Efficiency of immobilized *Saccharomyces cerevisiae* in remediation of Chromum

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SUMMARY

The chromium remediation ability of *Saccharomyces cerevisiae* was studied, as it is non-pathogenic and easily available as wastes from fermentation industries. Normal baker's yeast, mutants and genetic strains with and without metallothioneins were used in their immobilized forms. Their chromium remediating ability was studied and their efficiencies were compared. Flame Atomic Absorption Spectroscopy and diphenyl carbazide method were used to quantify chromium in the effluents. The work also focused on determining whether mutagenesis enhances or lessens the organism's ability to remediate chromium and establishing the role of metallothioneins. The chromium content of the effluent was around 5600ppm before remediation, after which it reduced to around 28ppm. The best activity was observed in the genetic strain containing multiple copies of metallothioneins and the mutants were still efficient in spite of the damage in the *de novo* pathway. Proteins isolated from the strains after bioremediation showed a similar banding pattern to metallothionein suggesting their activity in heavy metal stress.

Key words : Contamination, Chromium, Remediation, Mutagenesis, Immobilized, Metallothioneins.

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Chromium is the most common pollutant widely used in industries, resulting in the discharge of large quantities into the environment and therefore its contamination is extensive (Bartlett, 1991). Waste water containing chromium are generated by many industries particularly metal finishing industries, petroleum refineries, leather tanneries, iron and steel industries, textile manufacturing industries and others (German and Parrerson, 1974).

In the tanning industry large quantity of chromium or basic chromium sulfate is being used for chrome tanning (Pathe *et al*., 1995). Chrome tanning needs large amount of waste water to reduce residual salts as far as possible, which otherwise causes some problems during finishing of leather products. About 5-6 L of chrome waste water is produced per kg of hide and skin (Prasad and Nair, 1994). Spent chrome tan liquor is acidic in nature and greenish in color. Total suspended solids and BOD ranges were reported to be 1500 to 2400 mg/L and 800 to 3500 mg/L. The permissible limits of Chromium has been fixed at 2.0 mg/L and for industrial effluent discharged into inland surface water and public sewers, respectively.

Chromium is an essential element, required in trace amounts in the diet of some animals and human beings. However, exposure to chromium can damage cell membranes, alter enzyme specificity, disrupt cellular functions and damage the structure of DNA (Bruins *et al*., 2000).

The chromium in the effluents is primarily in the hexavalent form as chromate and dichromate. Hexavalent chromium is highly toxic and carcinogenic (Mearns *et al*., 1976; Nishiota, 1975; Petrilli and DeFlora, 1977; Vennit and Levy, 1974). It activates p53 by reactive oxygen species (ROS) mediated by free radical reactions that occur during the oxidative reduction of hexavalent chromium within the cell. Oxidative damage is considered to be an important mechanism in the genotoxicity of Cr (VI). Because Cr (VI) is a powerful oxidizing agent, exposure can cause irritation and corrosion. When inhaled Cr (VI) is a respiratory tract irritant and causes pulmonary sensitization. Chronic inhalation increases the risk of lung cancer. The other target organs of chromium are the kidneys, liver, skin and the immune system may also be affected. Trivalent chromium also plays a role in the prevention of arteriosclerosis. It stabilizes tertiary structure of proteins and the conformation of the cell RNA and DNA (Zetic *et al*., 2001). However, under certain extreme environmental conditions the trivalent chromium might get oxidized to the hexavalent form. Hence, the need arises to remediate chromium into its harmless form before being discharged.

The treatment of chromium bearing effluent has been reported through several methods such as reduction, precipitation, ion

exchange, reaction with silica, electrochemical reduction, evaporation, reverse osmosis, direct precipitation and adsorption on activated coal, alum, kaolinite and ash. These conventional methods used for the removal of hexavalent chromium uses chemical procedures, which are expensive and lack specificity (Katiyar and Katiyar, 1997). As an alternative, biological approaches utilizing microorganisms offer the potential for a highly selective removal of toxic metals coupled with considerable operational flexibility, hence they can be both *in situ* or *ex situ* in a range of bioreactor configurations (Lloyd and Lovely, 2001).

Immobilized cells as biofilms, beads or inert supports have been found to be most effective in designing bioreactors for heavy metal degradation (Bruce James, 2002). Immobilized non-living biomass and metal binding compounds can also be used to remove heavy metals (Katiyar and Katiyar, 1997).

Chromium remediation studies have been carried out with a variety of organisms like species of *Pseudomonas*, *Aeromonas*, *Bacillus*, *Micrococcus* and *Microbacterium* (Laxman and More, 2002). Of these, *Pseudomonas* species were the most efficient but pathogenic. Fungi are being exploited for heavy metal degradation as a result of their various characteristics; the most significant are the metallothioneins (MT).

Chromium degradation occurs under both aerobic and anaerobic conditions. The factors that affect the rate of chromium remediation are cell density, chromium concentration, electron donor, dissolved oxygen level, pH, temperature and the presence of other environmental compounds. *Saccharomyces cerevisiae* proved to be capable of accumulating chromium and cadmium (Brady and Duncan, 1994) and other metals. The organism is non-pathogenic and is a waste product of industrial fermentation activities (Volesky *et al*., 1995; Krauter *et al*., 1996). Hence, the present study exploited the immobilized forms of *Saccharomyces cerevisiae* in the remediation of chromium.

MATERIALS AND METHODS

Sample collection and pre-treatment:

Effluent from leather tanneries in and around Karur district of Tamil Nadu was collected. It was stored at 4°C with the idea of arresting any biological activity. The colour and appearance of the effluent was observed followed by measurement of pH using a pH meter. The chrome water obtained from the tanneries was filtered by Whatmann No. 1 filter paper. The pH was adjusted to < 2 using concentrated nitric acid.

Estimation of chromium:

FAAS (Flame Atomic Absorption Spectroscopy) was employed to analyze for trivalent and hexavalent chromium. AAS Photometer, EMerck SQ118 and "Spectragnant kit" was used. It was done in SITRA laboratories (South India Textile Research Association), Coimbatore. Diphenyl carbazide method (Clesoari and Green, 1995) was followed for the laboratory estimation of chromium where diphenyl carbazide solution was added for color development. Optical density was measured at 540nm using reagent water as reference and the value was plotted against the standard graph to determine the concentration of chromium in the effluent.

Microbial cultures:

Normal *Saccharmyces cerevisiae* was revived from dried baker's yeast, which is a commercially available product. Two genetic strains of S. *cerevisiae*, one with and the other without metallothioneins, numbered 308 and 309, respectively were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India.

308- Genetic stock, mating type 'a', trp ð ura 3, cup 1r (multiple chromosomal genes with MT genes)

309- Mating type 'a', arg 4-8, leu 2-3, leu 2-112, his 7-2, trp 1-289, ura 3-52, ade 5, cup 1ð (MT gene deleted)

The strains were cultured and maintained on Yeast peptone agar medium and sub-cultured once in 15 days. Their growth was aerobic, requiring a temperature of 30°C and an incubation period of 48 hours.

Mutagenesis and DNA analysis:

Hydroxylamine mutagenesis was carried out using 5M hydroxylamine hydrochloride (Yurgel and Michael, 2005) and MMS (Methyl Methane Sulfonate) using 1.5% and 3% MMS according to the protocol by Gasch *et al.* (2001). Plating the cells to analyze for auxotrophic markers followed both the procedures.

DNA was isolated from *Saccharomyces cerevisiae* (normal, mutant, 308 and 309) according to the protocol by Sambrook and Russel (1989) followed by electrophoresis on 0.8% agarose gel.

Immobilization and bioremediation:

To analyze the total chromium content, the pH of the chrome water was adjusted to <2 with concentrated Nitric acid. To analyze the hexavalent chromium content, the pH was made up to 8.0 with 1N sodium hydroxide and distributed in 100ml conical flasks (30ml each) and 100mM glucose was added. The pH was adjusted to 5.0 and incubated with the immobilized beads (50g/L) at room temperature for 72 hours. Chromium was estimated by the diphenyl carbazide method at an interval of 12 hours

for three days. From the concentration of chromium remediated, the percentage efficiency was found and the strains were rated accordingly. Simple statistics was used to calculate the coefficient of variation. This was done to rate the strains in terms of consistency in their activity.

Viability testing:

The cell viability of the test organisms after treatment with chrome water was assayed. The cells were revived from the immobilized state using 1% sodium citrate and were crushed. The homogenate was centrifuged for 4000rpm for 3 minutes and the supernatant was retrieved. It was plated and its viability was checked on the basis of its ability to form colonies on YPD (Yeast Peptone Dextrose) agar.

RESULTS AND DISCUSSION

Analysis of chromium in the tannery effluent:

The collected tannery effluent containing chromium was dark green in color with an acidic pH. The chrome water was filtered and pre-treated to adjust the pH to <2. When subjected FAAS, trivalent chromium was estimated to be about 5671.7ppm and hexavalent chromium to be about 2.3ppm and the total chromium as 5672.9ppm, which is the sum of the two former values. The chromium content obtained by laboratory estimation amounted to 5600µg/liter, similar to the result obtained by FAAS. As set by the National Pollutant Dischargeable Elimination System, the allowed dischargeable limit for hexavalent chromium is only 11ppb. The Environmental Protection Agency (EPA) has formulated the maximum permissible limits of Cr (VI) into water bodies as 50µg/ dm^3 and in drinking water as 3µg/dm^3 and that of Cr (III) as 100µg/ dm^3 (Lee and Lee 1998; Palmer and Puls, 1994). Hence, it was essential to evolve a method to remedy chromium.

Microbial culture and maintenance:

For the present study, one type of normal strain was revived from dried baker's yeast and two other genetic strains 308 and 309 were obtained from MTCC. They were cultured and maintained on YPD agar with routine sub-culturing once in 30 days and were evaluated for their remediation efficiencies.

Studies on the efficiencies of several microorganisms and plants and the mechanisms involved in remediation have been considered as a fundamental and potential study for *in situ* and *ex situ* conservation technologies, as well as to scavenge these heavy metals. Several microorganisms accumulating specific heavy metals have been characterized. Microorganisms interact with metals

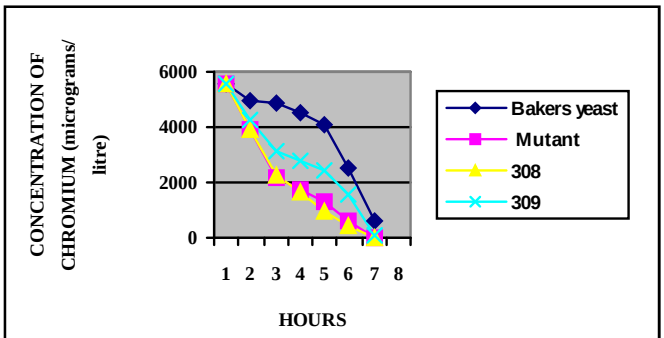

Fig. 1 : Chromium remediation by saccharomyces cerevisiae

by a number of processes including transport, biosorption to cell biomass, entrapment in extra cellular capsules, protein-DNA adduct formation and induction of stress, precipitation and oxidation reduction reactions and binding by cytosolic molecules (Gadd 1990; Lovely and Coates, 1997; Ksheminska *et al.*, 2003). A process of an initial rapid accumulation that is independent of metabolism and temperature has demonstrated bioaccumulation of metal cations, and by a process that internalizes the cation into the cell. Energy- independent uptake of metal cations has been described with influx being dependent on the electrochemical proton gradient across the plasma membrane (Katiyar and Katiyar, 1997). Gupta and Ahuja (2002) reports that the chromium remediation potential of fungi is attributed to the metal biosorption activity, which may also be the case with *Saccharomyces cerevisiae.*

Mutagenesis of Saccharomyces cerevisiae:

Mutagenesis was carried out using both hydroxylamine and MMS. The cells when plated after hydroxylamine mutagenesis, an auxotrophic marker for phenylalanine was obtained on exposure to 3ml of the mutagen for 3 hours and two other auxotrophic markers for isoleucine and tryptophan were obtained on exposure to 4ml of the mutagen for 3 hours. Following MMS mutagenesis, mutant colonies exhibiting a different phenotype was observed on plates containing phenylalanine and methionine (Fig. 2). Cells exposed to 1.5% of MMS, when plated on medium containing phenylalanine and methionine, produced colonies, which possessed a red sector within the raised moist colony (Fig. 2). This is due to mutation in the ade2 marker gene. Mutation was carried out in order to analyze whether DNA damage can alter the chromium remedial activity of the organism. Although the genetic material of most organisms is replicated and maintained with remarkable fidelity, it is a commonplace observation that mutations occur both spontaneously and in response to treatment with chemicals. The mutant allele accumulates a red

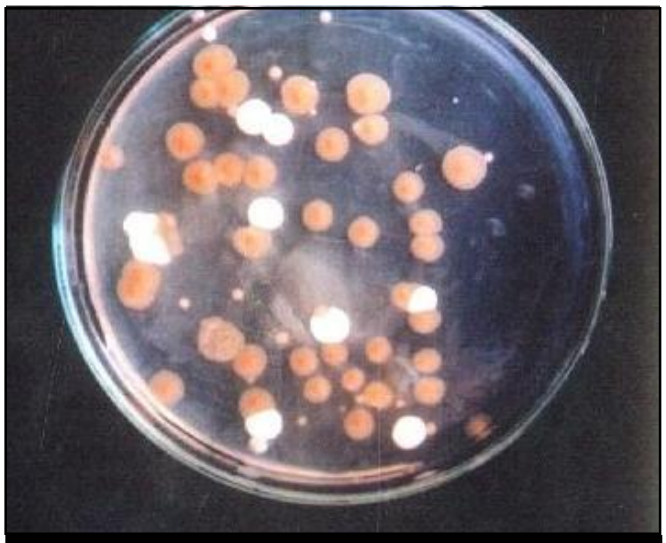

Fig. 2 : Mutant saccharomyces cerevisiae

pigment when homozygous but is recessive when heterozygous. This is due to mitotic recombination (reciprocal crossing over and non-reciprocal gene conversion), which can be induced to exposure to DNA damaging agents (Prakash *et al*., 1976; Bunsick *et al*., 1973; Christopher, 1970). There are several ways in which mutants sensitive to MMS could result in increased mitotic recombination (Prakash and Prakash, 1997). The mutant could be deficient in repairing single stranded breaks. Mutation may be induced at spontaneously occurring apurinic and apyrimidic sites or single strand breaks may be produced in excess in these mutants, the unpaired single strand breaks could then stimulate recombination and make the mutants hyper recombinants. Else, the mutants would exhibit enhanced mitotic recombination due to an indirect effect a decreased rate of DNA synthesis. This has been better implied for the explanation of the formation of red sector, which is due to the accumulation of a red pigment caused by the mutation in the enzymes involved in the *de novo* pathway of purine synthesis in the organism.

Genomic DNA was isolated and analyzed on 0.8% agarose gel. Bands were observed under UV and their molecular weights were determined by comparison with the known molecular weight of the DNA ladder (Lambda DNA isolated with *Hind III*) that was used. The DNA bands of the four test organisms possessed a molecular weight of 23,130 base pairs. The mutant organism also exhibited a single band indicating that mutagenesis using MMS did not lead to any DNA fragmentation and thereby produced only point mutations that altered a *de novo* pathway (Fig. 3).

Immobilization of Saccharomyces cerevisiae:

Baker's yeast, the mutants, strains 308 and 309 were immobilized. Immobilized cells have been reported to be very effective in heavy metal removal. Heavy metal toxicity and other extreme properties of waste effluents may limit the use of living cell systems. Freely suspended microbial biomass has disadvantages that include small particle size and low mechanical strength (Katiyar and Katiyar, 1997). Immobilized bioreactors using *Bacillus*

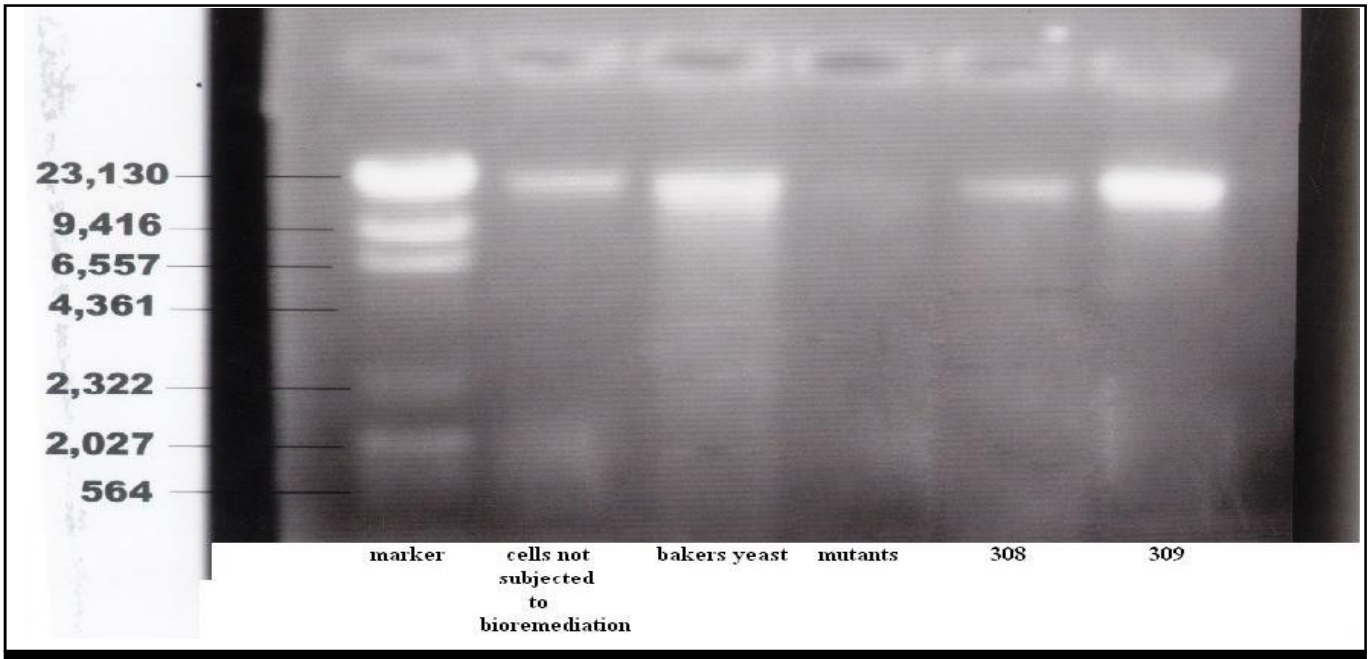

Fig. 3 : DNA-isolated from cells after remediation

coagulans have been tested for Cr (VI) reduction. *Pseudomonas* species, *Streptomyces* species and *Citrobacter* species immobilized on PVC have been reported to be very effective in heavy metal removal, namely vanadium, cadmium, copper, lead with high efficiency (Katiyar and Katiyar, 1997).

Chromium remediation:

All the immobilized forms of the test organisms were effective in chromium remediation. The gradual reduction in the absorbance value indicates the reduction in chromium concentration each hour. In 72 hours the immobilized beads of baker's yeast reduced the concentration of chromium in the effluent from 5600µg/l to 600µg/l, the mutants reduced the concentration to 37µg/l and metallothionein containing (308) and metallothionein-lacking strains (309) of *S. cerevisiae* reduced it to 28µg/l and 50µg/l, respectively. Their percentage efficiency was calculated to be 89.2%, 99.3%, 99.5% and 99.1%, respectively, from which it was concluded that the metallothionein containing strains were the most efficient followed by the mutants (Table 1 and Fig. 1). It has been illustrated that resistance to the toxic effects of copper ions in *S. cerevisiae* is mediated by the induction of a 6573 Da cysteine-rich protein, copper-metallothionein (Cu-MT). Since the protein is inducible only by copper it is referred to as Cu-MT. *Saccharomyces cerevisiae* contains a single Cu-MT gene, present in the CUP1 locus that is located on chromosome VIII, 42 centimorgans distal to the centromere. The basic repeat units codes for two genes, one of which codes for a 246-amino acid protein denoted as protein X as yet. The smaller ribonucleic acid (RNA) transcribed by the CUP1 locus encodes for a 61-amino acid cysteine rich protein, the yeast MT. Of the two genes present on the CUP1 locus, only the MT gene is transcriptionally induced after the addition of copper to the cells. A similar mechanism might be working in case of Chromium also.

MTs function as detoxifying agents by sequestering toxic metals and in the regulation and/ or metabolism of essential heavy metals (Hunt *et al*., 1984). Hence, the high efficiency of chromium remediation could be attributed to the presence of metallothionein, which causes the sequestration of chromium. Due to the formation of this complex it can be termed as Cr-metallothioneins. Further, the second best remedial organism was found to be the mutant, which illustrates the fact that the metallothionein gene does not get mutated due to hydroxylamine mutagenesis and thereby does not interfere with the Cr sequestering ability of the organism.

Alternatively, the strains lacking metallothioneins were also capable of efficient remediation and were rated third. This illustrates the fact that *S. cerevisiae* has other metal degradation mechanisms to remove chromium that is further enhanced in the presence of metallothioneins. Katiyar and Katiyar (1997) have reported that many fungi have high chitin content in their cell wall, which is an effective metal and radionuclide biosorbent. Fungal phenolic polymers and melanins contain phenolic units; peptides, carbohydrates, aliphatic hydrocarbons and fatty acids possess many potential metal-binding sites. In *Saccharomyces cerevisiae* Uranium is deposited as a layer of needle like fibrils on the cell wall reaching up to 50% of the dry weight of individual cells.

Studies on *Pseudomonas* species in remediation of chromium indicated an enzyme-mediated mechanism. The enzyme Cr (VI) reductase converts Cr (VI) to Cr (III) (Alvarez *et al*., 1999). However, this may not be the case with *S. cerevisiae*.

Coefficient of variation analysis was done to rate the organism's abilities in terms of consistency. It was found to be least for the yeast revived from baker's yeast (44.4) and highest for the metallothionein containing strains. However, this does not influence the remedial

Table 1 : Chromium remediation by the test organisms

Name of the immobilized test organism	Initial concentration of chromium (µg/ l)	Concentration of chromium after 72 hours (µg/ l)	Percentage efficiency	Coefficient of variation	Rank in terms of efficiency	Rank in terms of consistency
Normal *S. cerevisiae*	5600	600	89.2	44.81	4	1
Mutant *S. cerevisiae*	5600	37	99.3	89.16	2	3
S. cerevisiae 308	5600	28	99.5	94.26	1	4
S. cerevisiae 309	5600	50	99.1	63.56	3	2

efficiency of the strains.

Cell viability:

In the viability assay, the cells obtained from baker's yeast, mutant and metallothionein containing strains were beyond countable limits and for the strain lacking metallothionein the number of colonies were 256. The excellent viability of metallothionein containing and mutant strains after treatment enhances its potential for use as a bioremedial agent against chromium.

In summary, the study establishes the efficiency of *S. cerevisiae* in immobilized forms and the role of metallothioneins in chromium remediation. *In situ* bioremediation with biostimulation and bioaugumentation may prove to be highly efficient in chromium remediation. The role of metallothioneins in eliciting enhanced response against heavy metal stress could be employed to engineer super bugs for chromium remediation. *Pseudomonas* sp. due to their pathogenicity is less preferred as a bioremedial system. Hence, further studies are needed in an attempt to fuse the gene coding for Cr (VI) reductase enzyme e and the metallothionein genes that would lead to new strains that are highly efficient and stable in chromium remediation.

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