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Bioaccumulation of Zinc, Characterization and Quantitative Estimation of Zinc Binding Proteins in *Saccharomyces Cerevisiae*

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ABSTRACT

The discharge of industrial waste containing heavy metals and synthetic compounds into the environment causes significant environmental and health risks. Heavy metals can bind to metal binding sites on plasma membranes of the cells in living organisms. These binding sites are often specific for essential metals like zinc, iron, copper and calcium.The heavy metals may have a higher affinity for these binding sites compared to essential metals, leading to their displacement. By occupying metal binding sites, heavy metals can inhibit the normal functions of essential metals.They may interfere with the transport of ions across cell membranes, affecting critical cellular processes. This interference can disrupt enzymatic activities, as many enzymes require specific metal cofactors for their proper functioning. The substitution of essential metals by toxic heavy metals can lead to enzyme inactivation. The bioaccumulation of heavy metals in yeast species, particularly *Saccharomyces cerevisiae*, has gathered considerable attention. *S.cerevisiae* is an ideal model organism to identify the mechanism of biosorption. *S.cerevisiae* is a unicellular eukaryotic organism that shares many essential features with higher eukaryotes. Yeast was cultured in the presence of varying concentrations of Zn^{2+} (5, 10, 20, 50 and 100µg/ml).The accumulated zinc by *S.cerevisiae* was measured and correlated with changes in protein content. A decrease in protein content was observed when the concentration of Zn^{2+} exceeded 20µg/ml. Proteins extracted from yeast cells exposed to different concentrations of Zn^{2+} were separated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. SDS-PAGE allows for the separation of proteins based on their molecular weight.The molecular weight of individual proteins was estimated by comparing their migration patterns on the SDS-PAGE gel to molecular weight markers.The study aims to characterize Zn^{2+} binding proteins in *S.cerevisiae* by

analyzing the protein profiles under different zinc concentrations. Proteins that show changes in abundance or migration patterns in response to zinc concentrations may be postulant for Zn^{2+} binding proteins.The decrease in protein content with higher zinc concentrations suggests a potential negative impact of excess zinc on cellular protein synthesis or stability. Identification and characterization of Zn^{2+} binding proteins can provide insights into the cellular response to zinc stress and contribute to our understanding of metal homeostasis in yeast.

Keywords : Zinc, *Saccharomyces cerevisiae*, Bioaccumulation, SDS-PAGE.

I. INTRODUCTION

Elements, which are required as nutrients for maintaining normal growth of a microorganism may be metals or nonmetals. Metals have been classified for biological systems on the basis of their relative necessity and quantity in which they are required. Metals required in large amounts are major metals and those required in traces are trace metals respectively. Essential metals include major metals such as Na, K, Mg, Ca, Fe and trace metals such as Cu, Zn and Mo etc. Trace metals function as key components of essential enzymes or other proteins. Metal which has density greater than $5g/cm^3$ is termed as heavy metal[1-3]. Zinc is a transition metal having density $7.14gm/cm^3$.

Zinc is available in Earth's crust and on the surface as zinc ores and in seawater. It is amongst the most available of the trace elements. Zinc occurs exclusively as Zn^{2+} divalent cation in nature and found as ZnS in earth's crust. Zinc makes a major part of brass and is used as the negative electrode in sealed "dry batteries". Zinc ion is a good electron acceptor. Zn^{2+} being a symmetrical d^{10} ion is fairly hard and interacts strongly with oxygen and nitrogen donors. It polarizes groups to which it binds and either increases the attacking power of a bound base or increases the probability of attack on the bound group by acting as an acid. Zinc does not form free radical ions, so it has antioxidant properties[4]. Zinc is known for its ability to adopt various coordination geometries, including tetrahedral, trigonal bipyramidal, and octahedral. This flexibility arises from the intermediate size of the zinc ion and its ability to accommodate different ligands. Zinc passes readily from one symmetry of its surrounds to another without exchange and exchanges ligands quickly. The coordination geometry of zinc is essential for its interactions with biomolecules, such as proteins and nucleic acids. The ability of zinc to coordinate with proteins in different geometries allows it to participate in the catalysis of various biochemical reactions and stabilize protein structures. Such factors favor a higher catalytic activity since they allow rapid atom flow including substrate binding. Furthermore, zinc is redox inert enabling the formation of relatively stable associations within the cellular environment[4-7]. Zinc is essential for the normal growth and development of mammals as it is required for proper functioning of a large number of proteins, including various enzymes.

Zinc is a component in such a variety of enzymes and DNA binding proteins like zinc finger protein[8]. Zn^{2+} is found in Carboxypeptidase and Carbonic Anhydrase. Zinc enzymes in cells are involved in a great variety of enzyme reactions in the cytoplasm. Zinc digestive enzymes are active outside cells or in vesicles. Zinc appears in the enzyme Levulinic Dehydratase, which controls porphyrin synthesis and hence a vast range of

redox reactions. Zinc is very closely involved in the nitrogen metabolism of the plants. Its deficiency reduces the protein synthesis. However, Zn(II) is harmful at higher concentrations. Concentrations of zinc in the dry matter of plant tissue from 150-200µg/mg are considered as toxic.These heavy metals may be removed from the environment by some physical and chemical methods such as: reverse osmosis, ion exchange, electrolysis, precipitation and reduction[9-13]. Physico-chemical methods have several disadvantages such as high reagent requirement, unpredictable metal ion removal, formation of sludge and its disposal, high installation and operational costs etc.

The use of microbes as biosorbents for heavy metals offers a potentially inexpensive alternative compared to conventional methods of heavy metal removal from the water bodies[14]. Different microorganisms such as fungi, bacteria, yeasts and algae were used by many workers for removal of heavy metal ions[15,16]. Microorganisms accumulate metal by membrane transport and biosorption[17]. Biosorption based on location of the metal accumulated may be extra cellular, cell surface sorption or intracellular accumulation. Physicochemical interactions which occur between the metal and functional groups of the cell surface are based on ion exchange, complexation and physical adsorption which are independent of the metabolism. Accumulation of essential and non-essential metal ions may differ by excretion and storage processes.

Saccharomyces cerevisiae is an ideal model organism for studying various aspects of cell biology. *S.cerevisiae* shares essential cellular processes, such as DNA replication, repair and cell cycle regulation with higher eukaryotes. *S.cerevisiae* is asporogenous yeast found in the soil, in animal excretes, in food products like milk etc. *S.cerevisiae* is used in the baking and brewing industry. It is a readily available source of protein[18]. *S.cerevisiae* has well-defined mechanisms for metal homeostasis, including metal uptake, transport, and sequestration. Studying these processes in yeast can provide insights into how cells regulate metal concentrations, both essential and toxic, within their cytoplasm.The yeast genome contains homologs of metal transporters found in higher eukaryotes. These transporters play a crucial role in the uptake and efflux of metals. Understanding the function of these transporters in yeast can provide insights into similar processes in other organisms.

It has been reported that *S.cerevisiae* accumulate Zn^{2+} cations as well as Cu^{2+}, Pb^{2+}, Cd^{2+} and Co^{2+} ions[19]. For accumulation of essential metals it has been shown that cells produce metal binding proteins for metal accumulation. Metal binding proteins known as Metallothioneins (MT), have a low molecular weight and are cysteine-rich found in plants and eukaryotic microorganisms. MT can be induced by many substances, including heavy metal ions such as Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+} and Hg^{2+} etc[20]. It has been proposed that metallothioneins play an important role in cellular zinc uptake and metabolism. Zinc metabolism is transport metabolism and members of a variety of protein families transport zinc. Three transporter groups such as Cor-A transporter, Mgt-E and Mgt-A have been reported[21-24]. Cor-A transporter transports zinc in *S.cerevisiae*. It has been shown that ZRT-1p high affinity and ZRT-2p low affinity transporters of the ZIP family are involved in uptake of zinc into *S.cerevisiae*[25]. The metal transport system is influenced by the presence of heavy metal ions of the same charge and ionic radius[26]. Zn^{2+} may interact with Mg^{2+} thereby inhibiting its function into the yeast cells.

Characterization and determination of total proteins will show the involvement of different proteins in the accumulation of zinc. Variation in protein contents therefore will indicate the change in metabolic activities in-vivo. Identification and determination of individual protein molecules will enable us to explain the involvement of these proteins in transporting zinc inside and outside the cells. Quantitative estimation of proteins will give appropriate information about the effect of heavy metals on metabolic activities.

Molecular weights of proteins extracted from *S.cerevisiae* can be estimated using standard methods when grown in different concentrations of zinc.

II. Experimental

Inoculums preparation

Yeast can be easily cultured in simple and defined growth media. This simplicity allows for precise control over experimental conditions and facilitates the reproducibility of studies. Stocks of *Saccharomyces cerevisiae* were preserved on YEPD rich medium. It consists of 1% Yeast extract, 2% Peptone, 2% Dextrose and 2% Agar-Agar respectively. Yeast produces colonies on synthetic growth media (SGM). SGM was prepared by dissolving Glucose 0.5%, $(NH_4)_2SO_4$ 0.3%, KH_2PO_4 0.3%, $CaCl_2$ 0.025%, $MgSO_4$ 0.025% and Biotin 0.001% in double distilled water and autoclaved at 1 lb/inch^2 pressure.Yeast cells were cultivated in SGM by shaking on a horizontal shaker for a period of 15h at room temperature.

Growth characteristics

Optical density of SGM was determined per hour by using UV-Visible spectrophotometer at the wavelength of 570 nm at 25ºC temperature for 15h. A plot between time and optical density was drawn for finding out the mid log phase (Figure:1) at which cellular metabolic activities and essential nutrients accumulation become maximum. The mid log phase was obtained when growth of cells arrived at a period of 7h.

Determination of the dry mass of *S.cerevisiae*

Yeast cells were dispersed in SGM containing 5, 10, 20, 50 and 100 µg/ml Zn^{2+} respectively and grown for the period of 7h at 25ºC in aerobic conditions. At mid log phase of the yeast growth, SGM was centrifuged, cells were collected and harvested. These cells were washed using a citrate buffer (pH 4.8) followed by distilled water and then dried. The dry mass of the yeast cells, grown in various concentrations of Zn^{2+} were measured and results compared with the control (Figure:2).

Analysis of accumulated Zinc

Yeast cells were grown for 7h in SGM, having concentrations of Zn^{2+} as 5, 10, 20, 50 and 100 µg/ml respectively. After the period of 7h growth, yeast cells were applied for the centrifugation. After that cells were harvested and washed with the help of a citrate buffer having pH 4.8. These cells were dried and digested with 1% HNO_3 solution[27-29]. Accumulated Zinc by the cells was analyzed by the Atomic Absorption Spectrophotometer and results were compared with the control (Figure:3). Accumulated Zinc in terms of µg/mg of dry mass as well as µg/ng of total protein content was also calculated (Table:1).

Analysis of the total proteins

Yeast were grown in the presence of various concentrations of Zn^{2+} (5, 10, 20, 50 and 100)µg/ml for the period of 7h. Centrifugation was performed, cells were settled down and dried. The dried cells were treated with 10% trichloroacetic acid 5ml and ethanol-ether mixture 5ml, followed with addition of tris glycine buffer (0.2M, pH 8.6)10ml and boiled. The supernatants were used for total protein determination by Lowry's method[30] (optical densities were measured on UV-Visible Spectrophotometer at 610 nm). The total protein contents (µg/ml) were estimated with the help of standard protein curve (a plot of absorbance v/s concentrations of Bovine Serum Albumin) (Figure:4). The results obtained were compared with control (Figure:5). Total proteins in terms of ng/mg of dry mass was also calculated (Table:1).

Separation and molecular weights determination of Zinc binding proteins

The proteins extracted at different concentrations of Zn^{2+} were treated with a stacking buffer (0.5M Tris-HCl) and employed with sodium dodecyl sulphate polyacrylamide gel. The electrophoresis was performed with the help of a disk electrophoresis apparatus under the same conditions and chemical environment which results in the discrete protein bands.The standard protein molecular weight marker (14.3 kDa to 97.4 kDa) was also employed for electrophoresis simultaneously. The proteins get

separated on the basis of their molecular weights. The R_f (relative mobility) values for standards (Table:2) as well as for each band belonging to different proteins (Table:3) were calculated. A plot R_f v/s log M_r was drawn. The molecular weights of SDS-denatured polypeptides were estimated using R_f values by interpolation[31].

III.Results and Discussion

Effect of zinc on growth of *S.cerevisiae*

It is known that zinc has appeared as one of the most essential metals biologically[4]. Zinc enzymes have a significant role in carbohydrate, lipid and protein metabolism[7]. As we know, zinc is an essential trace element for the normal growth of living organisms due to its ability to form coordination geometries and participation in metabolic activities[4].

Increased growth of yeast cells in terms of the dry mass in the presence of Zn^{2+} was observed initially (Figure:2). It can be concluded from the results that 20µg/ml is the optimum concentration of zinc at which increase in the rate of metabolic reactions was observed. At the higher concentrations of Zn^{2+} ions, decreased dry mass values were obtained. At higher concentrations it may be accumulated by some nonspecific uptake systems and transported. In the cytoplasm it may interact with cellular entities affecting their activities. It has been suggested that Zn^{2+} ion has a greater affinity for imidazole groups than Mg^{2+} or Ca^{2+} ions[32] and binds effectively. Therefore Zn^{2+} may dislodge Mg^{2+} ions from its binding site[33]. Zn^{2+} ion has $3d^{10}$ and $4s^0$ configuration, so it can accept more charge from a given ligand than Mg^{2+}, resulting in the formation of a stable complex. Therefore at higher concentrations it results in the decreased growth.

Effect of accumulated zinc on total proteins in *S.cerevisiae*

Zinc is actively concentrated by most cells. It is an essential metal ion and plays a role in catalysis, protein structure and perhaps as a signal molecule in organisms. On analyzing the bioaccumulation results, we observed that accumulation of zinc by the yeast cells increased with increasing concentrations. It is fairly hard and interacts strongly with oxygen and nitrogen containing donors and forms complexes. Total protein contents of yeast were determined in the presence of different concentrations of zinc. From the results we see that there is a good correlation between accumulated zinc and decrease in protein content. These results clearly indicate that increased zinc concentrations hinder the normal growth of cells and start to show biochemical effects when the concentration increases more than 20µg/ml (Figure:5). This indicates that proteins may start degenerating or are involved in other metabolic reactions leading to the efflux of accumulated Zn^{2+} ions. It is established that even essential metals may be harmful to living organisms at higher concentration; their concentrations inside the cells are controlled by some systems. Zinc affects proteins because it is a constituent of a large number of enzymes and proteins. It is involved in various enzymatic reactions, nucleic acid metabolism, protein synthesis, maintenance of membrane structure and function, protection against free-radical damage, replication, transcription and translation of genetic material.

The conclusions drawn are indeed backed by the precise characterization of Zn^{2+} binding proteins through the assessment of their molecular mass. The outlined methodology begins with the cultivation of yeast cells in a Zn^{2+} free medium (SGM), followed by the extraction of proteins from these cells and their subsequent separation via the SDS-PAGE method. Four separated bands of proteins were observed (Figure:6). The molecular masses of these isolated proteins were measured by determining their R_f values (Table:3). First band represents a high molecular mass protein and the other three bands are of low molecular mass proteins respectively. Protein bands were isolated in the presence of varying concentrations of zinc (5, 10, 20, 50, 100) µg/ml. The molecular masses of these proteins were determined

using a standard protein curve plotted between R_f and log M_r.

The number of protein bands decreased upon exposure to zinc concentrations of 5µg/ml and 10µg/ml compared to the control. A comparison with the control indicated the influence of Zn^{2+} ions on protein mobility. Furthermore, the protein with a molecular mass of 22.08kDa disappeared in the presence of 5µg/ml and 10µg/ml Zn^{2+}. SDS-PAGE results of the proteins extracted from cells grown in (20, 50)µg/ml of Zn^{2+} revealed the presence of both low and high molecular weight proteins, ranging from 123kDa to 13.52kDa. One new protein of high molecular mass 92.4kDa was seen at 20µg/ml of Zn^{2+}. Occurrence of new protein bands is also an indication of de-novo synthesis of proteins. High molecular weight proteins were consistently observed across all concentrations. The binding of zinc with proteins potentially led to an increase in molecular weight, as evidenced by the emergence of a band at 123kDa.

This finding highlights the potential of utilizing N-doped TiO_2 and Methylene Blue dye as a promising combination for improving the performance of DSSCs.

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Figures and Tables

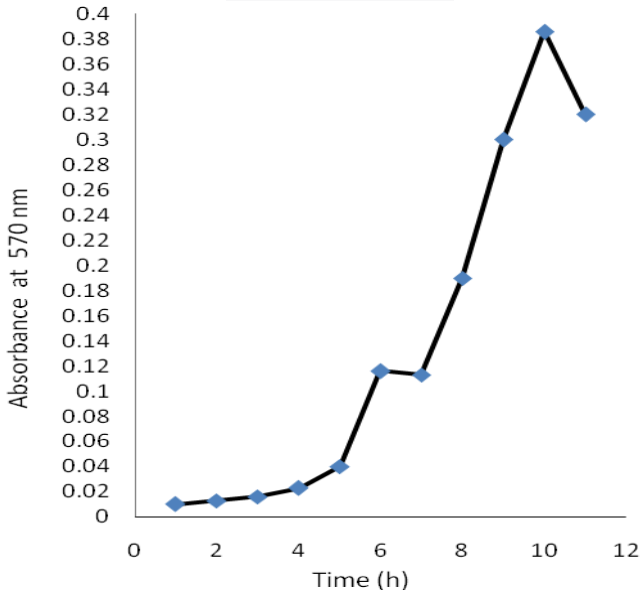

Figure 1 : Growth curve of *Saccharomyces cerevisiae* at 25 ^{0}C.

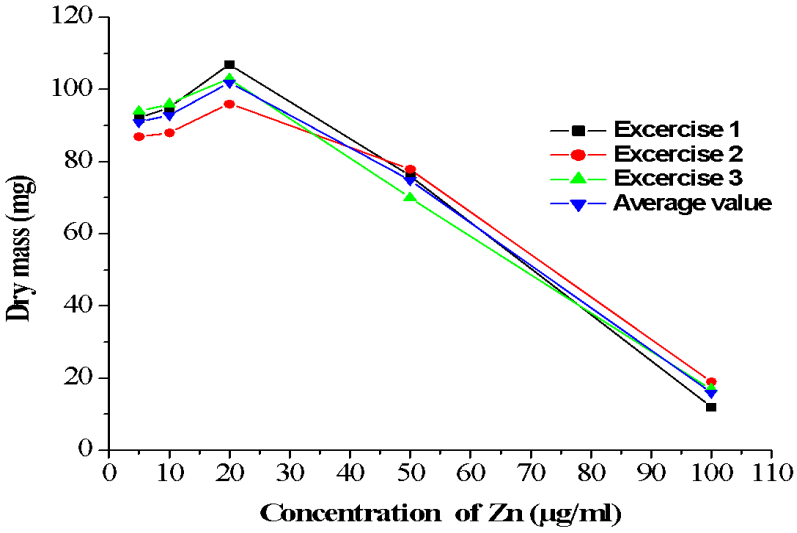

Figure 2 : Dried mass of *S.cerevisiae* when grown with Zn^{2+} ions.

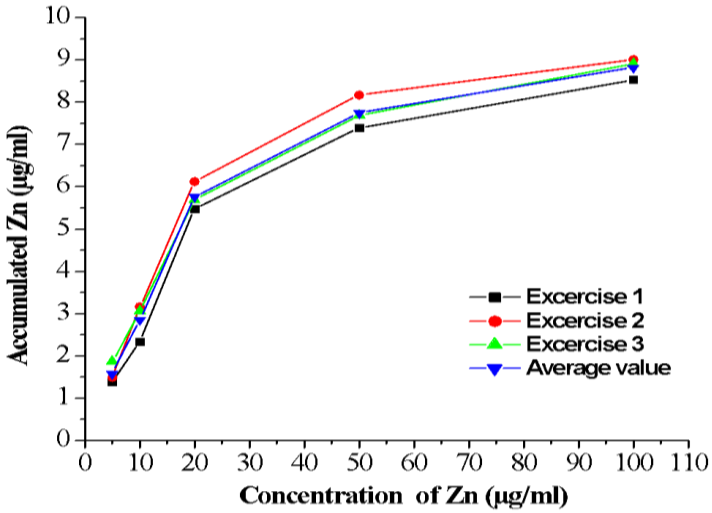

Figure 3 : Accumulated Zinc by *Saccharomyces cerevisiae*.

TABLE : 1

Accumulation of Zn^{2+} ions by yeast *Saccharomyces cerevisiae* in µg/ng of total proteins.

S. N.	Total Zn concentration supplemented (µg/ml)	Dry Mass (mg)	Total Protein (ng/mg of dry mass)	Absorbance on A.A.S.	Accumulated Zn (µg/mg of dry mass)	Accumulated Zn (µg/ng of proteins)
1	Control	86	1870.62	0.0001	0.0005	0.002×10^{-3}
2	05	92	1989.50	0.122	1.042	0.52×10^{-3}
3	10	96	1127.15	0.215	1.839	1.62×10^{-3}
4	20	105	1335.20	0.411	3.390	2.54×10^{-3}
5	50	77	2319.29	0.528	6.200	2.67×10^{-3}
6	100	18	2326.00	0.586	33.080	14.22×10^{-3}

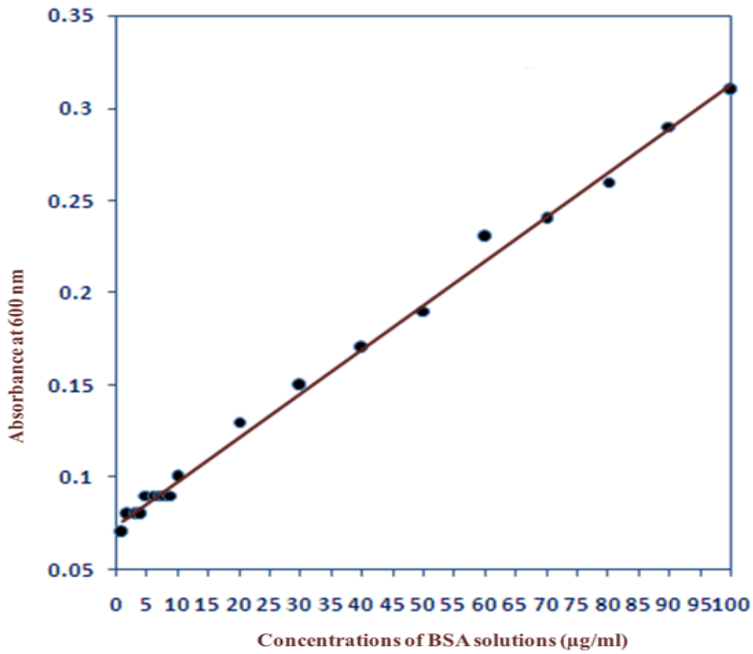

Figure 4 : Absorbance obtained for different concentrations of Bovine Serum Albumin at 600 nm.

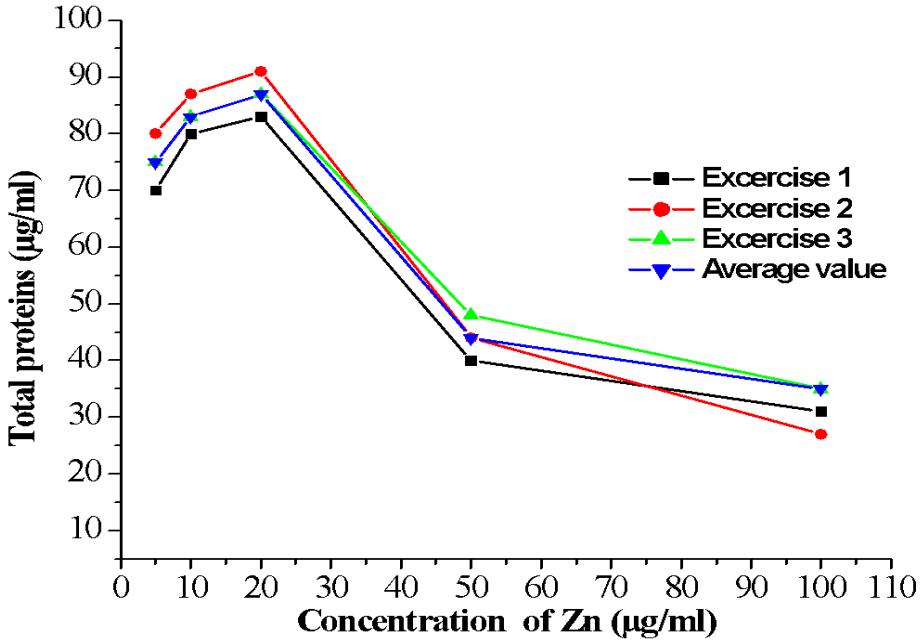

Figure 5 : Total proteins determined in *S.cerevisiae* when grown with Zn^{2+} ions.

TABLE: 2. R_f values of Standard Proteins from 14.3 kDa to 97.4 kDa

S. N.	Molecular mass of protein (kDa)	Log M_r	R_f
1	97.4	1.98	0.050
2	66.0	1.81	0.083
3	43.0	1.63	0.150
4	29.0	1.46	0.300
5	20.1	1.30	0.666
6	14.3	1.15	0.916

TABLE : 3. R_f values and molecular masses of proteins extracted from *S.cerevisiae* in the absence and presence of different concentrations of Zinc.

Concentrations (µg/ml)											
Control		5		10		20		50		100	
R_f	Mass (kDa)	R_f	Mass (kDa)	R_f	Mass (kDa)	R_f	Mass (kDa)	R_f	Mass (kDa)	R_f	Mass (kDa)
0.037	110.4	0.036	110.4	0.036	110.4	0.054	92.47	0.028	123	0.036	110.4
0.561	22.08	0.727	18.66	0.713	18.84	0.638	20.51	0.285	29.79	0.363	26.98
0.727	18.66	–	–	–	–	0.955	13.52	0.643	20.37	0.727	18.66
0.909	14.32	–	–	–	–	–	–	–	–	–	–

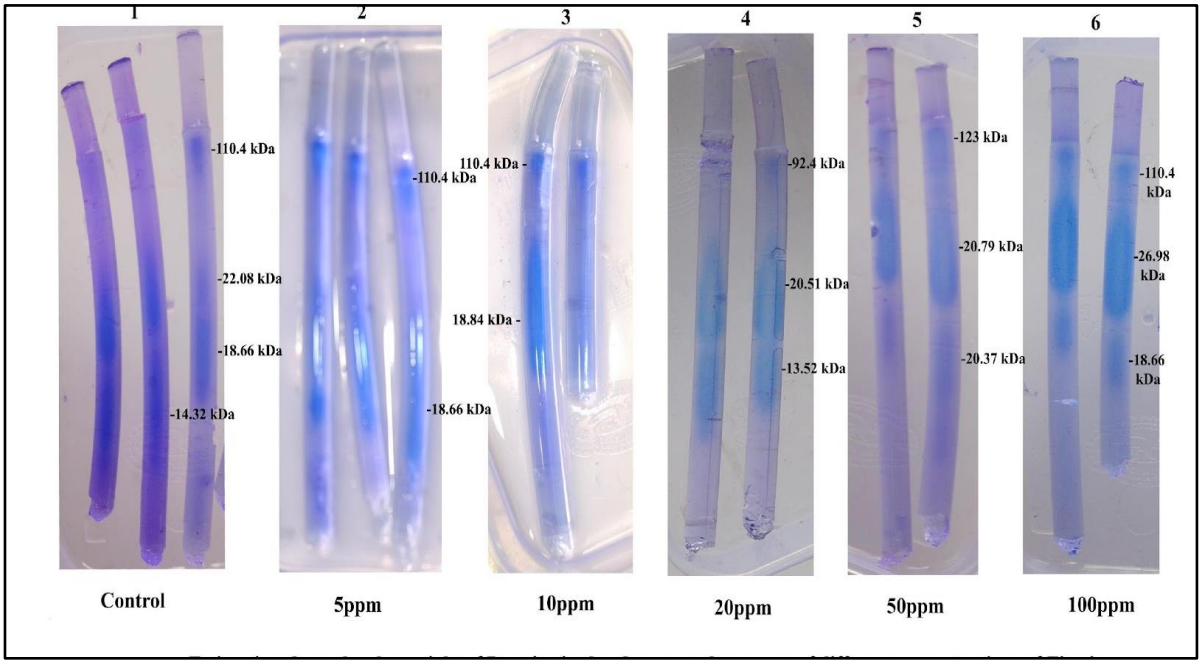

Figure 6 : Molecular weight of discrete protein bands estimated in the absence as well as presence of different concentrations of Zinc in *S.cerevisiae.*