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Research Paper

Study on heavy metals biosorption ability of *Saccharomyces cerevisiae*

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Abstract

Potential *Saccharomyces cerevisiae* was isolated from paper effluent for bioremediation of heavy metals. Morphological studies, physiological tests and molecular characterization confirmed isolated yeast colony was *S. cerevisiae*. *S. cerevisiae* has showed high biosorption of Cd^{2+} (67%), followed by Pb^{2+} (61%)> Ni^{2+} (64%)> Cr^{6+} (63%)> Cu^{2+} (57%)> Zn^{2+} (53%). SEM image of heavy metals treated *S. cerevisiae* showed patches of accumulated heavy metals and ED's spectrum of *S. cerevisiae* treated with heavy metals contained metal peaks. Elemental analysis by EDS confirmed that ion exchange mainly involved in heavy metals biosorption by *S. cerevisiae*. Optimization of parameters made maximum biosorption of heavy metals. The pH 4.0-5.0, temperature 20-25°C and contact time of 60 minute was found optimal for heavy metals biosorption. The metal biosorption was found maximum in single metal system compared to multi metal ions. Pre-treatment method enhanced the metal biosorption. Sorption isotherm studies fit into the model of Langmuir isotherm compared to Freundlich isotherm.

Keywords: *Biosorption, Heavy metals, Optimization, Saccharomyces cerevisiae, Sorption isotherm.*

1. Introduction

Lead, mercury, chromium, cadmium, copper, cadmium, nickel and zinc are the major hazardous heavy metals in terms of their environmental load and health effects. Their source is majorly by industrial activity and some natural causes like seepage from rocks and volcanic activity. Toxicity of heavy metals is of considerable concern in India because of their environmental burden (Sahni, 2011).

Most of the heavy metal salts are soluble in water to form aqueous solution and cannot be separated by physico-chemical methods of separation such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion exchange and membrane technologies (Tsekova and Petrov, 2002). These conventional processes used for metal removal are sometimes restricted because of technical and economic constraints (Akar and Tunali, 2006). Metal polluted environment contains fungi, which have adapted to toxic concentration of heavy metals and become metal resistant. Fungi affect the mobility and environmental fate of heavy metals. *Saccharomyces* sp. accumulates copper and zinc as micronutrients. Along with these micronutrients, it capable to accumulate non-nutrient metals likes nickel, cadmium, lead, chromium in amounts higher than the nutritional requirement (Jaeckel et al., 2005). Metals uptake by living fungi depends on species, culture conditions, metal concentration and cells in the solution (Melgar et al., 2007).

Fungal biomass can be used in the form of nanoparticles successfully for removal of heavy metals from wastewater. The main advantages in use of fungal strain for heavy metal removal from waste water is their size, texture and use as adsorbent without immobilize. Biosorption using fungi can be accomplished by simple physical methods without damaging the biosorbents structural integrity and accomplish a high efficiency in the removal of heavy metals from aqueous solution. In biosorption process, the fungal biosorbents have to be easily regenerated and used for number of sorption and desorption cycles without denaturation of biomass with the same removal efficiency (Adeyemi, 2009). Advantages of biosorption process compared to bioaccumulation process includes, growth independent, non-living biomass is not subject to toxicity limitation of cells. The removal of heavy metal ions by fungal biomass has been observed to be more than that by conventional adsorbents such as activated carbon, ion-exchange resins, even algae and seaweeds. Dead biomass can be subjected to physical and chemical treatments to enhance metal biosorption (Sag, 2001).

The objective of our study was to isolate and characterize potential *S. cerevisiae* for biosorption of major toxic heavy metals. Different parameters were optimized for increased biosorption of heavy metals.

2. Materials and methods

2.1. Isolation of *saccharomyces cerevisiae*

S. cerevisiae was isolated from paper industrial effluent using Malt extract medium. 50ml of the medium was dispensed to 250ml Erlenmeyer flask and 2ml of 1:10 diluted suspension of untreated paper industrial effluent was added and kept on a rotary shaker for 72 hr at 30°C. After incubation period, the content was allowed to settle, flask was tilted and a loop of sediment containing yeast cells was streaked on Yeast extract malt extract glucose agar media. Inoculated plates were incubated at room temperature

for 5-7 days for the growth of yeast colonies. Isolated *S. cerevisiae* was cultured on Yeast extract peptone dextrose agar (YEPD) media for identification (Saifuddin and Raziah, 2007).

2.2. Morphological identification

Growth of *S. cerevisiae* was confirmed by cultured the isolated colony in Yeast Nitrogen Base medium supplemented with nitrogen source, amino acids, vitamins, trace elements and salts. The morphological identification such as colour and colony characteristics was observed.

2.3. Physiological characterization

Physiological identification of *S. cerevisiae* was carried out by performing sugars fermentation test and nitrogen assimilation test (Warren and Shadomy, 1991). Identification of *S. cerevisiae* was further confirmed based on its utilization of 4% ethanol as carbon source and development of pseudo-mycelium by slide culture in a Petri dish (Cooke et al., 1960). Physiological tests, such as D-Glucose, sucrose, lactose fermentation test, catalase reaction, nitrate growth, ethanol growth, methanol growth, and urea hydrolysis tests were performed for confirmation of *S. cerevisiae* (Sofyan et al., 2000).Isolated pure culture was routinely maintained on YEPD agar media preserved at 4°C in refrigerator.

2.4. Molecular characterization

Molecular identification of *S. cerevisiae* was performed by gene sequencing of the ITS regions of ribosomal DNA (rDNA). The ITS 1 and ITS 4 region of the rDNA was amplified by polymerase chain reaction (PCR) using ITS 1 and ITS 4 primers. The ~700 bp amplicon was gel eluted and subjected to sequencing. The multiple sequence alignments were developed from the sequence data. ITS sequenceresults were assembled and compared with the sequences from Gene Bank (NCBI; http://www.ncbi.nlm.nih.gov) through Basic Local Alignment Search Tool (BLAST) searches. Distance tree of the isolate was determined and top 10 matches for the sequence derived from the isolate was compared with National Centre for Biotechnology Information (NCBI) blast nucleotide sequence (Marjeta et al., 2002; Nur et al., 2011).

2.5. Preparation of bio sorbent for heavy metals bio sorption

The growth medium used for preparation of *S. cerevisiae* biosorbent was Yeast Peptone Glucose (YPG) Medium (Chen and Wang, 2007). Five days old culture of *S. cerevisiae* grown on YEPD agar media plate at 30°C was used for inoculation (concentration of cell suspension used was 1x105 CFU/mL) in YPG liquid medium. The culture was grown in YPG medium at 30°C in conical flask kept on a rotary shaker agitated at 125 rpm. After 3-4 days of growth, *S. cerevisiae* was harvested by filtration using 150µm sieve and centrifugation. The harvested biomass was washed with generous amount of deionized water and stored at -20°C. The biomass of *S. cerevisiae* was treated with 0.5N NaOH for 30 min followed by washing with generous amount of distilled water until the pH of the solution reached to neutral range of 6.8-7.2. *S. cerevisiae* biomass was autoclaved at 15 lb/inch^2 for 20 min. Autoclaved biomass was dried at 60°C for 24 hr in hot air oven to get constant dry weight (Jaeckel et al., 2005; Iqbal et al., 2005).

2.6. Preparation of metal solution

Stock metal solution contained 1000mg/L concentration of Ni(II) ($NiSO_4.7H_2O$), Zn(II) ($ZnSO_4.6H_2O$), Cd(II) ($CdCl_2$), Pb(II) $(CH_3COO)_2Pb.3H_2O$, Cr(VI) ($K_2Cr_2O_7$) and Cu(II) ($CuSO_4.5H_2O$) was prepared by dissolving heavy metals salt in deionised distilled water. The reagents used were of analytical grade supplied by Merck. The working metal solution of 50, 100, 150 and 200mgL^{-1}concentration was prepared from the stock solution.

2.7. Biosorption experiments

Biosorption experiments for *S. cerevisiae* biomass was carried out using Cr^{6+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ni^{2+}metal solutions each contained 50, 100, 150 and 200 mgL^{-1} metal concentration. A batch experiment of biosorption was performed at constant temperature (25°C) in Erlenmeyer flasks stirred in a reciprocal shaker with 100 rpm for 02 hours. The equilibrium is reached significantly before two hours. In all sets of experiments, 0.1 gm of pre-treated S. cerevisiae biomass was inoculated into 100ml of each heavy metal solutions for biosorption study.
After biosorption process, the reaction mixtures were filtered to remove particulates and the filtrates were analyzed by Atomic absorption spectrophotometer for the determination of concentration of heavy metals biosorbed. Amount of heavy metals biosorbed by *S. cerevisiae* was calculated from the difference between total amount of heavy metal added and that remained in the supernatant after biosorption process. All the experiments were carried out on a triplicate basis (Kang et al., 2007).
The amount of metal ions (mg) bioadsorbed per gm (dry mass) of biomass was calculated using following equation:

$$Q= (C_i-C_f/m)\ V \quad (1)$$

Where Q = mg of metal ion bioadsorbed per gm of biomass, C_i = Initial metal ion concentration (mg/L), C_f = Final metal ion concentration (mg/L), m = Mass of biomass in the reaction mixture (gm), V = Volume of the reaction mixture.
The efficiency of heavy metal removal was calculated by following equation from the amount of metal ions adsorbed on biosorbent and amount of metal ions available in solution,

$$\% \text{ removal} = \frac{\text{Heavy metal ions removed (mg)}}{\text{Heavy metal ions available (mg)}} \times 100 \quad (2)$$

2.8. Sorption Isotherm analysis

The equilibrium of sorption is an important factor for evaluating the sorption process as unit operation. In order to investigate the sorption capacity and isotherm, two frequently used equilibrium models, such as Langmuir and Freundlich isotherms were studied and fitted to the data obtained. Initial concentrations of metal solutions used for isotherm analysis was between 50 and 200 mg/L metal concentration. 0.1 g of pre-treated S. cerevisiae biomass was mixed into 100 ml of metal solutions for sorption isotherm study.

2.8.1. Langmuir isotherm

Langmuir model is probably the best known and most widely applied sorption isotherm. This model defines a monolayer sorption with a homogeneous distribution of sorption sites and sorption energies without interaction between the sorbed molecules. It has produced good agreement with wide variety of experimental data and it can be represented as follows

$$q = \frac{q_{max} b C_f}{1 + bC_f} \quad (3)$$

Where, C is equilibrium concentration (m_{eq}/L), q is amount of metal ions sorbed (m_{eq}/g), q_{max} is maximum amount of metal ion sorbed per unit weight of biomass to form a complete monolayer (m_{eq}/g), b is sorption equilibrium constant (L/m_{eq}).
The Eq. (3) can be rearranged to the following linear form:

$$\frac{C}{Q} = \frac{C}{q_{max}} + \frac{1}{q_{max} b} \quad (4)$$

2.8.2. Freundlich isotherm

Freundlich empirical model can be applied to non-ideal sorption on heterogeneous surface as well as multilayer sorption and is expressed by Eq. (5). In contrast to Langmuir model, the Freundlich isotherm is more widely used but provides no information on monolayer adsorption capacity.

$$q = k_f C^{1/n} \quad (5)$$

Where, C is equilibrium concentration (m_{eq} /L), q is amount of metal ions sorbed (m_{eq}/g), k_f and n is Freundlich's adsorption constants.
The Eq. (5) is frequently used in the linear form by taking logarithm of both sides as follows:

$$\text{Log } q = \log k_f + 1/n \log C \quad (6)$$

All experiments were conducted at 27ºC and result of the data was applied to Equations (4) and (6). In order to determine how well the Freundlich model fitted with experimental data, the plot of log Ce versus log q_e was employed. The calculated values of Langmuir and Freundlich parameters were obtained using least squares method (Pinoa et al., 2006).

2.9. Scanning electron microscopy (SEM) analysis

SEM analysis gives morphological characteristics and confirmation of heavy metals accumulation by *S. cerevisiae*. During SEM analysis, a drop of sample was dried on clean silicon wafer and electron conductivity was created externally to the sample by sputtering with gold nanoparticles using a gold sputter coater (Jeol JFC 1100E Ion sputtering device) for 30min. Coated cells were applied with electron acceleration voltage of 20 keV and viewed under Field Emission-SEM (FEI-SIRION, Eindhoven, Netherland).

2.10. Energy dispersive x-ray spectrometry (EDS) analysis

ED's analysis was performed to analyse elemental peak and composition including the presence of metal ions on biomass of *S. cerevisiae* before and after treated with heavy metals. EDS of *S. cerevisiae* was taken using NORAN EDS system attached with SEM. Point and region analyses were performed at 15 keV. The EDS spectra with metal peaks were recorded during analysis period of 60 second (Xue and Lu, 2008).

2.11. Optimization of parameters

Batch experiments were performed to study the effect of different factors such as pretreatment, time period, pH, and temperature, presence of single and multi-metal ions on biosorption process (Kapoor et al., 1999).

2.11.1. PH

The optimal pH value required by *S. cerevisiae* for heavy metals biosorption was determined. *S. cerevisiae* was inoculated into series of 250 ml conical flasks each contained 50 mgL^{-1} of Cr^{6+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ni^{2+} metal solution. *S. cerevisiae* inoculated flasks were incubated in rotary shaker incubator at 25°C for 120 minutes in100 rpm. The pH of each metal solution varied from 02 to 07 (02, 03, 04, 05, 06 and 07). pH of the medium was adjusted using dilute HCl and NaOH. Amount of heavy metals biosorbed by *S. cerevisiae* biomass was calculated from difference between total amount of heavy metal added and that remained in the supernatant after biosorption. For each pH point the percentage of metal removal was calculated using equation 1.

2.11.2. Temperature

The optimal temperature value required by *S. cerevisiae* for biosorption of heavy metals was determined. *S. cerevisiae* was inoculated into series of 250 ml conical flasks each contained 100ml of 50mgL^{-1} Cr^{6+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ni^{2+} metal solution. *S. cerevisiae* inoculated metal solutions were incubated at varied temperature of 15°C, 20°C, 25°C, 30°C, and 35°C in rotary shaker incubator for 120 minute at 100 rpm. The pH of metal solution was adjusted to 4.0 using dilute HCl and NaOH. Amount of heavy metals biosorbed by *S. cerevisiae* biomass was calculated from the difference between total amount of heavy metal added and that remained in the supernatant after biosorption. For each temperature point the percentage of metal removal was calculated using equation 1.

2.11.3. Contact time

The optimal contact time required by *S. cerevisiae* for biosorption of heavy metals was determined. *S. cerevisiae* was inoculated into series of 250 conical flasks each contained 100 ml of 50mgL^{-1} concentration Cr^{6+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ni^{2+} metal solution. *S. cerevisiae* inoculated metal solutions were incubated at varied incubation period of 30, 60, 90, and 120 minute in rotary shaker incubator at 25°C of 100 rpm. PH of the metal solution was adjusted to 4.0 using dilute HCl and NaOH. Amount of heavy metals biosorbed by biomass after specific period of incubation was calculated from difference between total amount of heavy metal added and that remained in the supernatant after biosorption. The percentage of metal removal was calculated using equation 1.

2.11.4. Single and multi-metal system

0.1 g of *S. cerevisiae* biomass was treated with 50mgL^{-1}concentration of 100 ml Pb, Cd and Cr metal solution. 100ml of multi-metal solution each contained 50mgL^{-1} concentration (Pb+Cd), (Pb+Cr) and mixture of Pb+Cd+Cr metal solution was treated with 0.1g of *S. cerevisiae* biomass. Amount of heavy metals biosorbed by *S. cerevisiae* biomass was calculated from the difference between total amount of heavy metal added and that remained in supernatant after biosorption.

3. Results

3.1. Isolation and identification of *saccharomyces cerevisiae*

Due to continuous enrichment and adaptation in heavy metals polluted environment, potential *S. cerevisiae* was isolated from paper industrial effluent by standard plate count method using Malt extract agar (MEA) media. The presence of moist to waxy colonies was observed on Malt extract agar media. The stereobinocular microscopic observation showed the predominance of budding cells. The suspected yeast isolate was pure cultured and subjected for physiological and molecular identification.
Morphological characters observed for identification of *S. cerevisiae* were, budding or long oval shaped cells, pseudohyphae, and spore formation. The physiological tests were performed for identification of *S. cerevisiae* (Table 1).

Table 1: Physiological Characterization of *Saccharomyces cerevisiae*.

Sl. No	Result	Test
1	Ellipsoid	Morphology
2	No hyphae	Hyphae
3	Positive	Catalase-reaction
4	Positive	D-Glucose fermentation
5	Positive on	Sucrose fermentation
6	Negative	Lactose fermentation
7	Positive	Citrate growth
8	Positive	Ethanol growth
9	Negative	Methanol growth
10	Positive	Nitrate growth
11	Negative	Urea hydrolysis

Physiological tests such as glucose fermentation, galactose fermentation, maltose fermentation, sucrose fermentation, lactose fermentation, nitrate assimilation test and urease utilization tests were performed for the identification of *S. cerevisiae*. D-Glucose and sucrose fermentation medium inoculated with *S. cerevisiae* showed the production of gas in Durham tube and medium colour turned from red to yellow. Whereas the absence of gas production and without change in medium colour was observed in both control medium without inoculated with *S. cerevisiae* and lactose fermentation medium inoculated with *S. cerevisiae*. The *S. cerevisiae* inoculated citrate medium colour was changed from green to blue after incubation period. Citrate medium without inoculated with *S. cerevisiae* was retained their original green colour. The appearance of gas bubbles was observed in catalase test performed with isolated *S. cerevisiae*. Urea hydrolysis test medium inoculated with *S. cerevisiae* was showed the colour change from yellow to red after incubation period.

Molecular identification was performed for the confirmation of *S. cerevisiae*. The sequence of ITS region for *S. cerevisiae* was determined (Table 2).

Table 2: The Sequence of ITS Region for *Saccharomyces cerevisiae*.

1	aattcctagt aagcgcaagt catcagcttg cgttgattac gtccctgccc tttgtacaca
61	ccgcccgtcg ctagtaccga ttgaatggct tagtgaggcc tcaggatctg cttagagaag
121	ggggcaactc catctcagag cggagaattt ggacaaactt ggtcatttag aggaactaaa
181	agtcgtaaca aggtttccgt aggtgaacct gcggaaggat cattaaagaa atttaataat
241	tttgaaaatg gatttttttg ttttggcaag agcatgagag cttttactgg gcaagaagac
301	aagagatgga gagtccagcc gggcctagtc ttaagtgcgc ggtcttgcta ggcttgtaag
361	tttctttctt gctattccaa acggtgagag atttctgtgc ttttgttata ggacaattaa
421	aaccgtttca atacaacaca ctgtggagtt ttcatatctt tgcaactttt tctttgggca
481	ttcgagcaat cggggcccag aggtaacaaa cacaaacaat tttatttatt cattaaattt
541	ttgtcaaaaa caagaaattt cgtaactgga aattttaaaa tattaa

The assembly of ITS 1 and ITS 4 sequencing for *S. cerevisiae* was analyzed (Table 3).

Table 3: The Assembly of ITS 1 and ITS 4 Sequencing for *Saccharomyces cerevisiae*.

```
Query 1 AATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACA 60
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1 AATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACA 60

Query 61 CCGCCCGTCGCTAGTACCCATTGAATGGCTTAGTGAGGCCTCAGGATCTGCTTAGAGAAG 120
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61 CCGCCCGTCGCTAGTACCCATTGAATGGCTTAGTGAGGCCTCAGGATCTGCTTAGAGAAG 120

Query 121 GGGGCAACTCCATCTCAGAGCGGAGAATTTGGACAAACTTGGTCATTTGGAGGAACTAAA 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121 GGGGCAACTCCATCTCAGAGCGGAGAATTTGGACAAACTTGGTCATTTGGAGGAACTAAA 180

Query 181 AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAAAAGAAATTTAATAA 240
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181 AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAAAAGAAATTTAATAA 240

Query 241 TTTTGAAAATGGAttttttGTTTTGGCAAGAGCACAGAGCTTTTACTGGGCAAGAAGAC 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241 TTTTGAAAATGGATTTTTTTGTTTTGGCAAGAGCACAGAGCTTTTACTGGGCAAGAAGAC 300

Query 301 AAGAGATGGAGAGTCCAGCCGGGCCGCGCTTAAGTGCGCGGTCTTGCTAGGCTTGTAAGT 360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301 AAGAGATGGAGAGTCCAGCCGGGCCGCGCTTAAGTGCGCGGTCTTGCTAGGCTTGTAAGT 360

Query 361 TTCTTTCTTGCTATTCCAAACGGTGAGAGATTTCTGTGCTTTTGTTATAGGACAATTAAA 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361 TTCTTTCTTGCTATTCCAAACGGTGAGAGATTTCTGTGCTTTTGTTATAGGACAATTAAA 420

Query 421 ACCGTTTCAATACAACAAACTGTGGAGTTTTCATATCTTTGCAACTTTTTCTTTGGGCAT 480
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421 ACCGTTTCAATACAACAAACTGTGGAGTTTTCATATCTTTGCAACTTTTTCTTTGGGCAT 480

Query 481 TCGAGCAATCGGGGCCCAGAGGTAGCAAACACAAACAATTTTATCTATTCATTAAATTTT 540
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 481 TCGAGCAATCGGGGCCCAGAGGTAGCAAACACAAACAATTTTATCTATTCATTAAATTTT 540

Query 541 TGTCAAAAACAAGAATTTTCGTAACTGGAAATTTTAAAATATTAAAAACTTTCAACAACG 600
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 541 TGTCAAAAACAAGAATTTTCGTAACTGGAAATTTTAAAATATTAAAAACTTTCAACAACG 600

Query 601 GATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTG 660
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 601 GATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTG 660

Query 661 CAGAATT 667
          |||||||
Sbjct 661 CAGAATT 667
```

ITS sequencing of isolated yeast culture was showed 100% identity at 100% coverage with the sequence from *S. cerevisiae* during NCBI BLAST nucleotide sequence analysis (Table 4).

Table 4: Top 10 Matches for the Sequence Derived from *Saccharomyces cerevisiae*.

Sequences producing significant alignments:

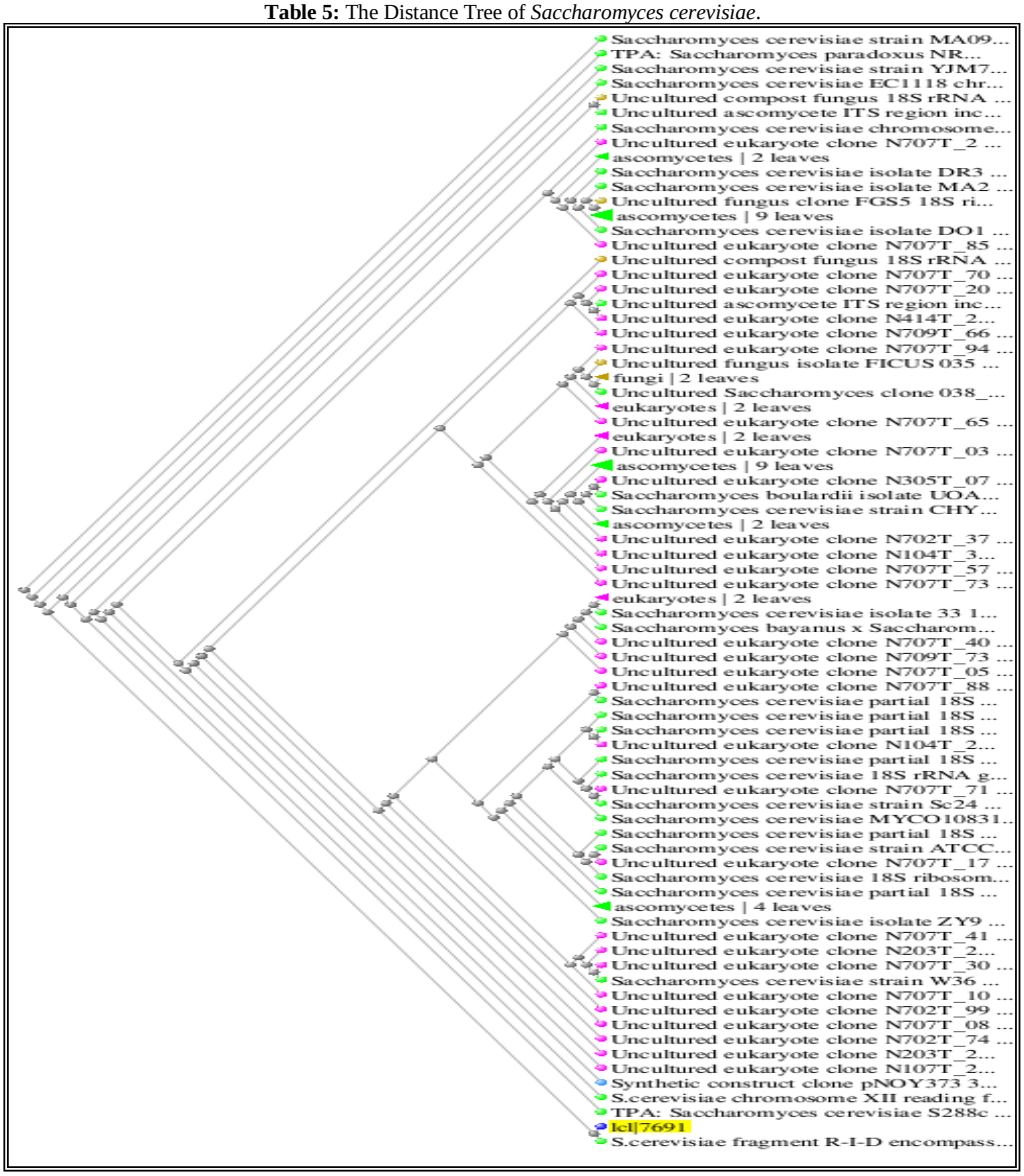
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident
S.cerevisiae fragment R-I-D encompassing 18S and 5.8S ribosomal RNA genes and their spac	1232	1232	100%	0.0	100%
TPA: Saccharomyces cerevisiae S288c chromosome XII, complete sequence	1188	2377	100%	0.0	99%
S.cerevisiae chromosome XII reading frame ORF YLR154c	1188	1188	100%	0.0	99%
Saccharomyces cerevisiae chromosome XII cosmid 9634	1188	2377	100%	0.0	99%
Uncultured compost fungus 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRN	1182	1182	99%	0.0	99%
Saccharomyces cerevisiae EC1118 chromosome XII, EC1118_1L10 genomic scaffold, whole c	1179	2358	100%	0.0	99%
Uncultured ascomycete ITS region including 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 2	1179	1179	99%	0.0	99%
Saccharomyces cerevisiae strain YJM789 35S ribosomal RNA gene, external transcribed spac	1173	1173	100%	0.0	98%
Synthetic construct clone pNOY373 35S ribosomal RNA, 18S ribosomal RNA, 5.8S ribosomal R	1166	1166	100%	0.0	98%
TPA: Saccharomyces paradoxus NRRL Y-17217 genes for 25S rRNA, 5S rRNA, 18S rRNA, 5.	1151	1151	100%	0.0	98%
Uncultured ascomycete ITS region including 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 2	1136	1136	99%	0.0	97%
Uncultured compost fungus 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRN	1129	1129	99%	0.0	97%

The distance tree of isolated yeast colony was corresponded to *S.cerevisiae* (Table 5). There fore the isolated yeast colony was confirmed as *Saccharomyces cerevisiae*.

Table 5: The Distance Tree of *Saccharomyces cerevisiae*.



3.2. Bio sorption of heavy metals

The percentage of metal biosorption in *S. cerevisiae* was decreased with increased in treated metal solution concentration to 50, 100, 150 and 200mgL^{-1}. *S. cerevisiae* showed metals biosorption at 50 mgL^{-1} concentration in the order of, Cd (67%)>Pb(61%)> Ni(64%)>Cr(63%)>Cu(57%)>Zn(53%). The biosorption of metals showed by *S. cerevisiae* in100 mgL^{-1} metals concentration was in the order of, Cd (63%)>Ni (59%)>Pb (55%)> Cu (54%)>Cr (53%)>Zn (49%). The order of metals biosorption observed in *S. cerevisiae* at 150 mgL^{-1}metals concentration was, Cd (51%)> Ni (47%) ≥Cu (47%)>Pb (43%)> Cr (41%)>Zn (38%). The order of metals biosorption observed in *S. cerevisiae* treated with 200 mgL^{-1} concentration was, Cd (35%)>Ni (31%)>Pb (29%)>Cr (25%)>Cu (39%)>Zn (21%) (Figure 1).

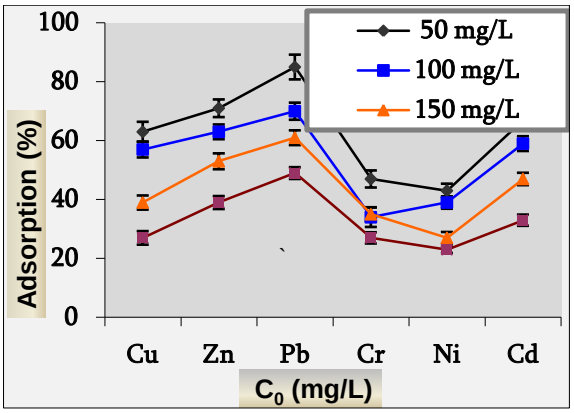


Fig. 1: Biosorption of Heavy Metals by *Saccharomyces cerevisiae*.

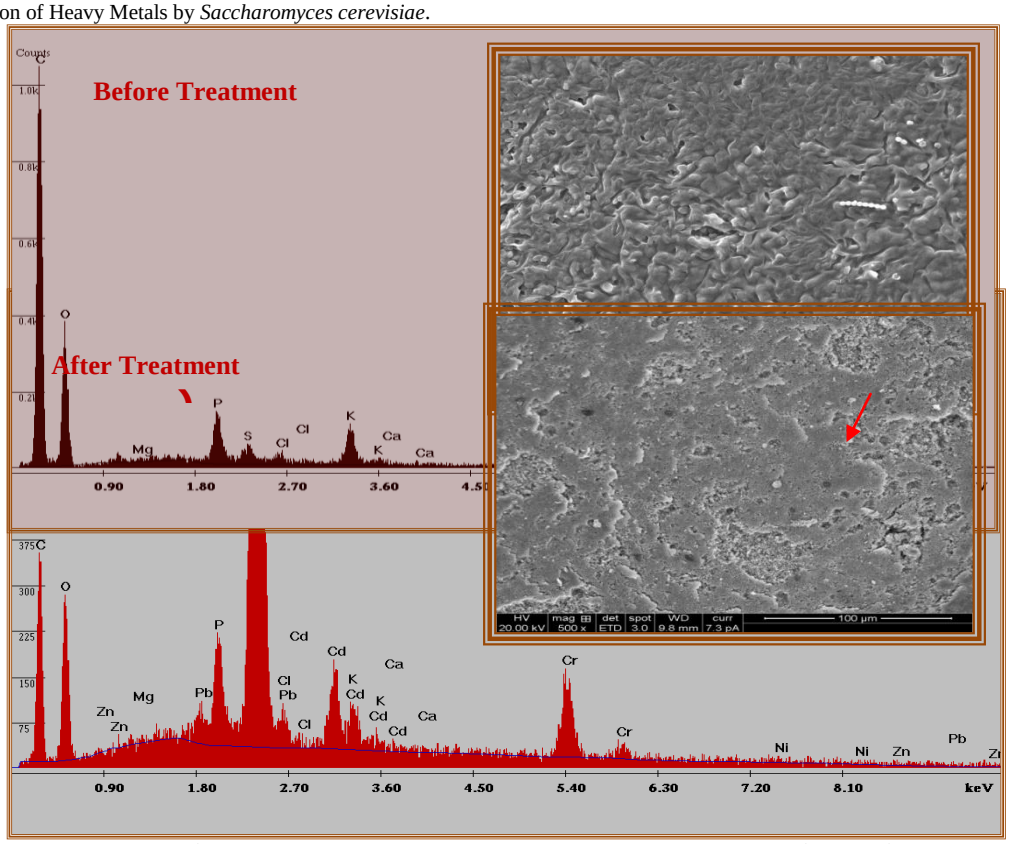


Fig. 2: SEM Photographs and EdS of *Saccharomyces cerevisiae* Showing Elemental Composition in (A) before (B) after Treated with Heavy Metals.

SEM coupled with EDS analysis was used to study the interaction of metal ions with *S. cerevisiae* (Figure 2).

SEM analysis revealed that metals was uniformly bound and formed flocculation or patches of heavy metals on S. cerevisiae treated with metals compared to control biomass without treated with metals SEM image of *S. cerevisiae* treated with metals showed altered morphology with dense particles of accumulated heavy metals compared to control showing absence of metal accumulation.

3.3. Sorption isotherms analysis

The correlation coefficients of Langmuir and Freundlich isotherms are 0.9979 and 0.8170 respectively (Table 6).

Table 6: Calculated Langmuir and Freundlich Coefficients.

	Langmuir parameters		Freundlich parameters	
1	q_{max}(mg/g dry biomass)	155.45	n	2.97
2	b(L/mg)	0.0623	k	16.8
3	r^2	0.9979	r^2	0.8170

The correlation coefficients of Langmuir isotherm are higher than that of Freundlich model for pre-treated *S. cerevisiae*. This indicates that the Langmuir isotherm for pre-treated *S. cerevisiae* is fitted better and is accepted for this adsorbent. Adsorption process could be well defined by Langmuir isotherm with r^2values 0.9979.

3.4. Scanning electron microscopy (SEM)

3.5. Energy dispersive x-ray spectrometry (EDS)

Quantification of metals within *S. cerevisiae* was performed by EDS analysis. When metals treated *S. cerevisiae* was applied for EDS analysis, formation of metals peak was detected compared to control *S. cerevisiae* without treated with metals. Peak of EDX analysis revealed that a metal ion of Zn, Pb, Cd, Cr and Ni has bind to *S. cerevisiae* cell wall at different keV. High ED's spectrum peak of Pb^{2+} indicates the maximum biosorption of Pb^{2+} by *S. cerevisiae* compared to other metals. Followed by Pb^{2+} decreased spectrum peak was observed for other metal ions in the order of, Cr > Cd >Zn ≥ Ni.
Elemental composition in EDS for metal treated *S. cerevisiae* was changed after treated with heavy metals (Table 7).

Table 7: Elemental Composition in EDS of *Saccharomyces cerevisiae* without Treated and Treated with Heavy Metals.

Sl. No.	Elemental composition	Control (wt %)	Metal treated (wt %)
1	Carbon (C)	66.80	28.59
2	Oxygen (O)	22.07	9.15
3	Magnesium (Mg)	0.23	0.08
4	Phosphorus (P)	3.63	1.19
5	Sulfur (S)	1.50	1.57
6	Chlorine (Cl)	1.02	0.77
7	Potassium (K)	4.35	0.81
8	Calcium (Ca)	0.41	0.17
9	Cadmium (Cd)	0.00	4.11
10	Chromium (Cr)	0.00	4.93
11	Nickel (Ni)	0.00	1.21
12	Zinc (Zn)	0.00	0.94
13	Lead (Pb)	0.00	46.48
Total		100.00	100.00

The ion exchange mechanism resulted in replacement of K, Ca, Mg ions by metal ions such as Pb, Cr,Cd,Ni, and Zn during biosorption of heavy metals by *S. cerevisiae*. The reduced C, O, Mg, P, K, Ca content was observed in control biomass of *S. cerevisiae* without treated with heavy metals compared to metals treated *S. cerevisiae* biomass due to exchange of these ions with heavy metals.

3.6. Optimization of parameters

3.6.1. PH

Bio sorption of heavy metals observed in *S. cerevisiae* biomass was high at pH ranging from 4.0 to 5.0 (Figure 3).

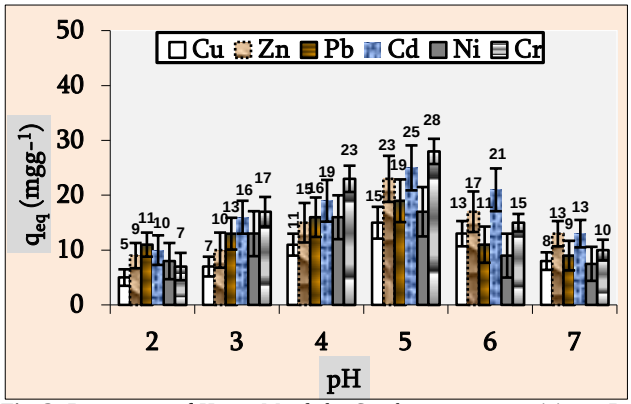


Fig. 3: Biosorption of Heavy Metals by *Saccharomyces cerevisiae* at Different pH.

The decreased metal biosorption was observed at very low acidic pH of 2.0 and 3.0. The biosorption ability of *S. cerevisiae* was decreased with increased in pH to 6.0 and above.

3.6.2. Temperature

Temperature is the factor influence on biosorption of heavy metals by *S. cerevisiae* biomass (Figure 4).

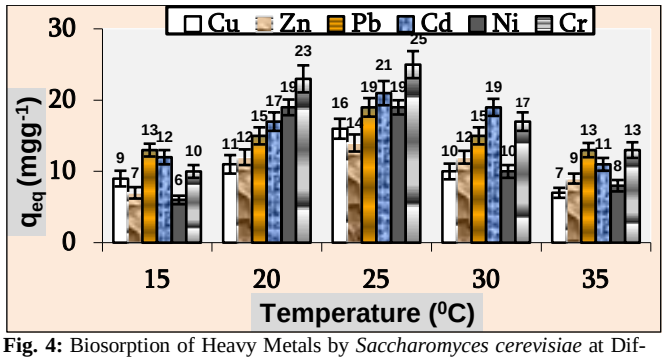


Fig. 4: Biosorption of Heavy Metals by *Saccharomyces cerevisiae* at Different Temperature.

The observed metal biosorption by *S. cerevisiae* varies at different temperature ranging from 15 to 35°C. Difference of 05°C in metal solution causes increased or decreased metal biosorption. The metals biosorption observed was high in the temperature ranging from 20 to 30°C. Biosorption of metals was decreased at low temperature between 15 to 20°C and in high temperature between 30 to 35°C.

3.6.3. Contact time

Contact time between biomass and metal solution influence on biosorption process. A sufficient contact time should be provided to the biosorbent (*S. cerevisiae* biomass) for sorption of biosorbate (metal solution). Contact time period between biomass and metal solution was studied from 15 to 120 min (Figure 5).

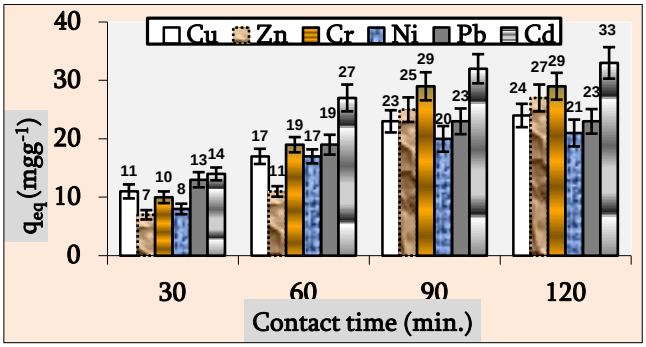


Fig. 5: Bio Sorption of Heavy Metals by *Saccharomyces cerevisiae* at Different Contact Time.

It was found that heavy metals biosorption by *S. cerevisiae* biomass was high during the contact time period of 60 to 90 min. Metals biosorption observed was less during initial contact time period of 15 to 30 min and remained constant after 90 min and above time period.

3.6.4. Single and multi-metal system

Bio sorption of heavy metals by *S. cerevisiae* was performed using single, double and multi heavy metals solution of lead, cadmium and chromium (Table 8).

Table 8: Biosorption of Heavy Metals by Fungi in Single and Multi-Metal System.

Organism	Metal type	mg of metal ion biosorbed per gram of biomass			
S.cerevisiae	Single metal	Pb+Cr	Pb+Cd	Cr+Cd	Pb+Cr+Cd
	5.34±0.19 (Pb)	4.12±0.19	3.99±0.11		1.73±0.07
	4.23±0.15 (Cr)	3.71±0.13		3.01±0.10	1.94±0.09
	7.93±0.37 (Cd)		5.93±o.23	4.27±0.22	2.19±0.15

Note: Results are expressed in Mean ± Standard Error (n=3)
This study determined the effect of more than one heavy metal on biosorption process. The maximum percentage of metal biosorp-

tion was achieved with single metal solution (Pb, Cr, Cd) compared to double (Pb+Cd) and multi metal solutions (Pb+Cd+Cr). The percentage of metal biosorption was decreased with increase in number of metal ions in solution in the order of, (Pb, Cr, and Cd) > (Pb+Cd) > (Pb+Cr+Cd).

4. Discussion

Fungi indigenous to paper mill effluent can be used for bioremediation of heavy metals (Vara et al., 2010; Shanthi et al., 2012). The presence of most dominant yeast genera belonging to *S. cerevisiae* in paper effluent indicates their ability to resist toxic pollutants and used for heavy metals bioremediation studies. It was found that *S. cerevisiae* isolated from highly metal contaminated sites has shown high metal resistance than those isolated from metal uncontaminated sites. Thus, the presence of metal may act as a selective pressure for metal resistance in *S. cerevisiae* (Madhu et al., 2013).

4.1. Identification

The morphological and physiological tests are commonly performed for identification of yeast strains (Kambe et al., 1997). S. cerevisiae inoculated into D-Glucose and sucrose medium, pH indicator (phenol red), which was red at neutral pH (7.0), turned to yellow in acidic pH (below 6.8) due to acid production. Non-fermentation ability of lactose and control medium without inoculated with *S. cerevisiae* shows the absence of indicator colour change and acid production. In positive citrate growth test, citrate is the only source of carbon and energy. When citrate is utilized, CO_2 generated and combines with sodium to form an alkaline product. This product change the Bromothymol blue indicator colour, from green (pH 6.8 and below) to blue (pH 6.8 and above). The enzyme catalase presents in *S. cerevisiae* breaks the hydrogen peroxide into water and oxygen for survive of *S. cerevisiae*. The release of oxygen gas bubbles is an indication for positive catalase test. S. cerevisiae produces urease enzyme, it attacks the carbon bond and liberates ammonia. The produced ammonia raises the pH of urease medium. Hence the colour of phenol red indicator changes from yellow (pH 6.8) to red (Aneja, 2003).

Yeast has much reduced morphological characters compared to other fungi. Hence, the physiological tests and molecular characterization have been used for identification of S. cerevisiae. The main characteristic features of *S. cerevisiae* are ellipsoid shape cell, have no hyphae, budding cells and do not produce lactose-digesting enzymes. Positive catalase test and negative results of lactose fermentation confirmed the presence of *S. cerevisiae* (Kurtzman, 1988).Morphological and physiological tests have commonly been used for phenotypic characterization of yeast species. These methods are often unreliable due to strain variability and therefore do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular techniques provides more powerful method for identification of *S. cerevisiae* compared to physiological and morphological tests (Marjeta et al., 2002).

Species conformation was carried out by ITS sequence using ITS 1 and ITS 4 primers and produced single pattern band around 524 to 626 bp. The BLAST result through Gene Bank showed the regions of local similarity between sequences of yeast isolate with *S. cerevisiae* (Nur et al., 2011).

4.2. Scanning electron microscopy (SEM) and energy dispersive x-ray spectrometry (EDS)

All treated metals biosorbed by *S. cerevisiae* biomass because the biosorption process is less dependent on factors like metabolic energy, substrate composition, enzymatic system and presence of metabolic inhibitors. The technique of SEM coupled with EDS analysis has been confirmed that chelation, ion exchange, adsorption and precipitation are involved in metal biosorption by *S. cerevisiae* due to multi-laminate, micro febrile cell wall structure (Dostalek et al., 2004). EDS analysis confirmed that ion exchange is one of the most predominant mechanism involved in heavy metals biosorption by *S. cerevisiae*. Presence of mannan-glucan cell wall with positively charged chitin and negatively charged phosphate and glucuronic acid residues offer extensive possibilities for binding of metals through ion exchange and coordination in *S. cerevisiae* (Sag, 2001).These functional groups have higher and more covalent affinity towards toxic transition metals like Cu, Cd, Co, Ni compared to alkali earth metals such as Na, K, Ca (Rao et al., 2005). Additional support for ion exchange is that the metal biosorption capacity of *S. cerevisiae* decreased with decreasing in pH due to metal cations and protons compete for same binding sites on the cell wall as pH decreased (Shadia et al., 2012).

EDS is useful for qualitative analysis because complete spectrum of elements present in specimen can be obtained very quickly. The accelerating voltage used in EDS indicates types of elements present on specimen. Materials containing heavy elements give higher detection limits in EDS due to higher background. Therefore the presence of heavy metals can be easily detected in EDS analysis (Goldstein et al., 2003).The cellular content in EDS analysis revealed that *S. cerevisiae* cell wall is rich in C, O, K and P contents to enhance the metal accumulation. ED's analysis of metal treated S. cerevisiae is rich in metal ions due to ion exchange with other ions (Engl and Kunz, 1995). The SEM can be used for element mapping, if X-ray spectrometer is added. This made considerable significance for identification of elements by overlap in the functions of these instruments (Huma et al., 2001).

4.3. Biosorption

Metal ion concentrations, adsorption time, pH and co-ions have great influence on metal biosorption. The kinetics of metal binding indicated that biosorption is rapidly reached 60% of the final uptake value within 15 min of contact period (Engl and Kunz, 1995). The metal uptake capacity was significantly affected in the presence of the co-existed ions. The presence of metal ions in two-component aqueous solution decreases metal uptake capacity of fungi. Metal biosorption in multi-metals aqueous solution is found less compared to single metal solution (Abhishek, et al., 2010).

This confirmed that extent of metal sorption depended on metal chemistry, affinity for binding sites and type of metal. The order of metal biosorption in a multi-metal system could be predicted well based on Langmuir parameters evaluated in binary metal system (Sarabjeet and Dinesh, 2007). High metal biosorption was obtained at initial metal concentration ranging from 50-100 mgL^{-1} at 30°C temperature and pH 5.0 (Ozer and Ozer, 2003).

Dark melanin pigment and extracellular polymers located in *S. cerevisiae* can reduce the toxic effect of heavy metals. This property is of great importance to *S. cerevisiae* growing in polluted habitats for binding and removal of heavy metals. Therefore, yeast tolerance towards heavy metals is of high importance for their application in heavy metals bioremediation (Shazia et al., 2012). Biomass of *S. cerevisiae* can be procured from the existing fermentation industries, which is essentially a waste after fermentation. As non-living biomass behave as an ion exchange, the process is very rapid and takes place between few minutes to few hours. Metal loading on biomass is often very high, leading to very efficient metal uptake. Due to non-living cells, processing conditions are not restricted to those conducive for the growth of cells. A wider range of operating conditions such as pH, temperature and metal concentration is possible. No aseptic conditions are required for biosorption process. Metal can be desorbed easily from the biomass and can be reused. Once metals bound, acts as a template for further heterogeneous nucleation and crystal growth (Srivastava and Thakur, 2006).

S. cerevisiae that have been heat killed and pre-treated have lost their membrane integrity and this intern helps in exposure of various functional groups on the surface of *S. cerevisiae* for binding and removal of heavy metals. Effluents containing multiple heavy metals like Cu, Ni, Zn, Cr, and Zn can be effectively treated using

S. cerevisiae. The external layer of *S. cerevisiae* influences global electric charge, hydrophobicity and wall porosity for the macromolecules. The presence of macromolecules allows yeast cells to display several functional groups that can interact with heavy metals. The external layer of the cell wall, which is wall porosity for macromolecules and mannoproteic in nature, seems to be more important for metal accumulation than the innermost layer of glucans. SEM results confirmed that several macromolecules present on yeast cell surface allow the yeast cells to bind with heavy metals to form flocculation of heavy metals. Formation of cell aggregate or biofilms during biosorption of heavy metals was confirmed by SEM image. Presence of external energy source like glucose for preparation of biosorbent greatly enhanced three fold biosorption processes. These features made yeast cells as desirable for heavy metals biosorption (Soares and Soares, 2012).

Metabolism-independent binding of metal ions in yeast cell is usually rapid and large amounts of heavy metals may be bound. The heavy metals biosorption capacity of dead biomass may be greater, than that of living cells at low metal concentrations. With increase in metal concentrations from $50mgL^{-1}$ to $200mgL^{-1}$, the percentage of biosorption decreased due to increased electrostatic interactions of metal ions on cell surface (Yun-guo et al., 2006). *S. cerevisiae* can successfully adsorb toxic Zn^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+} and Cu^{6+} metals from aqueous solution compared to other fungi, which are in their metals biosorption (Ahluwalia and Goyal, 2007). Biosorption of metals decreases with increase in number of metal ions due to number of metal ions compete for same binding sites available on biomass surface. Lack of metal binding sites also results in decreased metal biosorption (Shazia et al., 2012).

4.4. Effect of pH

The dependence of metal uptake on pH is related to both surface functional groups present on biomass and metal chemistry in solution. At low pH of 2.0-3.0, the surface ligands are closely associated with the hydronium ions (H_3O^+) and restricted the approach of metal cations. As a result of repulsive force causes decreased metal sorption takes place at low pH. The pH dependency of metals uptake by *S. cerevisiae* biomass is due to association and dissociation of certain functional groups like carboxyl and hydroxyl groups. At low pH, most of the carboxylic groups is not dissociated and cannot bind the metal ions to fungal cell wall (Choudhary and Sar, 2009).

At high pH value metals get precipitate, cadmium ions precipitate as Cd $(OH)_2$ and trivalent chromium ions precipitate as $Cr(OH)_3$. This causes very low biosorption of metal ions in high pH value (Pinoa et al., 2006). The metal binding sites on cell surface and availability of metal ions in solution are affected by pH. At low pH, the cell surface sites are closely linked to the H^+ ions, thereby making these unavailable for metal cations. However, with increase in the pH, there is an increase in ligand with negative charges which results in increased binding of cations. The increase in pH causes increased negative charge on cell surface which favoured electrochemical attraction and adsorption of metals (Johncy et al., 2010). The optimal pH for biosorption of heavy metals by fungal biomass is between 5.0–5.2 (Shankar et al., 2007).

Ionic strength influences the adsorption of solute on biomass surface. When two phases, e.g. biomass surface and solute in aqueous solution are in contact, they are bound to be surrounded by an electrical double layer owing to electrostatic interaction. Thus, adsorption decreases with increase in ionic strength. Some inorganic ions such as chloride, may form complexes with some metal ions and thereby affect the sorption process. The dosage of a biosorbent strongly influences the extent of biosorption (Price et al., 2001).

4.5. Effect of temperature

Temperature affects biosorption only to a lesser extent within the range from 20 to 35°C (Veglio and Beolchini, 1997). Higher temperatures usually enhance sorption due to increased surface activity and kinetic energy of the solute (Sag and Kutsal, 2000). Physical damage to the biosorbent and exothermic nature of some adsorption processes reduce biosorption capacity of the biomass in high temperature (Srivastava and Thakur, 2006). It is always desirable to conduct biosorption at room temperature, as this condition is easy to replicate (Vijayaraghavan and Yeoung, 2008).

4.6. Sorption Isotherms

Freundlich and Langmuir models are used to fit the experimental data in order to assess maximum adsorption capacity correspond to biomass surface saturation and adsorption intensity of sorbent towards the biomass. These classical adsorption models have been extensively used to describe the equilibrium established between adsorbed metal ions on the biomass (q_{eq}) and metal ions remaining in solution (C_{eq}) at constant temperature (Ho et al., 2000). Monolayer biosorption as well as heterogeneous surface conditions may co-exist under applied experimental conditions. Hence, the overall sorption of metal ions on biomass may involve more than one mechanism such as ion exchange, surface complexation and electrostatic attraction. The value of correlation coefficient (r^2) presented that the Langmuir model adjusts the experimental data very well for the adsorption of metal by pre-treated S. cerevisiae biomass. The equilibrium time is one of the important parameter in biosorption

The distribution of bonded ions on the sorbent surface is greater than unity, indicating that the metal ions are favourably adsorbed under all the examined experimental conditions. Both isotherms can be used to model biosorption data from dilute aqueous solutions. It is suggested that the sorption of metals is more correctly described by more than one model kinetic analyses. This not only allows the estimation of sorption rates but also lead to suitable rate expressions characteristic of possible reaction mechanisms (Smith, 1996).

5. Conclusion

Saccharomyces cerevisiae has shown great potential to treat all major toxic heavy metals through biosorption. Non-living biomass exhibited higher metal uptake ability. The potential advantages in use of non-living fungal biomass compared to already-existing treatment technologies for metals removal is, easily and certainly can be applied for treatment of large scale heavy metals without the generation of toxic sludge. Non-living biomass also eliminate the problem of metal toxicity, possible adverse operating conditions, economic burden of culture maintenance and nutrient supply for the living cells. The selection of *S. cerevisiae* for biosorption study made easy genetic manipulation to increase the biosroption of heavy metals.

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