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Bioleaching of copper from electronic waste using *Acinetobacter* sp. Cr B2 in a pulsed plate column operated in batch and sequential batch mode

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ABSTRACT

The amount of metal content present in electronic waste (e-waste) such as printed circuit boards (PCBs) exceeds that present in rich minerals thus allowing the use of PCBs as artificial ores. The copper content in PCBs is 10–30 mass %, which is the highest among all the metallic elements. The recovery of copper from e-waste serves dual fold benefit of conservation of metal resources and overcoming environmental hazard due to e-waste accumulation. In the currently reported study, a pulsed plate bioreactor in which the inter-plate spaces were packed with e-waste material was effectively employed for bioleaching of copper from e-waste using *Acinetobacter* sp. Cr B2. Various factors such as inoculum size, e-waste loading, frequency and amplitude of pulsation that significantly affected the bioleaching efficiency were studied. Inoculum size of 9% (v/v), frequency of 0.2 s^{-1}, amplitude of 6.5 cm and total e-waste loading of 40 g with 10 g/stage were found to provide maximum bioleaching of Cu. Around 23% of Cu bioleaching was achieved under these conditions by batch mode of operation. Increasing the number of sequential cycles of operation in sequential batch mode further improved the bioleaching efficiency, by overcoming the maximum copper solubility and growth limitations of the single batch operation. With five cycles of sequential batch operation around 63% leaching of Cu could be achieved. The bioleaching was found to be mediated both by the action of extracellular enzymes and metabolites. The study demonstrated the potential application of pulsed plate bioreactor for larger scale application of copper bioleaching from PCBs.

1. Introduction

The United Nations University (UNU) has calculated that about 42 Mt (million metric tons) of e-waste was generated globally in 2014 and expected to rise to around 50 Mt in 2018 [1]. According to its study, Asia generated around 16 Mt of e waste with India generating 1.7 Mt in 2014. The study revealed India as the fifth largest producer of E-waste. According to the report of Comptroller and Auditor General of India published in 2011 [2], around 400,000 tons of e-waste is being generated in India annually. E-waste is increasing at the compounded rate of 25% per annum in India and a meager 4% of total E-waste gets recycled in India [3]. E-waste contains an array of hazardous substances including heavy metals that can threaten life and the environment if not handled or disposed properly [4,5]. The amount of metal content present in e-waste such as printed circuit boards (PCBs) was found to exceed that present in rich minerals thus allowing the use of PCBs as "artificial ores" as reported [6]. E-waste contains approximately 40% metals, including base metals and precious metals [4,7]. The copper content in printed circuit board (PCB) is 10–30 mass %, which is the highest among the metallic elements [8]. Copper is an industrially significant metal, widely used in building construction (wiring, plumbing and weatherproofing), electrical and electronic products, transportation equipment and industrial machinery [9]. Thus, the recovery of copper from e-waste is important in order to conserve the environment and metal resources.

The possibility of a solution that is both economically feasible and also environmentally sustainable has encouraged a variety of active research in the area of metal recovery from e-waste in recent years. The most promising of these technologies is through the use of naturally occurring microorganisms in extracting valuable metals from the waste [10,11]. In an effort to develop an

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environmentally sound process with a huge potential to lower operational cost and energy requirements, the biohydrometallurgical route for reprocessing wastes is gaining a lot of interest. Bioleaching is the leaching of metals using microorganisms. Bioleaching process utilizes naturally occurring microorganisms. The process does not use toxic or hazardous chemicals and can be carried out under ambient conditions of temperature and pressure. Thus it is an environmental friendlier,less energy intensive and economical process. Thus the recovery of valuable metals from e-waste by applying microbes, is being researched and facilitated. Microbial strains including Acidot *hiobacillus thiooxidans* [12], *Aspergillus niger*, [13], *Acidothiobacillus ferroxidans* [13–15], *Leptospirilum ferriphilum* [15], *Sulfobacillus sulfoxidans, Chromobacterium violaceum* [16], *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* [17] have been predominantly used for bioleaching, either as a pure culture or a mixed consortium. The bacteria used for bioleaching of metals generally belong to the class of chemolithoautotrophs. Very little reported data is available on the use of heterotrophic organisms which could perform leaching of metals. In the present study, *Acinetobacter* sp. (Cr B2) a gram negative bacteria which was isolated from aerator liquid of an activated sludge process in a wastewater treatment facility of a dye/pigment based specialty chemical industry was employed to leach metals from e-waste [18]. Several strains belonging to the genus of *Acinetobacter* have been attracting growing interest from researchers for environmental and biotechnological applications. These bacteria are involved in biodegradation of xenobiotic organic compounds [19–23], Manganese leaching, biopolymer production, phosphate removal for wastewater treatment [22,23] and removal of metal ions such as hexavalent chromium from wastewater [24,25].

Acinetobacter sp. are widespread in water, soil and in living organisms. They are strictly aerobic, oxidase-negative, catalase positive, non-motile and appear as gram negative coccobacilli in pairs under the microscope [26]. They can use various carbon sources for growth, and can be cultured on a relatively simple media, including nutrient agar or trypticase soya agar [27]. They can grow with a single carbon or energy source and are being able to use any one of the large number of organic compounds such as aliphatic alcohols, amino acids, dicarboxylic acids, fatty acids, n-alkanes, alicyclic compounds and many aromatic compounds [28].

The primary characteristic of any bacteria to be used for bioleaching of metals is its high resistance to the metals which are present in E-waste. *Acinetobacter* sp. used in the present study has been found to be highly resistant to most of the metals like Cu, Ag, Pb, Ni, Cd,Fe and Zn [15,24] and the strain was also found to be able to leach metals from e-waste through shake flask studies [29]. Thus this strain was chosen for bioleaching.

Limited information is available on reactor studies and scale up possibilities of bioleaching. Column bioleaching has earlier been adopted using a packed bed column to extract metals [30]. Pulsed plate bioreactor has been proven advantageous over packed bed reactors in biotechnological applications [31]. Applying perturbations in the form of pulsations on to a fixed bed solves several problems of a packed bed reactor, including proper mixing and distribution of substrates, renewal of interfacial area and mass transfer limitations [32,33].

Pulsed plate bioreactors have been previously employed for a range of microbial processes including wastewater treatment, fermentation to produce exopolymers and growth of fungi and bacteria, thus proving to be a versatile and effective bioreactor [33,34]. Pulsed plate column with fixed bed of solids in the interplate space has proven to offer efficient oxygen mass transfer [35], solid-liquid mass transfer and mixing characteristics [36]. The bioleaching of metals from e-waste using an aerobic bacteria involves solid (e-waste and bacteria), liquid (water as a bioleaching medium) and gas (air) phases which necessitates the use of a contactor that can provide good solid-liquid (e-waste-water for metal transfer and bacteria-water for nutrient transfer) mass transfer and oxygen or gas-liquid (oxygen in air-water) mass transfer characteristics. Thus in the present study, the space between the plates of the pulsed plate column is packed with size-reduced printed circuit boards (PCBs). Pulsations, an alternate ascending and descending motion, are provided at varying frequencies and amplitudes to the plates. The column, containing the microorganism, is used as a bioreactor to effectively bioleach copper from the electronic waste into water containing nutrients for the bacteria.

2. Materials and methods

2.1. E-waste (PCB) characterization

Electronic waste was collected in the form of mobile phone PCBs from local mobile phone service centres. The e-waste was size reduced using Wiley mill and sieved to obtain a uniform particle size. The average particle size of reduced e-waste particles was analysed by cumulative sieve analysis [37] and was found to be 3.79 mm.

The e-waste material was then acid digested by treating four random grab samples of 2 g of e-waste with 20 mL of concentrated HCl and 5 mL of concentrated HNO_3 for 20–30 min till complete digestion was observed [38]. The concentrations of Cu, Fe, Ag, Zn and Ni ions present in the digest was measured using Atomic Absorption Spectrometer (GBC 932 *plus*) and the amount of each metal present in the e-waste was then calculated. The average amount of metals per gram of e-waste was found to be Cu 119.8 mg/g; Ag 0.274 mg/g; Fe 40 mg/g; Zn 3.71 mg/g and Ni 3.5 mg/g.

The PCB particles to be used in bioleaching experiments were washed and sterilized in an autoclave at 121° C for 15 min, dried in hot air oven at 100 °C for 2 h followed by UV sterilization to avoid microbial contamination.

2.2. Microorganism and bioleaching media

The bacteria used for bioleaching experiments, *Acinetobacter* sp. CR B2 (GenBank accession number: JF461086) was isolated [25] and the lyophilized cultures were available in the laboratory of Chemical Engineering Department at National Institute of Technology Karnataka (NITK), Surathkal. It was further subcultured on Lysogeny Broth (LB) at regular intervals. Bacterial inoculum for bioleaching experiments was prepared by growing the bacteria in LB broth for 24 h before inoculating into the bioleaching media.

The bioleaching media used was the optimized media for bioleaching of copper [29] which consisted of glucose 10 g/L; $CaCl_2$ 0.1 g/L; $MgSO_4{\cdot}7H_2O$ 0.1 g/L; NH_4Cl 5 g/L; NaCl 0.5 g/L; KH_2PO_4 3 g/L; and 6 g/L of NaH_2PO_4 in deionized water and the pH was adjusted to 12 using 0.1N NaOH and HCl. The optimum initial pH for bioleaching of copper by this strain of bacteria has been reported as pH 12 by Manasa [29]. The bioleaching media was prepared in distilled water, sterilized in an autoclave and used for bioleaching experiments in the pulsed-plate column.

2.3. Pulsed-plate bioreactor and bioleaching experiments

The experimental pulsed plate bioreactor shown in Fig. 1 consists of vertical Perspex column of 7 cm inner diameter, 7.2 cm outer diameter and 77 cm height. The plate stack consists of number of perforated brass plates of 6.8 cm diameter and 1.2 mm thick mounted over a central steel rod of 1 cm diameter inserted into a hole at the centre of each plate. The spacing between the

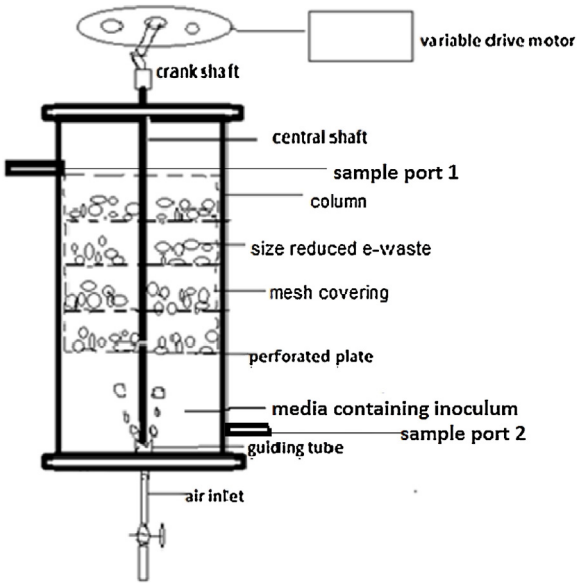

Fig. 1. Experimental pulsed plate bioreactor.

plates was kept at 3 cm. The entire circumference of the plate stack was covered with a 1 mm × 1 mm nylon mesh of thickness 0.5 mm. The space between the plates, forming each stage in the bioreactor, was filled with size reduced, sterilized e-waste particles, as per the requirement for the study. The pulsation of the plate stack was generated by running the flywheel using a variable speed motor with gear reduction and frequency controller, through the slider/crank arrangement. The required frequency of pulsation was set using variable voltage speed regulator and the amplitude was set, by changing the position of the crankshaft.The entire stack of plates was pulsated at the required frequency and amplitude through this arrangement. The column was filled with the working volume of previously optimized bioleaching media [29] in aqueous solution containing fresh inoculum of the bacteria under study. The media was sterilized in an autoclave at 121° C before adding into the column and inoculum was then added. Two sample ports were provided at 10 cm and 37 cm from the bottom of the column. The working volume of the reactor was 1.5 L. The Perspex column and the metal interiors of the column were ethanol washed and UV sterilized before they were assembled. Compressed air was continuously provided from a nozzle at the bottom at 1.5 LPM to ensure proper supply of oxygen to the microorganism. Air flow rate was regulated using an air pressure regulator and rotameter.

Samples were withdrawn from the sample ports every 3 hours during the leaching process and analyzed for metal content using atomic absorption spectrometer (AAS). The average concentration of the two samples (sample volume = 5 mL) taken from both the sample ports were considered for the study. Sample withdrawal continued at regular intervals for over 60 h during the bioleaching process.

Percentage of metal bioleached (Percentage of bioleaching) was calculated using Eq. (1)

$$\textit{Percentage of bioleaching of copper} = \frac{W_{BL}}{W_{AD}} * 100 \quad (1)$$

Where,

W_{AD} is amount of metal content per gram of e-waste as obtained by acid digestion (mg/g)

W_{BL} is amount of metal bioleached from 1 g of e-waste (mg/g) calculated using Eq. (2)

$$W_{BL} = \frac{C}{M_P} \times V \quad (2)$$

C is the concentration of metal in the leachate (by bioleaching) as analyzed using AAS (mg/L)

M_P is the total weight of e-waste packed in the pulsed-plate column (g)

V is the working volume of media in the pulsed-plate column.

The experiments were performed in duplicate and for each experiment the samples were analyzed in triplicates and the mean values were reported. The values in duplicate runs deviated by less than 2% around the mean values.

2.4. Batch experiments to study the effect of various parameters

Batch experiments were conducted in the pulsed plate column bioreactor by varying the inoculum size, e-waste loading, frequency and amplitudes of pulsation to obtain optimum conditions. Batch experiments were conducted at e-waste loading of 30 g/stage; frequency of 0.2 s^{-1} and amplitude of 6.5 cm with different inoculums sizes of 3%, 5%, 7% or 9% v/v to obtain an optimum size. Further batch experiments were conducted at the optimized inoculum size, e-waste loading of 30 g/stage, amplitude of 6.5 cm and at different frequencies of pulsation (0.2, 0.4, 0.6 or 0.8 s^{-1}). Thus, the optimum frequency was obtained. An experiment was also conducted under non-pulsed condition to study the effect of pulsation on bioleaching. Batch bioleaching experiments were also conducted at different e-waste loading conditions of 5, 10, 20 or 30 g per stage to determine the optimum e-waste loading at an amplitude of 6.5 cm, at the optimum conditions of inoculums size and frequencies obtained by earlier experiments. Finally, the optimization of amplitude of pulsation was carried out by conducting batch bioleaching experiments at optimized conditions of inoculums size, e-waste loading and frequency of pulsation with different amplitudes of 3.5, 5, 6.5 or 8 cm. One parameter was allowed to be varied keeping the other parameters constant. The optimum value was chosen based on the maximum bioleaching achieved.

2.5. Sequential batch experiments in pulsed plate bioreactor

A batch experiment was conducted in pulsed plate bioreactor for bioleaching of Cu from e-waste under the optimum conditions of inoculums size of 9% (v/v), frequency of 0.2 s^{-1}, amplitude of 6.5 cm and e-waste loading of 10 g/stage. At the end of 48 h of batch run, the bioleaching media with the leached metal was drained, and the reactor was filled with a fresh bioleaching media. The e-waste material was not removed from the column after the first run. The media was inoculated with the inoculum with 9% v/v size. The column was again run in batch mode under same set of frequency and amplitude conditions. The bioleaching media was drained at the end of 24 h and the concentration of Cu in the media was measured. The cumulative percentage of bioleaching after the second run was calculated using Eq. (3). The reactor was again filled with the fresh bioleaching media and the third batch run was conducted with the same e-waste material which was processed earlier in first and second runs. Totally five such sequential batch runs were conducted and the cumulative percentage of bioleaching after each run was calculated using Eq. (3).

$$\textit{cumulative percentage of bioleaching of Cu after } n^{th} \textit{ run} = \frac{\sum_{i=1}^{n} W_i}{W_{AD}} \times 100 \quad (3)$$

Where W_i is the amount of Cu bioleached in i^{th} run, (mg/g) [Calculated from Eq. (2)]

n is the run number, W_{AD} is the total amount of Cu present in the e-waste initially (mg/g)

2.6. Mechanism of bioleaching of Cu by Acinetobacter sp. Cr B2

200 mL of optimized bioleaching media was prepared in duplicate and 6 mL of cell culture was inoculated into each. The bacteria were allowed to grow at room temperature of 30 °C at 150 rpm for 5 days in an incubator shaker. The broth was centrifuged at 15,000 rpm at 4 °C for 10 min. The supernatant thus obtained from the duplicate broth were subjected to two sets of experiments, one by eliminating extracellular enzymes by means of heat sterilization and the other without eliminating the extracellular enzymes. To remove possible enzyme activity, the supernatant was autoclaved at 110 °C for 20 min. This would effectively denature all bacterial enzymes. 2 g of e-waste was added to the supernatant (heat treated or untreated). The flasks were kept in shaker and the percentage of bioleaching was determined by measurement of copper concentration in the samples after every one day of incubation for a period of seven days. These experiments were performed in duplicate and the mean values with error bars are reported.

3. Results and discussion

3.1. Effect of inoculum size on bioleaching of Cu from e-waste

The effect of inoculum size on bioleaching of copper from e-waste in a pulsed plate bioreactor under batch mode of operation was studied at a frequency of 0.2 s^{-1} and amplitude of 6.5 cm. Inoculum size was varied from 3% to 11% in increments of 2% to study its effect on bioleaching of Cu. Fig. 2 shows the time course variation of percentage of bioleaching of Cu at various inoculums size. 9% v/v inoculum size showed the highest leaching of Cu as compared to lower inoculum sizes. Abiotic control experiments were conducted and less than 0.4% copper was leached into the media in 100 h showing that the media components are contributing negligibly to leaching of copper. Experiments were also conducted in the absence of e-waste particles with the bacterial inoculum in the optimized media and no copper or other metals were detected in the media, nullifying the possibility of leaching of copper from the brass plates in the column. These results confirm that the copper present in the reactor liquid is that bioleached from the e-waste. Clogging of sample ports and air supply ports occurred with an inoculum size of greater than 9%, making it difficult to analyse the samples. At lower inoculum sizes of 3, 5 and 7% v/v, the maximum percentage of bioleaching was less than 3%. During the time course variation, frequent fluctuations in percentage of bioleaching were observed after the 21st hour with 9% inoculums size. When e-waste is brought in contact with the cells, two phenomena may be taking place. Heterotrophic bacteria secrete the enzymes such as glucose oxidase which converts glucose into gluconic acid [39]. The metabolites such as citric acid and gluconic acid [40,41] act as oxidizing agents and help in metal dissolution thus resulting in leaching of metals from e-waste. At the same time, the cells may also adsorb the bioleached metals from the solution, owing to the capability of bacterial cell surfaces to adsorb metals [42,43] like Cu. The Energy dispersive Spectra (Images not shown) of the bacterial samples from the reactor at various time periods have shown the presence of Copper peak and the mass percentage indicating the presence of copper on bacterial surface. The mass percentage of copper increased with time, thus confirming the occurrence of adsorption of Cu ions from the solution onto bacterial cell surface during the bioleaching operation.

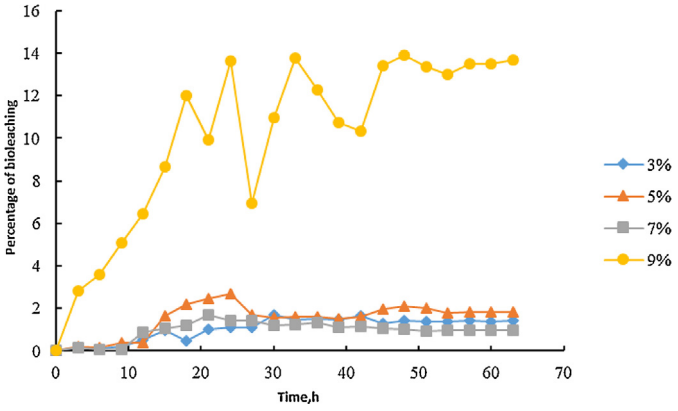

Fig. 2. Time course variation of copper bioleaching with different inoculum sizes. Conditions: pH = 12; frequency = 0.2 s^{-1}; amplitude = 6.5 cm; e-waste loading = 30 g/stage.

If the rate of bioleaching is higher than the rate of adsorption, then the accumulation of metals in the solution occur, leading to net increase in the metal concentration in solution, whereas if the rate of bioleaching is lower than the rate of adsorption then there appears a net decrease in metal concentration in solution. As observed in Fig. 2, with 9% inoculum size, the percentage of bioleaching increased up to 21st hour showing that the rate of leaching is higher than the rate of adsorption of Cu by the bacterial cells. Lower adsorption rate may be owing to lower concentration of Cu in the solution initially, which keeps the concentration gradient between the bulk liquid and cell surface lower. But as the leaching proceeds, the concentration of Cu in bulk liquid increases, thus increasing the concentration gradient and the rate of adsorption. Higher rate of adsorption as compared to the rate of leaching may have led to reduction in percentage of bioleaching from 21 to 24 h. These two parallel phenomena continuously occur leading to fluctuation in percentage of bioleaching up to 48 h and the increase or decrease in percentage of bioleaching occurs as governed by the dominating rate.

With inoculum sizes of 3–7%, the percentage of bioleaching was almost negligible up to 15 h. With low inoculum size, the number of cells initially present in the reactor is lesser and the rate of growth is lower. Lower growth rate leads to reduced production of enzymes, thus leading to lower bioleaching. After 15 h, the percentage of bioleaching increases, reaches a maximum in around 24–27 h and then decreases. But the slow increase in bioleaching percentage till it reaches the maximum indicate that the rate of bioleaching is very low. This is a result of lower rate of growth of the cells in the bioreactor. But further, the percentage of bioleaching decreases owing to adsorption of Cu by the cells and reaches a constant value after around 30 h. The percentage of bioleaching observed at the inoculum sizes of 3–7% is less than 9%, indicating that these lower inoculum sizes do not produce sufficient biomass to bring about bioleaching and thus are not suitable for bioleaching.

After 48 h of growth, the bioleaching percentage stabilized at all the inoculum sizes, indicating that leaching of Cu into the solution has stopped. This may be due to either or both of the possibilities that occur: (i) the bacterial cells have reached the stationary phase of its growth cycle after which, viable cells start to decrease thus reducing the overall leaching capacity (ii) the maximum solubility of Cu in the solution has been attained and further leaching into the solution has stopped. Under these circumstances, the rate of leaching equals the rate of adsorption/utilization leading to constant percentage of bioleaching.

Fig. 2 shows that the highest percentage of bioleaching (14%) of Cu was achieved with 9% inoculum size. The time at which the maximum percentage of bioleaching occurred was 48, 24, 21 and 48 h with 3%, 5%, 7% and 9% inoculum sizes respectively.

With higher inoculum size, initial number of cells present in the system is higher and hence the amount of organic acids released to solubilize the metal is also higher [43]. Increase in percentage of bioleaching with increase in inoculum size has been reported in bioleaching of metals by various researchers [14,43,44]. However, inoculum sizes greater than 9% caused an overgrowth of cells. 11% (v/v) inoculum size was also studied for bioleaching. Sample could only be collected for a few initial hours of time course and it was found that the percentage leaching was only 0.5% at 24 h. The bioleaching percentage achieved was lower than the maximum bioleaching achieved with 3–9% inoculums size. This could be due to high rate of adsorption of leached copper ions on large amount of bacterial biomass surface. Thus, 9% inoculum size is considered as the optimum.

3.2. Effect of frequency of pulsation

The effect of variation of frequency of pulsation (0.2 s^{-1}, 0.4 s^{-1}, 0.6 s^{-1} and 0.8 s^{-1}) in pulsed plate bioreactor was studied at an amplitude of 6.5 cm and with inoculum size of 9% (v/v). The experiment was also conducted under non-pulsed conditions. The time course variations of bioleaching of Cu at various frequencies are presented in Fig. 3. As observed, under non-pulsed conditions, the rate of bioleaching was very slow and only around 3.8% bioleaching was achieved in 27 h and around 6% at the end of 63 h. The maximum percentage of bioleaching was around 14% under non-pulsed condition and it occurred only after 168 h (not shown in figure). Under minimum pulsing condition of 0.2 s^{-1}, the maximum bioleaching of around 14% was achieved in around 27 h.

As the frequency was increased from 0.2 s^{-1} to 0.4 s^{-1}, the maximum bioleaching of around 4% was achieved in around 15 h and thereafter the percentage of bioleaching was constant indicating the attainment of stationery bioleaching phase. With further increase in frequency to 0.6 s^{-1}, the percentage of bioleaching slowly increased to around 4% in around 40 h before reaching the stationery phase. The time taken to attain maximum percentage of bioleaching were 168, 48, 28 and 48 h with non-pulsed, 0.2 s^{-1}, 0.4 s^{-1} and 0.6 s^{-1} frequencies respectively.

As observed from Fig. 3, pulsing action enhances the bioleaching efficiency. Under non-pulsed conditions, mass transfer limitations exist in the column, leading to lower growth and bioleaching rates. But as the frequency is increased to 0.2 s^{-1}, the mass transfer limitations are overcome and oxygen mass transfer rate and rate of solid-liquid mass transfer increases [35,36]. Higher

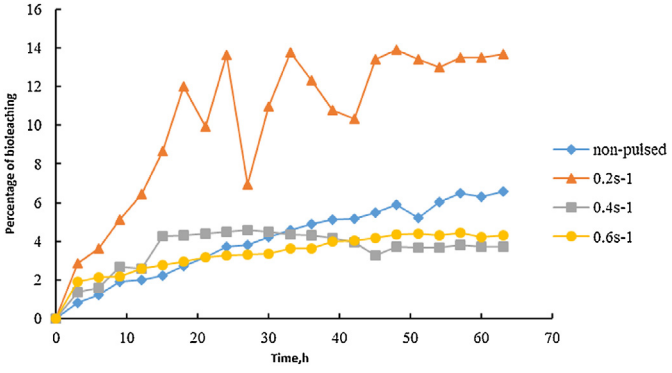

Fig. 3. Time course variation of copper leaching under non-pulsed condition and at different frequencies; Conditions: inoculum size = 9%; amplitude = 6.5 cm; e-waste loading = 30 g/stage.

mixing efficiency and mass transfer rates in the column result in enhanced rate of supply of nutrients and oxygen to the cells and thus leading to increased growth of the cells. The production of enzymes or metabolites responsible for bioleaching increases under higher growth conditions, thus resulting in higher rate of bioleaching of metals. But, with further increase in frequency to 0.4 or 0.6 s^{-1}, the bioleaching rate reduced considerably as observed in Fig. 3. The initial rates of bioleaching were calculated at non-pulsed condition and at different frequencies. It was found that the initial rates were 0.35, 1.333, 0.931 and 0.744 mg L^{-1} h^{-1} under non-pulsed condition and at frequencies of 0.2, 0.4 and 0.6 s^{-1} respectively. It implies that the rate of bioleaching has enhanced on provision of pulsing action. However with increase in frequency to greater than 0.2 s^{-1}, the rate has decreased. This could be due to the possible fact that at very high frequencies, the microorganisms undergo high shear and the viable cells begin to decrease slowly. Subjecting the microorganisms to continuous shear could lead to disintegration of the cells [45], low growth and thus the overall leaching decreases. High degree of pulsation may also lead to improper contact between the cells and the surface of the e-waste particles due to very fast movement of the liquid media containing the cells over the surface of e-waste particles. Very high frequencies provide more number of contacts but less contact time per stroke which may result in insufficient effective contact between the cells and e-waste. Reduced contact of cells and the e-waste may reduce the rate of bioleaching. The reduction in rate of leaching due to the combined effect of reduced contact and the microbial cell disintegration may override the increase in rate of bioleaching due to enhanced mixing and mass transfer characteristics, thus leading to net reduction in rate of bioleaching at very high frequencies. It indicates that there exist a threshold value of frequency below which the effect of enhanced mass transfer and mixing characteristics are dominant over the effect of reduced contact and cell disintegration. The pulsed plate column when operated at frequencies below the threshold value, the bioleaching efficiency increases with the increase in frequency. In the present study under the range of conditions studied, the frequency of 0.2 s^{-1} is the optimum frequency resulting in maximum percentage of bioleaching and higher rate of bioleaching.

As observed in Fig. 3, the fluctuations in percentage of bioleaching over the time period of bioleaching was found to be minimal at higher frequencies of 0.4 and 0.6 s^{-1}, whereas at 0.2 s^{-1} large fluctuations were observed before reaching the stationery phase. At higher frequencies of 0.4 and 0.6 s^{-1}, the rate of bioleaching is lower, thus the metal concentrations in media is low, leading to negligible adsorption rate during the period of bioleaching. Thus in the course of study, the metal concentration slowly increases. No reduction in bioleaching percentage is observed under these high frequency conditions as the increase in metal concentration has not reached such a value that leads to considerable adsorption phenomena to occur during 63 h of the experimental run. When the bioreactor was run at a frequency of 0.8 s^{-1}, no leaching of metals occurred owing to high shear conditions that led to negligible growth.

3.3. Effect of e-waste loading on bioleaching

The effect of e-waste loading on the bioleaching efficiency was studied with a range of e-waste loading of 5 g, 10 g, 20 g and 30 g per stage. These e-waste loadings occupied 11.1, 22.2, 44.4 and 66.7% by volume of each stage respectively. The total e-waste loading in the column were 20 g, 40 g, 80 g and 120 g respectively. Fig. 4 shows the time course variation of percentage of bioleaching of Cu at different e-waste loading conditions.

The initial rates of bioleaching at e-waste loading of 5, 10, 20 and 30 g/stage were found to be 0.89, 1.63, 0.74 and 0.73 mg L^{-1} h^{-1}

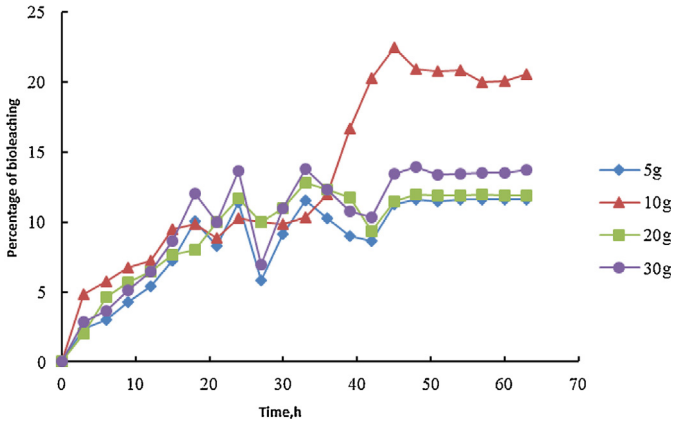

Fig. 4. Time course variation of copper leaching with different e-waste loading; Conditions: inoculum size = 9%; amplitude = 6.5 cm; frequency = 0.2 s^{-1}.

respectively. Maximum rate was achieved with 10 g/stage e-waste loading. The time taken to attain maximum percentage of bioleaching was 63, 48, 50 and 48 h with 5 g, 10 g, 20 g and 30 g per stage of e-waste loading respectively. As the amount of e-waste packed per stage increased from 5 g to 10 g, the rate of leaching of Cu increased. Above this value, the bioleaching decreased. There are several competing factors which yield an optimum e-waste loading. With increase in e-waste loading, surface area increases; tendency of partial fluidization of bed reduces; ratio of e-waste loading to inoculum amount increases. Increase in surface area renders positive effect on bioleaching rate, whereas the reduced fluidization and increase in ratio of e-waste loading to inoculums amount offers negative effect on bioleaching rate. Oxygen mass transfer performance is the overall rate-limiting step in bioleaching processes and at higher solids concentration the demand for oxygen may exceed the maximum possible supply of bioreactors [46], thus leading to lower leaching efficiency. The optimum value of e-waste loading is governed by several factors such as surface area availability, occurrence of partial fluidization of bed, the ratio of e-waste loading to inoculums quantity and oxygen transfer rate. The optimum is achieved when the net effect is the maximum. Thus, 10 g per stage of e-waste loading is considered the optimum, as it resulted in maximum percentage of bioleaching of 22.45% Cu as compared to other loadings.

3.4. Effect of amplitude of pulsation on bioleaching

Fig. 5 shows the effect of amplitude of pulsation on time course variation of percentage of bioleaching at frequency of 0.2 s^{-1}, e-waste loading of 10 g/stage and inoculum size of 9% (v/v). The initial rates of bioleaching are 0.67, 1, 1.87 and 1.67 mg L^{-1} h^{-1} at amplitudes of 3.5 cm, 5 cm, 6.5 cm and 8 cm respectively. It is the minimum at the amplitude of 3.5 cm, indicating lower rate of leaching and the rate of bioleaching increased with an increase in amplitude of pulsation.

The maximum bioleaching was achieved at 32 h with 3.5 cm amplitude and at other amplitudes it was achieved in 48 h. Fig. 5 shows that the maximum bioleaching increased from around 5 to 23% as the amplitude of pulsation was increased from 3.5 cm to 6.5 cm. The solid-liquid mass transfer coefficient and mixing efficiency increases with the increase in amplitude of pulsation since the amplitude of oscillation controls the length of eddy generated along the column [36]. Similarly the oxygen mass transfer coefficient increases with the increase in amplitude of pulsation [35]. The increase in amplitude of pulsation increases the mass transfer and mixing characteristics of pulsed plate column [35,36,47].

Increased mixing and mass transfer at higher amplitudes lead to better contact between the e-waste, cells and nutrients, thus leading to higher bioleaching efficiency. However, further increase in amplitude from 6.5 cm to 8 cm did not result in considerable change in rate of leaching and the bioleaching efficiency. Thus, amplitude of 6.5 cm is considered as the optimum for Cu bioleaching.

3.5. Effect of vibrational velocity on bioleaching of copper

To understand the combined effect of frequency and amplitude of pulsation, a graph of maximum bioleaching vs. vibrational velocity (product of Amplitude and frequency) was plotted and shown in Fig. 6. The data of the experimental runs to study the effect of frequency (Section 3.2) and amplitudes of pulsation (Section 3.4) were used for this study. Increase in vibrational velocity can result in increased mass transfer characteristics and the shear on bacterial cells. At lower vibrational velocity values, the effect of increased mass transfer with increase in vibrational velocity was much higher than the shear to bacterial cells, hence % bioleaching increased. Beyond a certain vibrational velocity, damage caused by shear to the biomass dominated over the increased mass transfer characteristics and hence decrease in % bioleaching was observed owing to disintegration of the cells.

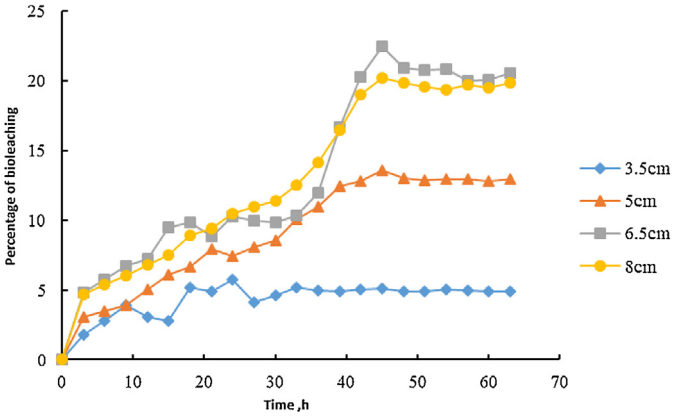

Fig. 5. Time course variation of copper leaching at different amplitudes; Conditions: inoculum size = 9%; Frequency = 0.2 s^{-1}; E-waste loading = 10 g/stage.

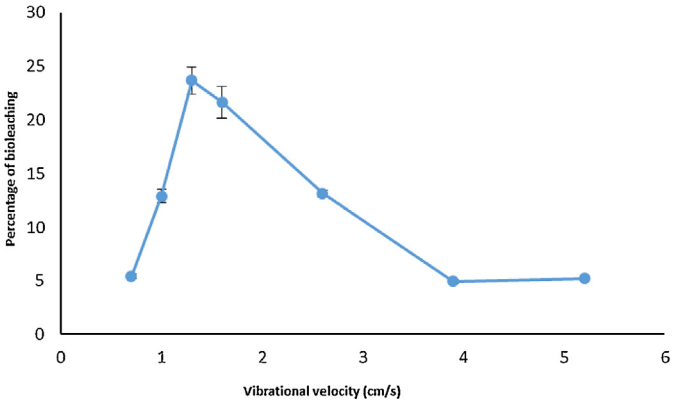

Fig. 6. Effect of vibrational velocity on bioleaching of copper.

3.6. Bioleaching of Cu from e-waste in pulsed plate bioreactor operated under sequential-batch mode

The results of batch experiments on bioleaching of Cu presented in Sections 3.1 to 3.4 showed that the optimum conditions for bioleaching of Cu from e-waste in pulsed plate column are inoculum size 9% (v/v), frequency 0.2 s^{-1}, amplitude 6.5 cm and the e-waste loading of 10 g/stage. Under these optimum conditions around 23% of Cu was bioleached from the e-waste. Low bioleaching of Cu from e-waste may be the result of inactivation of the enzymes present in the media by the leached copper ions, thus inhibiting the rate of metabolic processes and the secretion of metabolites which bring about bioleaching or due to death of cells in the media caused by nutrient limitations leading to limited metabolic activity. So, in order to test the possibility of further bioleaching, a second set of experiment was conducted with the used e-waste from the first batch and with fresh nutrient media. The sequential batch trials were conducted five times, each time with the used e-waste from the previous run and with fresh nutrient media and inoculum in the pulsed plate bioreactor. All the sequential batch experiments were conducted under the optimum set of conditions of inoculum size 9% (v/v), frequency 0.2 s^{-1}, amplitude 6.5 cm but with the used e-waste from the earlier batch. Table 1 shows the amount of copper bioleached in each run and the cumulative percentage of bioleaching of Cu achieved after 24 h of run time in each of the sequential batch runs.

It was found that further bioleaching of Cu from e-waste was possible with repeated leaching in fresh bioleaching media and a considerable amount of Cu continued to get leached in the subsequent sequential batch reactor runs. This indicates that the e-waste can be continued to be leached for Cu, however due to growth constraints and limited solubility of Cu in the broth, it can only attain a particular percentage of bioleaching in a single run. After five sequential- batch runs, around 63.5% of Cu could be bioleached from e-waste. Increasing the number of sequential batch cycles can result in higher bioleaching. These studies proved the effectiveness of operation of pulsed plate bioreactor in sequential batch mode and confirmed the possibility of complete leaching of Cu from electronic waste using *Acinetobacter* sp. by employing sequential batch operation with increased number of stages. Around 23% of Cu bioleaching was achieved under the optimum conditions within 48 h by batch mode of operation whereas 63.5% of Cu was bioleached from 40 g total e-waste by operation with five cycles under sequential-batch mode of operation in a total duration of 144 h. These results seem to be superior to or on par with those reported in recent literature. Hong et al. have achieved 98% copper bioleached from e-waste pulp in 14 days using acidophilic sulphur oxidising bacteria [48]; Xiang et al. have achieved 95% copper bioleached from 2 g e-waste in 16 days in a 200 mL flask by bacterial consortium enriched from natural acid mine drainage [6]; Karwowska et al. could get 53% copper bioleached from 10 g e-waste in 14 days using a culture of sulphur-oxidising bacteria and a mixed culture of biosurfactant-producing bacteria [12]. 60% Cu bioleached in 3 days and 98% copper was leached in 21 days from 1 g e-waste in 100 mL (10 g/L)

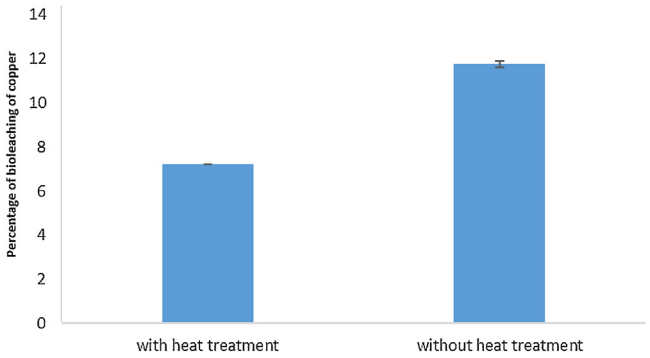

Fig. 7. Bioleaching of copper with cell free supernatant (with and without heat treatment).

by Willner et al. [14] using bacteria of the genus *Acidithiobacillus* in shake flasks. Around 60% copper was bioleached in a two step process after 21 days with only 5 g/l electronic scrap using *A. niger* by Brandl et al. [41]. Jorge Enrique Madrigal-Arias [49] could achieve only 26% bioleaching of Cu in 14 days with 200 mg of e-waste in 50 mL (0.4 g/L) with Asperigilus species. Chen et al. [50] have performed column bioleaching for 28 days resulting in 94% copper recovery. The amount of e-waste processed for leaching copper in these cases were very less and took very long time for the leaching. Jujun et al. [51] have obtained around 88% leaching of copper, but they used a mixture of pure copper and gold in their experiments to replace the electronic waste. They have also used certain additives in the bioleaching media. But in the present study with 40 g e-waste (10 g/stage) in 1.5 L (i.e. 26 g/L e-waste loading) around 63% recovery was possible in total around 6 days by sequential batch runs. In the present study higher e-waste loading was handled and the time for bioleaching was smaller. With these conditions around 63% bioleaching obtained is better than those reported in the study even in terms of the time for bioleaching, thus implying higher rate of bioleaching. If the number of cycles was increased, higher bioleaching could have been obtained. Thus, in the present study pulsed plate bioreactor could be effectively used to process large quantity of e-waste. The *Acinetobacter* sp. Cr B2 has proved to efficiently leach copper from e-waste at a faster rate.

3.7. Mechanism of bioleaching of Cu by Acinetobacter sp. Cr B2

To determine if the bioleaching is enzyme mediated ot metabolite mediated, bioleaching was performed using the bacterial supernatant after heat treatment and without heat treatment. Maximum bioleaching was achieved on the fourth day. The percentage of bioleaching of Cu on the fourth day with heat treated supernatant and untreated supernatant are presented in Fig. 7. Around 7.2% and 11.6% of bioleaching occurred in four days with and without heat treatment respectively. It is observed from these results that bioleaching could also occur with the heat treated supernatant indicating that metabolites are involved in bioleaching. In the absence of heat treatment, higher bioleaching

Table 1
Cumulative percentage of bioleaching of Cu achieved after 24 h of run time in each sequential batch run. Conditions: inoculum size = 9%; frequency = 0.2 s^{-1}; amplitude = 6.5 cm. Initial e-waste loading in run 1 = 10 g/stage.

Sequential batch run No.	Amount of Cu bioleached from i^{th} run, W_i mg/g	Cumulative percentage of bioleaching of Cu in each sequential batch run (time of run)
1 (first run with the fresh e-waste)	22.77	22.45 (after 48 h)
2	18.44	37.55 (after 24 h)
3	12.94	49.37 (after 24 h)
4	5.5	54.39 (after 24 h)
5	9.95	63.5 (after 24 h)

was observed, showing that enzymes also contribute to bioleaching along with the metabolites. In the presence of both the enzymes and metabolites, bioleaching is almost doubled. Overall leaching seems to be greatly affected by the presence of both enzymes and metabolites. It can be inferred that in the absence of enzymes, metabolites cause bioleaching to occur. The organic acids such as lactic acid, oxalic acid, citric acid and gluconic acid or the compounds with at least two hydrophilic reactive groups such as phenol derivatives are excreted into the culture medium as metabolic products and dissolve heavy metals to form soluble metal complexes and chelates [52–54]. The dissolution occurs by direct displacement of metal ions from the ore matrix by hydrogen ions [55]. Brombacher et al. [56] have also reported lower bioleaching efficiency in the heat treated cell free supernatant than that carried out without heat treatment for bioleaching of metals from fly ash using Thiobacillus sp. They attributed the higher bioleaching obtained without heat treatment to several components involved in the electron transport chain of Thiobacillus such as rusticyanin, cytochromes, iron–sulfur proteins) which are located in the periplasmic space [57] and might, therefore be present in the cell free spent medium catalyzing the oxidation of metal compounds. The higher bioleaching of copper in the present study by the cell free supernatant in the absence of heat treatment may thus be attributed to certain enzymes/proteins which may catalyse the process of oxidation of metals by the metabolites.

4. Conclusion

Pulsed plate column can be effectively employed for bioleaching of copper from e-waste. *Acinetobacter* sp. Cr B2 has exhibited a tremendous potential in efficiently leaching copper from e-waste. Bioleaching of copper has been brought about by the combined action of extracellular enzymes and the metabolites. Inoculum size of 9% (v/v), frequency of 0.2 s^{-1}, amplitude of 6.5 cm and total e-waste loading of 40 g with 10 g/stage were determined to be the optimum conditions for maximum bioleaching of Cu. Around 23% of Cu bioleaching was achieved under these optimum conditions within 24 h by batch mode of operation, whereas 63.5% from 40 g total e-waste was bioleached with five cycles of operation under sequential-batch mode within a duration of 5 days. Bioleaching efficiency can be enhanced by increasing the number of sequential batch cycles. These studies proved the effectiveness of operation of pulsed plate bioreactor in sequential batch mode and confirmed the possibility of complete leaching of Cu from electronic waste using *Acinetobacter* sp. by employing sequential batch operation with increased number of stages. This process being conducted under ambient condition is advantageous as it is less energy intensive, easily controllable and offers benefits of lower pollution risks.

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