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Feasibility of cheese whey as an energy source for the growth of *Aspergillus* sp. and for the removal of heavy metals in batch reactor

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ABSTRACT

In the present work, batch biosorption of Cu, Zn, and Ni ions by *Aspergillus* sp. was investigated. The effect of initial metal ion concentration (0–500 mg/l), pH (2.0–6.0), inoculum concentration (v/v), and different concentrations of total sugar in cheese whey (2, 4, 6, 8, and 10 g/l) on the biosorption of Cu, Zn, and Ni was studied separately. In the absence of metals and at pH 5, a maximum concentration of 5.62 g/l of biomass was observed. However, a decrease in the concentration of biomass was observed in the presence of Cu, Zn, and Ni. The concentration of Cu, Zn, and Ni was increased from 50 to 500 mg/l, and the maximum specific uptake was found to be 9.4 ± 0.15–62.2 ± 0.20 mg/g, 9.5 ± 0.1–64.0 ± 0.25 mg/g, and 7.2 ± 0.2–29.43 ± 0.08 mg/g, respectively. The biomass concentration increases with the increase in total sugar concentration in cheese whey. Scanning electron microscopy and X-ray Energy Dispersion Analysis depicted the possible cell–metal ions interaction.

Keywords: Biosorption; Heavy metal; Cheese whey

1. Introduction

Anthropogenic activities are the main sources of heavy metal contamination in the environment [1]. The removal of these hazardous materials from wastewater has been reported using various techniques including precipitation, membrane filtration, ion exchange, sorptive flotation, etc. [2,3]. However, these techniques are not economically viable for small- and medium-scale industries. Biosorption of heavy metals has received serious attention in recent years as a low-energy process [4]. Micro-organisms (algae/fungi/yeasts) have been reported as potent bioremediators for heavy metals [5,6]. The removal of metal using living cells may involve metal uptake into the cell across the cell membrane, which is dependent on the cell metabolism, and is referred to as the intracellular uptake or active uptake or bioaccumulation [7,8]. In most of the laboratory studies on biosorption of heavy metals using growing cells of micro-organisms, glucose has been used as an energy source [9–12]. For the development of an economically viable bioremediation process, there is a need to look for a cheaper energy source.

Cheese whey can act as a cheaper substrate than glucose for the growth of micro-organisms [13]. The major components of whey, the milk serum obtained from manufacturing of cheese, are lactose (44–52 g/l), proteins (6–8 g/l), and mineral salts (4–9 g/l) [13]. The composition of whey, however, varies according to the

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source from which it is obtained. The global production of whey is estimated at about 85 million tons. About 40% of the total global production of whey is either disposed into rivers, lakes or other water bodies or loaded onto land [14]. Due to the presence of high mineral and organic matter content, the raw whey causes environmental pollution such as eutrophication [15,16]. It has been estimated that whey has a biological oxygen demand (BOD) values of 40–60 g/l and chemical oxygen demand (COD) values of 50–80 g/l, respectively [14,17]. Lactose contributes to 90% of the COD and BOD content of whey. Considerable efforts have been made to develop improved procedures for recycling of whey [13,18]. The biological treatment of whey is a feasible process because the composition of whey is very suitable for the growth of certain micro-organisms [19,20]. A very little information is available on utilization of whey as a cheaper energy source in metal bioremediation process. Whey, being a waste from food industries, is expected to make the bioremediation of metals economically more viable process with simultaneous benefit of minimization of water pollution caused by the disposal of whey in surface water sources.

In the present study, an attempt was made to examine the potential of *Aspergillus* sp. (isolated in the laboratory from industrial wastewater) for the removal of heavy metals (Cu, Zn, and Ni) from aqueous solution and using cheese whey as an energy source for the growth of *Aspergillus*. The effects of pH, concentration of cheese whey, and initial metal concentration on the growth of the microorganism were examined along with the removal of metals in batch reactors. Scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX) were carried out in order to have an insight into the adsorption of the metal ions onto the surface of the cells.

2. Material and methods

2.1. Media and growth condition

The fungal strain (*Aspergillus* sp.) isolated in the laboratory by dilution and streak plate method from an industrial wastewater was used in the present study.

In order to have substantial amount of biomass, the isolated organism was grown in a sterile growth media at 30˚C and 150 rpm. The organism was then cultured in Potato Dextrose Agar plates and stored at 4˚C. To maintain the potency and metabolic activity of the organism, the stock culture was subcultured every 1–2 weeks.

For preparation of an inoculum, *Aspergillus* sp. was initially grown in 250-ml Erlenmeyer flask at 30˚C and pH-5.6, with shaking at 180 rpm using 100 ml of growth media. The growth media consisted of (g/l): glucose, 10; $K_2H_2PO_4$, 0.5; NaCl, 1; $MgCl_2$, 0.5; NH_4NO_3, 0.5; and yeast extract, 5. A 10% (v/v) of 1-day-old culture was used as an inoculum for the growth of *Aspergillus* sp. in cheese whey growth media which contained 48 mg/l total sugar. The *Aspergillus* sp. was cultivated in 250-ml Erlenmeyer flask with 100 ml working volume using 10% (v/v) cheese whey at 30˚C with shaking at 180 rpm. A 10% (v/v) of 1-day-old culture was used for all batch biosorption experiments.

2.2. Batch biosorption experiments

2.2.1. Preparation of solution

Stock solutions of Cu, Zn, and Ni (1,000 mg/l) were prepared by dissolving appropriate quantities of pure metal salts $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$, and $NiCl_2{\cdot}6H_2O$, respectively, in double-distilled water. The solutions of different concentrations were obtained by suitable dilution of the stock solution.

2.2.2. Batch biosorption

Batch biosorption studies were carried out in 250-ml Erlenmeyer flasks with a working volume of 100 ml. The sterile cheese whey (10% v/v) was used as the growth media for *Aspergillus* sp. The metal solutions containing different concentrations of Cu, Zn, and Ni were introduced in the growth medium individually to get the desired concentration of metal. The growth media was inoculated with 10% v/v inoculum of *Aspergillus* sp. The pH was adjusted to 5. Each flask was incubated at 30˚C with shaking at 180 rpm. The samples were drawn at a time interval of 12 h. The samples were analyzed for biomass concentration and residual concentration of metals (Cu, Zn, and Ni). The biosorption process was monitored for 8 d till the equilibrium condition for metal removal was achieved. All the experiments were carried out in triplicate.

The effect of pH on the growth of the organism and on the removal of Cu was studied at different fixed pH values. The effect of pH was studied in the range between 2.0 and 6.0. The initial concentration of Cu was 50 mg/l. A similar study was carried out using 50 mg/l initial concentration of Ni and Zn in separate batch experiments. The effect of different concentrations of total sugar contained in cheese whey (2, 4, 6, 8, and 10 g/l) on the biosorption of Cu, Zn, and Ni was studied individually at an initial concentration

Table 1
Characteristic properties of cheese whey

S. No.	Parameter	Range
1	pH	4.7–5.9
2	BOD (kg/l)	32–53
3	COD (kg/l)	64–86
4	Total phosphorous (g/l)	786–1,000
5	Total nitrogen (g/l)	367–484

of 50 mg/l. The effect of initial concentration of Cu, Ni, and Zn (50–500 mg/l) was also examined on the growth of the *Aspergillus* sp.

2.3. Analytical methods

The characteristics properties of cheese whey are presented in Table 1. The values of different parameters of cheese whey composition measured in the present study lie in the range which conforms to those given in the literature [13]. The total phosphorous content was analyzed spectrophotometrically. Biochemical oxygen demand was analyzed by Winkler's method using iodometric titration. COD was analyzed by open reflex method using Ferrous Ammonium Sulfate titration. Total Kjeldhal nitrogen was estimated by acid digestion followed by distillation and titration. All the methods were performed according to the standard methods for wastewater examination [21]. The residual concentrations of Cu, Zn, and Ni ions were determined using Atomic Absorption Spectrophotometer (Perkin Elmer A Analyst 200). The weight of biomass was determined gravimetrically after drying it at 80°C until constant weight was achieved. The total sugar content in cheese whey was determined by hydrolyzing the non-reducing sugar to total reducing sugar. The analysis was carried out by adding 2.5 ml HCl (2 M) to 25 ml of cheese whey in an Erlenmeyer flask (50 ml), and then boiling the mixture for 5 min. The mixture was cooled and neutralized with 10% NaOH and the volume was made up to 50 ml with double-distilled water. The hydrolysate was then analyzed for the reducible sugar by 3,5-dinitrosalicylic acid method. The absorbance of the solution was measured using UV/vis spectrophotometer 117 (Systronics) at 540 nm.

2.4. SEM and EDX analysis

In the present study, SEM and EDX spectral analysis were carried out to examine the surface of the cell before and after metal sorption. The scanning electron micrographs and energy dispersive X-ray analysis before and after biosorption of metal were recorded using ZEISS EVO Series Scanning Electron Microscope EVO 50. The samples for SEM and EDX were mounted on a stainless steel stub by coating them with a thin layer of silver under vacuum in order to increase the electron conduction and to improve the quality of the micrographs. The magnification and acceleration voltage for the micrographs were same. The instrumental operation conditions were EHT (acceleration voltage from secondary electrons) = 20.00 kV, WD (width) = 19.00 mm, signal A SE1, Magnification = 3.63 KX (Fig. 6(a)), and conditions were EHT = 20.00 kV, WD = 19.50 mm, signal A SE1, Magnification = 3.62 KX (Fig. 6(b)).

All the biosorption experimental conditions (pH 5, Initial metal concentration 50 mg/l, inoculum 10% v/v, and temperature 30°C) were same throughout the experiment. The *Aspergillus* sp. was exposed for the same length of time (8 d) in the medium, both in the presence and in the absence of metal content.

Table 2
Comparison of biomass conc. (g/l) and % Cu and Ni removal using cheese whey as a substrate at different initial conc. of Cu and Ni

Initial metal conc. (mg/l)	Biomass concentration (g/l)		% Cu removal		Biomass concentration (g/l)		% Ni removal	
	Growth media (glucose)	Cheese whey	Growth media (glucose)	Cheese whey	Growth media (glucose)	Cheese whey	Growth media (glucose)	Cheese whey
0	6.72	5.22			6.72	5.17		
50	6.4	4.91	100	94	6.07	4.4	96	55
100	5.58	4.78	96	90	5.21	4.15	90	50
150	5.3	4.57	84	79	4.74	3.94	87	48
250	4.2	4.1	76	71	3.2	3.03	51	26
500	3.12	2.65	42	30	1.9	1.61	21	6

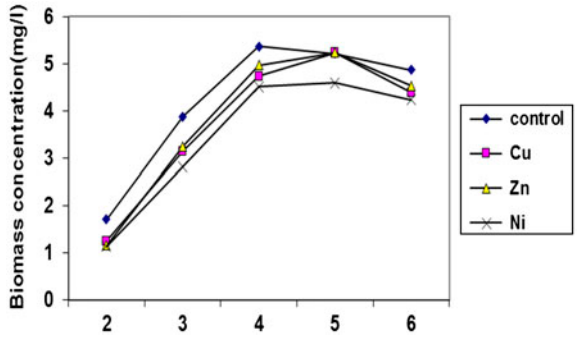

Fig. 1. Effect of pH on biomass concentration (g/l) in the presence of Cu, Zn, and Ni.

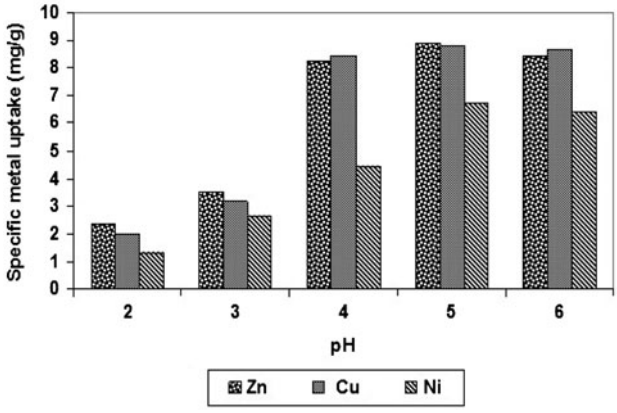

Fig. 2. Effect of pH on specific metal uptake.

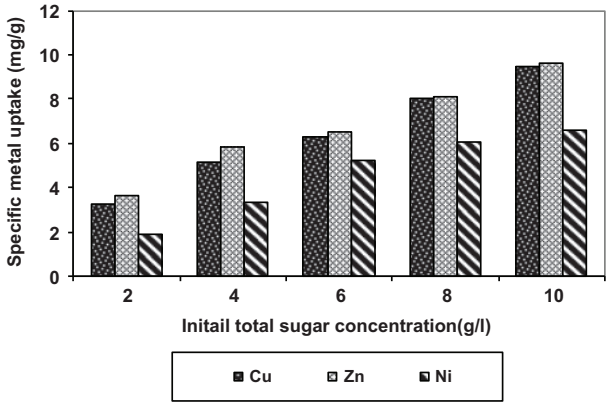

Fig. 3. Effect of initial total sugar concentration (g/l) on specific Cu, Zn, and Ni uptake (mg/g) in cheese whey.

3. Results

3.1. Effect of pH

Fig. 1 shows the effect of initial pH (2.0–6.0) on biomass concentration (g/l) of *Aspergillus* sp. in sterile cheese whey in presence of 50 mg/l Cu and Ni, respectively. The initial pH of the cheese whey was 5.6. The biomass concentration increased with increase in pH up to 5 and then decreased with further increase in pH up to 6.0. A similar trend was observed earlier using Zn and Ni in the present work. A higher biomass concentration was observed in the presence of Cu (5.12 g/l) in comparison to 4.67 g/l in presence of Ni in cheese whey. The findings in presence of Zn are comparable with the findings in presence of Cu. The specific uptake was observed to be 8.7, 8.8, and 6.7 mg/g for Cu, Zn, and Ni respectively in cheese whey at pH 5 (Fig. 2).

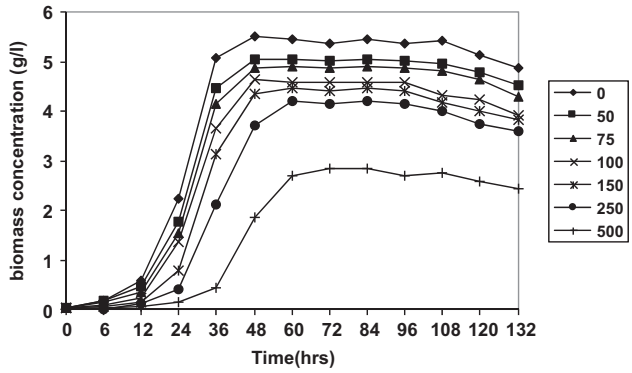

Fig. 4. Change in biomass concentration (g/l) with time at different initial Cu concentration (mg/l) in cheese whey.

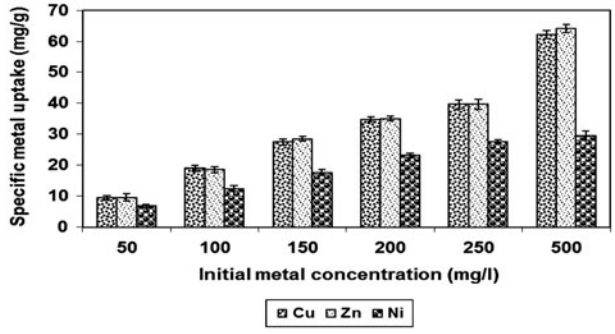

Fig. 5. Specific metal uptake at different initial metal concentrations (mg/l) in cheese whey.

3.2. Effect of total sugar concentration

Fig. 3 shows the effect of total sugar concentrations of cheese whey on specific Cu, Zn, and Ni uptake by the organism. The studies at different concentrations of total sugar (2–10 g/l) contained in cheese whey clearly indicate an increase in biomass concentrations

with the increase in total sugar concentration (Fig. 3). Due to the increase in biomass concentration, the specific Cu uptake increased from 3.2 to 9.4 mg/g, specific Zn uptake from 3.5 to 9.6 mg/g, and specific Ni uptake from 1.8 to 6.6 mg/g.

3.3. Effect of initial metal concentration

Fig. 4 shows the change in biomass concentration with time at different initial Cu concentrations (0–500 mg/l) in cheese whey. It is clear from the figure that the lag phase increased with the increase in initial Cu concentration in cheese whey. In the absence of Cu, the lag phase of growth was observed to be 6 h. This was followed by the exponential phase of growth (12–48 h). The growth of the organism reached stationary phase within 60 h in cheese whey. The lag phase was found to be nearly the same up to 100 mg/l initial Cu concentration, beyond which it increased with the increase in initial Cu concentration. The biomass concentration decreased from 5.62 to 2.82 g/l with the increase in initial concentration of Cu from 0 to 500 mg/l. Similar findings were obtained during the growth of the organism in the presence of Zn and Ni in cheese whey (shown in Figs. 5 and 6, respectively). A significant reduction in biomass concentration was observed from 5.62 to 2.8 g/l with the increase in initial Zn concentration from 5.62 to 1.82 g/l with the increase in initial concentration of Zn from 0 to 500 mg/l (Figs. 5 and 6), respectively.

In the presence of Cu, the specific growth rate (μ) in cheese whey decreased from 0.162 to 0.116 h^{-1}, and in the presence of Ni to 0.103 h^{-1} by increasing initial metal concentration from 0 to 500 mg/l. The reduction in the growth rate was observed to be 28% in the presence of Cu and 37% in the presence of Ni in cheese whey. The results indicate that metals have an inhibitory effect on the growth of the organism and Ni is more toxic to the organism as compared to Cu. An increased specific Cu uptake by the micro-organism was observed with an increase in initial concentration of Cu in cheese whey (Fig. 5). The maximum Cu uptake was found to increase from 9.4 ± 0.15 to 62.2 ± 0.20 mg/g and a decrease in Cu removal from 94 to 35% was observed by increasing initial Cu concentration from 50 to 500 mg/l. Fig. 5 shows that the maximum Ni uptake increased from 7.2 ± 0.2 to 29.43 ± 0.08 and the percentage removal of Ni was found to decrease from 70 to 11% with the increase in initial Ni concentration from 50 to 500 mg/l. The results clearly indicate that the organism can grow and remove metal efficiently in cheese whey media. The maximum specific Zn uptake increased from 9.5 ± 0.1 to 64.0 ± 0.25 mg/g by increasing initial concentration of Zn from 50 to 500 mg/l. The higher specific uptake values were obtained for Cu and Zn as compared to Ni. This is due to the enhanced biomass yield obtained in presence of Cu and Zn (Table 2).

3.4. SEM and EDX analyses

The morphological changes observed on the *Aspergillus* sp. in the presence of Cu were studied through SEM micrographs, which clearly indicate the deformation of the surface of the fungal biomass. The cell wall of the fungal biomass was found to be smooth in control, (Fig. 6(a)) but in the presence of Cu some morphological changes were observed on fungal cell wall (Fig. 6(b)). The fungal mycelia became tightly packed and shortly spectated, and there was an

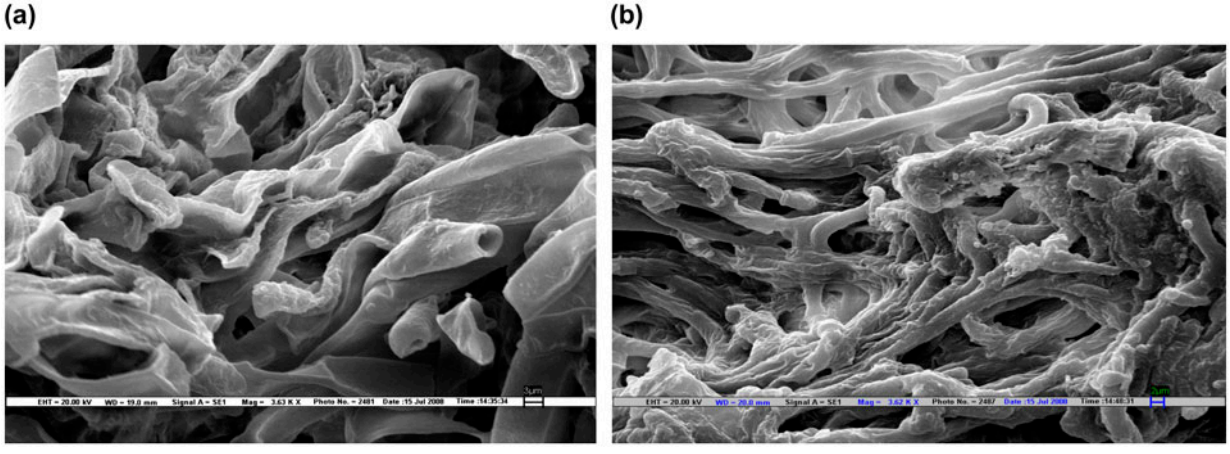

Fig. 6. SEM of *Aspergillus* sp. grown in cheese whey (a) in absence of metal (b) in presence of Cu (Magnification 3.62 KX).

increase in the surface area of the cell wall. This may be attributed due to the fact that changes were formed inside the cell wall and not outside.

4. Discussion

4.1. Effect of pH

At high acidic pH (2.0), the growth of the organism was inhibited resulting in lower metal uptake. Beyond pH 2, the organism started growing and maximum growth of the organism was obtained in the pH range 4–5. The lower metal uptake value was obtained between pH 2–4 due to lower biomass production and also due to the competition for binding sites between metal ions and hydrogen ions. At low pH, the cell surface sites are closely linked to the H^+ ions, thereby making these unavailable for other cations. However, with an increase in pH, there is an increase in ligands with negative charges, which resulted in increased binding of cations. In the study reported on biosorption of zinc, cadmium, and lead using live fungal biomass, it was observed that metal biosorption was inhibited below pH 3.0 due to the repulsion between metal cations with the positively charged metal binding ligands [6]. The studies carried out on removal of copper using growing cells of *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*, and *Candida* sp. also showed that the optimum pH for metal removal was in the range of 4.0–5.0 [22]. In the present study, due to the decreased solubility of heavy metals at pH beyond 6.0, further studies could not be carried out at higher pH values.

4.2. Effect of total sugar concentration

Sugar is present in the form of monosaccharides and polysaccharides in cheese whey. For the growth of the micro-organism an energy source is required [8,9]. The sugar present in cheese whey is providing source of energy for the growth of fungal biomass. The growth of the fungal biomass increases with the increase in sugar concentration up to a certain level and becomes constant in the media [10]. The surface of the fungal biomass increases with the growth of the fungal biomass. The surface binding sites also increases with increase in the fungal biomass resulting in higher specific metal uptake.

4.3. Effect of initial metal concentration

The increase in specific uptake with increase in metal concentration could be due to the availability of more metal ions for binding. Further, the growth of the micro-organisms decreased significantly at higher concentration leading to higher values of specific metal uptake. The high concentration of metal shows the mutagenic effect on the growth of the organisms [23]. The specific uptake values of Cu, Zn, and Ni observed in the present study are compared with the values reported for other micro-organisms (*S. cerevisiae*, *K. marxianus*, *S. pombe*, *Candida* sp., and *Rhizopus oryzae*) using glucose as the energy source [24]. The metal uptake depends not only on the initial metal concentration, but also on the availability of active sites for metal binding. As long as the active sites are free, the specific metal uptake increases with an increase in metal concentration. Metal ion uptake in fungi has been reported to involve an initial binding of metal ions to negatively charged sites on the cell wall with various functional groups, such as carboxyl, hydroxyl, phosphate, amide, and sulfhydryl groups followed by a slower energy-dependent entry [25,26]. The binding of different metal ions also depends on the ability of the metal ions and different physical and chemical properties of the metals. In the present work, the differences in the extent of removal of Cu, Zn, and Ni by the *Aspergillus* sp. may be due to the difference in electro negativity, charge, and ionic radius of the metal ions. However, more detailed studies are required in this direction.

4.4. SEM and EDX analyses

Copper is an element essential for all living organisms as a cofactor for a variety of enzymes; however, excess of this element can be mutagenic. Although most fungal isolates showed increased growth in the presence of low concentrations of Cu, this effect is mostly reversed and progressively deleterious above 30–50 mg/kg of Cu [27]. A variety of mechanisms that attenuate metal toxicity have been reported [28,29]. The metals are usually bound to the fungal cell wall [30], or precipitated outside hyphae. Chitin (b-1,4-linked polymer of N-acetyl glucosamine) is the active component responsible for the biosorption of metal cations by the fungal cell wall [31]. The cell wall of fungi is composed of polysaccharides, proteins, and lipids, which contain functional groups with potential metal chelating capacities [30]. The copper forms complexes with polysaccharides within the walls rather than precipitated salts which may cause the changes in the cell wall. This may be reason for the morphological changes observed during Cu sorption by the *Aspergillus* sp. in the present study. More in-depth study is required in this area. The confirmation of

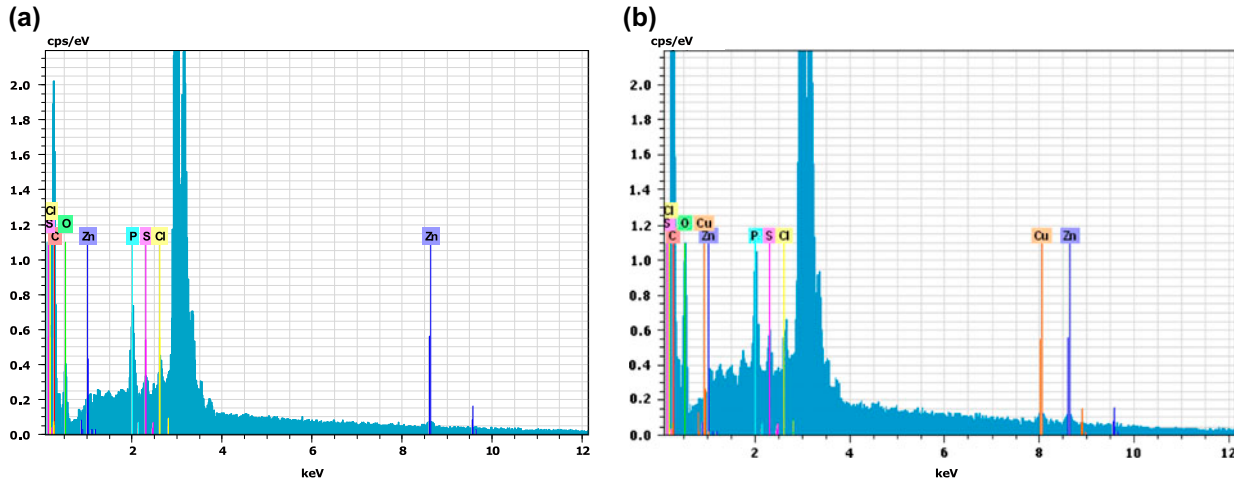

Fig. 7. EDX Micrograph of *Aspergillus* sp. (a) grown in absence of metal and (b) in the presence of Cu.

metal present on the fungal biomass was observed as peaks of the particular metal by EDX analysis. Fig. 7(a) and (b) shows the peaks of Cu, before and after the biosorption of Cu along with the peaks of silver. The organism was isolated from industrial waste of Zn electroplating industry, so the peaks of Zn were also observed along with the peaks of Cu on EDX analysis in Fig. 7(a) and (b). The samples were coated with peaks of silver so prominent in both the figures. This observation was also confirmed by EDX analysis for Zn and Ni signals together with the silver peak in the spectra (Figure not shown for Zn and Ni).

5. Conclusions

From the results of the present study, it can be concluded that *Aspergillus* sp. was able to grow in cheese whey growth media without external addition of nutrients, thus having a great potential for the removal of Cu, Zn, and Ni. The metal removal is dependent on pH, concentration of cheese whey, and initial concentration of metals. The percentage removal and specific uptake of metals using cheese whey is in close agreement with the percentage removal of metals using glucose and the necessary nutrients. Therefore, the waste cheese whey is expected to make the process of metal removal cost-effective. Further, the removal of metals by the organism is clearly indicated in SEM and EDX spectral analysis.

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