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De-mercurization of wastewater by *Bacillus cereus* (JUBT1): Growth kinetics, biofilm reactor study and field emission scanning electron microscopic analysis

Sanjukta Ghoshal, Pinaki Bhattacharya, Ranjana Chowdhury*

Chemical Engineering Department, Jadavpur University Kolkata 700 032, West Bengal, India

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ABSTRACT

Removal of mercuric ions by a mercury resistant bacteria, called *Bacillus cereus* (JUBT1), isolated from the sludge of a local chlor-alkali industry, has been investigated. Growth kinetics of the bacteria have been determined. A multiplicative, non-competitive relationship between sucrose and mercury ions has been observed with respect to bacterial growth. A combination of biofilm reactor, using attached growth of *Bacillus cereus* (JUBT1) on rice husk packing, and an activated carbon filter has been able to ensure the removal of mercury up to near-zero level. Energy dispersive spectrometry analysis of biofilm and the activated carbon has proved the transformation of Hg^{2+} to Hg^0 and its confinement in the system.

1. Introduction

Mercury, a highly toxic heavy metal has drawn the global attention due to its extensive application, wide spread distribution, high toxicity and the bio magnification [1–4]. Power plants based on combustion of fuels, mining, some pharmaceutical industries and mercury-cell chlor-alkali plants are the main sources of mercury appearing in either solid or liquid or gaseous effluents. Usually mercury is deposited in Hg^{2+} form. Toxicity of mercuric ions is due to its capacity to bind with sulphhydryl, thioester and imidazole groups of enzymes and proteins inactivating vital cellular function [5]. Once discharged to the environment, Hg^{2+} gets transformed to methyl mercury – a highly toxic form. Methyl mercury is taken up by aquatic organisms and is biomagnified through food chain [6]. The health of higher predators, namely fish, birds, animals and human belonging to the food chain gets threatened. The methyl mercury in fish standard, as proposed by joint FAO/WHO expert committee, is given in terms of provisional tolerable weekly intake (PTWI) for an individual. The value of PTWI is set as 1.6 μg/kg [7] of body weight for an individual. As per the WHO recommendation, although PTWI may be exceeded somewhat in case of adults, this is not permissible in case of pregnant mothers, children and young adolescents. As the people in West Bengal, India, consume fish daily, a recent survey [8] of mercury concentration of fish from markets of Kolkata and neighbouring areas indicate a risk of exceeding PTWI in case of normal adults. The main source of mercury in fish is the water bodies facing mercury effluents from industries. Therefore, the removal of mercury from the industrial effluent must be carefully handled. Although several chemical and physical methods [5,9] are available for the removal of Hg^{2+}, they are usually expensive, labour intensive and generate a concentrated waste stream. Application of biotransformation process of soluble bivalent mercury to its less toxic, potentially volatile elemental metallic form may be a promising route to protect the environment from the threat of mercury pollution as the mercury resistant bacteria offer a possible cost-effective method [10] of removing mercury. Removal of toxic mercuric (Hg^{2+}) ions by mercury-resistant bacteria is actually a biphasic process in which Hg^{2+} ions in the abiotic wastewater phase are transported to the biotic intracellular environment and are acted upon by specific enzymes, called mercury reductase, to form metallic mercury. Metallic mercury, a less toxic form, diffuses out of the cellular environment to the bulk phase. Several works have already been reported on the isolation of mercury resistant bacterial strains from industrial effluents and sludge [11,12], soil, river and lake and estuary sediments [1,6,11–14], marine environment [2,15,16] and oil contaminated sites [10]. A few successful research works have already been undergone on the removal of mercuric ions from wastewater in bioreactors in laboratory and industrial scales using mercury-resistant bacteria either in free and in immobilized (biofilm) [1,17–19] forms. In the Indian perspective, only a few works on isolation of mercury-resistant marine bacteria have been reported. No work on the mercury-resistant bacteria isolated from sludge of Indian chlor-alkali industries has yet been reported. It is an established fact that ubiquitous and indigenous microorganisms present in heavy metal contaminated sites are more effective for the removal of that harmful heavy metal [20].

* Corresponding author. Tel.: +91 033 2414 6378.
E-mail address: ranjana.juchem@gmail.com (R. Chowdhury).

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Therefore, in this study a new bacterial strain *Bacillus cereus* (JUBT1) isolated from the sludge of a local chlor-alkali industry has been used to remove mercuric ions from a simulated wastewater stream. Growth kinetics including specific transformation rate of mercuric ions and the growth rate of *Bacillus cereus* (JUBT1) has been determined using the data of batch studies in Erlenmeyer flasks. Same bacterial strain has been utilized in a laboratory scale 1 m long and 0.5 m diameter biofilm reactor. The metallic mercury in the treated water has been removed using an activated carbon based filter. The performance of the reactor as well as the carbon filter has been studied using inlet concentration of Hg^{2+} in feed stream and feed rate of the simulated wastewater as parameters. The characteristic of biofilm has been investigated using FESEM.

2. Experimental

2.1. Materials

2.1.1. Bacterial strain and culture condition

Mercury-resistant bacterial strain was isolated from the sludge of a local chlor-alkali industry (Hindustan Heavy Chemicals, West Bengal, India) initially as a mixed culture. A pure monoculture was prepared from the mixed culture using conventional microbiological techniques. The isolate was cultured in laboratory condition in mercury specific growth medium composed of (per liter) sucrose (10 g), yeast extract (10 g), NaCl (30 g) dissolved in sodium phosphate buffer (0.25 M). Temperature and pH of the culture broth were maintained at 30 °C and 7 respectively. At first the selective sterile culture broth (0.2 Mpa, 121 °C, 15 min) was inoculated with the sludge of chlor-alkali industry. The growth medium was supplemented with $HgCl_2$ solution at a concentration of 0.002 g/m^3. The broth was incubated in a rotary shaker at 30 °C for 7 days at 150 rpm. The enriched culture was obtained by repeated inoculation of the previous bacterial culture in a fresh selective medium containing $HgCl_2$. The isolate was established as *Bacillus cereus* (JUBT1) using 16S-rDNA technique and the analysis was performed at Genei, Bangalore.

2.1.2. Chemicals

Sucrose (SRL, India), yeast extract powder (LOBA Chemie, India), NaCl (Merck, India), $HgCl_2$ (Merck, India), NaH_2PO_4 (Merck, India) and Na_2HPO_4 (Merck, India) were used in the experiment.

2.2. Methods

2.2.1. Dry weight method for the determination of bacterial mass

The biomass concentration in the reaction broth was determined by dry weight method. In this method the broth was centrifuged at the rate of 10,000 rpm for 15 min at 4 °C. The bacterial mass was then transferred to a pre-weighed aluminum cup and dried at 50 °C overnight. The exact weight of the bacterial mass was determined by subtracting the weight of dry cup from that of the cup containing dry bacterial mass.

2.2.2. Atomic absorption spectrophotometry for mercury measurement

Mercury concentrations in reactor and filter outlet as well as in samples of batch experiments (after centrifugation at 10,000 rpm for 15 min) were determined by atomic absorption spectrophotometer (A-analyst, 200; Perkin-Elmer) using the cold vapour technique. The measuring wavelength for mercury was 253.7 nm.

2.2.3. Field emission scanning electron microscopy and energy dispersive spectrometric analysis (FESEM-EDS)

FESEM analysis (JSM 6700F) of reactor packing material, rice husk containing bacterial biofilm collected after 25 days, 120 days and 150 days of operation, was performed. EDS analysis of some selected bacteria of the biofilm and packing material from activated carbon filter were also performed.

2.3. Experiments

2.3.1. Batch experiment

Growth kinetics of *Bacillus cereus* (JUBT1) with respect to sucrose was determined by conducting batch experiments varying initial sucrose concentration from 1000 to 10,000 g/m^3 in Erlenmeyer flasks under shaking condition in absence of mercury. Separate batch experiments were performed varying initial concentration of Hg^{2+} from 5–30 ppb, the sucrose concentration being kept at 10,000 g/m^3. A constant temperature of 30 °C and a neutral pH were maintained. Samples were withdrawn at an interval of 8 h over 72 h period.

2.3.2. Bioreactor design and experimental set up

A laboratory scale stainless steel packed bed reactor of 1 m length and 0.5 m diameter was used in order to treat the sterile simulated $HgCl_2$ solutions. The reactor was filled with rice husk. Separate experiment was conducted to see the adsorption capacity of rice husk with respect to mercury. It was observed that rice husk, in its natural form, did not adsorb any mercury. However, there are reports [21–23] available in the literature that rice husk ash obtained at 700 °C was successfully used as an adsorbent to reduce mercury level in wastewater. Isolated bacteria were grown as biofilm on the packing matrix inside the reactor. A 1 m long and 0.25 m diameter filter packed with activated carbon, was subsequently placed after the reactor to adsorb the residual mercury from the reactor outlet. Simulated waste water and air were fed continuously into the reactor in downward direction using a peristaltic pump and a compressor respectively. Performance of the bioreactor was studied using different parameters like inlet concentration of mercuric ions (5–30 ppb) and volumetric flow rate (0.00942–0.0176 m^3/h) of the feed solution etc. Samples collected from the reactor as well as from the carbon filter were analyzed for concentration of Hg^{2+} using atomic absorption spectrophotometer.

3. Results and discussion

As discussed in many literature [3,6,24–29], bacteria having mercury-resistance (mer) determinants are capable of reducing mercuric ions. Resistance to bivalent mercury ions in bacteria is conferred by the NADPH dependent cytoplasmic flavoenzyme mercuric reductase [3,5,18,25,26,28]. This enzymatic reduction of bivalent mercuric ions to insoluble, volatile elemental mercury by mercury-resistant bacteria is an energy-dependent process and this important mechanism of bacteria plays a great role to detoxify their surrounding environment [17,24]. Hg^{2+} ions enter the bacterial periplasm with the aid of small periplasmic protein mer P in the periplasmic compartment. Subsequently, inner membrane transporter protein mer T transfers Hg^{2+} to the active site of the NADPH – dependent enzyme mercuric reductase, encoded by the gene mer A [3,18,26]. Ultimately, Hg^{2+} is reduced to Hg^0 by electron transfer from $FADH_2$ in the active site of the enzyme and NADPH is transformed to $NADP^+$. The enzyme, mercuric reductase, catalyzes the reaction [26] mentioned below

$$Hg^{2+} + NADPH_2 \rightarrow Hg^0 + NADP^+ + 2H^+$$

A schematic diagram of the mechanism of bacterial mercury removal is shown in Fig. 1.

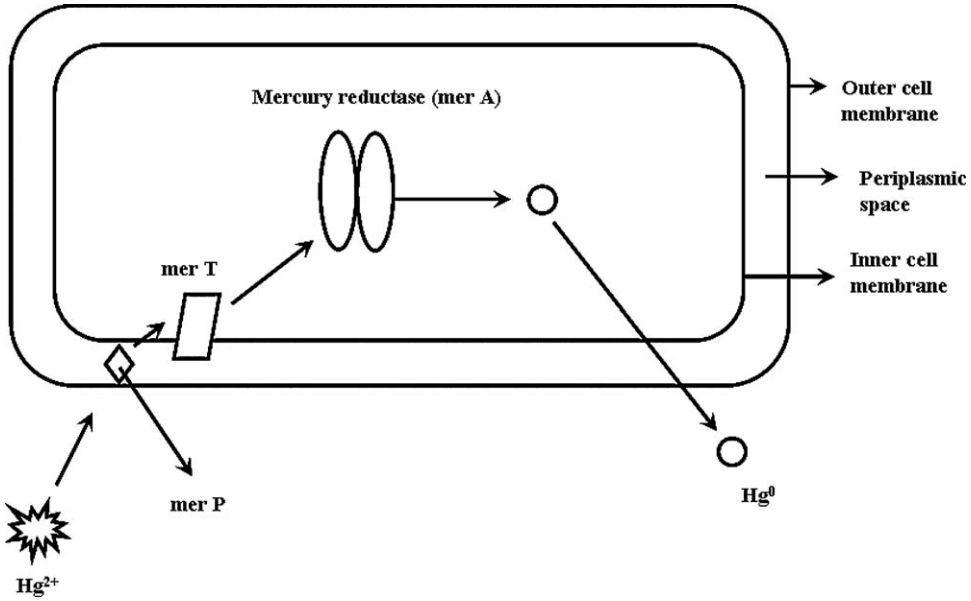

Fig. 1. Schematic representation of mercury uptake by mercury resistant bacterial strains.

3.1. Growth kinetics of *Bacillus cereus* (JUBT1)

Growth patterns of mercury resistant bacteria play the vital role with respect to their efficacy to remove Hg^{2+} from the bulk phase. From the batch mode experiments, it has been observed that the growth of *Bacillus cereus* (JUBT1) is dependent on concentrations of carbon source as well as mercuric ions. To study the growth solely on carbon source, Monod type model [30,31], applicable for unstructured growth, has been attempted. This may be represented as follows,

$$\mu = \frac{\mu_{max}\ C_{S1}}{K_{S1} + C_{S1}} \tag{1}$$

where, μ = specific growth rate of bacterial cell, μ_{max} = maximum specific growth rate of bacterial cell, C_{S1} = sucrose concentration and K_{S1} = saturation constant for sucrose.

In double reciprocal form, the equation becomes

$$\frac{1}{\mu} = \frac{K_{S1}}{\mu_{max}} \times \frac{1}{C_{S1}} + \frac{1}{\mu_{max}} \tag{2}$$

The linearity of the plot of $1/\mu$ versus $1/C_{S1}$ (Fig. 2) establishes the applicability of Monod model for the growth of *Bacillus cereus* (JUBT1) with respect to carbon source. The values of μ_{max} and K_{S1} have been determined from the plot as well as through non-linear regression analysis. The value of the correlation coefficient was 0.957. These are given in Table 1. Figs. 3 and 4 show the time history of bacterial growth and uptake of mercury respectively at initial sucrose concentration of 10,000 g/m^3, initial mercuric ion concentration being varied in the range of 5–30 ppb. Close analysis of the figure reveals that addition of mercuric ion enhances the growth of *Bacillus cereus* (JUBT1) within the range of Hg^{2+} concentrations under study. This suggests that similar to multiple-substrate systems, non-competitive relationship between carbon source and

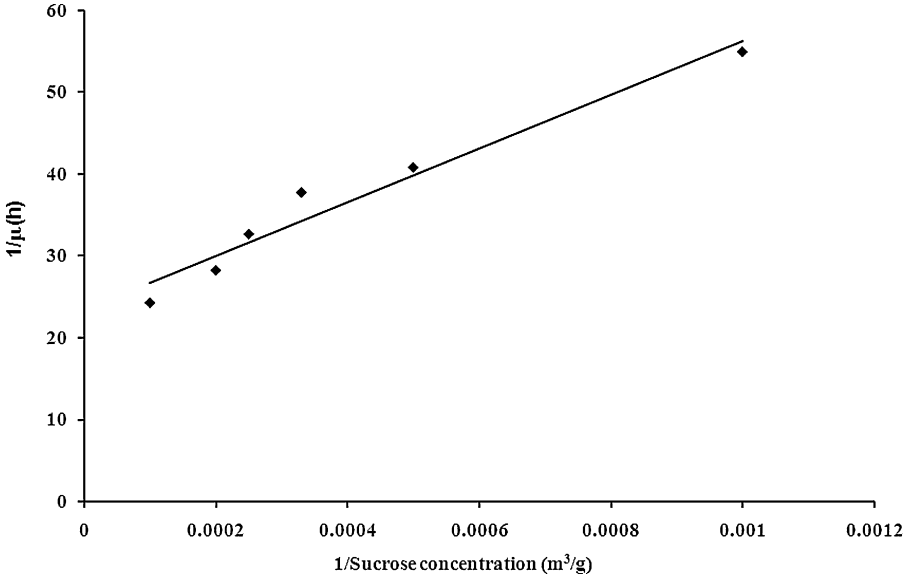

Fig. 2. Double reciprocal plot of specific growth rate versus sucrose concentration.

Table 1
Values of the kinetic parameters.

Kinetic parameter	Value	Value of correlation coefficient
μ_{max} (h^{-1})	0.043	0.957
K_{S1} (g/m^3)	1410.787	0.957
K	0.6	0. 971
K_{S2} (ppb)	3.95	0.971

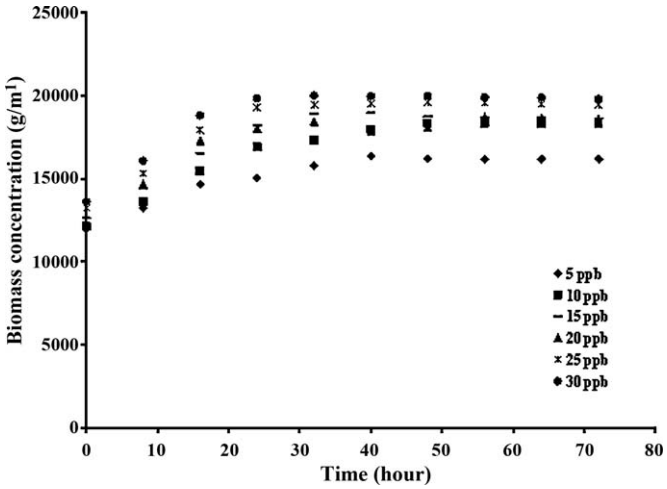

Fig. 3. Time history of biomass concentrations with initial mercury concentration as parameter ((♦) 5 ppb; (■) 10 ppb; (—) 15 ppb; (▲) 20 ppb; (*) 25 ppb and (●) 30 ppb).

Hg^{2+} exists. It has also been observed that a multiplicative [32] relationship of following type holds good for the system.

3.2. Non-competitive kinetics of multiplicative nature

$$\mu = \frac{\mu_{max}\, C_{S1}}{K_{S1} + C_{S1}} \times \frac{K\, C_{S2}}{K_{S2} + C_{S2}} \tag{3}$$

where C_{S2} = mercury concentration, K_{S2} = saturation constant for mercury and K = constant.

On rearrangement, Eq. (3) reduces to,

$$\frac{\mu}{(\mu_{max}\, C_{S1})/(K_{S1} + C_{S1})} = \frac{K\, C_{S2}}{K_{S2} + C_{S2}} \tag{4}$$

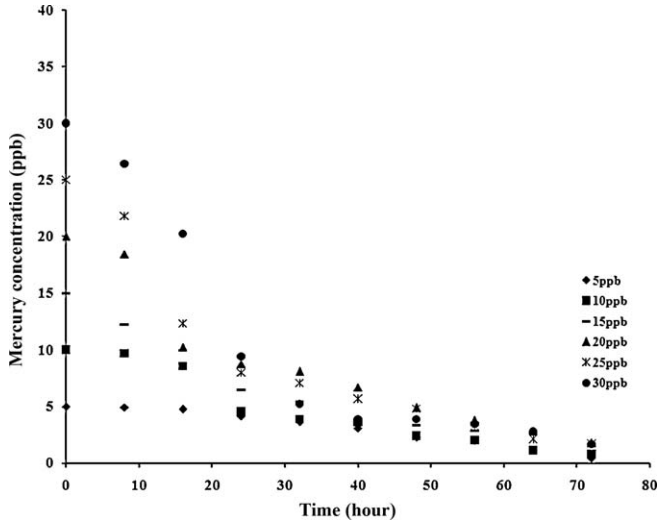

Fig. 4. Time history of mercury concentration with initial mercury concentration as parameter ((♦) 5 ppb; (■) 10 ppb; (—) 15 ppb; (▲) 20 ppb; (*) 25 ppb and (●) 30 ppb).

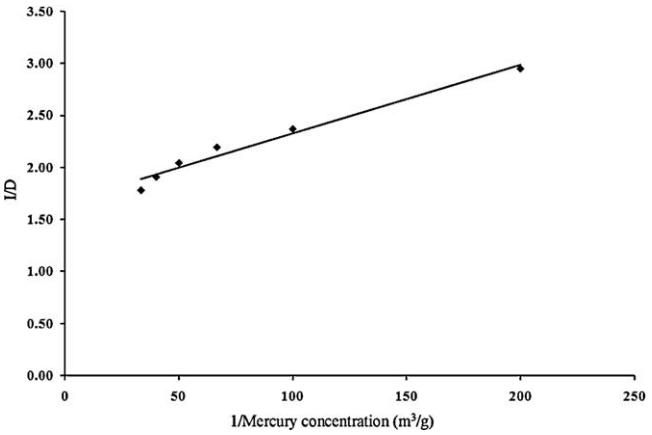

Fig. 5. Plot of reciprocal of "*D*" versus reciprocal of mercury concentration.

A new parameter '*D*' may be introduced

$$\frac{\mu}{(\mu_{max}\, C_{S1})/(K_{S1} + C_{S1})} = D \tag{5}$$

Relevant kinetic parameters have been determined using the following technique,

$$\frac{1}{D} = \frac{K_{S2}}{K} \times \frac{1}{C_{S2}} + \frac{1}{K} \tag{6}$$

Reciprocal of D (Eq. (5)) is plotted against reciprocal of mercury concentration (C_{S2}) (Fig. 5) and the value of K and K_{S2} have been determined from the slope and the intercept and through non-linear regression analysis. The value of the correlation coefficient was 0.971. The values of K and K_{S2} have also been shown (Table 1).

3.3. Reactor performance

The biofilm reactor (Fig. 6) was operated continuously for five months without any trouble. The process undergoing in the biofilm reactor is based on the reduction of mercuric ions by the biofilm of the isolated strain already grown on the packing materials i.e., rice husk. The actual reactor operation is started after the development of biofilm on the packings. Therefore, feed wastewater containing mercuric ions is treated by the microorganisms present in the biofilm right from the entrance of the packed zone of the reactor. The waste stream does not have to wait for the growth of microorganisms during its propagation through the bed before being demercurized. However, the biofilm is sustained by the nutrients supplied through the simulated wastewater under aerobic condition maintained by the supply of air stream in the reactor. Superficial velocity and initial mercuric ion concentration were identified as the main operating parameters. Effects of variation of these parameters on the efficacy of removal of mercuric ions from wastewater were investigated. Fig. 7 elucidates the trend of percentage removal of mercury as a function of initial mercuric ion concentration (0.005–0.03 g/m^3) with superficial velocity (0.048–0.089 m/h) as a parameter. The residence times were 8 h, 10 h and 15 h when superficial velocity was maintained at 0.089 m/h, 0.072 m/h and 0.048 m/h respectively. The system reached saturation, i.e., in other words, attained steady state after elapsing a period of twice the residence time. Data of Figs. 7 and 8 were obtained at thrice the residence time, i.e., at 24 h, 30 h and 45 h in respective cases. Time evolution of effluent concentration might be divided into two regimes, one following time invariant nature, exhibited (Figure not shown) beyond twice the residence time, and another following time dependent nature when the process time is less than this (twice the residence time). It is observed that at each superficial velocity, efficacy of mercury removal increases with

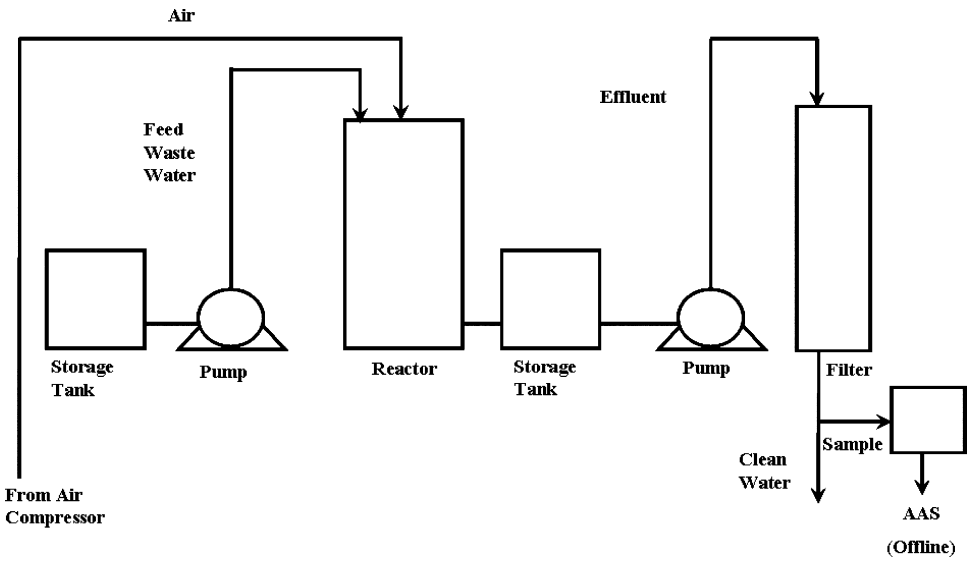

Fig. 6. Schematic diagram of bioreactor set up.

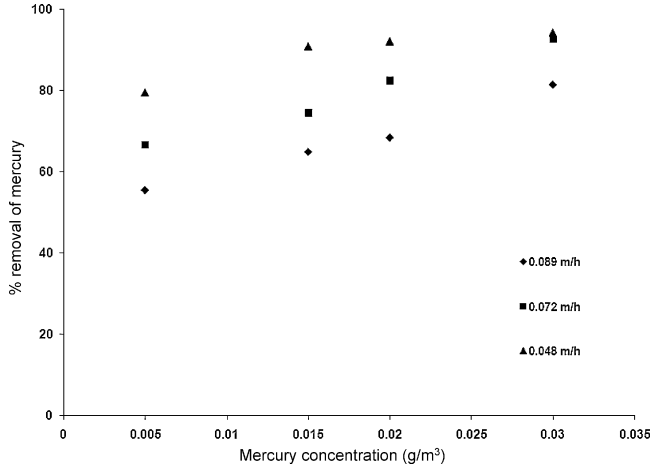

Fig. 7. Plot of removal of mercury in the biofilm reactor against initial mercuric ion concentration with superficial velocity as a parameter ((♦) 0.089 m/h; (■) 0.072 m/h and (▲) 0.048 m/h).

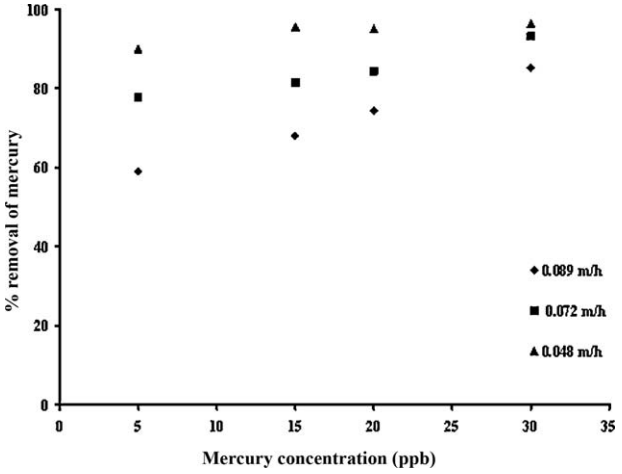

Fig. 8. Plot of removal of mercury in the bioreactor-carbon filter assembly against initial mercuric ion concentration with superficial velocity as a parameter ((♦) 0.089 m/h; (■) 0.072 m/h and (▲) 0.048 m/h).

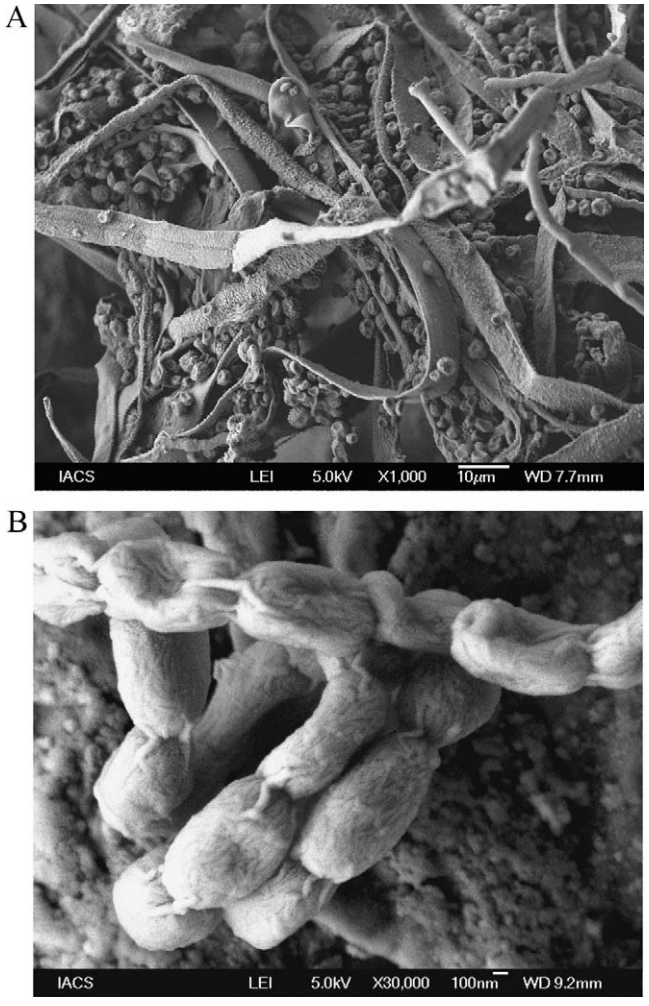

Fig. 9. FESEM of *Bacillus cereus* (JUBT1) grown on packing material (A. Magnification – ×1000; B. Magnification – ×30,000).

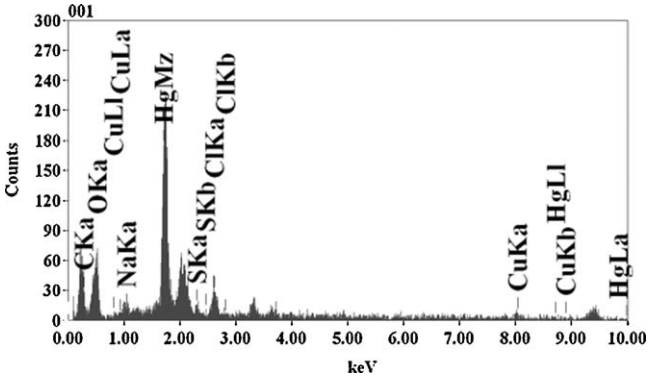

Fig. 10. Energy dispersive spectra of biofilm after 120 days of operation.

the increase of initial mercuric ion concentration. This is expected because of the fact that in the present range of concentration, mercuric ion serves as a growth enhancer and hence results in increase in mercury removal. The experimental trends, as depicted in Fig. 7, also show that at each initial mercuric ion concentration, removal efficiency becomes higher for lower value of superficial velocity. This is due to the increase in residence time with the decrease in superficial velocity. However, at the highest value of mercuric ion concentration, removal efficiency of mercury at superficial velocity 0.072 m/h and 0.048 m/h are equal. This may be due to the achievement of saturation at these conditions. The maximum value of mercury removal achieved after the treatment by the biofilm reactor was 94.4%. This corresponds to the inlet concentration of Hg^{2+} of 30 ppb and superficial velocity of 0.048 m/h. As it was experimentally verified that rice husk did not serve as an adsorbent for mercury, the total reduction of mercuric ions in the feed wastewater was due to the microbial conversion of Hg^{2+} to Hg^0. In all the cases mercury concentration in the treated water decreased after being passed through the carbon filter. For same set of operating parameters, the experimental trends of variation of overall removal efficiency after the passage of wastewater through the biofilm reactor and carbon filter have been shown (Fig. 8). From both the figures, it is evident that while the biofilm reactor could ensure removal efficiency of Hg^{2+} in the range of 91–94.4%, the subsequent passage through the carbon filter enhances the efficiency in the range of 95–96.4%.

3.4. Analysis of biofilm and carbon filter

From assessment of performance of the biofilm reactor and carbon filter it becomes obvious that the system is capable of removing mercury from ppb level to near-zero level. In the bioreactor, biofilm is the active component to remove mercuric ion. Thus FESEM analysis of biofilms taken from the reactor after 25 days, 120 days and 150 days was done. The micrographs of biofilm of 120 days age are shown (Fig. 9A and B). The analysis of the micrographs indicates thick growth of bacteria on rice husk. While biofilm of 25 days age (not shown) indicated much less bacterial growth, characteristics of biofilm of 150 days age (not shown) were same as that of 120 days. Elemental analysis provided by EDS (Fig. 10) of selected bacteria in the 120 days biofilm showed the presence of mercury in. The EDS analysis (Fig. 11) of packing material from activated carbon filter ensured the presence of residual mercury. Thus mercury was absolutely confined in the combined system of biofilm reactor and activated carbon filter.

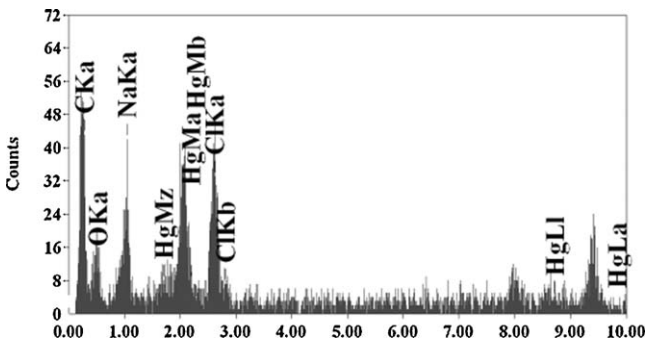

Fig. 11. Energy dispersive spectra of carbon filter after 120 days of operation.

4. Conclusion

Bacillus cereus (JUBT1) isolated from sludge of a local chlor-alkali industry has been successfully used to remove Hg^{2+} from simulated waste water stream both in free and in attached (biofilm) forms. Batch studies have been performed to determine the growth kinetics of the mercury resistant bacteria with respect to sucrose- the carbon source and mercury. A biofilm reactor has been operated using the microorganism's attached growth on rice husk packing to treat simulated Hg^{2+} contaminated waste water stream. A combination of the biofilm reactor followed by a carbon filter was used to reduce the mercury concentration of treated water to near zero level. The EDS analysis of the biofilm and the carbon filter indicates that mercury is confined in the biofilm and in the activated carbon. It is expected that similar systems will be very effective to treat mercury contaminated waste water in an effective and economic route.

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