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Characterization of sophorolipid biosurfactant produced by *Cryptococcus* sp. VITGBN2 and its application on Zn(II) removal from electroplating wastewater

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Abstract

The present study aimed at elucidating the role of biosurfactant produced by yeast for the removal of Zn(II) ions from electroplating wastewater. The yeast species isolated from CETP, Vellore, Tamilnadu was identified as *Cryptococcus* sp.VITGBN2, based on molecular techniques, and was found to be potent producer of biosurfactant in mineral salt media containing vegetable oil as additional carbon source. Chemical structure of the purified biosurfactant was identified as acidic diacetate sophorolipid through GC-MS analysis. Interaction of Zn(II) ions with biosurfactant was monitored using FT-IR, SEM and EDS analysis. Zn (II) removal at 100 mg l^{-1} concentration was 84.8 % compared were other synthetic surfactants (Tween 80 and sodium dodecyl sulphate), yeast mediated biosurfactant showed enhanced Zn (II) removal in batch mode. The role of biosurfactant on Zn(II) removal was evaluated in column mode packed with biosurfactant entrapped in sodium alginate beads. At a flow rate of 1 ml min^{-1} and bed height of 12 cm, immobilized biosurfactant showed 94.34 % Zn(II) removal from electroplating wastewater. The present study confirmed that Zn(II) removal was biosurfactant mediated. This is the first report establishing the involvement of yeast mediated biosurfactant in Zn(II) removal from wastewater.

Key words

Cryptococcus sp. VITGBN2, Sophorolipid, Zn (II) removal

Introduction

Zn(II) is a metal ion which is released into the environment through industrial activities such as manufacture of alloys, sheet metal galvanization, TV picture tubes etc. In the Dangerous substances Directive (76/464/EEC) of the European Union, zinc has been registered as the List 2 dangerous substance. The World Health Organisation (WHO) recommends 5.0 mg l^{-1} as maximum acceptable concentration of zinc in drinking water (Mohan and Singh, 2002). Beyond this level, zinc is toxic and can lead to health problems such as irritability, gastrointestinal distress, lung disorders, metal fume fever, growth retardation and even cancer (EPA, 2005). It is phytotoxic, and the recommended level of zinc for disposal on agricultural land is 2.5 mg g^{-1} of dried sludge solids. Wastewater contain high concentration of zinc and discharging these wastewaters into natural systems adjoining land masses and sewer systems is a normal practice in small and medium scale industries which poses serious problems to the environment and ecosystems. Therefore, there is a significant need for zinc removal from wastewater (Mishra *et al.*, 2012).

Conventional methods for zinc removal include reverse osmosis (Aljendeel, 2011), ion exchange (Him *et al.*, 2009), electrofloatation (Casquaeria *et al.*, 2006), chemical precipitation (Ghosh *et al.*, 2011), ultrafiltration (Rahmanian, 2010) and adsorption (Katsou *et al.*, 2011). However, application of such methods has been always expensive and ineffective in terms of energy and chemical product consumption, especially at low concentration of 1-100 mg l^{-1}. Therefore, an alternate cost effective treatment strategy is required which will be eco- friendly.

Novel technologies involving microorganisms and their products to remove heavy metals have been successfully applied

to waste streams such as sewage sludge, industrial effluent, and mine water. Use of biosurfactants to improve the removal of heavy metal contaminants from aqueous media and soils has received increasing attention in recent years (Acikel, 2011; Ramani *et al*., 2012).

Biosurfactants are biological surfactants that are produced extracellularly or as part of the cell membrane by yeast bacteria, fungi or marine microorganisms inhabiting various substrates including sugars, oils, alkanes and wastes (Acikel, 2011). In general, their structure includes a hydrophilic moiety consisting of amino acids, peptides, anions or cations, mono-di or polysaccharides and a hydrophobic moiety consisting of unsaturated or saturated fatty acids. Biosurfactants can be classified into several groups viz. glycolipids, lipopeptides, lipopolysaccharides, phospholipids and fatty acids/ neutral lipids. Biosurfactants reduce interfacial tension by accumulating at the interface of immiscible fluids or of a fluid and solid and increase the surface area of insoluble compounds leading to increased mobility and bioavailability (Rahman *et al*., 2003). In case of metals, the anionic biosurfactant carries a negative charge, an ionic bond is formed that is stronger than the metal's bond with soil.

Biosurfactants have been reported to be effective in the remediation of heavy metal contaminated environments (Singh and Cameotra, 2013). However, these studies were mostly restricted to metal removal from soil (Asci *et al*., 2007, 2008; Deepika and Kannabiran, 2010). There are reports on the use of bacterial biosurfactants for remediation of heavy metals from aqueous solution (Das *et al*., 2009; Ramani *et al*., 2012). Report on the removal of metals from soil by biosurfactant of yeast origin scanty (Rufino *et al*., 2012). To the best of our knowledge, no report is available on metal remediation from wastewater using biosurfactants produced by yeast. Hence, the present work was undertaken to establish the potential of biosurfactant produced by yeast species for removal of Zn(II) ions from industrial wastewater.

Materials and Methods

Isolation and identification of yeast strain : Effluent sample was collected aseptically from the Common Effluent Treatment Plant (CETP) located in Ranipet, India. Yeast colonies were isolated by pure culture techniques and maintained on Yeast Extract Peptone Dextrose (YEPD) agar plates at 4°C. Gene sequencing and identification of yeast was done following the methodology reported by Altschul *et al.* (1990). The partial and complete 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA sequences of strain VITGBN2, after the assembly, had been deposited in GenBank under accession number KC135884.

Analysis of wastewater: Wastewater was collected from Krishna electroplating works, located at Kolkata, West Bengal, India. The physico-chemical characteristics of effluent were analyzed using standard analytical methods (APHA, 2005). The concentration of zinc, copper, nickel and cadmium, present in wastewater, were analyzed using Atomic Absorption Spectrophotometer (AAS) (Varian AA-240, Australia).

Isolation, optimization and characterization of biosurfactant: Minimal media (MM) with vegetable oil was used for optimization and production of biosurfactant by yeast strain. The yeast strain was cultured at different temperatures (20 ºC to 50 ºC), substrate concentrations (1.0 % - 4.0 % v/v of vegetable oil) and pH (3.0-9.0). All experiments were carried out in 250 ml Erlenmeyer flasks, containing 50 ml MM. The MM composed of the following components: NH_4NO_3 (0.1 %), KH_2PO_4 (0.02 %), $MgSO_4$. $7H_2O$ (0.02%), yeast extract (0.3 %) and glucose (5 %). The culture was maintained in a water bath shaker at 120 rpm for a time period of 160 hr. The crude biosurfactant was extracted, purified and characterized following the procedure of Chandran and Das (2011). The performance of biosurfactant was compared with those of commercial surfactants, Sodium dodecyl sulphate (SDS) and Tween 80.

Metal chelating activity of the biosurfactant and synthetic surfactants: Biosurfactant and synthetic surfactants viz. SDS and Tween 80 at different concentrations *i.e.* (2.0 - 10.0) x CMC were incubated with initial Zn(II) concentration of 10 mg l^{-1} for 12 hr. The optimized concentration of biosurfactant and synthetic surfactants was then incubated with various initial concentration of Zn(II) ions ranging from 10-120 mg l^{-1} in order to compare the efficacies of Zn(II) removal by biosurfactant and synthetic surfactants. The supernatant was analyzed by AAS for residual Zn(II) concentration. All experiments were performed in triplicates and the data presented were mean of triplicates. The Zn(II) removal percentage using yeast biosurfactant was calculated from the following expression :

$$\text{Zn(II) removal \%} = \frac{C_i - C_f}{C_i} \times 100$$

Where C_i is the initial concentration of Zn(II) ion (mg l^{-1}). C_f is the final concentration of Zn(II) ion (mg l^{-1}).

The chemical nature of native and Zn(II) interacted biosurfactant was determined by FT-IR spectrophotometer (Thermo Nicolet Co., USA). Zn(II) removal by biosurfactant was further confirmed using SEM (Hitachi, Model: S-3400N) and EDS (Peltier cooled x-ray head from Thermo, USA).

Treatment of wastewater in column mode using yeast mediated biosurfactant immobilized in alginate beads: A glass column with an internal diameter of 3 cm and height 15 cm was employed in the column experiments. The column was packed with yeast biosurfactant immobilized in sodium alginate beads. Wastewater collected from electroplating industry containing 85 mg l^{-1} Zn(II) ions was used in this experiment. Before passing through the column, the pH of wastewater was

Table 1 : Physicochemical characterization of biosurfactant produced by yeast strain *Cryptococcus sp.* VITGBN 2

Characterization	Surface tension (mNm^{-1})	CMC ($mg\ l^{-1}$)	Oil displacement (cm^2)	Drop collapse test
Distilled water	72 ± 0.5	Negative	Negative	Negative
Crude biosurfactant	33 ± 0.5	280	73 ± 0.5	+++
Tween 80	39.4 ± 0.3	1248	67 ± 0.9	++
SDS	36.7 ± 0.6	382	51 ± 0.4	++

adjusted to 6.0 and was fed through the column at a desired flow rate using a peristaltic pump. The effect of bed height on Zn(II) removal was studied at three different bed heights *viz.* 4, 8 and 12 cm. The effect of flow rate on Zn(II) ion removal was studied at three different flow rates *viz.* 1, 3 and 5 ml min^{-1}. Samples were collected from the exit of column at different time intervals and analyzed by AAS. Wastewater was passed through the column till values reached the US EPA standard (5.0 $mg\ l^{-1}$).

Results and Discussion

Colonies of strain VITGBN2 on YEPD agar after 48 hr of incubation were cream colored and about 2-5 x 3-7 µm in size, ovoid, mucoid, glossy colonies. Staining revealed ovoid cells in the presence of a thin layer of glycoprotein capsular material that had a gelatin-like consistency surrounding each cell (figure not shown). Using designed primers for PCR, the18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA regions were amplified and sequenced. The size of the sequence was 615 nucleotides long. The sequence analysis of 18S rRNA gene showed 99 % sequence coverage and 96 % homology with *Cryptococcus laurentii* strain RY1(accession no: EF063363.2) in similarity search using the BLAST programme. The ITS1 and ITS2, 5.8S rRNA region showed 98 % sequence coverage and 96 % homology with *Cryptococcus aff. laurentii* IMUFRJ 51980 (accession no: FN428898.1). Also the sequence of 26S rRNA gene (D1/D2/D3 region) showed 98 % sequence coverage and 96 % homology with *Cryptococcus laurentii* (accession nos: FN428934.1, FN428921.1, FN428909.1 and FN428903.1). Therefore, the isolate VITGBN2 was identified as *Cryptococcus* species designated as *Cryptococcus sp.*, strain VITGBN2. Phylogenetic analysis (figure not shown) revealed that the strain VITGBN2 was closely related to *Cryptococcus laurentii* strain RY1 (accession no.EF063363.2).

Table 2 : Physico-chemical analysis of electroplating wastewater treated with immobilized *Cryptococcus sp.* VITGBN2 biosurfactant in packed bed column

Parameters	Before treatment	After treatment
pH	7.63±0.17	6.2±0.63
Conductivity (µΩ)	5.68±0.20	2.4±0.02
TSS ($mg\ l^{-1}$)	1310±3.7	634±5.2
TDS ($mg\ l^{-1}$)	1137±4.1	589±4.5
COD ($mg\ l^{-1}$)	61±1.2	28±0.64
Zn(II) ($mg\ l^{-1}$)	85±0.64	4.8±0.4 (94.34 %)*
Cd(II) ($mg\ l^{-1}$)	10±0.54	1.91±0.09
Ni(II) ($mg\ l^{-1}$)	21±0.76	2.92±0.17
Cu(II) ($mg\ l^{-1}$)	20±0.43	2.72±0.15

* Percent Zn(II) removal

Biosurfactant production on vegetable oil was found to be maximum at pH 7.0, 30 ºC and substrate concentration of 2% (v/v) vegetable oil substrate. In all culture conditions tested, biosurfactant concentration was highest at early stationary phase, 80 hr.

The yeast species *Cryptococcus sp.*VITGBN2 showed high cell surface hydrophobicity, 78 % over a time period of 80 hr and almost constant for a period of 160 hr. The cell surface hydrophobicity was related to the biosurfactant secreted on the

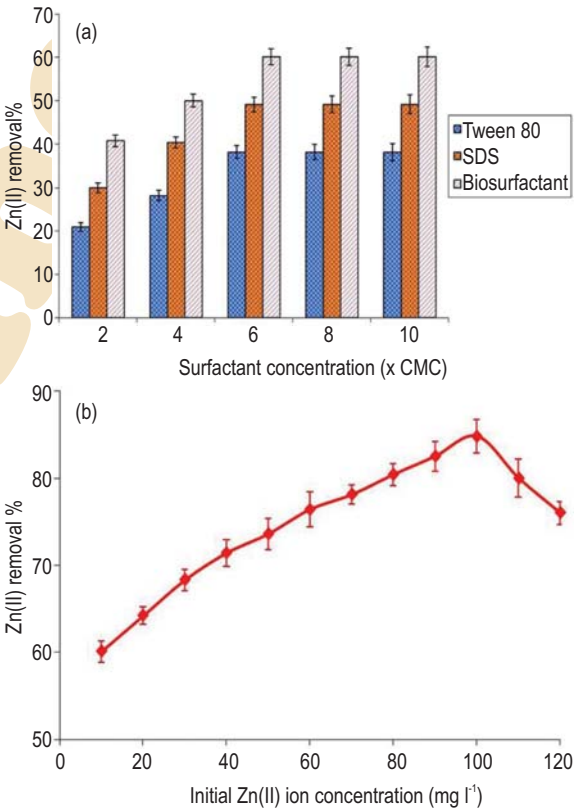

Fig. 1 : Percentage of Zn(II) removal (a) at various concentrations by biosurfactant compared with commercial surfactants (b) At various concentrations of Zn(II) ion by 6×CMC of biosurfactant

cell surface, helping adhesion of bacteria to hydrocarbons, and resulting in effective degradation (Maneerat, 2005). The drop collapse test and oil displacement test were also conducted for the primary screening of biosurfactant production. The oil displacement test is an indirect measurement of surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution. The surface activity of crude biosurfactant was investigated in comparison with that of Tween 80 and SDS. Drop collapse test and oil displacement test were highly positive for crude biosurfactant than synthetic surfactants, Tween 80 and SDS, which indicated high surface activity (Table 1). The ability of crude biosurfactant along with Tween 80 and SDS to reduce surface tension of distilled water was compared. The biosurfactant produced by *Cryptococcus sp.* VITGBN2 reduced the surface tension of distilled water to a minimum value with low value of CMC. Tween 80 and SDS reduced the surface tension but had high CMC values (Table 1). The results suggested that the isolated biosurfactant provided excellent properties in terms of reduced surface tension and a low value of CMC. The

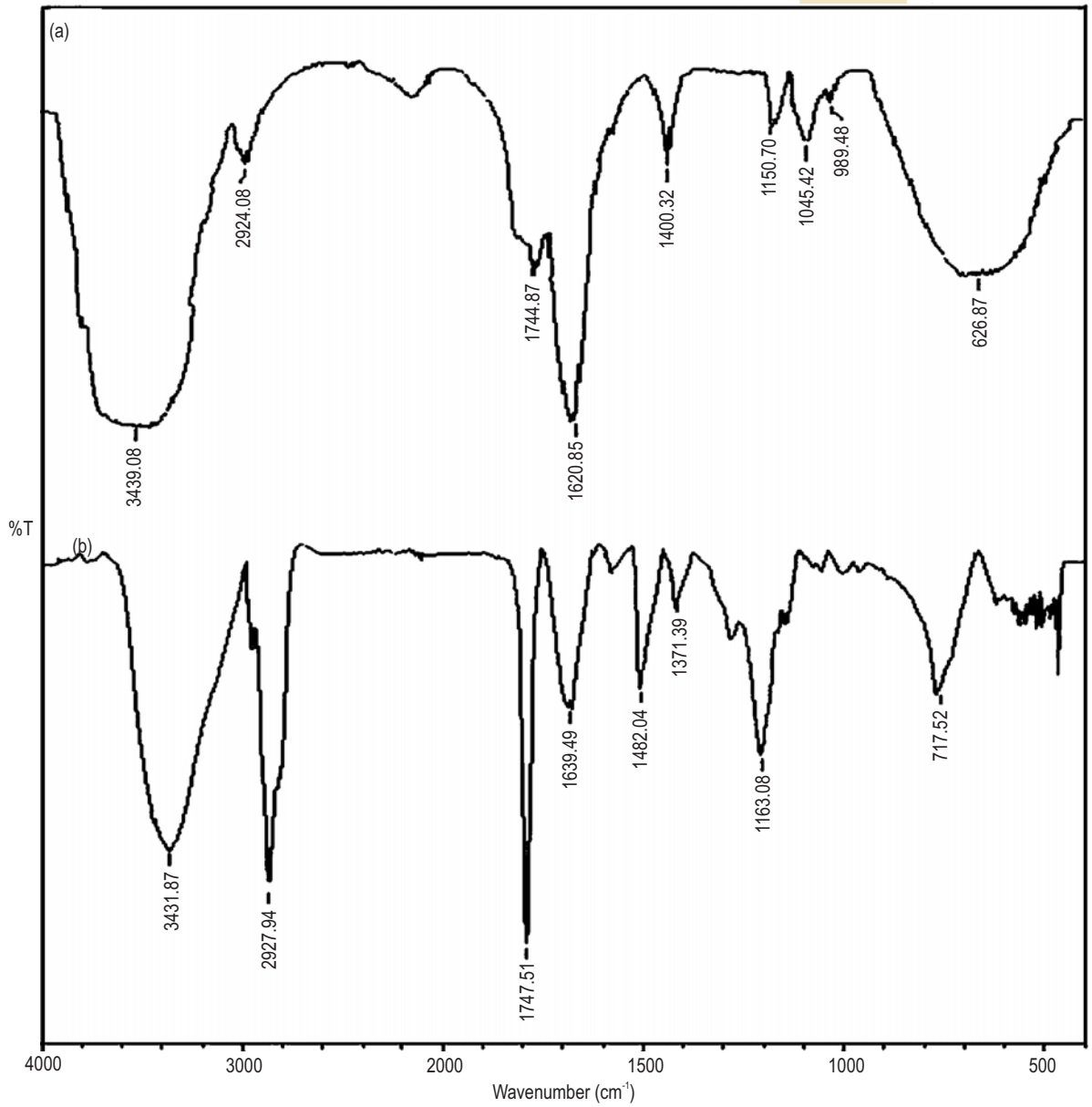

Fig. 2 : FTIR spectra of (a) native biosurfactants and (b) Zn(II) interacted biosurfactant

emulsification index of biosurfactant was evaluated by determining the emulsifying activity with respect to vegetable oil. The biosurfactant exhibited different stabilization property with the vegetable oil tested as expressed in terms of emulsification index of 78 ± 0.4 %.

The biosurfactant produced by the yeast contained carbohydrate (37.97 %) and lipid (51.66 %) as major constituents. MS spectra of biosurfactant isolated from the yeast species *Cryptococcus sp.* VITGBN2 showed significant ions at m/z 706 on mass spectra corresponding to chemical structures were determined as diacetate acidic sophorolipid with fatty acid moiety C18:1. The yeast species *Cryptococcus sp.* VITGBN2 was found to be the potent producer of diacetate acidic sophorolipid.

The results showed higher Zn(II) removal efficiency with increase in the concentration of biosurfactant and both the synthetic surfactants (Fig. 1a). Enhanced Zn(II) removal was noted for all concentrations of the biosurfactant as compared to synthetic surfactants. The Zn(II) removal percentage of 10 mg l^{-1} of Zn(II) ion at 2× CMC of biosurfactant, SDS and Tween 80 was 40.7, 30.2 and 21.4 % respectively; while the percentage removal of Zn(II) ion was increased to 60.1, 49.3 and 38.2 % respectively for biosurfactant, SDS and Tween 80 at 6× CMC. This trend of enhancement of metal remediation capacity with increasing amount of biosurfactant was also reported earlier but the concentration of biosurfactant used for metal removal was higher as compared to that used in the present study (Kim and Vipulanandan, 2006).

The effect of initial Zn(II) concentrations on the removal of Zn(II) ions from aqueous solution in batch process by optimized biosurfactant concentration (6× CMC) was determined by varying Zn(II) ion concentration (Fig. 1b). The acidic diacetate sophorolipid biosurfactant showed higher Zn(II) removal efficiency of 84.8 % at 100 mg L^{-1} of Zn(II) ion. Zn(II) ion-biosurfactant co-precipitate could be seen as an off-white precipitate after addition of biosurfactant to Zn(II) solution followed by proper incubation. At a concentration higher than 100 mg l^{-1}, Zn(II) removal efficiency decreased. This might be explained due to decreased ratio of forming co-precipitation of biosurfactant to Zn(II) ions at higher concentration.

FT-IR studies were performed for confirmation of removal of Zn(II) ion by acidic diacetate sophorolipid biosurfactants. FT-IR spectrum of native biosurfactant (Fig. 2a) showed the presence of O-H stretching at 3439.08 cm^{-1}. A band at 2924.09 cm^{-1}, resulting from C-H stretching mode reflected the presence of an aliphatic chain. The absorption regions at 1744.87 and 1629.85 cm^{-1} were due to carbonyl stretching of C=O group. The absorption bands noted at 1400.32 and 1158.78 cm^{-1} indicated OH stretch in carboxylic acid and C-O stretch in lactones respectively. Similar result was reported which also showed the presence of hydroxyl, carboxyl and carbonyl groups on the surface of biosurfactants produced by *Candida rugosa* (Chandran and Das,2011).

FTIR spectra of biosurfactant interacted with Zn(II) ion showed that the peaks expected at 3439.08, 2924.09, 1744.87, 1629.85, 1400.32 and 1158.78 cm^{-1} had shifted, respectively to 3437.81, 2927.94, 1747.51, 1639.49, 1426.04 and 1163.08 cm^{-1}, respectively (Fig. 2b). Spectral analysis of native and Zn(II) interacted biosurfactant clearly indicated that hydroxyl group (–OH), carboxyl (–COOH) and carbonyl (C=O) groups were the predominant contributors in Zn uptake for the biosurfactant produced by *Cryptococcus sp.* VITGBN2. Huang and Liu (2013) reported the involvement of hydroxyl, carboxyl groups for removal of Cd(II) and Pb(II) from aqueous solution using biosurfactants produced from bacteria.

Removal of Zn(II) ion from the aqueous solution was further confirmed using SEM. Fig. 3a. shows the morphology of native biosurfactant, indicating self assembly of sophorolipid molecule conforming hexagonal structure. Fig.3b. shows the sequestered Zn(II) ions onto biosurfactant. The projected spherical nodules in SEM images confirmed the anchoring of Zn(II) ions with the biosurfactant molecule. EDS of native biosurfactant (Fig. 3c) and Zn(II) ion interacted biosurfactant (Fig. 3d) served as a direct proof of metal attachment to the micellar structure of the biosurfactant.

Zn(II) removal percentage was evaluated by acidic diacetate sophorolipid under free and immobilized condition. Zn(II) removal was found to be maximum (88.9 %) and 84.8 % in the case of immobilized and free biosurfactant respectively, whereas alginate beads showed Zn(II) removal percentage of 4.1 %. Therefore, the biosurfactant immobilized in alginate bead was used for further experiment.

The immobilized acidic diacetate sophorolipid was employed for treatment of electroplating wastewater in a column mode. The effect of flow rate on Zn(II) removal was evaluated using three different flow rates (1, 3 and 5 ml min^{-1}) at a bed height of 4 cm. Fig. 4a. showed the breakthrough curves of Zn(II) ion removal at different bed heights at an optimum flow rate of 1ml min^{-1}. Fig. 4b. showed that the breakthrough time (t_b) and exhaustion time (t_e) increased with increase in bed height.

It was seen that with increase in flow rate, there was a decrease in breakthrough and exhaustion times due to insufficient residence time of metal ions in the column (Muhamad *et al*., 2010) Therefore, maximum Zn(II) removal of 94.34 % was obtained at highest bed height of 12 cm and the lowest flow rate of 1ml min^{-1}, with total residence time of 200 minutes leaving 4.8 mg l^{-1} residual Zn(II) ion in treated electroplating wastewater (Table 2). The residual concentration of Zn(II) in treated effluent was less than US EPA standard (5.0 mg l^{-1}). There was significant difference in the physico-chemical properties of electroplating wastewater after biosurfactant treatment. The residual

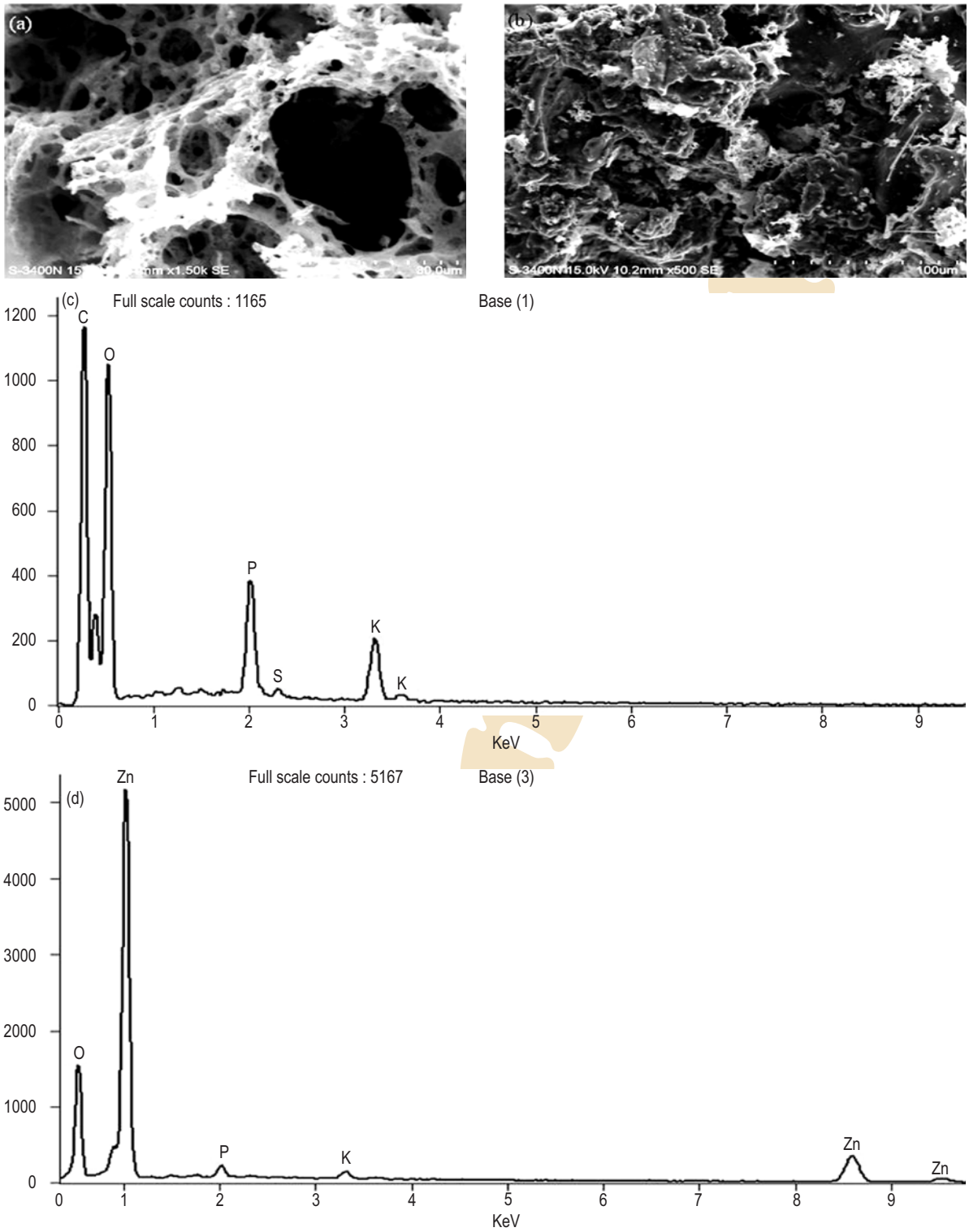

Fig. 3 : SEM images of **(a)** native biosurfactant **(b)** Zn(II) ions interacted biosurfactant **(c)** EDS analysis of native biosurfactant **(d)** Zn(II) ions interacted biosurfactant

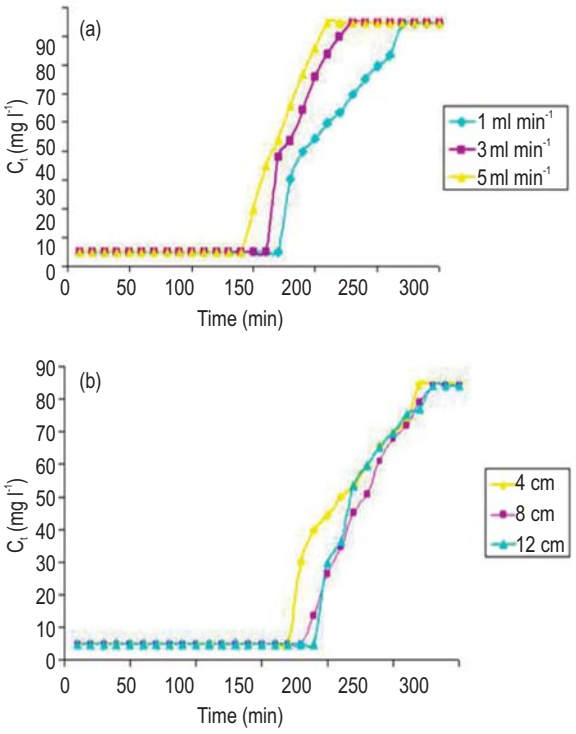

Fig. 4 Breakthrough curve for Zn(II) removal by biosurfactant **(a)**different flow rates **(b)** different bed heights

concentrations of other metals *viz.* Ni(II), Cd(II) and Cu(II) were found to be 2.92 mg l^{-1}, 1.91 mg l^{-1} and 2.72 mg l^{-1} respectively (Table 2), which were below the permissible limits.

Therefore, the present study confirmed that *Cryptococcus sp.*VITGBN2 produced sophorolipid biosurfactant which showed high physico-chemical properties in terms of surface activities compared to synthetic surfactants, *viz.* Tween 80 and SDS. Biosurfactant could remove Zn(II) ions from wastewater successfully. The property of sophorolipid biosurfactant to chelate Zn(II) ions forming an insoluble precipitate may be focused towards its application for the removal of other heavy metal ions from industrial wastewater.

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