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DETOXIFICATION OF MERCURY POLLUTANT BY IMMOBILIZED YEAST STRAIN *CANDIDA XYLOPSOCI*

AATIF AMIN AND ZAKIA LATIF*

Department of Microbiology and Molecular Genetics, University of the Punjab, 54590 Lahore, Pakistan
**Corresponding author's e-mail: zakia.mmg@pu.edu.pk; umna123@yahoo.com.*

Abstract

Keeping in mind the toxicity of mercury, this study was designed to isolate and evaluate the yeast strains for the remediation of mercury from the environment. Yeast strains, from various sources, were isolated on Yeast Extract Peptone Dextrose (YEPD) medium supplemented with different concentrations of Mercuric chloride ($HgCl_2$). Well plate method was used for preliminary screening of strains resistant to mercury. Seven strains were selected for phenotypic characterization on YEPD medium supplemented with $HgCl_2$. Mercury resistant strains were also evaluated for Hydrogen sulfide (H_2S) production on Lead acetate (LA) medium. H_2S producer yeasts co-precipitated the divalent mercury (Hg^{+2}) in the form of mercuric sulfide (HgS) which is less toxic and insoluble in water. Quantification of mercury from selected strains was done by Dithizone method. Characterization of selected strains was also done by 18S rRNA (Ribosomal ribonucleic acid) sequencing. Two strains, *Candida xylopsoci* (Z-HS 51), with highest ability and *Candida rugosa* (Z-HS 13) with lowest ability to remediate mercury, were entrapped in sodium alginate. The immobilized *C.xylopsoci* (Z-HS 51) was also compared with free cells for its ability to reduce mercury pollutant. The reusability and shelf life of immobilized cells of *C. xylopsoci* (Z-HS 51) was also checked.

Introduction

Mercury is one of the most toxic heavy metal in the environment (Nascimento & Chartone, 2003; Oehmen *et al.,* 2009) but still it has significant industrial and agricultural uses. These uses have led to severe localized mercury pollution. Reports of Hg poisoning because of industrial, agricultural, and laboratory exposure as well as its suicidal use are numerous. Methyl mercury (CH_3Hg^+), mercuric chloride ($HgCl_2$) are the forms of mercury, problematic for living organisms as it causes serious neurological disorders (Brodkin *et al*., 2007; Holmes *et al.,* 2009). Metal mining, fossil combustion, chloralkali and industries have raised mercury levels in water bodies and soils. Main source of mercury into the environment is industry (Barkay *et al.,* 2003; Wagner-Do¨bler, 2003; Fatta *et al.,* 2007). No doubt, increasing level of heavy metals in the environment remains a major problem worldwide. Environmental decontamination of polluted sites is one of the main challenges for sustainable development. Experts are using various techniques to reduce the level of mercury in industrial effluents. Numerous physicochemical processes for heavy metal removal have been applied to fulfill the environmental regulations. But these methods have some limitations such as very expensive, less efficient and sometime released hazardous by-products (Manohar *et al*., 2002; Zhang *et al*., 2005). Thus new technologies to remove toxic mercury are of great interest. In this regard, hydrogen sulfide producing yeasts have been applied for the remediation of mercury pollution as an alternative to physiochemical processes (Deckwer *et al.,* 2004). It co-precipitates the mercury in the form of HgS resulting the conversion of divalent mercury (Hg^{+2}) to Hg^o (non-toxic form). In this context, microbial bioremediation is an attractive technology for the remediation of mercury for the sustainable development and it seems a potential approach due to low cost, simple and environmentally friendly (Wagner-Do¨bler, 2003).

The potential of using immobilized cells for bioremediation of heavy metal pollutants in industrial processes is regarded as a valuable application. The immobilization of whole growing cells by techniques of encapsulation, entrapment in polymer gels and adhesion onto the surface of carriers has been applied for valuable matrices (Latif *et al*., 2007; Sinha & Khare, 2011). In the current study, hydrogen sulfide producing yeast strains have been isolated which have the ability to remediate the mercury and can survive at high levels of mercury as well. *Candida xylopsoci* (Z-HS 51) was immobilized in sodium alginate and evaluated for its potential for the bioremediation of mercury. Each experiment was done three times and controls were run parallel for comparison.

Materials and Methods

Isolation and screening of mercury resistant, hydrogen sulfide producing yeast strains: Samples of rotten fruits, commercial yeasts, poultry waste, sewage water, poultry feed, sugarcane, yogurt, sunflower, tenaries, rose, mango tree bark and garden soil from different areas of Lahore, Pakistan were collected and stored at 4ºC in airtight bags. Yeasts were isolated and purified on YEPD medium (glucose 2%, peptone 2%, yeast extract 1%, agar 2%). Preliminary screening was carried out on the basis of their resistance against $HgCl_2$ by well plate method (Zeroual *et al*., 2001). Selected mercury resistant strains were further checked out qualitatively for H_2S production on LA medium (Guimaraes *et al*., 2006). Mercury resistant H_2S producing yeast strains were further screened out by growing in YEPD liquid medium supplemented with 20 µg/ ml $HgCl_2$ at 30°C on continuous shaking. After 36 h, the quantification of mercury remediation in YEPD broth was done by Dithizone method (Elly, 1973; Khan *et al*., 2005). The control culture medium supplemented with the same concentration of Hg as in the treated one but without yeast isolates were also processed for comparison.

Phenotypic characterization of selected yeast strains: The growth behavior of seven yeast strains (Z-HS 1, Z-HS 5, Z-HS 13, Z-HS 25, Z-HS 33, Z-HS 51 and Z-HS 62) was observed on YEPD agar plates supplemented with different concentrations of $HgCl_2$ (50, 75 and100 µg/ml) and on LA plates. Selected yeast strains were grown on YEPD liquid medium overnight at 30ºC on continuous shaking at 150 rpm. Optical density of overnight culture was measured at 600 nm and tenfold serial dilutions (10^{-1} to 10^{-6}) of all strains were prepared with the same liquid medium. 2 µl of each dilution was spotted on YEPD plates containing different concentrations of mercuric chloride and LA agar plates and incubated at 30ºC. Results were recorded after 24 h for YEPD plates with $HgCl_2$ and after 72 h for LA agar plates.

Molecular characterization of yeast strains: Seven strains including H_2S and non-H_2S producers were selected for 18S rRNA ribotyping and sequences were submitted to NCBI.

Immobilization of yeast cells by encapsulation with sodium alginate: Encapsulation of hydrogen sulfide producing (Z-HS 51) and non-hydrogen sulfide producing (Z-HS 13) was done by following the methods described by (Carvalho *et al.,* 2002; Latif *et al.,* 2007). Cultures were grown for overnight at 30ºC, in 50 ml YEPD liquid medium at 150 rpm. When the optical density of the culture at 600 nm was reached at 1.0, the cultures were pellet down by centrifugation at 14,000 rpm for 5 min at room temperature and encapsulated by mixing pellet with 50 ml of sterilized 2% (w/v) Sodium alginate solution containing 3% sucrose. Yeast-alginate mixture from the height of 15 cm was drop wise dropped into a beaker containing 75 mM Calcium chloride solution under continuous stirring and kept for 30 min on the same condition for hardening the coating of sodium alginate around the cells. Synthetic encapsulated beads were washed with 5 mM $CaCl_2$ solution for one hour and collected by filtration. Beads were stored at 4ºC until further use.

Remediation of mercury by using immobilized / free cells and their usability: Reduction of mercury by encapsulated yeast strains Z-HS 51(mercury resistant) and Z-HS 13 (non-resistant) was done by inoculating 4 g synthetic beads (100 beads from 5 ml culture of O.D. 1.0 at 600 nm) in 50 ml YEPD liquid medium (to make the final OD_{600nm} 0.1) containing 20 µg / ml $HgCl_2$. After every 36 h growth at 30ºC, with continuous shaking, cultures were filtered and washed (X3) beads with autoclaved 5 mM $CaCl_2$ to reuse for the subsequent inoculation in fresh 50 ml YEPD liquid medium. Estimation of mercury was done by Dithizone method (Elly, 1973; Khan *et al*., 2005). Overnight grown, free cells culture (5 ml of OD_{600nm} 1.0) of the same strain was also used in 50 ml YEPD liquid medium along with encapsulated yeast strain for comparative studies.

Shelf life of immobilized yeast cells: Four aliquots (4 g /aliquot) of immobilized cells of Z-HS 51 (H_2S producer) were used to study the shelf life. Each aliquot was processed at two weeks interval, by growing in YEPD liquid medium containing 20 µg / ml $HgCl_2$ for 36 h with continuous shaking at 30ºC. Quantification of mercury in beads free extract was carried out by Dithizone method (Elly, 1973; Khan *et al*., 2005).

Results

Isolation and screening of yeast strains: Seven mercury resistant and non-resistant strains were selected out of 70, by well plate method showing zone of inhibition ranging from 09 mm to 16 mm. For mercury processing ability of yeast isolates was checked by adding $HgCl_2$ at a concentration of 20 µg/ml in YEPD medium. Decrease in concentration of mercury was checked by Dithizone method. According to our observations, Fig. 1 clearly shows that bioremediation of mercury varied in different isolates of yeast. Yeast isolates Z-HS 01, Z-HS 33, Z-HS 51 and Z-HS 62 resistant to $HgCl_2$ decreased Hg by 83-93.5% from the medium while isolates Z-HS 05 and Z-HS 13 were non-resistant to Hg removed mercury in traces which is non-significant as compared to other isolates.

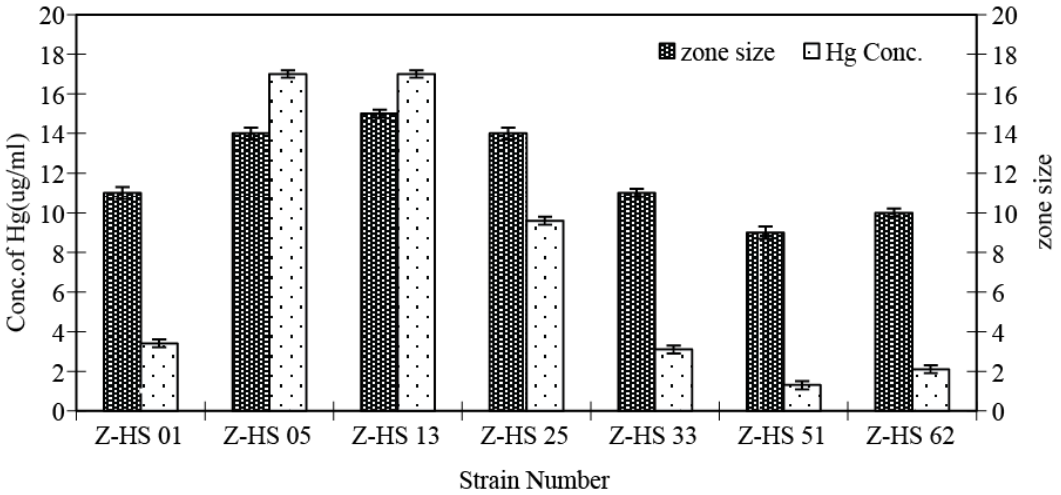

Fig. 1. Measuring of inhibition zone (mm) in well plate method after 24 hours of incubation at 30ºC (gray bars) and remainder of mercury (µg/ml) in culture medium inoculated with yeast isolates, after 36 hours incubation at 30ºC determined by Dithizone method (white bars).

Phenotypic characterization of yeast isolates: The phenotypic behavior of seven selected yeast strains was checked on YEPD plates supplemented with 50 µg/ ml of $HgCl_2$ (Fig. 2). There was complete inhibition of non-resistant yeast on YEPD agar plates with 75 and100 µg/ml $HgCl_2$ (data not shown). It is concluded from the results that under starvation condition with the increase in concentration of mercury, significant suppression in growth was observed in case of mercury sensitive strains as compared to mercury resistant yeast strains.

These results were further confirmed by qualitative assay for hydrogen sulfide production on LA plates (Fig. 3). Mercury resistant yeast isolates Z-HS 01, Z-HS 33, Z-HS 51 and Z-HS 62, having smaller zone of inhibition (Fig. 1) gave darker color on LA medium specify high level of hydrogen sulfide (H_2S) production whereas non resistant isolates Z-HS 05 and Z-HS 13 exhibiting larger zone of inhibition appeared as white colonies specifying the non hydrogen sulfide producing yeast.

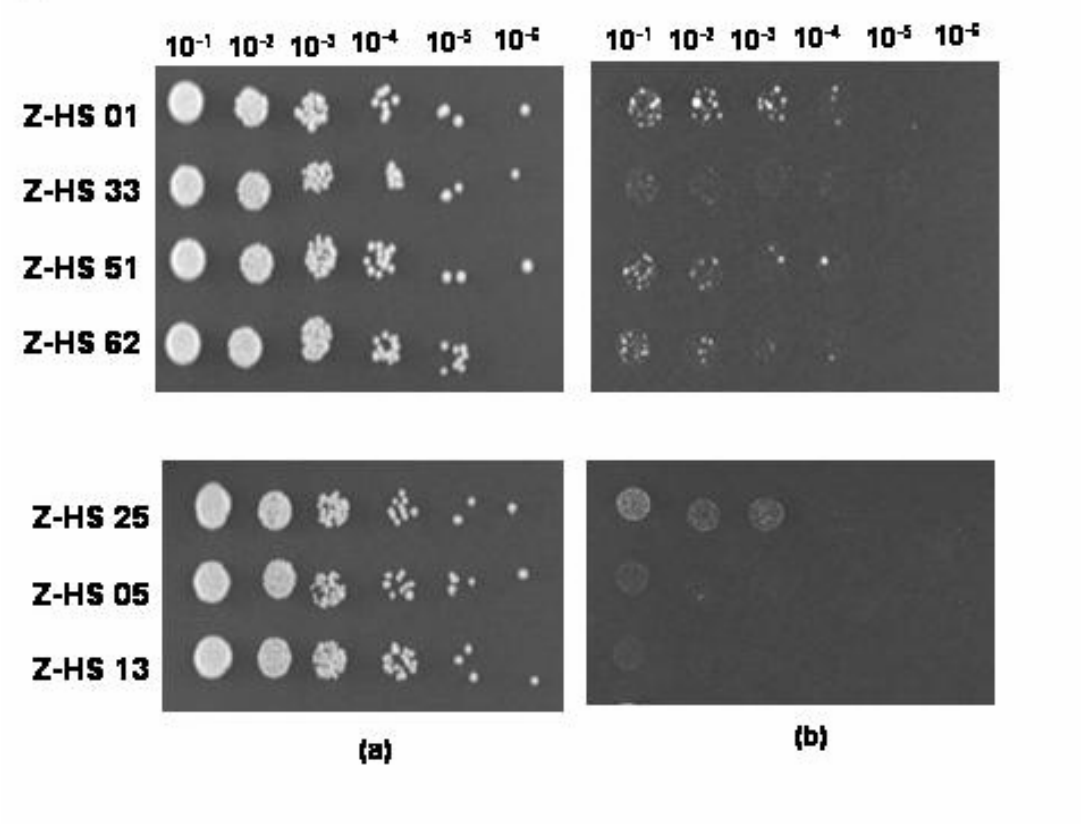

Fig. 2. Growth of serially diluted yeast isolates Z-HS 01, Z-HS 33, Z-HS 51, Z-HS 62, Z-HS 25, Z-HS 05 and Z-HS 13 on YEPD agar (a): $\Delta HgCl_2$ (b): $+HgCl_2$ (50 µg/ml for phenotyping of seven yeast strains varying in mercury tolerance).

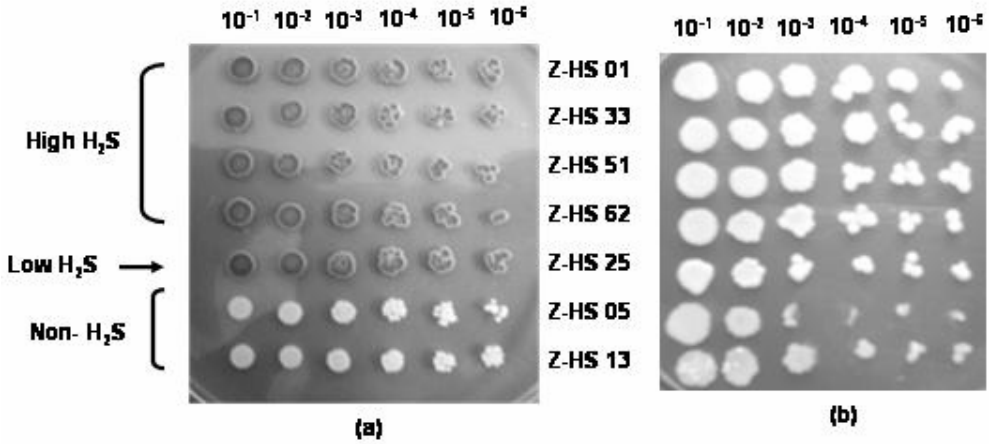

Fig. 3. Qualitative test for Hydrogen sulfide (H_2S) production by seven yeast isolates on LA agar plate (a). Spotted yeast cells (2µl) were incubated at 30^oC for 3 days. High intensity color by mercury resistant isolates indicate the production of H_2S as compared to non-resistant, light color colonies, indicate non-H_2S producers. White color colonies appeared on control plates (b) without LA parallel for comparison to the experimental plates.

Molecular characterization of yeast strains: Mercury resistant yeast strain Z-HS 51 was characterized on the basis of 18S rRNA gene and identified as *Candida xylopsoci* (JF896574) and mercury sensitive Z-HS 13 as *Candida rugosa* (JF896571).

Reduction in mercury concentration by using immobilized / free cells and their usability: Highly resistant yeast strain *Candida xylopsoci* immobilized in Sodium alginate as synthetic beads (Fig. 4) reduced 18.7 µg / ml of mercury out of 20 µg/ml from the medium within 36 h incubation at 30°C whereas the free cells remediate 15 µg/ml. Non-resistant *Candida rugosa* remediate only 2 µg/ml in the same time period (Fig. 5). When alginate beads were reused continuously in four cycles, the beads were dissolved gradually in subsequent cycles but the capacity of mercury reduction was same in all cycles (Fig. 5). In case of immobilized *C. xylopsoci*, the remediation of mercury was constant in all subsequent cycles in repeated experiments. Results showed that entrapment has no effect on the ability of strain to reduce the mercury in the medium.

Shelf life of immobilized yeast cells: Bioremediation frequency of synthetic beads stored at 4°C was checked up to two months with the interval of 15 days. Results reported in Fig. 6 showed that the entrapment of mercury remediation strain *C. xylopsoci* has no effect on the ability of the strain to reduce the mercury even after 2 months storage.

After every 15 days interval, one hundred entrapped cells (4gm) obtained from 5ml overnight culture of $OD600_{nm}$ at 1 were incubated in 50ml YEPD medium supplemented with 20 µg/ml $HgCl_2$ for 36 hours at 30°C on continuous shaking and quantified mercury in the remainder cell free extract by Dithizone method.

Fig. 4. Sodium alginate synthetic beads of mercury resistant *Candida xylopsoci* (Z-HS51).

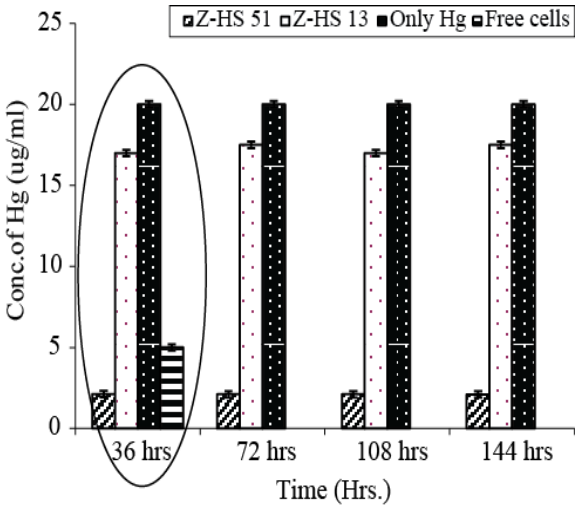

Fig. 5. Repeatedly use of immobilized *Candida xylopsoci* (Z-HS51) and *C.rugosa* (Z-HS 13) to check the potential of mercury remediation (four constitutive cycles). Reduction in mercury concentration (µg/ml) is shown by bars and encircled one shows the comparison of immobilized beads with free cells of *C.xylopsoci* for the remediation of mercury from the culture medium supplemented with 20 µg/ml $HgCl_2$.

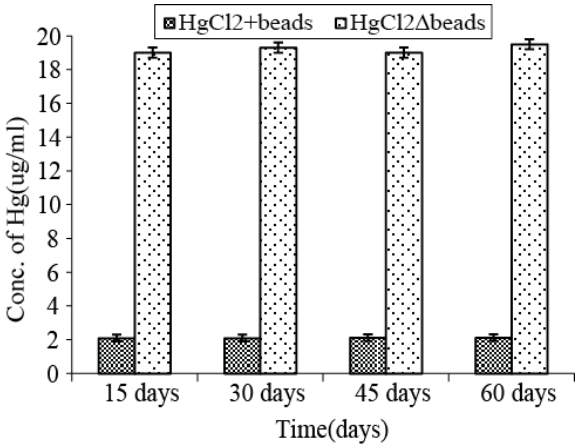

Fig. 6. Evaluation of shelf life of immobilized *C.xylopsoci* with sodium alginate for the potential to remove mercury.

Discussion

Unlike organic pollutants, heavy metals do not degrade and thus create a different kind of challenge for remediation. The major source of environmental contamination of mercury arises from various anthropogenic activities such as chlor-alkaly industries, fluorescent lamps, in the recovery of gold, burning coal, natural gas, metal processes and petroleum products, and use of mercurial fungicides in paper making. All activities have increased mercury level in the atmosphere and it is increasing day by day, therefore it is important to remove these hazardous pollutants from the environment. Nature has developed amazing range of devoted resistance phenomena in microorganisms to overcome the poisonous burden on environment (Barkay *et al*., 2003; Kabir *et al*.,

2011). The bioremediation of the heavy metal is the well known phenomena in certain microorganisms when challenged with the environmental pollutions (Valls & de Lorenzo, 2002; Thakkar *et al.,* 2010). Bioremediation of heavy metals using microorganisms have received a great attention in recent years for its potential application in the industry, as it is non-destructive, cheaper and economical (Rise-Roberts, 1998; Munir *et al*., 2010). There is also a large interest in microorganisms that can facilitate with the transformation and removal of metal contaminants. Few types of yeast especially *Saccharomyces* species resistant to mercury has been reported previously but were not used as a bioremediation agent for mercury removal. But other yeast species are reported and well known for having great role in the remediation of other metals (Shakoori *et al.,* 2005; Rehman & Anjum, 2010). Yeasts are more efficient and have ability to accumulate a broad range of heavy metals to varying degrees under a wide range of external conditions (Villegas *et al*., 2005). Microbial detoxification of metal ions is achieved by several mechanisms including regulation of uptake, transformation into less toxic forms, and intracellular immobilization (Rama-Rao *et al*., 1997; Rehman & Anjum, 2010). In the present investigation, *Candida xylopsoci* (Jf896574) identified by 16S rRNA sequencing was found to be highly resistant to mercury and H_2S producer which was determined by qualitative test giving dark color on LA agar plate. Zafar *et al*., (2007) isolated filamentous fungi from metal contaminated agriculture soil and reported their metal tolerance and biosorption potential. Rehman & Anjum (2010) reported that *Candida tropicalis* was found to be resistant to Cd up to the concentration of 2,800 mg/L and the order of resistance regarding the metal resistance was $Zn^{2+} > Ni^{2+} > Hg^{2+} > Cu^{2+} > Cr^{2+} > Pb^{2+}$. *C. tropicalis* and *C.albicans* are well known for resistance to the Hg2+, Cd2+, Pb2+, SeO_3^{2-}(selenite) and AsO_4^{3-} (arsenate) (Berdicevsky *et al*., 1993). Other *Candida* species have the capacity to remediate heavy metals from their surroundings (Podgorskii *et al*., 2004). *Candida xylopsoci* isolated from local environment showed in the present study are not only highly resistance to mercury but also have good potential to remediate mercury. It remediated 18 µg/ml of mercury in 36 hours from the synthetic culture medium but its potential increased when entrapped in sodium alginate. Metal uptake is generally a prior step to detoxification by microbial cells. The mercury resistance has been previously reported in many *Candida* spp. but mercury remediation by alginate immobilized yeast cells has never been attempted by *Candida xylopsoci.* Entrapped yeast cells in sodium alginate have more remediation potential as compare to free cells. It might be due to the polymeric nature of the alginate, which capture metals non-specifically to transport into the cells. Reusability of immobilized *Candida xylopsoci* was checked repeatedly in four continuously cycles. It was observed that reduction in mercury concentration was constant up to 90% in all cycles from the culture medium supplemented with $HgCl_2$. It indicates no loss of cells viability to remediate mercury. Sinha & Khare, (2010) reported reusability of immobilized cells but the efficiency of mercury removal by immobilized *Enterobacter* sp. was100% and 97% in first two cycles but in the next two successive cycles decreased. The effect of entrapment on mercury detoxification potential by *Candida xylopsoci* has no effect on its shelf life up to 2 months.

Conclusion

It is concluded from the present studies that immobilization of yeast cells responsible for the detoxification of mercury have numerous advantages over free suspended culture. There is other promising advantage of immobilization of yeast cells over the free cells, such as the reuses of entrapped yeast strains to remediate mercury remain constant after using multiple times. The most important finding is that no residues remain in the medium because they re-dissolved at the end. The same strategy can be applied in any polluted reservoir because the immobilized cells never loose their ability to reduce the pollutants from the environment and also there would not be any need to dispose off entrapped microorganism from the bioreactor because they re-dissolve within the system. The immobilization do not effect the shelf life of microbes, but provides favorable micro-environmental conditions for the organisms, protect against harsh environment, improve genetic stability and can be transferred easily and safely at any time and place.

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