Growth Parameters and Protein Content of Yeast *Saccharomyces Cerevisiae* on Increasing Concentration of Cadmium (Cd)

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Abstract:

The paper presents an effect of Cadmium(Heavy metal) on growth characteristics and protein content of yeast Saccharomyces cerevisiae. The review of literature on effect of heavy metals on living organisms gives the information about their toxicity and reduction in growth of organism. Lead is a common heavy metal and mankind has known its utility for centuries. It shows toxic effects when accumulated by organism. Saccharomyces cerevisiae is selected for experiment as it is easy to grow in normal laboratory conditions. The study may predict the use of S.cerevisiae in controlling metal ion concentration in the environment.

Keywords

Cell Membrane, nutrients, heavy metals, growth parameters, metallothioneins

1. Introduction

All living organisms depend upon external environment for essential nutrients. Air, water and soil are the constituents of our atmosphere which remains in contact with organisms and constitutes eternal environment [1].Essential nutrients like Oxygen, Nitrogen, Phosphorous; Potassium, Carbon, Sodium Plants and animals accumulate essential nutrients from environment [2]. Carriers and permeases are the protein molecules which are found in the plasma membrane of living organisms are helpful in the accumulation. Along with essential nutrients other substances present in the environment are non-essential and have toxic effects, when accumulated by organisms [3].Higher concentrations of these are not tolerable for organisms and they do not survive[4].Industrialization and human activities are the major sources of non-essential elements. The metals having a density more than five are known as heavy metals. This class of heavy metals includes about 38 elements. Toxic levels of heavy metals in environment are found due to the various reasons like soil near roads contains a high level of Lead concentrations; heavy metals are components of fungicides, pesticides and disinfectants.

Influx of heavy metals into the living cells may occur by different ways. It may be diffusion controlled or carrier mediated process. The protein molecules are responsible for the transportation of nutrients as these protein molecules have binding sites, which bind to the metabolites, transporting them across the semi-permeable plasma membrane [5]. Heavy metal binding proteins have been identified and characterized by workers in lower plants[6] Actual mechanism of heavy metal uptake is still not known. Proteins are involved in the action of most heavy metal ions at normal and toxic concentrations. Generally, a metal ion concentration of 1 μg/ml is satisfactory for specific binding. Proteins in intra nuclear inclusion bodies bind Lead, when present at high concentration and much of Cadmium and Mercury absorbed is sequestered by soluble kidney protein, metallothionein.Protein molecules generally act as a multidentate ligand and bind with metal ion at different binding sites. When the essential metals are replaced by toxic metal ions the activity of the protein is affected. Some microorganisms appear to be able to withstand metal concentration. Resistance power of fungi towards heavy metals has been studied but examples are relatively rare. They show that growth was retarted at high concentrations of heavy metals.

The work done on the tolerance of heavy metals in fungi shows that these are able to grow in extreme conditions. Several workers have isolated micro-organisms tolerant to high metal concentration from contaminated soils. Seal (1970) showed that fungus, *Aspergillus,* from Copper mine soil has a greater Copper tolerance than related fungus from normal soil [7]. Wide variety of bacteria and algae has also been studied for heavy metal tolerance. The most common species of bacteria that are able to resist extreme conditions (high concentrations of Cu and Fe and extreme pH) are *Thiobacillus ferroxidans.Chlorella vulgaris,* green algae, is tolerant to Ba, Mn, Pb and Cu.Higher concentration of heavy metals, therefore, may not prevent the growth of micro-organisms. Some micro-organisms appear to be able to withstand metal concentration.

2 Cadmium

It has been estimated that in modern times anthropogenic sources of atmospheric cadmium exceed natural sources. High environmental concentrations of cadmium are mainly a localized problem. Amongst the heavy metals, cadmium is regarded by many as one of the toxic trace element in the environment. It has now become a ubiquitous contaminant of human environment and is labelled as "dissipated element". The increased emission from production units and waste disposal combined with long-term persistence in the environment and its relatively rapid uptake and accumulation by food chain crops contribute to its potentially hazardous nature[8]. Themain source of Cadmium pollution in the environment is metal smelters. It is interesting to note that dietary factors, such as quantities of calcium, vitamin D and proteins influence absorption of cadmium.. Its uptake depends much on soil pH and the presence of other cation species. Its uptake is, however, depressed by Ca and Zn. The uptake process is probably a passive one. In most organisms, its transport is usually directly proportional to the external concentration [9].

2.1 Cadmium Chemistry

Cadmium is a relatively rare metal and is not found in the pure form in nature. It was discovered in 1817 as a constituent of smithsonite ($ZnCO_3$) obtained from a Zinc ore by German chemist F. Stromeyer. The new metal was named Cadmium after 'kadmia' the ancient Greek name for calamine or zinc carbonate. Cadmium is a soft, ductile, silver white metal that falls in II-B group of the periodic classification. It has a specific gravity of 8.642 and melting point of 320.9° C. Its atomic weight (112.41) is derived from a mixture of eight stable isotopes with mass numbers 106, 108. 100-114 and 116. Cadmium is concentrated in sulphide minerals and is found mainly in zinc, Lead-zinc and Lead-copper-zinc ores. It is recovered as a by-product from the smelting and refining of Zinc ore concentrate.

The average concentration of Cadmium in the lithosphere is 0.098g/g. Soil and plant contents of this element are generally low except where soils are formed from rocks, with high concentration of the metal such as shades or where pollution has occurred due to industrial activity. As these cadmium-rich rocks are not widely distributed in Earth's crust, they are not likely to be of much significance in their effects on soil plant-animal relationship on a worldwide scale. However, at the local level, crops, grown on soil developed on these rocks could result in significantly larger amounts of metal reaching man and animals.

Cadmium in batteries is largely used along with electroplating pigments, chemicals and alloys. Growth in the manufacture of products, which utilize cadmium batteries, has increased substantially during the last two decades. Cordless, household items such as brushes, electric shavers, flashlights and communication equipments, utilize sealed nickel-cadmium batteries. Vented batteries, on the other hand, are used in aircrafts, buses and diesel locomotives. Other cadmium uses include fungicides, phosphorescent dials, and compounds in photography, lithography, process engraving, curing rubber and miscellaneous uses. From the limited data available, cadmium does not appear to have accumulated in natural eco-systems to extreme levels as reported for many other elements such as Lead, zinc, nickel *etc.* The main sources of cadmium pollution are industrial activity. The atmosphere plays an important role in the dispersion of cadmium in the environment. Sink processes for atmospheric cadmium are wet and dry deposition, which Leads to cadmium enrichment of soil, vegetation and surface waters [10].

The atmospheric source may provide important cadmium in-put into these media and consequently contribute to human exposure through food and drinks as well as by the breathing of polluted air.

The effectiveness of a metal that enters a biological system and proceeds to influence and interact with other molecules in the organism depends upon its physiochemical properties like charge, radius, charge to radius ratio, polarisability, electronic configuration, coordination number, stereo-chemistry, hydration and hard/soft character. Metal ions linked to biological molecules primarily through oxygen, nitrogen sulphur.

Cadmium with atomic no. 48 has five complete 'p' sub-shells. The electronic configuration reveals that there are two electrons in the outermost shell. Thus, cadmium has an electro valence of two. The general chemistry of cadmium suggests that cadmium has high affinity for sulphur and toxic effects of metal are the result of reaction with essential sulfhydryl group in proteins. Among the amino acids found in proteins, Cd^{+2} has the highest affinity for cysteine, histidine and moderately high for methionine. Other amino acid components are also binding sites for cadmium. Cadmium is a soft metal as external electrostatic field deforms its electrons.

Increasing use of cadmium compounds in industries causes the Increase in natural contamination. Cadmium concentration increases in air and water by mining, from smelters, alkaline accumulators, alloys, paints, plastic, burning of coal, sewage sludge, use of fertilizers, pesticides and insecticides and insecticides. The use of fertilizers in agriculture, especially super-phosphates as well as cadmium containing pesticides are potential contributors to the environmental contamination. Recent outbreak of cadmium pollution affecting large populations in Japan and other parts of the world have aroused much concern about the toxicity of this heavy metal. Strict controls have been laid down and top priority has been given in setting up the safe standards for the industrial contamination by the metal (W.H.O. 1980).

2.2 Biological effects

Cadmium and Zinc are chemically very similar, and hence cadmium is able to mimic the behavior of the essential element Zinc in its uptake and metabolic functions. Unlike Zn, however, cadmium is both to plants and animals. The basic cause of the toxicity probably lies in much higher affinity of cadmium for thiol grouping in enzymes and other proteins. Therefore, its presence disturbs enzyme activity [11].

Cadmium is reported as a cumulative poison. Cadmium present in the food stuff is sequestered in the intestinal mucosa. In this process, becomes vulnerable to the effects of cadmium. Cadmium is stored in the kidneys and to some extent in the liver and spleen.

Excess cadmium results in the damage to kidney tubules, rhinitis (inflation of the mucous membrane of the nose), emphysema (a chronic disease of the lungs in which the alveoli become excessively distended) as well as other chronic disorders.

Cadmium toxicity causes itai-itai disease (Itai means pain) in the Toyama city region of Japan. Excess amount of Cadmium in diet found to impair kidney functions and disturb the metabolism of calcium and phosphorous and cause bone diseases.

During the last two decades it has been established that long 'exposure to cadmium oxide dust may Lead to chronic poisioning by the metal.

In early 1960's it was found that injecting cadmium compounds in mice could induce carcinomas. It was also noted that tumors also be produced in testes and pancreas. Chronic inhalation of $CdCl_2$, aerosols were found to induce lung carcinomas in rats. The long-term exposure with CdO, CdS and $CdSO_4$ resulted in the induction of lung carcinomas. Thus, it appears that inhalation of different cadmium compounds in rats can cause lung cancer.

3 Materials

Yeast strain *'Saccharomyces cerevisiae'* was obtained from NCIM, Pune. It is easy to culture in moderate laboratory conditions and serves as an important tool to study the vital metabolic activities going on inside the cell. Saccharomycetes are the ascomycetous yeasts. (The term yeast refers to simple morphological forms of Ascomycetes.) Ascosporogenous yeasts are known particularly for their ability to ferment carbohydrates, hence the name Saccharomycetes. These are well distributed over the surface of Earth and are particularly abundant in sugar-rich substrates such as nectar of flowers and surfaces of fruits, vegetative parts of plants *etc.* In contrast to most other ascomycetes, Saccharomycetes are unicellular organisms and do not forms a mycelium. Cells occur in variety of shapes such as globose, elongated or rectangular. Individually, cells are colourless but on solid media they produce coloured colonies. There are three main functions of yeast plasma membrane. Firstly, it forms an expandable cover and protective barrier for the protoplast, secondly, it controls the entry and exit of solutes to and from the cells, in other words, allows communication of cells with the external environment, and thirdly, it works as an organelle on which enzyme reactions leading to synthesis of many important molecules take place. The structure of plasma membrane is best explained by fluid mosaic model.

According to this model, phospholipid and sterols form a lipid bilayer in which the non-polar tail parts of lipids face each other at the core of the bilayer and polar head parts face outwards forming the basic matrix in which two types of proteins are embedded at irregular intervals. First type of proteins is loosely attached extrinsic proteins which are relatively easy to remove from the membrane. While the second type of proteins are the proteins which cannot be easily removed. These proteins are asymmetrically oriented in the lipid bilayer. The individual lipid and protein subunits in a membrane form a fluid mosaic and these are free to move laterally in the plane of the membrane. Most of these proteins are carriers and permeases, responsible for the influx and efflux of nutrients.

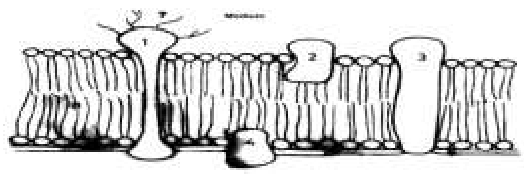


Fig: 1 Fluid mosaic model of plasma membrane

Previous Work done on Yeast S.Cerevisiae

The literature reveals that many workers have used the yeast cells as the model system in the past study the structure and functional relationships.

Lipids modulate the activities of several microbial membrane proteins, including those related to cellular permeability. In membrane transport amino acid uptake in *S.cerevisiae* has been studied by Keenan *et al* (1982). Ion transport in *S.cerevisiae* has been studied by Kleinhans *et al,* (1979). Sugar transport has been studied by Franzusoff and Arillo (1983). Thiamine transport has been studied by Iwashima and Nose (1975). Dye uptake has been studied by Bard *et al,* (1978).Polyene antibiotic senstivity has been studied by Gale *et al,* (1975).In membrane bound enzymes the AT pase activity in *S.cerevisiae* was studied by Bottema *et al* (1983). L-Kynurenine hydroxylase activity was studied by Janki *et al,* (1975), Cytochrome oxidase activity has been studied by Thompson and Parks (1974).

All glass apparatus used was of Corning/ Borosil make. They were washed first with dil. Chromic acid and then successively with distilled water.

Agar, Peptone and Yeast extract were obtained from Difco, U.S.A. Bovine serum albumin and Coomassie Brilliant Blue - G were obtained from Sigma Chemical Company, U.S.A. All the chemicals used were of Anal R, CDH grade.

The yeast cells were grown in SGM containing different concentrations (like 5 ppm, 10ppm, 50ppm, 100ppm and 200ppm) of Cadmium. Temperature was noted during the experiment and the flask containing the cells was kept on a magnetic stirrer for 10 hrs.

2. Methods and Observations

To study the growth characteristics of *Saccharomyces cerevisiae,* the cells were transferred from the YEPD (which is the solid medium in the form of slants containing Glucose, Agar agar, Yeast extract and peptone) to SGM (which is a suitable liquid media) in sterile condition of laminar flow. Growth of cells was monitored by taking the Optical Density every hour and drawing the growth curve. In growth curve time period between the points A and B is called the lag phase, in which the cells start the growth and the metabolic activities are very slow. The stage from point B to C is the mid log stage and point Z is mid log phase which is 10 hours at 27°C. Mid log phase is the time period in hours at which the rate of multiplication is the maximum and indicates maximum metabolic activity as well as maximum accumulation of nutrients by cells. Stage between point C to D is the saturation phase in which the metabolic activity shows the constant rate. All the experiments were done at mid log phase i.e./after the growth of 10 hrs. in SGM.

The essential conditions for the normal growth of yeast cells

1. Suitable media which contains Carbohydrates, Nitrogen source, Agar-agar (for the solidification), if required, source of vitamin, metals etc.
2. Temperature between 20°C to 30°C.
3. pH of the media between 3.5 to 4.5 i.e. acidic condition. pH above and below the above range may retard the growth of cells.
4. Completely sterilized media to avoid the interference of other microorganisms.
5. Continuous stirring of the liquid media is necessary, for the continuous supply of oxygen to the yeast cells during the growth. If the media is kept stable, the cells die.

Observations for the growth characteristics:

From the growth curve the time at which cells were growing at the maximum rate was estimated. This is called midlog phase. All the experiments were performed at mid log phase. Growth was monitored by observing Absorbance at 460 nm at different time interval

Growth parameters of *Saccharomyces cerevisiae*

Table-1

S. No.	Time in hours	Absorbance at 460 nm.
1	0	0.09
2	1	0.11
3	2	0.12
4	3	0.13
5	4	0.14
6	5	0.15
7	6	0.18
8	7	0.19
9	8	0.22
10	9	0.29
11	10	0.35
12	11	0.41
13	12	0.43
14	13	0.44
15	14	0.44
16	15	0.45
17	16	0.45

p-ISSN: 2348-6848
e-ISSN: 2348-795X
Volume 04 Issue 07
June 2017

18	17	0.45

RECORD OF THE WEIGHT OF *S.CEREVISIAE* IN THE PRESENCE OF Cd $(NO_3)_2.4H_2O$

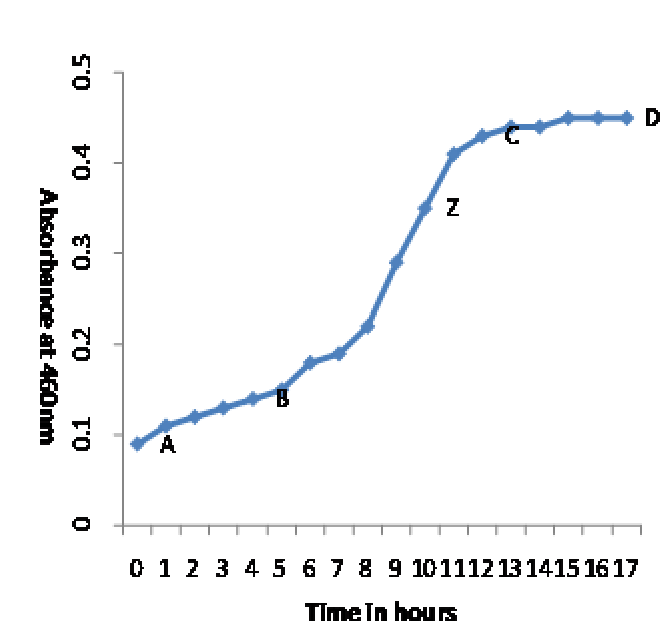
The growth of *S.cerevisiae* cells was also monitored by determining the dry weight of the cells in the presence and absence of Cadmium concentrations. The results were compared and it was observed that the dry weight of cells reduced remarkably at heavy metal concentration of 200µg/ml

Table-2

S.No.	Cadmium Concen-tration (µg/ml)	wt. of emtpy weighing tube (A) gm.	wt.of weighing tube + dry yeast(B)gm.	wt. of yeast cells(B-A) gm.
1	Control	3.7205	4.0216	0.3011
2	5	3.5063	3.6075	0.1012
3	10	3.7453	3.8334	0.0881
4	50	3.5350	3.6112	0.0762
5	100	2.8754	2.9304	0.0550
6	200	3.7105	3.7417	0.0312

Protein contents in yeast *saccharomyces cerevisiae*

To study the total proteins of yeast *Saccharomyces cerevisiae,* extraction of proteins were done. For this purpose the yeast cells were grown separately in SGM in the absence as well as in the presence of Pb in different concentrations for 10 hours. The culture was centrifuged, and treated with 5 ml of 10% TCA and again centrifuged for 3min. The mixture was centrifuged now with ethanol ether mixture, (1:1 v/v) to remove the excess of TCA. Cells were then boiled in glycine buffer on water bath for 3 min and centrifuged. Supernatant liquid was used as the source material for protein estimation and separation. To determine the total protein contents, Follin's



reagent was used (also called Follin - Ciocalteau reagent). It is quite complex and contains phosphomolybdic acid and tungstate. The aromatic amino acids-tyrosine and tryptophan-present in the proteins react with these chemicals and produce a dark blue colour. A number of reagents listed below were also prepared for the experiments under study.

A. 2% Sodium Carbonate in 0.1 N NaOH
B. 0.5% Copper Sulphate solution in 1% Sodium potassium tartrate solution (Freshly prepared solution was used.)
C. C, 50 ml of reagent A was mixed with 1 ml of reagent B, just prior to the use.
D. Follin-Ciocalteau reagent—The reagent was commercially available, however it had to be diluted with equal volume of water just before use. The reagent can also be prepared in the laboratory into a 2 liter flask by mixing out 100 gm. sodium tungstate, 25 gm. sodium molybdate, 50 ml 85% phosphoric acid and 100 ml. concentrated HC1 with 500 ml. distilled water. The mixture" was refluxed gently for about 10 hours with air condenser. After cooling, 150 gm. of lithium sulphate, 50ml of distilled water and a few drops of Bromine were added and boiling continued for another 10 minutes without the condenser. This helps in removing excess Bromine. After cooling, the volume was made up to 1000 ml and the solution filtered, if necessary. It was taken care

that the filtrate did not have any greenish tint. If greenish tint persisted it was boiled with Bromine once more. This was the stock reagent, which was diluted with equal volume of water just before use.

E. The standard protein Bovine Serum Albumin (BSA) solutions having different concentrations were prepared.

0.5 ml of sample solution was diluted with 0.5 ml of distilled water to make the total volume to 1 ml. to each experimental test tube, 5.5 ml. of the alkaline mix (reagents) was pipette out, mixed well and allowed to stand at room temperature for 15 minutes. 0.5 ml of the reagent was pipette out into each experimental test tube, mixing rapidly after each addition. The test tubes were left for 30 minutes and readings of the solutions formed were taken at 650 nm.

A proper blank solution without protein sample was prepared side by side, by mixing 1 ml distilled water and the reagents as that of the sample. From the absorbance of sample solutions and blank, standard curves between concentration of protein and absorbance were drawn.

Different solutions of known concentrations of BSA were prepared from the stock solution. Then 5.5 ml of reagent C and 0.5ml of Follins reagent were mixed. Distilled water was added to make the final volume to 7.0 ml. Optical Density (O.D.) was determined at 650 nm for different concentrations and standard curve drawn. Similar process was followed for proteins extracted from yeast cells and O.D. observed at 650 nm. From the standard curve total proteins were then estimated. The above method is known as Lawry's method [12].

Graph-Standard curve for protein estimation
Table &Graph-2 for standard curve (protein estimation)

S.no.	Concentration(ml of BSA solution) (with conc. 1 μg/ml)	Dilution with Distilled water in ml	Absorbance at 650nm
1	0	1000	0.07
2	20	980	0.1
3	40	960	0.11
4	60	940	0.13
5	80	920	0.14
6	100	900	0.15
7	120	880	0.19
8	140	860	0.21
9	160	840	0.22
10	180	820	0.25
11	200	800	0.28
12	220	780	0.29
13	240	760	0.3
14	260	740	0.32
15	280	720	0.35
16	300	700	0.36
17	320	680	0.38
18	340	660	0.4
19	360	640	0.42
20	380	620	0.45
21	400	600	0.46

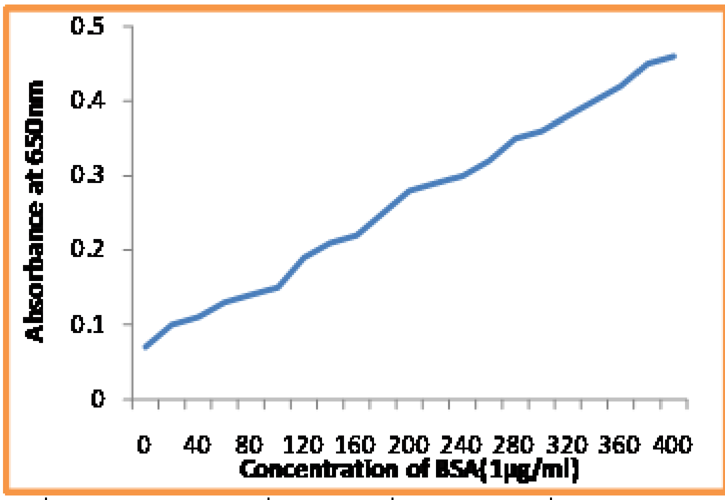


Table-3

Total protein contents in S. Cerevisiae in the presence of different concentrations of Cadmium

Experiment No 1.

S.No.	Final Concentration of Cadmium(µg/ml)	Absorbance at 650nm	Total Proteins(µg/ml)
1	Control	0.44	380
2	5	0.33	256
3	10	0.30	240
4	50	0.25	180
5	100	0.21	140
6	200	0.15	80

Experiment No 2.

S. No.	Final Concentration of Cadmium(µg/ml	Absorbance at 650 nm	Total Proteins (µg/ml)
1	Control	0.44	380
2	5	0.32	250
3	10	0.29	230
4	50	0.25	180
5	100	0.20	130
6	200	0.15	80

Experiment no.3

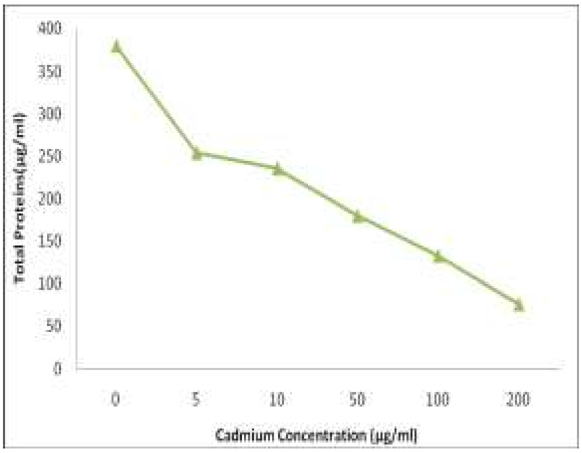
S.No.	Final Concentration of Cadmium(µg/ml)	Absorbance at 650 nm	Total Proteins (µg/ml)
1	Control	0.44	380
2	5	0.32	256

3	10	0.30	240
4	50	0.25	180
5	100	0.20	130
6	200	0.14	70

Average Total Protein Content

S. No.	Final Concentration of Cadmium(μg/ml)	Absorbance at 650 nm	Total Proteins (μg/ml)
1	0	0.44	380
2	5	0.32	254
3	10	0.29	236
4	50	0.25	180
5	100	0.20	133
6	200	0.14	76

Variation of Total Proteins with different concentration of Cadmium



Results and Discussion

It was observed that on increasing the concentration of Cadmium from 5μg/ml to 200μg/ml, the mass and protein contents in the yeast decreased continuously. The minimum dry weight at 200 (μg/ml) concentrations was found, 0.03gm and protein content is 76(μg/ml). It shows that the Cadmium interference reduces the dry weight as well as total protein content of the cells considerably.
The metal toxicity is a reasonable determinant of reduction in growth and that the degree of response of the organism is dependent on the amount of metal which traversed through the cell membrane and was not bound by detoxifying mechanism [13].
Total protein contents after treatment with Cadmium 76 μg/ml at 200μg/ml concentration. The control had 380μg/ml protein contents under the same conditions. These results confirm the findings of other workers. The protein concentration decreased due to Cadmium deposition
Occurrence of metallothioneins (MTs) in the organisms exposed to toxic heavy metals has been reported long back. Experiments have given information concerning heavy metal protein interactions. Metallothioneins and MT like protein molecules and polypeptides containing high cysteine contents have also been identified in higher plants. Induction of protein synthesis and accumulation of metllothionein in liver and kidney of some fishes has also been reported. All these reports suggest that the presence of heavy metal concentration inside the cells and in the immediate environment of the cells have metabolic effects on the organisms.
Present observations suggest that heavy metals may bind to certain proteins with enhancement, modification or inhibition of the normal biological activity. Protein molecules undergo a regulation in response to changes in the microenvironment. The total binding proteins decrease in proportion to heavy

metal concentration. It is also confirmed in the past that proteins are utilized in response to the uptake of heavy metal ions. The other possible reason for the decreased protein synthesis could be the replacement of metal ions in protein synthesizing enzymes. Binding of heavy metals with specific protein molecules might 'therefore' affect its structure and function significantly. These metal bonded protein molecules are either excreted out by the cells or may be stored in some organelles of the cells. The heavy metals as environmental contaminants are toxic virtually to every system in the organisms. These effects lead to the decreased protein contents of the cells, and in turn in decrease of the body weight.

It is quite likely that cells come under great stress to synthesize new molecules to face the environmental stress to remove heavy metals accumulated inside the cells. It may be possible that free metal ions denature the protein portion or may have inhibitory effect on the interaction between the proteins and lipids in cell membrane.

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