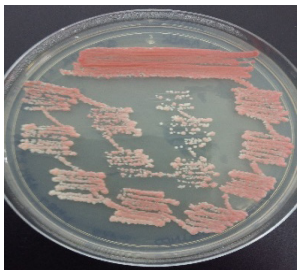

Fig 7: [PSI^-] [PIN^-] on SD –URA +1/4 YPD as control.

The colony colour differences were observed for strain with and without Aβ42 amyloid. The appearance of pink colonies in fig: 6 may be due loss of plasmid.

3.2 Prion induction study with stress conditions

Normally when stress is not applied, the yeast grows pink on YPD with least copper concentration while all white with 50µM $CuSO_4$. But when stress is applied, the colony grows into white as well as pink on YPD with $CuSO_4$. The appearance of white colonies may be due to aggregation of amyloid induced by stress applied.

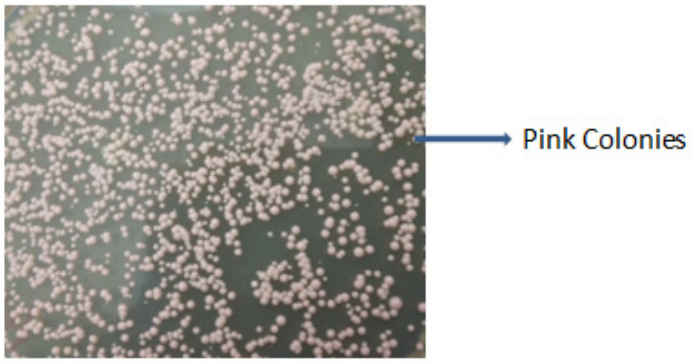

Fig 8: Aβ42 –MRF L3149 on YPD (without any stress)

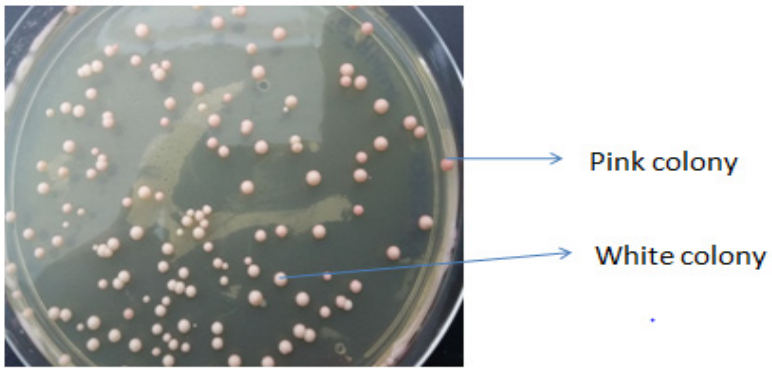

Fig 9: Aβ42 –MRF L3149 on YPD (with 2mM H_2O_2)

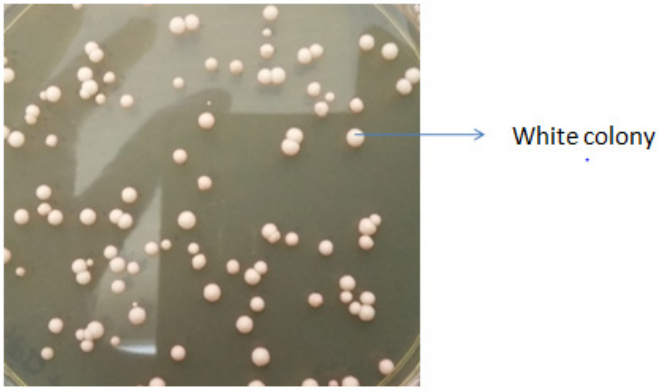

Fig 10: Aβ42 –MRF L3149 on YPD + 1µM $CuSO_4$

(with 2mM H_2O_2)

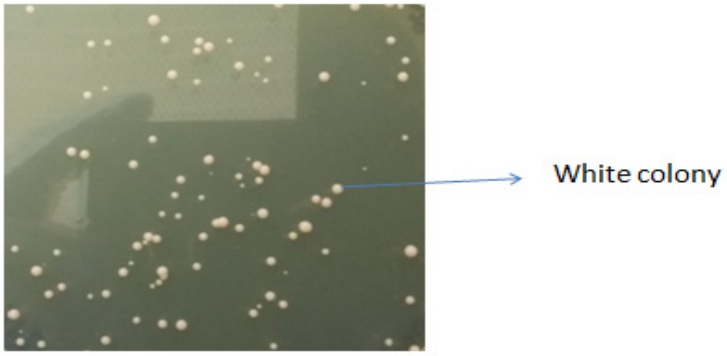

Fig 11: Aβ42 –MRF L3149 on YPD (with 10mM H_2O_2)

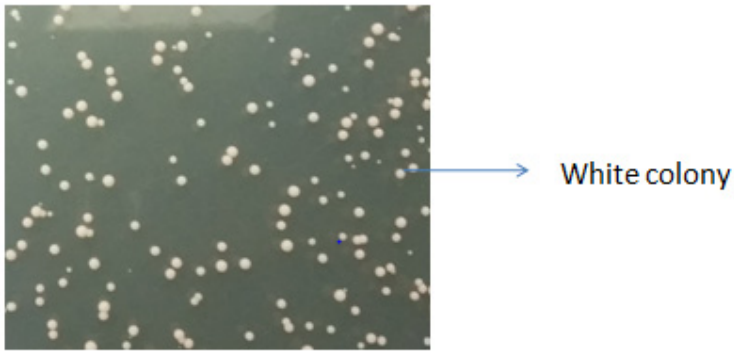

Fig 12: Aβ42 –MRF L3149 on YPD + 1µM $CuSO_4$

(with 10mM H_2O_2)

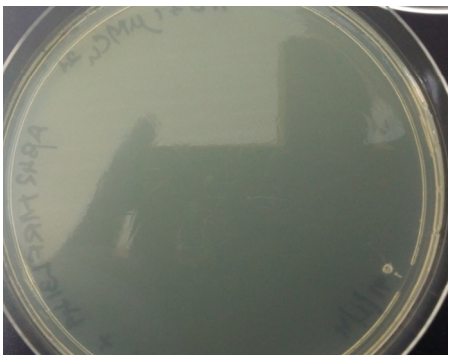

Fig 13: Aβ42 –MRF L3149 on YPD + 1μM $CuSO_4$

(with 12mM DTT)

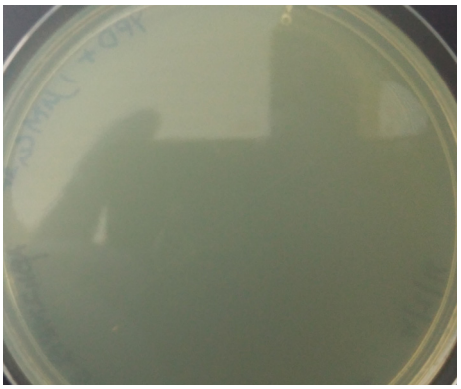

Fig 14: Aβ42 –MRF L3149 on YPD + 1μM $CuSO_4$

(with 1M NH_4Ac)

Table 2: Amyloid Aggregation with H_2O_2

	Stress Conditions	Total no. of colonies	No. of pink colonies	No. of white colonies	Percentage of white colonies (%)
YPD	H2O2 (2mM)	146	22	124	84
YPD	H2O2 (2mM)	156	35	121	77
YPD + 1µM CuSO4	H2O2 (2mM)	161	4	157	97
YPD + 1µM CuSO4	H2O2 (2mM)	178	9	169	94
YPD	H2O2 (10mM)	141	-	141	100
YPD	H2O2 (10mM)	167	-	167	100
YPD + 1µM CuSO4	H2O2 (10mM)	176	-	176	100
YPD + 1µM CuSO4	H2O2 (10mM)	165	-	165	100

There were white colonies formed with 2mM and 10mM H_2O_2 while no growth was observed when treated with 12mM DTT and 1M NH_4Ac. The latter may have been lethal to the cells hence no growth was observed. From Table 2, Treatment with H_2O_2 have caused aggregation of amyloid thus forming white colonies.

Chapter 4

Conclusion

In this study, amyloid aggregation of Aβ42 was found when treated with H_2O_2 . The percentage of white colonies formed were higher with higher concentrations of H_2O_2. There was no growth or aggregation observed with DTT or NH_4Ac. The Duo might have been lethal to the cells hence no growth of cells.

Stress conditions are found to increase induction of [*PSI* $^{+}$] formation in yeast, thus there is also a chance for its link with AD when exposed to various stress conditions [16].

References

1. Chiti, Fabrizio, and Christopher M. Dobson. "Protein misfolding, functional amyloid, and human disease." *Annual Review Biochem.* 75 (2006): 333-366.

2. Levine 3rd, H. (1993). Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein science: a publication of the Protein Society*, *2*(3), 404.

3. Sunde, M., Serpell, L. C., Bartlam, M., Fraser, P. E., Pepys, M. B., & Blake, C. C. (1997). Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *Journal of molecular biology*, *273*(3), 729-739.

4. Divry, P. Etude histochimique des plaques seniles [Histochemical study of senile plaques]. *J Belge de Neurologie et de Psychiatrie* 1927. 27:643–657.

5. Forman, Mark S., John Q. Trojanowski, and Virginia MY Lee. (2004) "Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs." *Nature medicine* 10.10: 1055-1063.

6. Botstein, David, Steven A. Chervitz, and J. Michael Cherry. (1997) "Yeast as a model organism." *Science (New York, NY)* 277.5330: 1259-1260.

7. Outeiro, Tiago Fleming, and Paul J. Muchowski. (2004) "Molecular genetics approaches in yeast to study amyloid diseases." *Journal of Molecular Neuroscience 2*3.1-2: 49-59.

8. Liebman, Susan W., and Yury O. Chernoff. (2012)"Prions in yeast." *Genetics* 191.4: 1041-1072.

9. Park SK, Pegan SD, Mesecar AD, Jungbauer LM, LaDu MJ, Liebman SW. (2011) "Development and validation of a yeast high-throughput screen for inhibitors of $A\beta_{42}$ oligomerization". *Diseases Models and Mechanisms*. 4.6.: 822-31.

10. Outeiro, Tiago Fleming, and Susan Lindquist. (2003) "Yeast cells provide insight into alpha-synuclein biology and pathobiology." *Science* 302, no. 5651: 1772-1775.

11. Mason, Robert P., and Flaviano Giorgini. "Modeling Huntington disease in yeast." *Prion* 5.4 (2012): 269-76.

12. Johnson, Brian S., J. Michael McCaffery, Susan Lindquist, and Aaron D. Gitler. (2008) "A yeast TDP-43 proteinopathy model: Exploring the molecular determinants of TDP-43 aggregation and cellular toxicity." *Proceedings of the National Academy of Sciences* 105, no. 17: 6439-6444.

13. Sviatoslav Bagriantsev and Susan Liebman, "Modulation of Aβ42 low-n oligomerization using a novel yeast reporter system". *BMC Biology* 2006, **4**:32.

14. Jossé, L., Marchante, R., Zenthon, J., von der Haar, T., & Tuite, M. F. (2012) "Probing the role of structural features of mouse PrP in yeast by expression as Sup35-PrP fusions." *Prion* 6.3: 201-210.

15. Knopman DS. Alzheimer disease and other dementias. In: Goldman L, Schafer AI, eds.*Goldman's Cecil Medicine*. 25th ed. Philadelphia, PA: Elsevier Saunders; 2016:chap 402.

16. Jens Tydmers *et al.*, "Prion Switching in Response to Environmental Stress". *PLoS Biology* 2008, 6, 2605-2613.

17. Sei-Kyoung *et al.*, "Development and validation of a yeast high-throughput screen for inhibitors of Aβ42 oligomerization". *Disease Models & Mechanisms* 4 2011.