



Studies on growth parameters and accumulation of lead content on increasing concentration of lead (PB) by yeast *Saccharomyces cerevisiae*

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Abstract

The paper presents an effect of Lead (Heavy metal) on growth characteristics and accumulated content Lead by 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, 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 external 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, There is also evidence of increasing general environmental pollution from widespread use of compounds of Lead and Mercury [5].

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 [6]. Heavy metal binding proteins have been identified and characterized by workers in lower plants [7] 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 retarded 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) [8] showed that fungus, *Aspergillus*, from Copper mine soil has a greater Copper tolerance than related fungus from normal soil [8]. 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 ferrooxidans*. *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. Lead

Lead naturally occurs in the form of mineral deposits, mainly as galena (Lead sulphide). It has low melting point and is malleable and relatively inert. These properties of Lead make it an ideal material for many applications. Man has used lead since prehistoric times. It was known even in second century B.C. that working with Lead is a health hazard [9] Later during the technological revolution of the 18th and 19th centuries the wide spread use of Lead in manufacturing processes resulted

in industrial Lead poisoning and serious cause of ill health and deaths.

2.1 Chemistry of lead

The alchemist believed Lead to be the oldest known metal and associated it with the planet Saturn. The atomic weight of Lead is 207.19; atomic no. 82, melting point 327°C, boiling point 1744°C and sp.gr. 11.35 at 20°C. It has a valence of 2 and 4. Lead is obtained chiefly from galena (PbS) by roasting process. It is a bluish white metal of bright luster, very soft, highly malleable and ductile and a poor conductor of electricity. It is very resistant to corrosion

Uses: Lead is very effective as a sound absorber, and is used as a radiation shield around x-ray equipment and nuclear reactors. It is used to absorb vibrations. White Lead, the basic Lead carbonate, sublimed white Lead (PbSO₄) chrome yellow (PbCrO₄), red Lead oxide (Pb₃O₄) and other Lead compounds are extensively used in paints. Lead oxide is used in producing fine crystal glass and flint glass of high index of refraction for achromatic lenses. Its nitrate and acetate are soluble salts and are used in chemical laboratories. Lead salts are sometimes used in medicines, as antiseptic and astringents. Care must be taken while using Lead as it is a cumulative poison ^[10].

2.2 Atmospheric Lead

In the early part of this century smelting and refining were the main sources of atmospheric Lead. Today the combustion of Leaded petrol in automobiles is the largest contributor of Lead in our atmosphere.

Approximately 90% of air borne Lead in the United States, which can be traced, comes from the combustion of Petrol (Data from Nat Inventory Air Pollution Emission and control 1972). Three Lead isotopes ²⁰⁶Pb ²⁰⁷Pb ²⁰⁸Pb from radioactive decay of the Uranium chain elements contributes to total environmental Lead. Forest fires are known to be another source of atmospheric Lead. Refuse incineration is also a significant source of Lead. A negligible natural source of Lead may be from the process of evaporation and volatilization of elements associated with "bubble bursts" from surface of ocean waters.

2.3 Surficial Lead

Significant quantities of Lead get deposited on surface from waste and sewage. Soils near highways and industrial areas are also important source of Lead. Lead compounds are widely spread on the surface of soil and during the rainy season due to the flow of water, salts of Lead accumulate in water bodies. Precipitation from the atmosphere tends to increase the Lead contents of soil and aquatic systems. Most of the Lead compounds entering aquatic system are not water-soluble and are removed by sedimentation. Plants through their roots also absorb lead; hence there is a direct relationship between natural contents of Lead in plants and soil ^[11].

Street dust is a potential source of ingested Lead, especially in children. Continuous exposure to Lead, results in its gradual accumulation in the body. No beneficial role of Lead has yet been known and there is no substantial evidence to suggest that it is an essential trace element. On the contrary, at the cellular level Lead is found to interfere with the respiratory

pigment, energy production and membrane function.

2.4 Biological Effects

Biological interest in Lead was initiated as a result of its toxic properties as an industrial hazard to man and plants. The concentration of Lead estimated to produce Lead poisoning is well documented. Lead is toxic because it mimics many aspects of the metabolic behavior of Ca and inhibits many enzyme systems. Attention has, therefore, been confined to the biological consequences of long-term exposure to low concentration of Lead present in the environment and factors that influence the retention of Lead in living organisms.

It was observed that exposed children have lower score on psychometric intelligence, auditory and speech processing and show more non-adaptive behavior. Lead accumulates in bones and tissues and in high concentration causes characteristic symptoms of Lead poisoning resulting in anemia. Anemia in man is caused by disorders in the porphyrin metabolism. It seems that harm affects virtually all steps in the process of harm synthesis. Other problems created by high concentration of Lead are impairment of the function of liver, kidney, spleen, spinal deformities and ultimately death. Both the central and peripheral nervous systems are affected by absorbed Lead. In man one of the chief concern of Lead toxicity is its effect in causing brain damage particularly in the young. Lead pollution can also induce aggressive behavior in animals.

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.) Ascosporeogenous 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 form 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 are 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.

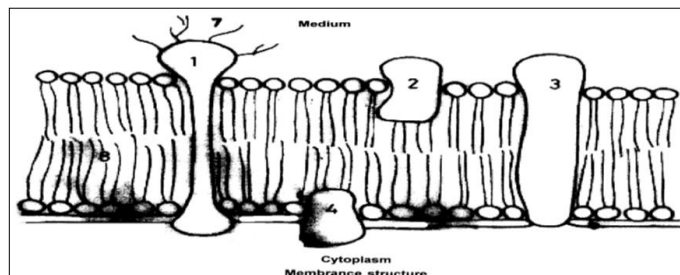


Fig 1: the lipid globular protein mosaic model with a liquid matrix (the fluid mosaic model) 1-6-polypeptides, 7-carbohydrates, 8-lipids

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 sensitivity 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 Lead. Temperature was noted during the experiment and the flask containing the cells was kept on a magnetic stirrer for 10 hrs.

5. 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.

Table 1: Growth parameters of *Saccharomyces cerevisiae*

| 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 |
| 18 | 17 | 0.45 |

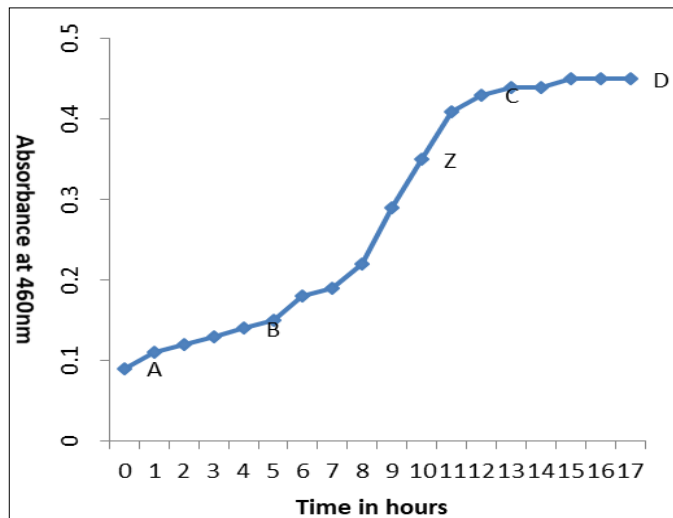


Fig 2

Record of the weight of *S. Cerevisiae* in the presence of Pb (No₃)₂

The growth of *S. cerevisiae* cells was also monitored by determining the dry weight of the cells in the presence and absence of lead 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. | Lead Concentration (µg/ml) | wt. of empty 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 | 3.6194 | 3.8206 | 0.2012 |
| 3 | 10 | 3.7453 | 3.9368 | 0.1915 |
| 4 | 50 | 3.4505 | 3.5448 | 0.0943 |
| 5 | 100 | 2.8754 | 2.9596 | 0.0842 |
| 6 | 200 | 3.7105 | 3.7705 | 0.06 |

Atomic absorption spectrophotometry

The absorption of light by atoms in the gaseous state forms the basis of Atomic Absorption spectrometry. When a solution containing metallic species is introduced into a flame the vapour of metallic species is obtained. Some of the metal atoms may be raised to higher energy level and will emit the characteristic radiation of the metal. A large percentage of the metal atoms, however, remain in the non-emitting gaseous state. These atoms of the metal absorb the light radiation of their own specific wavelength, which is the same wavelength, as they would emit, if excited. When radiation with this wavelength is allowed to pass through a flame having atoms of the metallic species, portion of that radiation will be absorbed and the absorption will be proportional to the density of atoms in the flame. Therefore, AAS determines the amount of light absorbed. From absorption the concentration of metallic element can be calculated because the absorption is proportional to the density of the atoms in the flame. The method of calculation of final concentration in µg/ml is as follows:

Concentration of the metal in ppm =

$$\frac{\text{Reading of AAS } (\mu\text{g/ml}) \times \text{Dilution factor}}{1000 \times \text{Weight of sample taken (g)}}$$

Atomic Absorption Spectrophotometry is a standard method for the determination of heavy metal concentration in universal and biological samples. In spectrometric analysis, light of definite wavelength extending to the ultraviolet region of the spectrum constitutes the source of light and thus the use of a spectrophotometer. It may be regarded as a combination of two instruments spectrometer and a photometer in one cabinet. A photometer is an instrument, which is used, for the production of coloured light of selected colour or wavelength when it is used as a part of spectrophotometer it is usually known as monochromator. A photometer is a device, which is used, for measuring the intensity of light and when attached with spectrophotometer, it is used to provide the intensity of the monochromatic light produced by the monochromator. The most important advantage is that analysis provides a significant method of determining minute quantities of substances. In an atomic absorption analysis, the element being determined must be reduced to the elemental state, vaporised and imposed in the beam of radiation from the source.

This process is frequently accomplished by drawing a solution of the sample as a fine mist, into a suitable flame. The flame thus serves as a function analogous to that of the cell and solution in conventional spectroscopy.

There are two fundamental laws related to the absorption of monochromatic radiant energy by homogenous solutions. The Lambert law expresses the relationship between the light absorptive capacity and thickness of the absorbing system. It states that "when a beam of monochromatic light passes through a transparent medium, layers of equal thickness of that homogenous absorbing medium absorb equal proportion of incident radiations.

The second law formulated by Beer states that "the fraction of monochromatic radiant energy absorbed on passing through a solution is directly proportional to the concentration of the absorber." It is thus a relationship between the light absorptive capacity and the concentration of the solute in solution.

Choice of suitable flame i.e. air-propane or air-acetylene etc. depends upon the following factors :

1. Metal to be determined.
2. The presence of other materials

To perform the experiment the source lamp of required element was allowed to warm up. Monochromatic wavelength and slit width to resonance peak with all light cut off from the detector was selected and adjusted to zero transmission. With reference to zero concentration solution entering the flame was adjusted to 100. Standard samples were ignited and made them synthetically from a pure salt of the element to be determined and run the samples and observed the analyzed results with the help of computer.

For the determination of Lead, the gas used was a mixture of acetylene and air, voltage applied was 220 and the lamp used was electrode discharge lamp.

**Accumulation of lead by *saccharomyces cerevisiae*
In the presence of different concentrations of lead
In sgm. Lead was supplemented as pb (no₃)₂.**

Table 3: Experiment - 1

| S. No | Lead Concentration (µg/ml) | AAS Reading ppm | Lead accumulated (µg/ml) | Total protein (µg/ml) | Lead accumulated (µg/µg protein) |
|-------|----------------------------|-----------------|--------------------------|-----------------------|----------------------------------|
| 1 | Control | 0.01 | 0.01 | 380 | 2.63×10^{-5} |
| 2 | 5 | 0.02 | 0.02 | 320 | 6.25×10^{-5} |
| 3 | 10 | 0.04 | 0.04 | 290 | 13.79×10^{-5} |
| 4 | 50 | 0.05 | 0.05 | 250 | 20.00×10^{-5} |
| 5 | 100 | 0.09 | 0.09 | 200 | 45.00×10^{-5} |
| 6 | 200 | 0.14 | 0.14 | 170 | 82.00×10^{-5} |

Table 4: Experiment - 2

| S. No. | Lead Concentration (µg/ml) | AAS Reading ppm | Lead accumulated (µg/ml) | Total protein (µg/ml) | Lead accumulated (µg/µg protein) |
|--------|----------------------------|-----------------|--------------------------|-----------------------|----------------------------------|
| 1 | Control | 0.01 | 0.01 | 380 | 2.63×10^{-5} |
| 2 | 5 | 0.02 | 0.02 | 325 | 6.15×10^{-5} |
| 3 | 10 | 0.04 | 0.04 | 290 | 13.79×10^{-5} |
| 4 | 50 | 0.07 | 0.07 | 240 | 29.16×10^{-5} |
| 5 | 100 | 0.09 | 0.09 | 200 | 45.00×10^{-5} |
| 6 | 200 | 0.13 | 0.13 | 165 | 78.78×10^{-5} |

Table 5: Experiment - 3

| S. No | Lead Concentration (µg/ml) | AAS Reading ppm | Lead accumulated (µg/ml) | Total protein (µg/ml) | Lead accumulated (µg/µg protein) |
|-------|----------------------------|-----------------|--------------------------|-----------------------|----------------------------------|
| 1 | Control | 0.01 | 0.01 | 380 | 2.63×10^{-5} |
| 2 | 5 | 0.03 | 0.03 | 320 | 9.37×10^{-5} |
| 3 | 10 | 0.05 | 0.05 | 280 | 17.85×10^{-5} |
| 4 | 50 | 0.06 | 0.06 | 250 | 24.00×10^{-5} |
| 5 | 100 | 0.11 | 0.11 | 190 | 57.89×10^{-5} |
| 6 | 200 | 0.14 | 0.14 | 160 | 87.50×10^{-5} |

Table 6: Average Accumulation

| S.No. | Lead Concentration (µg/ml) | AAS Reading ppm | Lead accumulated (µg/ml) | Total protein (µg/ml) | Lead accumulated (µg/µg protein) |
|-------|----------------------------|-----------------|--------------------------|-----------------------|----------------------------------|
| 1 | Control | 0.01 | 0.01 | 380 | 2.63×10^{-5} |
| 2 | 5 | 0.01 | 0.01 | 321 | 7.25×10^{-5} |
| 3 | 10 | 0.02 | 0.02 | 286 | 15.14×10^{-5} |
| 4 | 50 | 0.06 | 0.06 | 246 | 24.38×10^{-5} |
| 5 | 100 | 0.09 | 0.09 | 196 | 49.29×10^{-5} |
| 6 | 200 | 0.13 | 0.13 | 165 | 82.76×10^{-5} |

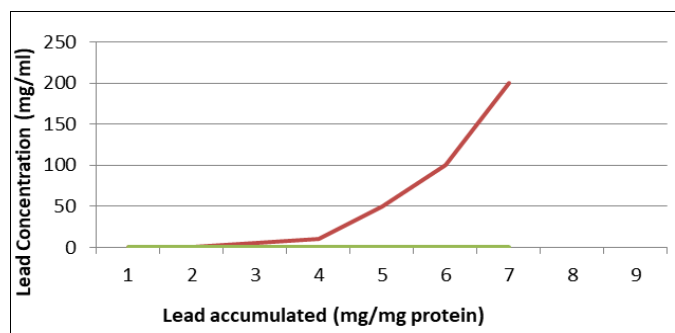


Fig 3: graph showing variation of accumulated lead

Results and Discussion

It was observed that on increasing the concentration of Lead from 5µg/ml to 200µg/ml, the mass of yeast decreased continuously. The minimum dry weight at 200 µg/ml concentration was found, 0.06gm in the case of Lead. It shows that the lead interference reduces the dry weight 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].

Accumulation of heavy metals by *S.cerevisiae* shows that at 200 µg /ml concentration Lead accumulation is 82.76×10^{-5} µg / µg protein.

These results confirm the findings of other workers. The protein concentration decreased due to lead exposition induced proteinemia In the case of Lead, it appears that it affects the metabolic activities of *S. cerevisiae*. Anemia produced by lead treatment in rats has also been reported [14].

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 metallothionein 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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