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Biosequestration of chromium (III) in aqueous solution using cationic and anionic biosurfactants produced from two different *Bacillus sp.*- A comparative study

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Abstract

Tannery wastewater discharged from chrome tanning section contains Cr (III) in the range of 2100-2300 ppm and a viable technique for its removal remains a greater concern for leather industry. The ability of a biosurfactant to chelate toxic heavy metal ions and form an insoluble precipitate may be exploited in the treatment of Cr (III) containing wastewater. In the present study, biosurfactant producing micro organisms, *Bacillus subtilis* and *Bacillus cereus* were isolated from tannery wastewater contaminated soil using palm oil and coconut oil as substrates respectively. The biosurfactants produced from palm oil (Palm oil BS) and coconut oil (Coconut oil BS) were characterized as anionic and cationic biosurfactants respectively using blue agar plate method, agar double diffusion technique and zeta potential measurement. The biosurfactants were characterized for their amino acid composition and elemental (CHNS) composition. The thermal behavior of the biosurfactant was characterized using TGA and DSC analyses. The Surface tension values of the anionic and cationic biosurfactants were 28.16±0.2 mN/m and 23.02±0.2 mN/m respectively. The SDS-PAGE and FT-IR analyses confirmed that both the biosurfactants were lipoprotein in nature. The binding ability of the lipoprotein anionic and lipoprotein cationic BS with chromium (Cr (III)) ions in the aqueous solution was then determined. The interaction of Cr (III) with BS was confirmed using FT-IR, SEM-EDX analysis and Atomic absorption spectrophotoscopy (AAS).

Keywords: Lipoprotein, cationic biosurfactant, Anionic biosurfactant, *Bacillus sp*., Cr (III), tannery wastewater.

1. Introduction

The leather manufacturing industry converts the putrescible raw skins and hides into non putrescible leather through various chemical and mechanical processes.[1] Basic chromium sulphate is the most common tanning agent used in leather industry as it enables faster and cheaper production of highly microbial resistant and durable leathers. More than 80% of finished leather goods are tanned using basic chromium sulphate.[2] The amount of trivalent chromium discharged in the effluent, while processing one ton of wet salted skins/hides, is in the range of 3 - 7 kg or 5 - 10 kg Cr_2O_3.[3] According to the available reports,[4] the recommended level for Cr(III) is raised in the range of 0.5-2.0 ppm regardless to fresh, marine, irrigation and drinking water. In many countries, the treated tannery wastewater is discharged onto the open land or into rivers.[5]

Cr(III) tends to be strongly bound by soil humic acid polymers, and this affinity restricts the availability of Cr(III) to be oxidized and reduces the decomposition of organic matter.[6] Cr(III) in soils could be leached into surface water or groundwater, and absorbed by plants.[5] Chromium(III) enters the human food chain through consumption of the vegetative plants. Symptoms of Cr (III) phytotoxicity include inhibition of seed germination or of early seedling development, reduction of root growth, leaf chlorosis and depressed biomass.[7]

Tannery wastewater from chrome tanning section contains Cr (III) in the range of 2100-2300 ppm.[8] Improper treatment of Cr (III) containing effluent, non scientific method of storage of chromium tanned solid waste and consequently leaching of Cr(III) onto the soil could release Cr(III) to the environment, causing groundwater contamination and thereby, adverse biological and ecological effects were the end results.[9] Precipitated Cr (III) hydroxides remain stable in the sediments under aerobic conditions while under acidic and anoxic conditions Cr(III) hydroxides

are soluble and remain as ionic Cr(III) species,[10] which would be leached into groundwater sources.

Cleaner technologies used to reduce Cr(III) in wastewater such as high exhaustion process, direct or indirect chromium recycling too could not eliminate Cr(III) completely from tannery wastewater.[11] Among the techniques known for the removal of Chromium from tannery wastewater , biosequestration of Cr(III) is known to be one of the proven technologies.

Biosurfactants are the structurally diverse group of surface-active substances produced by microorganisms. They consist of a polar hydrophilic moiety and a non-polar hydrophobic group. The hydrophilic group consists of mono, oligo or polysaccharides, peptides or proteins and the hydrophobic moiety contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols.[12, 13] The six major types of biosurfactants available are hydroxylated & cross linked fatty acids (mycolic acids), glycolipids, lipopolysaccharides, lipoproteins and phospholipids.[14] Biosurfactants are used in the remediation of pollutants due to their surface active properties.[15]

The chemical configuration of biosurfactant facilitates them to bind with metal ions. The characteristic feature of biosurfactants to chelate toxic heavy metals and form an insoluble precipitate may be exploited in the treatment of heavy metal containing wastewater.[16] Biosurfactants of anionic nature could capture the metal ions through electrostatic interactions or complexation. The cationic biosurfactants can replace the same charged metal ions by competition.[17] To our knowledge, there are no reports on removal of Cr(III) using cationic biosurfactants.

The focal theme of the present investigation was to compare the Cr (III) binding ability of anionic and cationic biosurfactants (BS) produced from two different substrates such as palm oil and coconut oil respectively.

2. Materials and Methods

2.1. Isolation of Microorganisms

Biosurfactants producing microorganisms were isolated from tannery wastewater contaminated soil. The soil was acclimatized with palm oil or coconut oil and they were serially diluted to isolate the microorganisms using nutrient agar by pour plate method, followed by incubation for 24 - 48 h at 35°C.

2.2. Screening and identification of biosurfactant producing microorganisms

The surface active properties of biosurfactant produced by isolated bacterial strains were screened by oil drop collapse activity[17] using four different oils namely palms oil, diesel oil, coconut oil and olive oil. The screened organisms from palm oil and coconut oil acclimatized soil were identified by 16S ribosomal DNA (16S rDNA) sequencing and phylogenetic analyses.

2.3. Optimization studies for production of biosurfactants

The various parameters that significantly affect the production of biosurfactants using palm oil and coconut oil as substrates such as time (24-120 h), pH (1-10), temperature (20-50ºC) and Concentration of substrate (10-60 g/L) were optimized using one parameter at a time while the other parameters were kept constant. The most significant range of parameters was further optimized using Response Surface Methodology (RSM) for the production of biosurfactant.

2.4. Production, extraction and purification of Biosurfactants

The production of BS was carried out at optimized conditions and the bacterial cells were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The cell free supernatant was adjusted using 2N HCl to get the final pH to 2.0 and kept for overnight at 4°C. The pellet was collected by centrifugation at 9000 rpm for 20 min at 4°C and the biosurfactant was extracted

with acetone. The extracted biosurfactant was further purified using silica gel column chromatography to obtain the purified biosurfactant.

2.5. Characterization of Biosurfactant

2.5.1. Determination of ionic character of BS

Blue agar plate method: The ionic nature of biosurfactant was characterized by slight modification of blue agar plate method.[18] Mineral salt agar medium supplemented with glucose as carbon source (2%) and methylene blue (MB: 0.2 mg/mL) were used for preparation of blue agar plate. The anionic and cationic nature of BS was identified by adding Cetyl trimethyl ammonium bromide (CTAB: 0.5 mg/mL) and Sodium dodecyl sulphate (SDS: 0.5 mg/mL) to the respective media. Biosurfactant of volume 50µl containing 10µg was loaded into each well made in blue agar plate and the plates were incubated at 37°C for 48-72h. A dark blue halo zone was considered positive result for anionic and cationic biosurfactants in their corresponding plates.

Agar Double Diffusion technique: The ionic character of the BS was determined using agar double diffusion technique.[19] In an agar plate (1% agar of low hardness), two regularly-spaced rows of wells were made. The purified BS of volume 100µL containing 20 µg was filled in the wells in the lower row while the upper row of well were filled with a pure compound (SDS or CTAB) of known ionic charge. The anionic and cationic nature of BS was identified by adding CTAB (0.5 mg/mL) and SDS (0.5 mg/mL) in the wells of upper row. The appearance of precipitation lines between the corresponding wells is considered to be the ionic nature of the biosurfactant.

2.5.2. Zeta potential of biosurfactants

The zeta potential of the biosurfactants was determined by mixing with 5 ml of buffer solution and the solution was agitated for 30 min using magnetic stirrer. The biosurfactants were

centrifuged and re-dispersed in 5 ml of pure water and agitated once again for 30 min. Water was used as the dispersant for the particles to measure their zeta potential using Zetasizer (Nano-ZS from Malvern Instruments, UK).

2.5.3. Emulsification Stability Index (E24%)

Emulsification stability of biosurfactant was determined in accordance with the method followed by Cooper and Goldenberg.[20] Palm oil, olive oil, diesel oil or coconut oil of volume 2 ml was added to the same amount of the cell broth and mixed using a vortex mixer for 2min and left to stand for 24h.

$$\text{Emulsification index (E24\%)} = \frac{\text{Height of the Emulsion layer (mm)}}{\text{Total height (mm)}} \times 100$$

2.5.4. Surface tension measurement

The most important property of the surfactants is the reduction in interfacial tension of water. The BS also reduces the surface tension of water similar to chemical surfactant. The biosurfactant containing fermentation broth was centrifuged at 10000 rpm for 20 min and then the surface tension of the supernatant was measured.[21] Surface tension measurements were recorded by Wilhelmy Plate method using Surface Tensiometer (NIMA Technologies Ltd., England).

2.5.5. Biochemical characterization of biosurfactants

The biochemical composition of BS such as carbohydrate, protein and lipid contents were estimated by phenol-sulphuric acid method,[22] Lowry's method[23] and phosphoric acid vanillin reagent method[24] respectively.

2.6. Amino acid composition of biosurfactant by HPLC

The amino acid composition of biosurfactant was determined by HPLC. The biosurfactant was hydrolyzed with 6 N HCl at 100°C for 20 h and neutralized with 1 M NaOH. The amino acid composition was analyzed using Agilent 1100 HPLC amino acid analyzer (Agilent Technologies, Middleburg, Netherlands) and the data analysis was performed by using HP chem station.[25]

2.7. Molecular weight determination of biosurfactant

The molecular mass of the biosurfactant was determined by using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), in accordance with the method of Laemmli,[26] on a 5% stacking gel and 12% resolving gel. The protein marker ranging from 14.3 to 94.7 KDa was used as a standard marker for the determination of molecular weight of biosurfactant.

2.8. Instrumental Characterization of Biosurfactant

2.8.1. Elemental compostion of biosurfactants

The elemental composition (Carbon, Hydrogen, Nitrogen and Sulphur) of the biosurfactants were determined using CHNS analyser (Euro vector EA 3000 series).

2.8.2. Thermal Behaviour of Biosurfactants (TGA and DSC)

The required quantity (8–10 mg) of BS was loaded onto a platinum TGA pan and gravimetric analysis was carried out under pure nitrogen atmosphere, from 0 ºC to 800 ºC using a temperature gradient of 10 ºC min^{-1}. Thermograms were routinely recorded in duplicate using TGA Universal V4.4A TA instruments.

For DSC analysis, BS samples (8–10 mg) was loaded in an aluminum DSC pan and gravimetric analysis was made under nitrogen atmosphere, from 0 ºC to 200 ºC using a

temperature gradient of 10 ºC min^{-1}. Scans were routinely recorded in duplicate using a DSC Q200 (V23.10 Build 79).

2.8.3. Circular Dichroism (CD) spectroscopy

The Circular Dichroism was used to study the secondary structures of BS in the range of 150- 250 nm with a path length, 1mm and width, 1 nm using a Peltier temperature controlled system (JASCO J715, Japan).

2.9. Interaction of chromium (III) ions with biosurfactants

The biosequestration of chromium (III) ion from aqueous solution was confirmed by atomic absorption spectroscopic analysis (AAS). Effect of time (2-12 h), pH (4-6), different concentration of anionic & cationic biosurfactants (0.5, 1.0, 1.5, 2.0 and 2.5g) and concentration of Cr (III) (100-500 mg/L) were studied. The time study was performed using anionic and cationic BS of 0.5g for the removal of Cr (III) from aqueous solution containing Cr(III) of 100 mg/L. At the optimized conditions, concentration of Cr (III) before and after sequestration by biosurfactants were measured using an atomic absorption spectrophotometer (AAS).

$$Biosequestration\ of\ Cr\ (III)(\%) = \frac{C_0 - C_t}{C_0} \times 100$$

C_0 = initial concentration of Cr(III) in mg/L; C_t = final concentration of Cr(III) in mg/L.

2.10. UV-Visible spectroscopy

The UV–visible scans of cationic BS, anionic BS and the BS after interaction with Cr (III) were recorded in the range $\lambda_{200–800}$nm using a UV–visible spectrophotometer (Cary varion; Agilent Technologies, Middleburg, Netherlands).

206 *2.11. Fluorescence spectroscopy*

207 The fluorescence spectra of cationic BS, anionic BS and the BS after interaction with Cr
208 (III) were recorded using a fluorescence spectrophotometer in the wavelength range $\lambda_{200-800}$nm
209 (Cary Eclipse; Agilent Technologies, Middleburg, Netherlands).

210 *2.12. FT-IR spectral Analysis*

211 The functional groups of anionic and cationic lipoprotein BS, before and after treating
212 with Cr(III), were characterized using FT-IR spectrophotometer (Perkin Elmer). The samples
213 were dried and made in the form of pellet with dimensions thickness, 1 mm and diameter, 13mm,
214 using spectroscopic grade KBr. The spectra were recorded in the spectral range of 400–4000 cm^{-1}
215 .

216 *2.13. SEM analysis*

217 The binding of chromium ions with BS was further confirmed using SEM analysis. The
218 biosurfactant and chromium bound biosurfactant [BS-Cr (III)] samples were coated with gold
219 foil of thickness 120–130 µm under Argon atmosphere. SEM images were recorded on a
220 scanning device attached to a JEOL JM-5600 electron microscope at 20 kV accelerating voltage
221 with a 5–6 nm electron beam.

222 *2.14. EDX analysis*

223 The biosurfactant and chromium bound biosurfactant [BS- Cr(III)] samples were coated
224 with gold foil of thickness 120–130 µm under Argon atmosphere. EDX spectra were recorded on
225 a scanning device attached to a JEOL JM-5600 electron microscope at 15 kV accelerating
226 voltage with a 5–6 nm electron beam.

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3. Results and Discussion

3.1. Isolation, screening and identification of biosurfactant producing microorganism

Enrichment of bacteria isolated from the tannery wastewater contaminated soil was carried out in M9 Minimal media (HiMedia) of volume 100 mL with palm oil or coconut oil (1 g) as the sole carbon source. Palm/coconut oil concentration in the media was increased from 1 to 5 g with an incremental increase of 1 g. The media was acclimatized for 4-5 weeks and then the microbes were serially diluted (10^{-1} to 10^{-10}). The biosurfactant producing microorganisms with non-identical morphology were isolated from palm oil or coconut oil acclimatized soil. About 6 biosurfactant producing microorganisms from palm oil as the substrate and 4 microorganisms from coconut oil as the substrate were isolated. Based on their zone diameter in oil drop collapse assay (Table 1), microorganisms with high biosurfactant activity (P3 & C2) were used for the further studies. The screened microorganisms were identified as Bacillus *sp.,* using 16s rDNA gene sequencing. The phylogenetic analysis shows the evolutionary relationships among various other *Bacillus* species based upon similarities and differences in their physical or genetic characteristics. The phylogenetic trees for identified organisms are presented in Fig.1 and confirmed that they belong to *Bacillus subtilis* and *Bacillus cereus*.

3.2. Statistical Optimization for production of biosurfactant

The production of biosurfactants from *Bacillus sp.* was studied under batch mode. The process parameters such as time (24-120 h), pH (1-10), temperature (20-50ºC) and concentration of substrate (10-60 g/L) was optimized using one parameter at a time. The most significant range of parameters was further optimized using RSM (Refer appendix).

RSM clearly states that the biosurfactant production from *B.subtilis* and *B.cereus* was significantly ($p<0.05$) influenced by pH, temperature and concentration of oil. Nonlinear

relationships were significantly ($p < 0.05$) fitted to the experimental data for describing the changes in biosurfactant yield, when all the experimental variables were simultaneously altered. Amongst all response variable effects, the interaction effect of pH and palm oil concentration had the most significant ($p < 0.05$) positive effect on the biosurfactant production from *B.subtilis* while the interaction effect of temperature and coconut oil concentration had the most significant ($p < 0.05$) positive effect on the biosurfactant production from *B.cereus.*

The *Bacillus subtilis* produced BS by 0.32 g per gram of palm oil (12.8 g/L) as the substrate at the optimized conditions such as time, 96h; pH, 5; temperature, 30 ^{o}C; and concentration of palm oil, 40g/L. The production of rhamnolipid biosurfactant using palm oil was reported by Thaniyavarn *et al*. (2.91g/L) [16] and using palm oil sludge by Nawawi (85g/L) [27]. Similarly, *Bacillus cereus* yielded 0.45 g of BS per gram of coconut oil (22.5g/L) as the substrate at the optimized conditions such as time, 96h; pH, 7; temperature, 40 ^{o}C; and concentration of coconut oil, 50g/L. The rhamnolipid biosurfactant produced using coconut oil was reported by Thaniyavarn *et al*. (2.93g/L)[16] and Patil *et al.* (2.8 g/L).[28] Kannahi and Sherley[29] also reported rhamnolipid biosurfactant of 44.2 g/L using mixed oil containing 2% coconut oil.

Activation energy

The activation energy required by *B. subtilis* and *B.cereus* for BS production using palm oil and coconut oil respectively was calculated from the Arrhenius equation

$$k = Ae^{\frac{-E_a}{RT}}$$

Where, k is the rate constant, A is the Arrhenius factor and E_a is the activation energy. The activation energy for the BS production from *B. subtilis* and *B.cereus* was calculated from the plot of ln k versus 1/T. The activation energy (E_a) for the production of BS from *B. subtilis* and *B.cereus* was found to be 80.59 kJ/mol and 87.95 kJ/mol respectively. The Arrhenius factor was

observed to be $6.88*10^{11}$ and $1.46*10^{13}$ h^{-1} for the production of BS from *B. subtilis* and *B.cereus* respectively.

3.3. Characterization of biosurfactant

3.3.1. Determination of ionic character of BS

Blue Agar Plate Method: The anionic and cationic BS were characterized by the formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited in dark blue color against the light blue background. The biosurfactants produced from palm oil and coconut oil as the substrates exhibited the dark blue halo zone in CTAB (positively charged surfactant) and in SDS (negatively charged surfactant) containing plates respectively (Fig. 2(I)). These results confirm that the biosurfactant from palm oil substrate was anionic in nature and the biosurfactant from coconut oil substrate was cationic in nature.[30]

Double Diffusion Agar Method: Agar double diffusion tests are based on the passive diffusion of two compounds bearing similar charges or opposite charges in a weakly concentrated gel. Fig. 2(II)a suggests that the precipitation lines were formed between the BS from palm oil and the cationic compound (CTAB) while no precipitation was found with BS from coconut oil. Similarly, the precipitation lines were observed between the BS from coconut oil and the anionic compound (SDS) while no precipitation line was observed between SDS and BS from palm oil (Fig. 2(II)b). Thus, the results confirm that the biosurfactants produced from *B.subtilis* and *B.cereus* were anionic and cationic in nature respectively.

.3.2. Zeta potential measurement of biosurfactants

Fig 3 shows that the zeta potential of the coconut oil BS was +54.7 mV and palm oil BS was – 33.01mV. The zeta potential value suggests the polarised nature of surfactants and degree of polarization. Furthermore, because of the high positive zeta potential, the cationic BS can be

298 expected to enhance the exchange of Cr (III) ions from the aqueous solution compared to the
299 anionic BS.

300 *3.3.3. Emulsification Stability Index (E24%)*

301 The emulsification stability indices of anionic and cationic biosurfactants were evaluated
302 by determining the emulsifying activity with different hydrocarbons. Both the biosurfactants
303 exhibited different stabilization properties with the hydrocarbons tested as expressed in terms of
304 emulsification stability index at 24h.[31] The BS produced from coconut oil substrate exhibited
305 better emulsification activity compared to the palm oil BS as shown in the Table 2.

306 *3.3.4. Surface Tension measurement*

307 A good surfactant can lower surface tension of water from 75 to 35 mN/m.[32] The cationic
308 and anionic BS were tested for the surface activity. The surface tension of aqueous solutions
309 containing cationic and anionic BS was 28.16±0.2 mN/m and 23.02±0.2 mN/m respectively.
310 Thus, the lipoprotein BS reported in this present investigation could be classified under efficient
311 and effective surfactants, as per norms suggested by Kim *et al*.[33]

312 *3.3.5. Biochemical assays of BS*

313 The carbohydrate, lipid and protein contents of cationic BS were 25mg/g, 157mg/g and
314 630mg/g respectively. But the anionic BS contained comparatively less amount of carbohydrate,
315 lipid and protein such as 30mg/g, 270mg/g and 600mg/g of respectively. The biochemical assays
316 suggests that the BS were lipoprotein in nature. This is in accordance with Nguyen *et al.*[34] that
317 the hydrophilic part of cationic BS contains aminoacids or peptides.

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3.4. Determination of amino acid composition of BS by HPLC

The amino acid composition showed that the anionic lipoprotein BS contained about 61.5% of polar amino acids and 38.5% of non-polar amino acids. The cationic lipoprotein BS contained polar amino acids by 53% and non-polar amino acids by 47% (Table 3).

3.5. Molecular weight determination of biosurfactants

The molecular weight of the purified lipoprotein BS was confirmed by using SDS-PAGE. The molecular weights of anionic and cationic BS were 18kDa and 90KDa respectively (Fig.4). The high molecular weight of biosurfactants confirms that both the biosurfactants were of giant molecular lipoproteins. The BS from coconut oil substrate showed high molecular weight lipoprotein compared to other biosurfactants from other sources reported in the literatures. [30, 35] The reported research on *Bacillus subtilis* strain indicated the production of low molecular weight biosurfactant[32] and the BS from *Bacillus cereus* strain was reported to produce plipastatins, a family of lipopeptides.[36]

3.6. Instrumental characterization of BS

3.6.1. Elemental analysis of BS

The elemental composition of cationic BS was Carbon, 41.87%; Hydrogen, 8.47%; and Nitrogen, 3.25% and that of anionic BS was Carbon, 20.25%; Hydrogen, 4.95%, and Nitrogen, 2.12%. The high nitrogen content imparts a certain degree of cationic nature to the BS as reported by Bognolo.[37]

3.6.2. Thermal Behaviour of BS (TGA-DSC)

Thermal stability may be regarded as an important property of biosurfactants for their varied applications. Both the cationic and anionic BS were observed to possess high thermal stability. TGA of anionic BS showed the initial weight loss by 1.51% at 39.51 ^{o}C due to moisture

removal and the maximum weight loss of BS by 41.43% at temperature 461.05 ^{o}C due to the decomposition of constituents of it. At the end of the scan (at 800 ^{o}C), 55.3% of the BS remained as residue. The DTG showed major weight loss of 0.296%/^{o}C at 215.58 ^{o}C (Fig5a).

TGA of cationic BS showed the initial weight loss by 2.42% at 81.97 ^{o}C due to the removal of moisture content. The maximum weight loss in BS by 52.25% was due to the decomposition of its constituents occurred in the temperature range from 255.59 ^{o}C to 408.74 ^{o}C. At the end of the scan, 37.7% of the BS remained as fixed residue, at 800 ^{o}C. The DTG showed major weight loss of 0.4376%/^{o}C at 353.59 ^{o}C(Fig.5b). This suggests that cationic BS was thermally stable compared to anionic BS.

Differential Scanning Calorimetry was used to characterize the phase transition occurred in lipoprotein over the temperature range of 30–300 ^{o}C. The transition, an exothermic process, was from amorphous solid to crystalline solid. DSC thermogram of anionic BS showed sharp crystallization temperature at 197 ^{o}C (Fig 6a). The DSC thermogram of cationic BS showed transition temperature at 107 ^{o}C and 197.70 ^{o}C (Fig 6b).

3.6.3. Circular Dichorism (CD) of lipoprotein BS

A far UV CD spectra of BS showed β- sheet form in phosphate buffer solution (Fig 7). The negative peak in the region of $\lambda_{200\text{-}225}$ nm indicates that the BS molecules were organized by β-sheet formation. The large number of carboxylic groups on the surface due to β-sheet organization may contribute to the special behaviors of BS such as ease of surface β-sheet micelles formation and ease of surface adsorption.[14] These surface adsorption properties were important features for the binding of biosurfactant with metals ions.

3.7. Interaction of trivalent chromium ions with biosurfactant

3.7.1. Effect of time

The biosurfactant for sequestration of Cr(III) ions from aqueous solution was confirmed by Chromium(III) estimation with AAS. The amount of Cr (III) ions remained at equilibrium after the sequestration with biosurfactant (0.5 g) was determined at different contact time viz. 2, 4, 6, 8, 10 and 12h. The maximum percentage removal efficiency of chromium was 50% and 80% by anionic BS and cationic BS respectively at contact time of 10h (Fig. 8a). The cationic biosurfactant showed higher sequestering efficiency for Cr (III) compared to that of anionic biosurfactant.[12]

3.7.2. Effect of pH

The effect of pH on sequestration of Cr (III) ions by biosurfactant was studied in the range of pH 4-6. It was observed that, the maximum removal of Cr (III) was observed at pH 6 by 50% with anionic BS and 82% with cationic BS at pH 5 (Fig. 8b). pH plays a very important role in the sequestration of trivalent chromium because of its influence on the protonation of different amino acids and rendering them to acquire charge to facilitate exchange for Cr(III) in solution. It was observed that BS was effective enough to sequester Cr (III) in the pH range 4–6.5. The sequestration efficiency of BS was reduced at pH above 6.5 due to the precipitation of Cr^{3+} as $Cr(OH)_3$.[38] The H^+- concentration in the pH range (4-6) would facilitate the cationic BS to sequester Cr (III) ions greater than the anionic BS.

3.7.3. Effect of biosurfactant Concentration

The effect of biosurfactant concentration on the removal of Cr (III) ions was studied at different concentration of BS i.e. 0.5, 1.0, 1.5, 2.0 and 2.5g. The sequestering efficiency of Cr (III) ions was increased with increase in concentration of biosurfactant. The cationic BS at

concentration of 2.5g removed Cr (III) ions by 98% while anionic BS at the same concentration removed the chromium ions by 85% from solution containing Cr(III) of 100 mg/L (Fig. 8c).

3.7.4. Effect of Cr (III) ions concentration

The effect of concentration of Cr (III) ion on its removal by fixed biosurfactant concentration of 2.5g was studied. While the percentage removal of Cr(III) using the anionic biosurfactant was 85, 78, 77, 75 and 75%, the cationic biosurfactant removed Cr(III) ion by 98, 97.5, 95, 93.7, and 91.6% at chromium(III) concentration of 100, 200, 300, 400 and 500 mg/L respectively (Fig. 8d). Hence, the biosurfactant concentration of about 2.5g was able to sequester Cr (III) by 458 mg from the aqueous solution while removal of Chromium by rhamnolipid biosurfactants were reported by Das *et al.*[13] and Juwarkar *et al*.[39] The other potential Cr (III) biosorbents reported were Sargassum seaweed biomass (40 mg/g),[40] Loofa sponge immobilized biomass of *Chlorella sorokiniana* (69.26 mg/g),[41] pretreated Bran rice (285.71 mg/g),[42] *Cassia fistula* biomass (107.5 mg/g)[43] and sawdust Acacia Arabica (111.61 mg/g).[43]

3.8. Instrumental Evidences for the removal of Cr (III) ions using BS

3.8.1. UV-Visible spectra of BS

The UV-Visible spectra (Fig.9a) of cationic and anionic BS showed the characteristic peak at λ_{290}nm. The delocalization of electrons from non-bonding to π anti-bonding site (π*) caused absorption at λ_{290}nm. This confirms the presence of aromatic amino acids (phenylalanine) in cationic BS as shown in Table 4. After interaction of cationic BS with Cr (III), the blue shift i.e to 285nm was observed with reduced intensity (Fig. 9b). The reduction in intensity of absorption may be attributed to the decrease in delocalization of electrons owing to interaction of cationic BS with Cr (III). The anionic BS after interaction with Cr (III) showed the characteristic peak at 289nm.

3.8.2. Fluorescence spectral analysis

The fluorescence spectra (Fig.10) of cationic and anionic BS show that BS is a fluorescent inactive compound. After interaction of cationic BS with Cr (III), the fluorescence spectra showed the presence of excitation and emission peaks at λ_{232} nm and λ_{357} nm respectively. Also interaction of anionic BS with Cr (III), showed the presence of excitation and emission peaks at λ_{232} nm and λ_{349} nm respectively, which confirms the bonding of Cr (III) ion with BS.

3.8.3. FT-IR spectral Analysis

The FT-IR spectrum of anionic BS (Fig. 11a) showed the presence of asymmetric N-H stretching of amide at 3451.03 cm^{-1}. C=O stretching vibration of the peptide group was observed at 1651.96 cm^{-1}. The peak at 2924.23 cm^{-1} resulting from -C-H stretching of methylene group reflects the presence of an aliphatic chain. The peak at 1466.10 cm^{-1} was due to the methylene bending. The absorption peak at 1750.11 cm^{-1} may be due to the presence of carbonyl stretching in ester groups. The shouldering at725 cm^{-1} may correspond to C-N stretching vibration. This may be attributed to the bonding of protein moiety with the lipid component in lipoprotein BS.[24] The peaks at 702 and 2511 cm^{-1} may be attributed to C-S and S-H stretching respectively of cysteine present in lipoprotein.

The FT-IR spectrum of cationic BS (Fig. 11c) showed N-H stretching of peptide bond at 3297.26 cm^{-1}, and C=O stretching of the peptide group at 1658.41 cm^{-1}. The bands at 2952.52 cm^{-1} resulting from the C-H stretching of methylene group reflects the presence of an aliphatic chain. The peak at 1465.90 cm^{-1} was due to methylene bending. The absorption region at 1743.28 cm^{-1} was due to the carbonyl stretching of ester group. The shouldering at 1107, 1156, 1121 &1248.28 cm^{-1} may correspond to C-N stretching vibrations. The presence of peptide bond with

esters confirms that the biosurfactants were lipoprotein. The characteristic stretching frequency of amides in the regions 3350 cm^{-1} and 1500–1650 cm^{-1} are not normally observed in the FT-IR spectra of rhamnolipid biosurfactants, which differentiates the unique nature of lipoprotein biosurfactant from rhamnolipid biosurfactant.[31]

Fig. 11b shows the FT-IR spectrum of BS- Cr (III) due to the binding of Cr (III) with NH group and thus N-H stretching band at 3451.03cm^{-1} was shifted to 3395cm^{-1}. The shift in frequency to a lower value confirms that the stabilized bond was formed between Cr (III) and the NH stretching of anionic BS. The characteristic peak of Cr (III) was observed at 620 cm^{-1}. The C-N stretching vibration was shifted from at 725 cm^{-1} to 713 cm^{-1} after ionic bonding of Cr (III) with anionic BS.

The FT-IR spectrum in Fig. 11d showed broadening of the OH stretching and shifting of amide bond at 3297.26 cm^{-1} to 3139 cm^{-1} due to the strong binding of Cr (III). The characteristic peak of Cr (III) was observed at 620 cm^{-1}. The shift in the C=O stretching of the peptide group from 1658.41 cm^{-1} to 1651.41 cm^{-1} was observed due to the strong binding of cationic biosurfactant with chromium (III) ions. Also the band at 1746 cm^{-1} due to C-O stretching was shortened. This becomes an evidence for the binding of Cr (III) ions with the peptide group of the biosurfactant.

3.8.4. SEM analysis of biosurfactant bonded with chromium ions

The sequestration of Cr (III) ions from the aqueous solution was further confirmed using Scanning Electron Microscopy. The SEM images (Fig. 12a, b) show the morphology of anionic and cationic BS, illustrating the assembly of lipoprotein molecules. The patches in the SEM images (Fig.12c, d) confirm the attachment of Cr (III) ions with biosurfactant molecules.

3.8.5. EDX analysis of biosurfactant bonded with chromium (III)

The EDX analysis further confirmed the removal of Cr (III) ions from aqueous solution. The EDX spectrum of anionic BS - Cr (III) showed Cr(III) was 0.18 weight% (Fig. 13a) and the cationic BS- Cr(III) showed 0.44 weight% of Cr(III) at 15 kV accelerating voltage with a 5–6nm electron beam(Fig. 13b). The results confirmed that the removal efficiency of cationic BS was comparatively higher than that of the anionic BS, thus indicating that cationic BS may be regarded as a more appropriate chelating agent than the anionic BS.

3.9. Mechanistic view for removal of Cr (III) ions in aqueous solution by anionic and cationic BS

The biosurfactant acquired cationic charge (+54.7 mV as evidenced from Fig. 3 relating zeta potential profile) due to the presence of tertiary amine in histidine, resonance structure of phenylalanine and tyrosine in lipoprotein. The amino acids such as histidine (pI 7.59), phenylalanine (pI 5.91) and tyrosine (pI 5.66) in cationic BS have isoelectric points much higher than the pH of BS (pH 5). At the optimized pH (pH 5) cationic BS removed Cr (III) by 98%. The positive charges acquired by the cationic BS due to over expression of above amino acids (15.63% as evidenced from the composition of amino acids, Table 3) which may be the contributing reason for the enhanced removal of Cr (III) from aqueous solution. At the optimized pH (pH 5) cationic BS is protonated as evidenced from FT- IR spectroscopy of histidine. The peak at 3082.6 cm^{-1} could be attributed to the presence of symmetric N-H stretching and protonated NH_2^+ group present in histidine.[44] The in-plane bending of C-H (ring) at 1121 cm^{-1} and 1156 cm^{-1} corresponds to cationic form of phenylalanine.[45]

The protonated amino acids (histidine, phenylalanine and tyrosine) at pH 5 which is lower than their isoelectric points release protons due to delocalization of electrons caused by

481 resonance (as evidenced from UV-Vis spectroscopy, Fig. 9a). The presence of O^- after
482 deprotonation is confirmed from stretching vibration at 1591.23 cm^{-1}.[46]

483 The non bonded electrons at nitrogen of histidine, tyrosine and phenyl alanine stabilize
484 bonding with Cr (III) through coordinate linkage (as evidenced from shift in frequency as
485 recorded by FT-IR spectroscopy).

486 After interaction of cationic BS with Cr (III) ions, the peaks such as 3443.35 cm^{-1} &
487 3082.6 cm^{-1} are broadened and also the O^- vibration peak disappeared. The in-plane C-H bending
488 vibration corresponding to phenylalanine disappeared. This clearly suggests that the Cr (III)
489 binds with nitrogen of histidine, oxygen of tyrosine and nitrogen of phenyl alanine to form a co-
490 ordinate bonding. The shift in the vibrational frequency corresponding to N-H stretching of the
491 peptide from 3297.26 to 3139 cm^{-1} and the shift in the C=O stretching of the peptide group from
492 1658.41 cm^{-1} to 1651.41 cm^{-1} were observed after interaction of Cr (III) with cationic BS. These
493 vibrational shifts in Cr (III) interacted cationic BS matrix is evident for stabilization of bonding
494 of Cr (III) with the respective amino acids. The pictorial representation of removal of Cr (III) by
495 cationic BS is shown in Fig. 14.

496 The anionic charge (– 33.01mV as evidenced from the zeta potential profile, Fig. 3) of
497 the BS could be due to the presence of sulphur containing amino acid, cysteine in lipoprotein.
498 Cysteine has isoelectric point (pI 5.02) much lower than the pH of BS. At optimized pH (pH 6),
499 the anionic BS acquires negative charge due to ionization of cysteine alone (2.85% as evidenced
500 from composition of aminoacid in anionic BS, Table 3). At the optimized conditions, anionic BS
501 ionically bonded with Cr (III). The Cr (III) has possible bonding only with cysteine of anionic
502 BS which is poorly expressed in anionic BS and thus accounting for poor removal (85%) of Cr
503 (III) by anionic surfactant.

The charged centre of cysteine coordinated with Cr (III) as evidenced from change in frequency as recorded by FT-IR spectroscopy. The FT-IR spectrum of anionic BS showed a band at 1560 cm^{-1} corresponds to N H bend in cysteine.[47] In addition, a weak broadened band at 2511 cm^{-1} may be attributed to S-H stretching in cysteine molecule. After the interaction of anionic BS with Cr (III), the shift in N-H bending vibration to 1552 cm^{-1} was observed. Also the disappearance of weak S-H stretching band, clearly indicates Cr (III) binds with the sulphur containing amino acid through ionic bonding. The vibrational shift in the peak corresponding to N-H stretching of the peptide group from 3451 to 3395 cm^{-1} was evident. The shift in C-N stretching vibration from 725 cm^{-1} to 713 cm^{-1} after ionic bonding of anionic BS with Cr (III) was observed. These vibrational shifts suggest that alteration in the peptide linkage as a consequence of interaction of Cr (III) with anionic BS.

The anionic BS containing cysteine (Table 3) alone is responsible for ionic bonding with the sulphur group present in BS .Thus , Cr (III) was removed only by 85% with anionic BS while cationic BS removed Cr(III) by 98% at the same concentration (2.5g). The mechanism for the removal of Cr (III) by anionic BS has been illustrated in Fig. 15.

This is evident from EDX spectra that anionic BS-Cr (III) contained Cr (III) by 0.18 % of ions (Fig. 13a) and the cationic BS-Cr (III) contained Cr(III) by 0.44 % (Fig.13b). Fluorescence spectra also confirmed the removal of Cr (III) by cationic and anionic BS (Fig.10).

4. Conclusions

In the present investigation, the production of anionic and cationic BS using palm oil and coconut oil as substrates respectively and their ability to remove Cr (III) ions from aqueous solutions were studied. The agar double diffusion technique and zeta potential measurement of biosurfactants confirmed the charge carried by the surfactants. The composition of the

biosurfactants confirmed that they belong to lipoprotein type. The biosequestration of Cr (III) ions by biosurfactants was confirmed using FT-IR analysis through their shifts in the peptide group of BS. The cationic biosurfactant showed that the maximum removal of Cr (III) ions from aqueous solution was 98% at the biosurfactant concentration of 2.5g through coordinate bonding, while the anionic biosurfactant removed Cr (III) by 85% using of the same concentration of biosurfactant through ionic bonding. To best of our knowledge, this forms the first report on interaction of cationic BS with Cr (III) ions in aqueous solution.

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References

[1] J. H. Sharphouse, Leather Technician's Handbook, Leather Producers Association, Buckland Press Ltd, London 1989.

[2] B. Basaran, M. Ulaş, O. Behzat and A. Band Aslan, Indian J. Chem. Technol., 2008, **15**, 511-514.

[3] Environment Commission of I.U.L.T.C.S.: IUE Recommendations on Cleaner Technologies for Leather Production, London 1997.

[4] Central Pollution Control Board, 1986. The Environment (Protection) Rules, India, pp 418.

[5] Z.X. Wang, J.Q. Chen, L.Y. Chai, Z.H.Yang, S.H. Huang and Y. Zheng, J. Hazard. Mater., 2011, **190**, 980 – 985.

[6] L. Di Palma, D.Mancini and E.Petrucci, Chem. Eng. Trans., 2012, **28**, 145-150.

[7] D. C. Sharma, C. Chatterjee, and C. P. Sharma, J. exp. Bot., 1995, **25**, 241-251.

[8]H.M. Abdulla, E.M. Kamal, A.H.Mohammed, and A.D. EI Bassuony, Proc. fifth Scien. Environ conf. Zagazig uni, 2010, 171-183.

[9] J. Kotas and Z. Stasicka, Environ. Pollut., 2000, **107**, 263-283.

[10] Ecological Analysts, Inc. 1981. American Petroleum Institute, 2101 L St., N.W., Washington, DC 20037. pp 207.

[11] M. M. Altaf, F. Masood, and A. Malik, Turk. J. Biol., 2008, **32**, 1–8.

[12] M.P. Plkociniczak, G. A. Płaza, Z.P. Seget and S.S. Cameotra, Int. J. Mol. Sci., 2011, **12**, 633-654.

[13] P. Das, S. Mukherjee and R. Sen, Bioresour. Technol., 2009, **100**, 4887–4890.

[14] G. Dehghan Noudeh, M. Housaindokht and B.S. Bazzaz, J. Microbiol. Methods, 2005, **43**, 272-276.

[15] A. Franzetti, I. Gandolfi, G. Bestetti and I. M. Banat, Trends in Bioremediation and Phytoremediation, 2010, 145-156.

[16] J. Thaniyavarn, A. Chongchin, N. Wanitsuksombut, S. Thaniyavarn, P. Pinphanichakarn, N. Leepipatpiboon, M. Morikawa and S. Kanaya, J. Gen. Appl. Microbiol., 2006, **52**, 215–222.

[17] V. Walter, C. Syldatk and R. Hausmann. Bioscience and Springer Science, Business Media 2010.

[18] R.D. Rufino, J. M. Luna, G. M. Campos-Takaki, S. R.M. Ferreira and L.A. Sarubbo, Chem Eng. Trans., 2012, **27**, 61-66.

[19] T. Meylheuc, C.J. Vanoss and M.M. Bellon-Fontaine, J. Appl. Microbiol., 2001, **91,** 832.

[20] D. G. Cooper and B. G. Goldenberg, Appl. Environ. Microbiol., 1987, **53**, 224–229.

[21] T. Varadavenkatesan and V.R. Murty, J. Microbiol. Biotech. Res., 2013, **3(4)**, 63-73.

[22] M. Dubois, K.A. Gills, J.K. Hamilton, P.A. Rebers and F. Smith, Anal.Chem., 1956, **28**, 350–356.

[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 1951, **193**, 265-275.

[24] J. Izard and R.J. Limberger, J. Microbiol. Methods, 2003, **55**, 411 – 418.

[25] S. Ramakrishnan, K. N. Sulochana, R. Punitham and K. Arunagiri, Glycoconj J, 1996, **13**, 519–523.

[26] U.K. Laemmli, Nature, 1970, **227**, 680–85.

[27]W.M.F.W. Nawawi, International Islamic University Malaysia, Kuala Lumpur, Malaysia, 2011.

[28] S. Patil, A. Pendse and K. Aruna, Int.J.Curr.Biotechnol., 2014, **2**, 20 -30.

[29] M. Kannahi and M. Sherley, Int. J. Chem. Pharma.Sci., 2012, **3**, 37-42.

[30] S.K. Satpute, B.D. Bhawsar, P.K. Dhakephalkar and B.A Chopade, Ind. J. Mar. Sci., 2008, **37**, 243–250.

[31] K. Ramani, S. Chandan Jain, A.B. Mandal and G. Sekaran, Colloids and Surfaces B: Biointerfaces, 2012, **97**, 254– 263.

[32] S.S. Bhadoriya, N. Madoriya, K. Shukla and M.S. Parihar, Biochem. Pharmacol., 2013, **2**, 113.

[33] S.H. Kim, E.J. Lim, S.O. Lee, J.D. Lee and T.H. Lee, Biotechnol. Appl. Biochem., 2000, **31,** 249–253.

[34] T. T. Nguyen, N. H. Youssef, M. J. McInerney, and D. A. Sabatini, Water res., 2008, **42**, 1735-1743.

[35] P.Saranya, S.Swarnalatha and G.Sekaran, RSC Adv., 2014, **4**, 34144-34155.

[36] C.N. Mulligan and B. F. Gibbs, Proc. Indian Natn Sci. Acad., 2004, **1**, 31-55.

[37] G. Bognolo, Colloids Surf. A: Physicochem. Eng. Asp., 1999, **152**, 41-52.

[38] E.Z. Ron and E.Rosenberg, Appl.Microbiol. Biotechnol., 1999, **52**, 154-162.

[39] A.A. Juwarkar, K.V. Dubey, A. Nair and S. Kumar Singh, Ind. J. Microbiol., 2008, **32**, 142-146.

[40] D. Kratochvil, P. Pimentel and B. Volesky, Environ. Sci. Technol., 1998, **32**, 2693-2698.

[41] A. Nasreen, I. Muhammad, Z. Saeed Iqbal and I. Javed, J. Environ. Sci., 2008, **20**, 231–239.

[42] K.K. Singh, R. Rastogi and S.H. Hasan, J. Colloid Interface Sci., 2005, **290,** 61–68.

[43] P. Miretzky, and A. Fernandez Cirelli, J. Hazard. Mat., 2010, **180**, 1–19.

[44] K. Rajarajan, K. Anbarasan, J. Samu Solomon, G. Madhurambal, J. Chem. Pharma. Res., 2012, **4**, 4060-4065.

[45] S. Olsztynska, M. Komorowska, L. Vrielynck and N. Dupuy, Appl. Spectrosc., 2001, **55**, 901-907.

[46] A. Barth, Prog. Biophys. Mol. Biol., 2000, **74**, 141–173.

[47] S. Aryal, B.K.C. Remant, N. Dharmaraj, N. Bhattarai, C.H. Kim and H.Y. Kim, Spectrochim. Acta A Mol. Biomol. Spectrosc., 2006, **63**, 160–163.

Figure Captions

Fig. 1: Phylogenetic tree for identified organisms from (a) Palm oil substrate (b) Coconut oil substrate.

Fig. 2: (I) Blue agar plate method (a) CTAB plate (Anionic BS) (b) SDS plate (Cationic BS); **(II)** Double diffusion on Agar (a) CTAB (b) SDS.

Fig.3: Zeta potential of a) Anionic BS (b) Cationic BS

Fig. 4: SDS PAGE for lipoprotein biosurfactant **Lane 1**: Marker **Lane 2**: Anionic BS- 18KDa **Lane 3**: Cationic BS-90KDa

Fig. 5: TGA and DTG thermograms of (a) Anionic BS (b) Cationic BS

Fig. 6: DSC spectra of (a) Anionic BS (b) Cationic BS

Fig. 7: Circular Dichorism Spectra of biosurfactant

Fig. 8: Removal percentage of Cr(III) ions (a) Time (pH: 7, Anionic BS and Cationic BS concentration: 0.5g, Cr(III) concentration: 100 ppm) ,(b) pH(Time: 10h, Anionic BS and Cationic BS concentration: 0.5g, Cr(III) concentration: 100 ppm) (c) Different Anionic BS and Cationic BS concentration(Time: 10h, pH: 5, Cr(III) concentration: 100 ppm), (d) Different Cr(III) ion concentration(Time: 10h, pH: 5, Anionic BS and Cationic BS concentration: 2.5g)

Fig. 9: UV-visible spectra of (a) Anionic BS and Cationic BS, (b) Anionic and Cationic BS with Cr(III) ions

Fig.10. Fluorescence spectra of (a) cationic BS, (b) anionic BS, (c) cationic BS with Cr(III) and (d) anionic BS with Cr(III) ions

Fig. 11: FTIR spectra of (a) Anionic BS, (b) Anionic BS with Cr(III) ions, (c) Cationic BS (d) Cationic BS with Cr(III) ions

642 **Fig. 12:** SEM images of (a) Anionic BS, (b) Anionic BS with Cr(III) ions (c) Cationic BS, (d)
643 Cationic BS with Cr(III) ions
644 **Fig. 13:** EDX spectra of (a) Anionic BS with Cr(III) ions (b) Cationic BS with Cr(III) ions
645 **Fig.14:** Mechanistic view for removal of Cr (III) by cationic BS in aqueous solution
646 **Fig.15:** Mechanistic view for removal of Cr (III) by anionic BS in aqueous solution
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Table legends

Table 1: Screening of microorganisms by oil drop collapse assay

Table 2: Emulsification stability Index of biosurfactant with different hydrocarbons

Table 3: Aminoacid composition of biosurfactant from *Bacillus Sp.*

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693

694 **Table 1:** Screening of microorganisms by oil drop collapse assay

Palm oil		Coconut oil	
Isolated microorganisms	**Zone diameter (cm)**	**Isolated microorganisms**	**Zone diameter (cm)**
P1	0.9±0.5	C1	1.5±0.4
P2	1.2±0.4	C2	**3.4±0.15**
P3	**2.8±0.3**	C3	0.6±0.08
P4	0.6±0.2	C4	1.8±0.21
P5	1.6±0.4		
P6	1.3±0.3		

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Table 2: Emulsification stability Index of biosurfactant with different hydrocarbons

Hydrocarbons	Emulsification stability Index, E24 (%)	
	Palm oil BS	Coconut oil BS
Palm oil	35±1.2	40±1.5
Coconut oil	30±1.0	40±1.0
Olive oil	22±1.8	35±1.5
Diesel oil	35±1.5	38±1.8

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724 **Table 3:** Aminoacid composition of biosurfactant from *Bacillus Sp.*

Amino acids	µmoles/g	
	Anionic BS	**Cationic BS**
Aspartic acid	-	10.9
Glutamic acid	23	45
Serine	7.4	5.1
Histidine	-	0.9
Glycine	0.44	8.5
Threonine	4.23	2.6
Arginine	34.5	13.8
Alanine	3.75	6.5
Tyrosine	1.58	15.5
Methionine	0.14	1.6
Valine	1.64	3.1
Phenylalanine	-	2.9
Isoleucine	-	2.6
Leucine	11.3	4.5
Lysine	-	2.2
Cysteine	2.85	-
Glutamine	1.22	-
Asparagine	0.85	-

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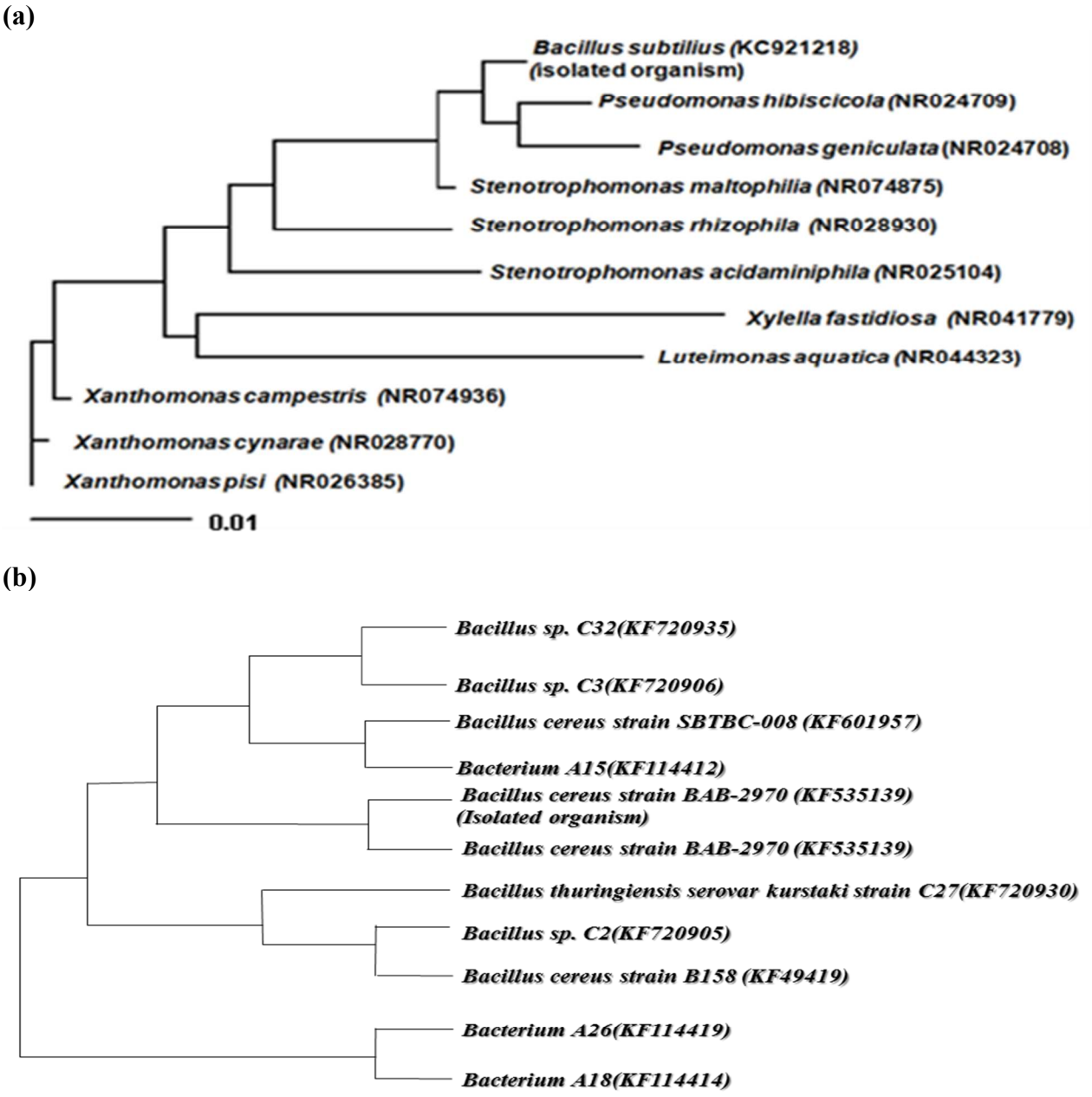

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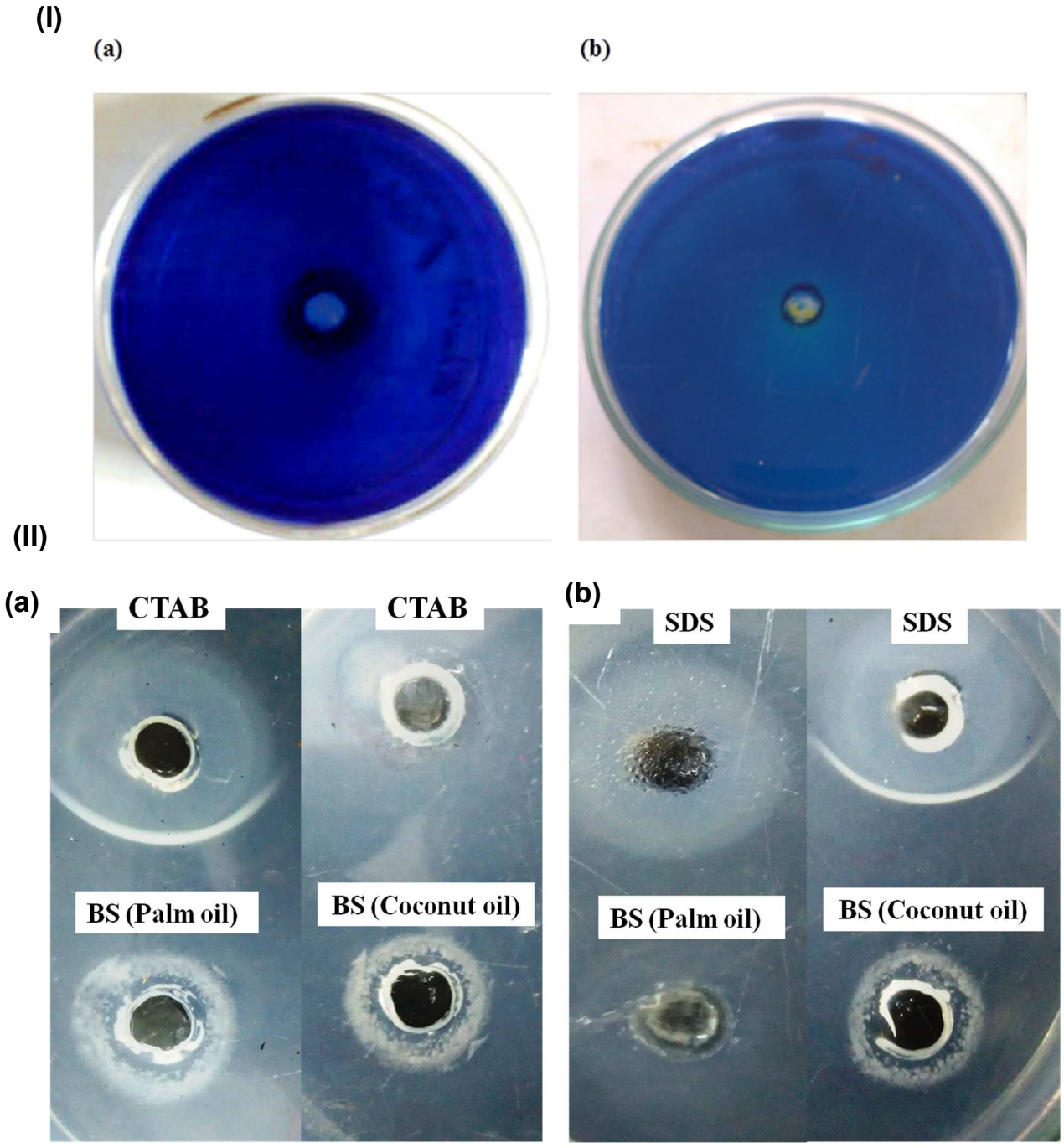

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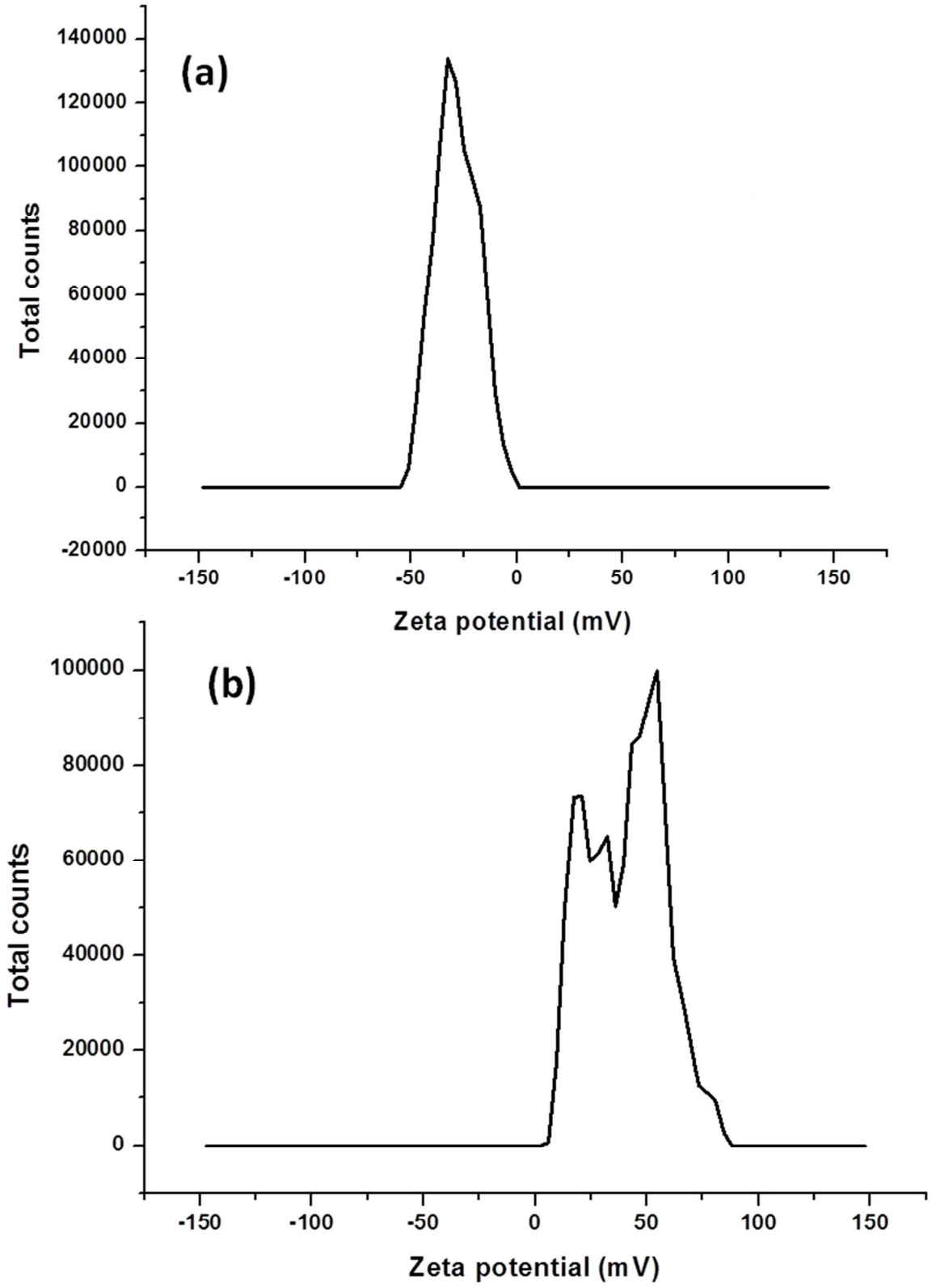
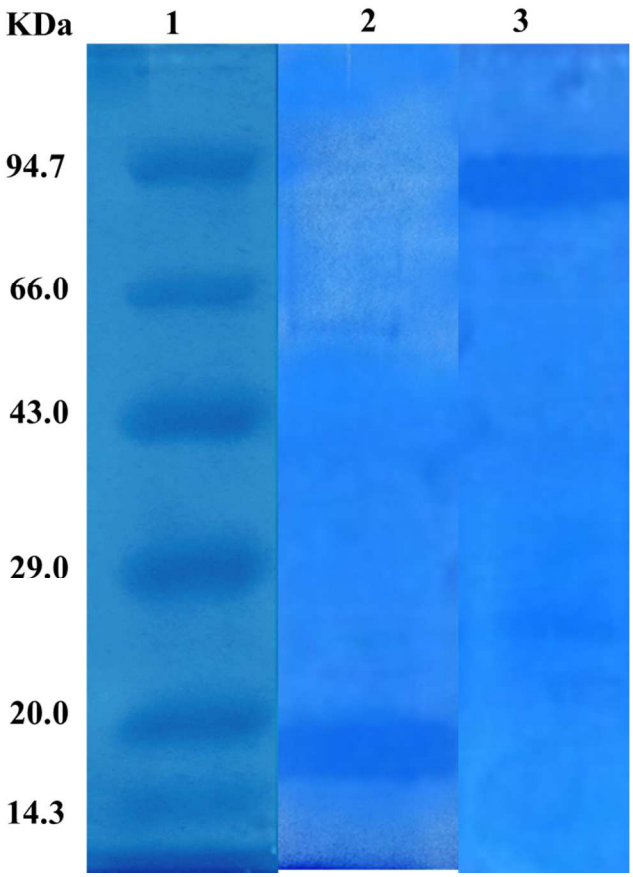


Fig. 3: Zeta potential of (a) Anionic BS (b) Cationic BS



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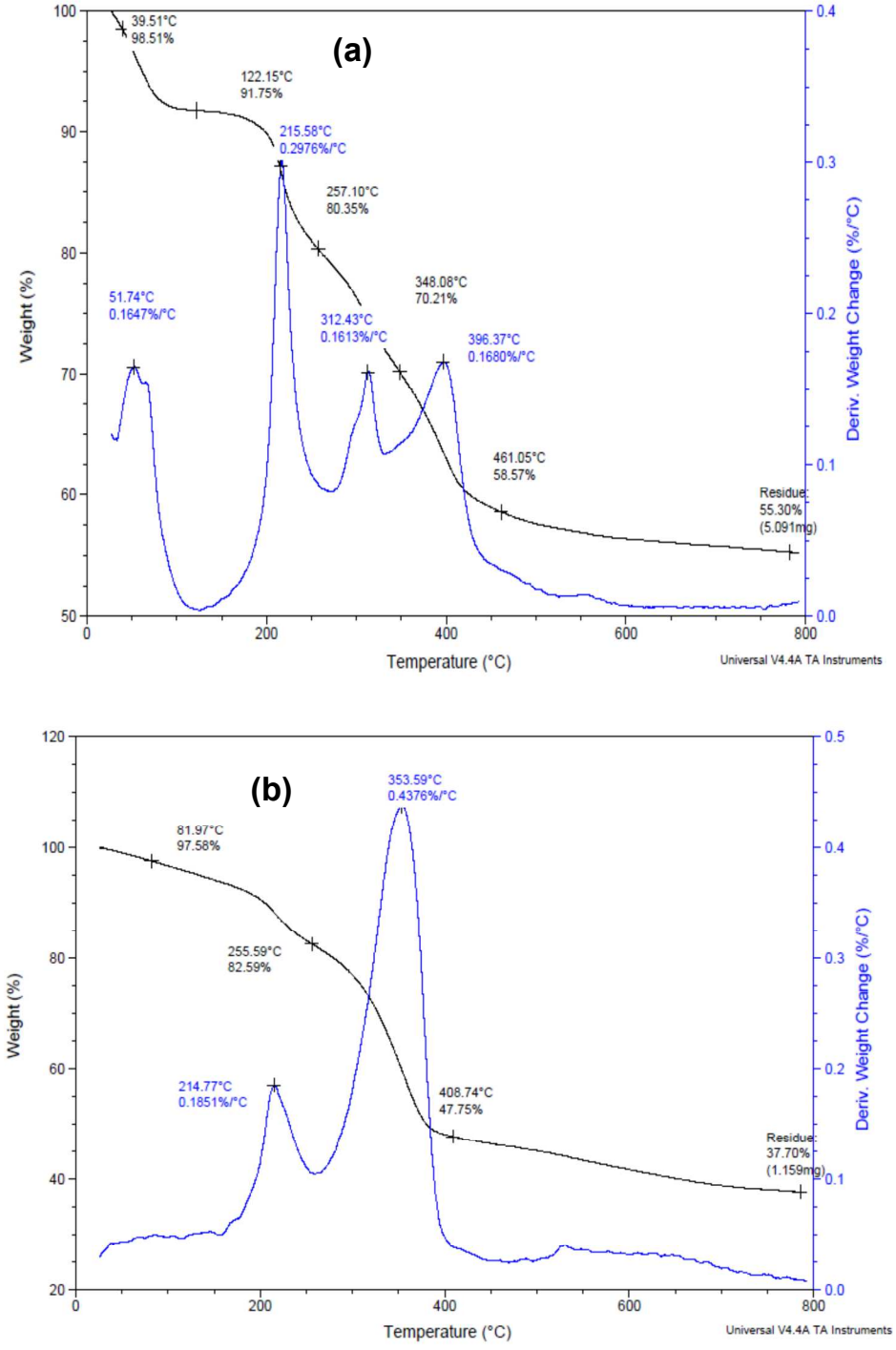

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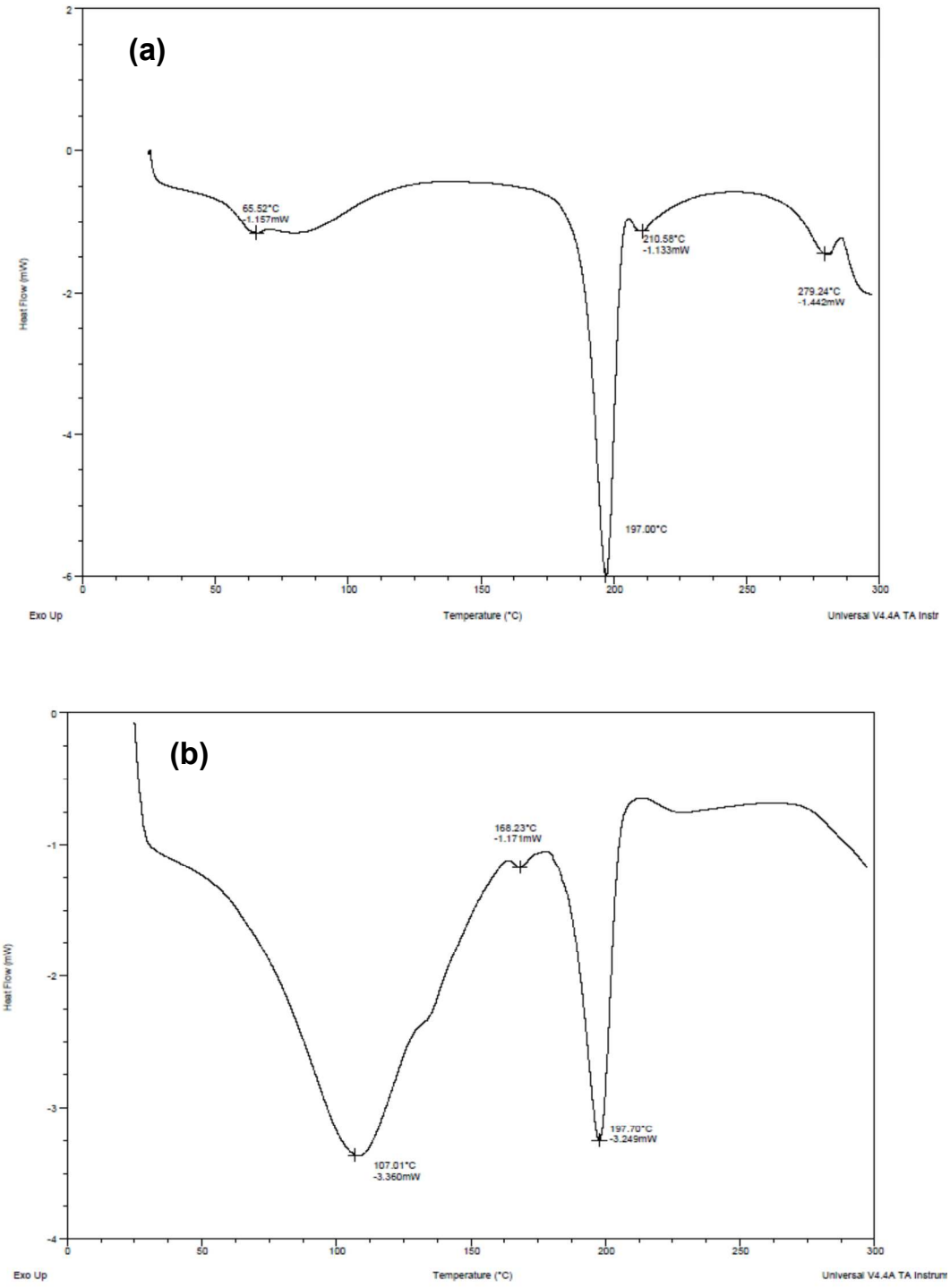
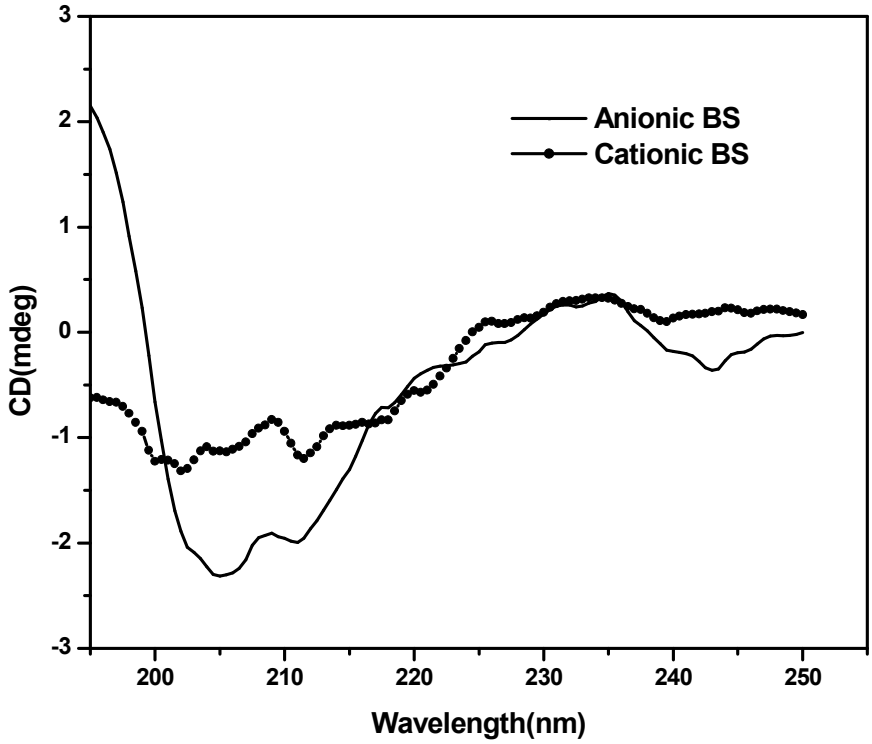


Fig. 6: DSC spectra of (a) Anionic BS (b) Cationic BS



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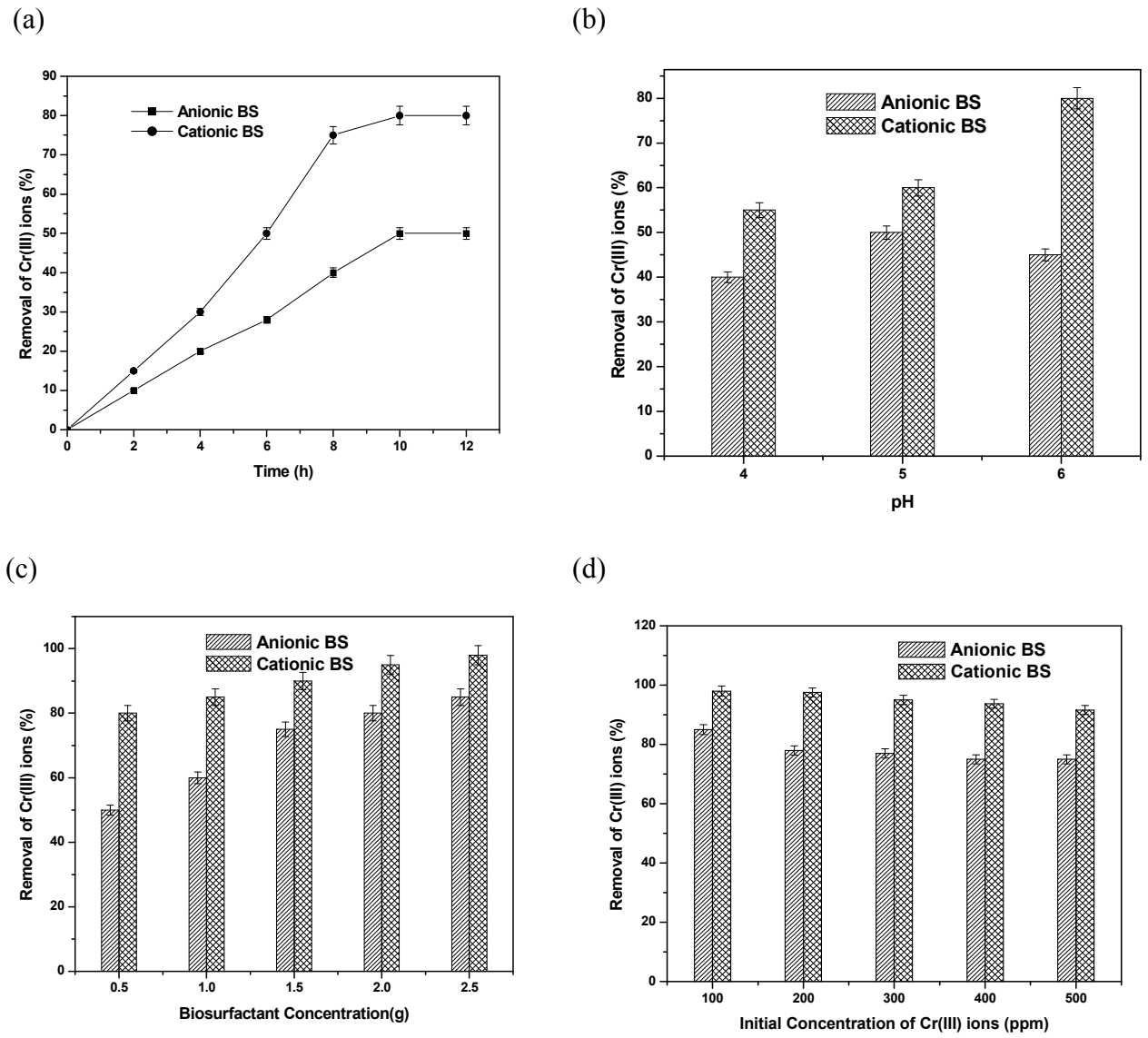

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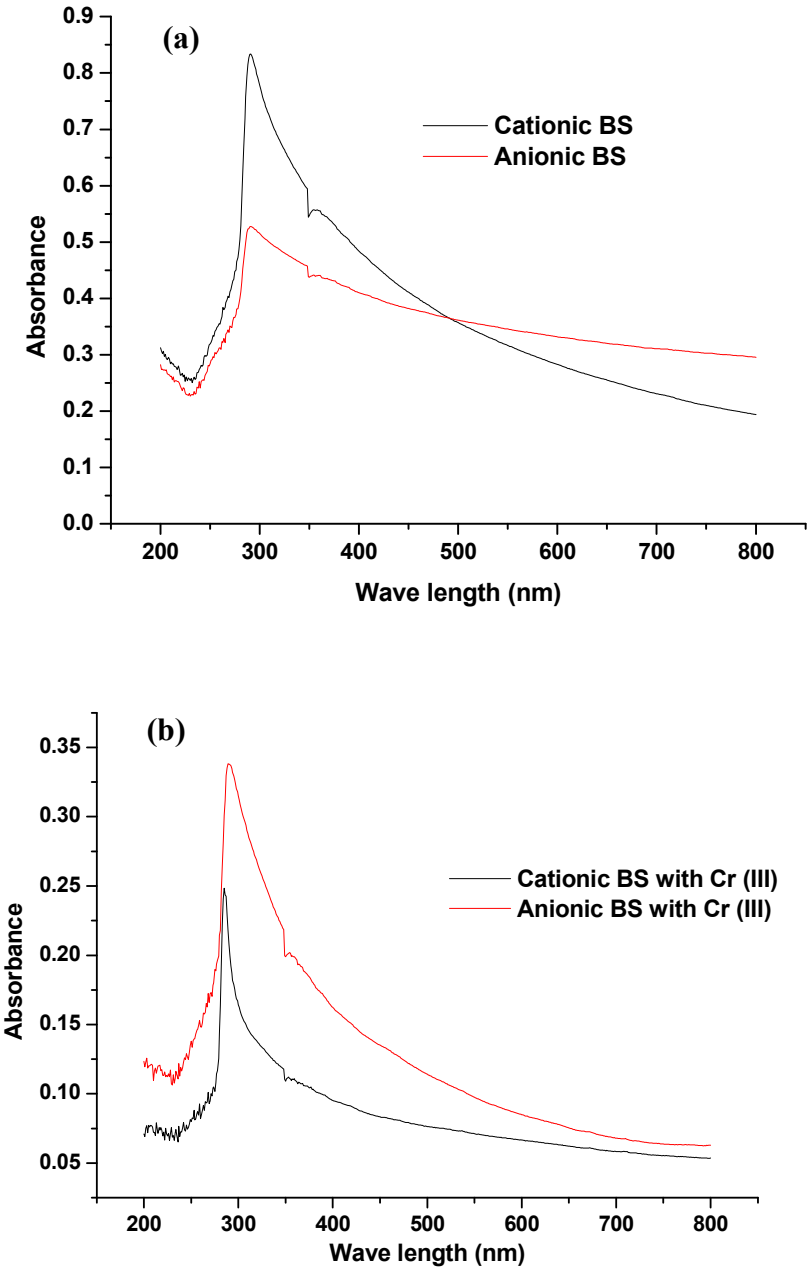

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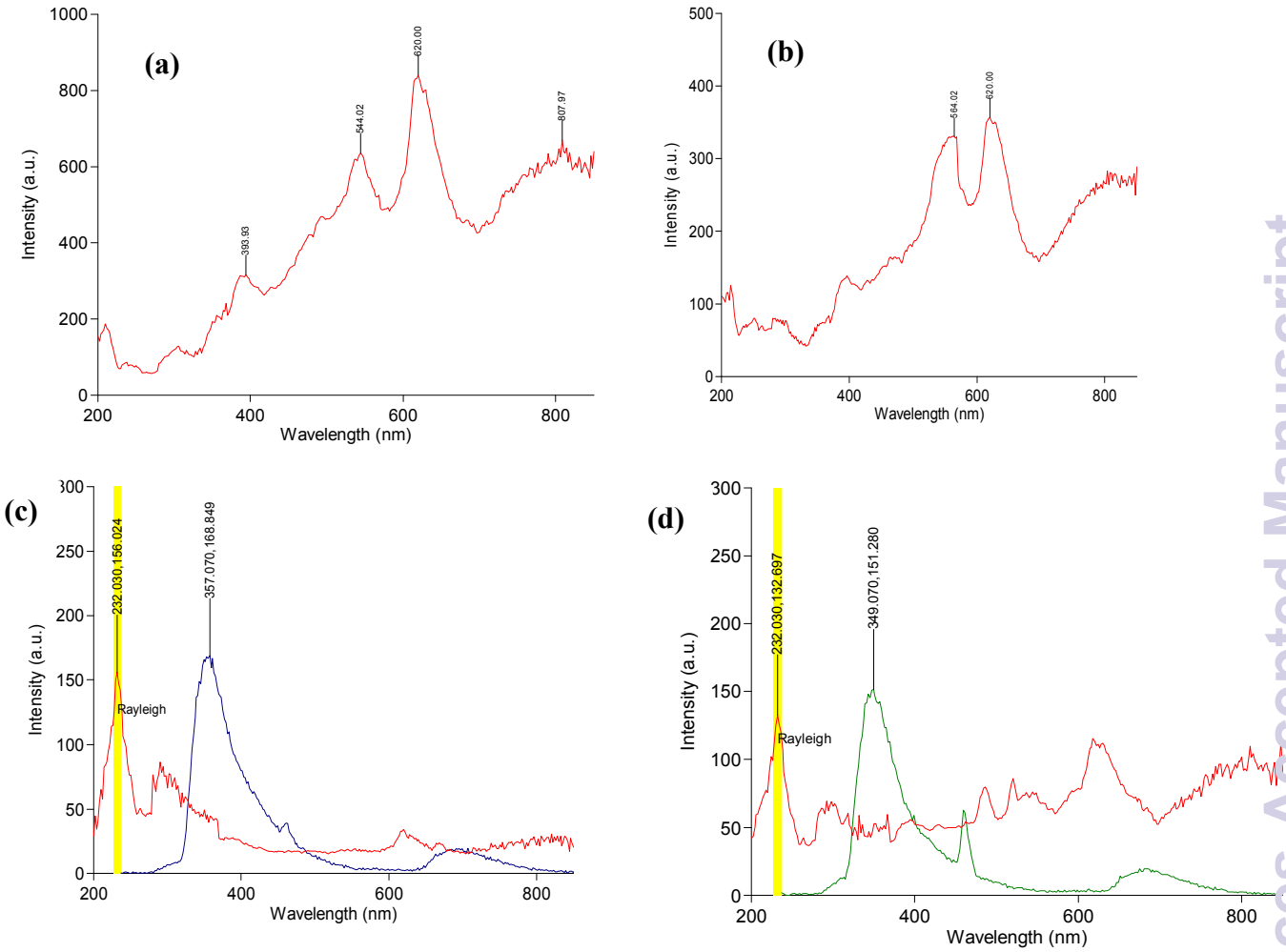

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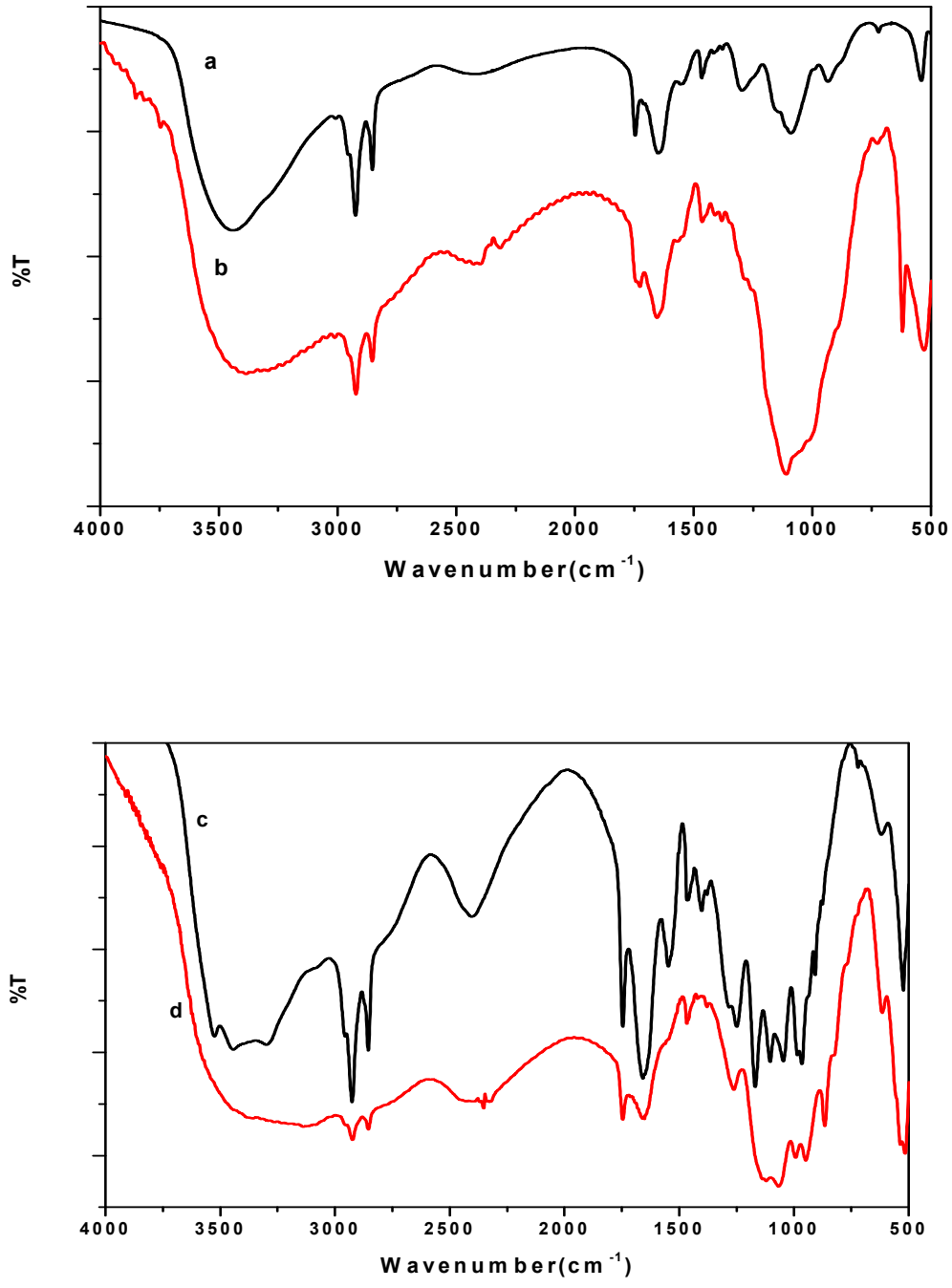

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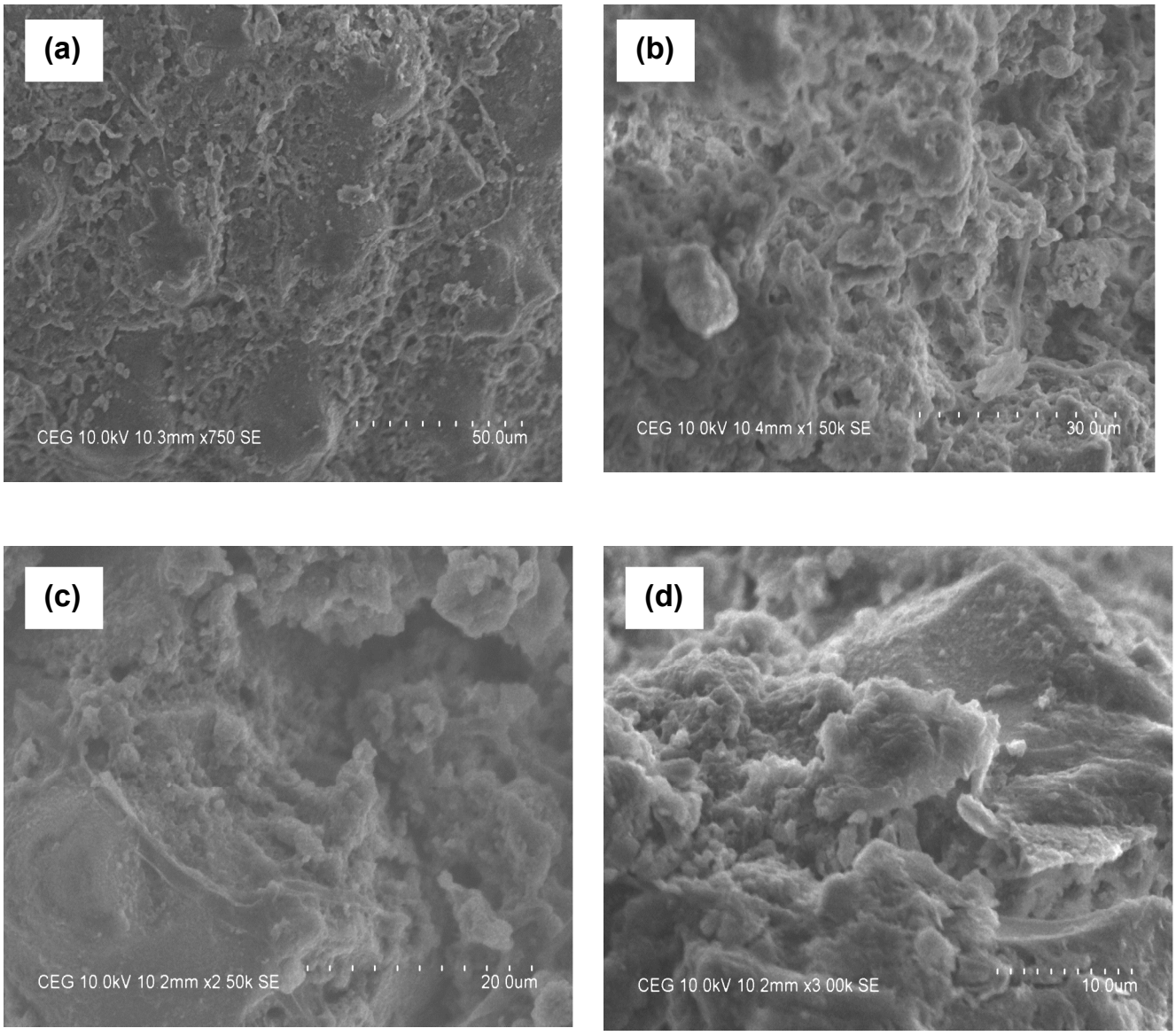

Fig. 12: SEM images of (a) Anionic BS, (b) Anionic BS with Cr(III) ions (c) Cationic BS, (d) Cationic BS with Cr(III) ions

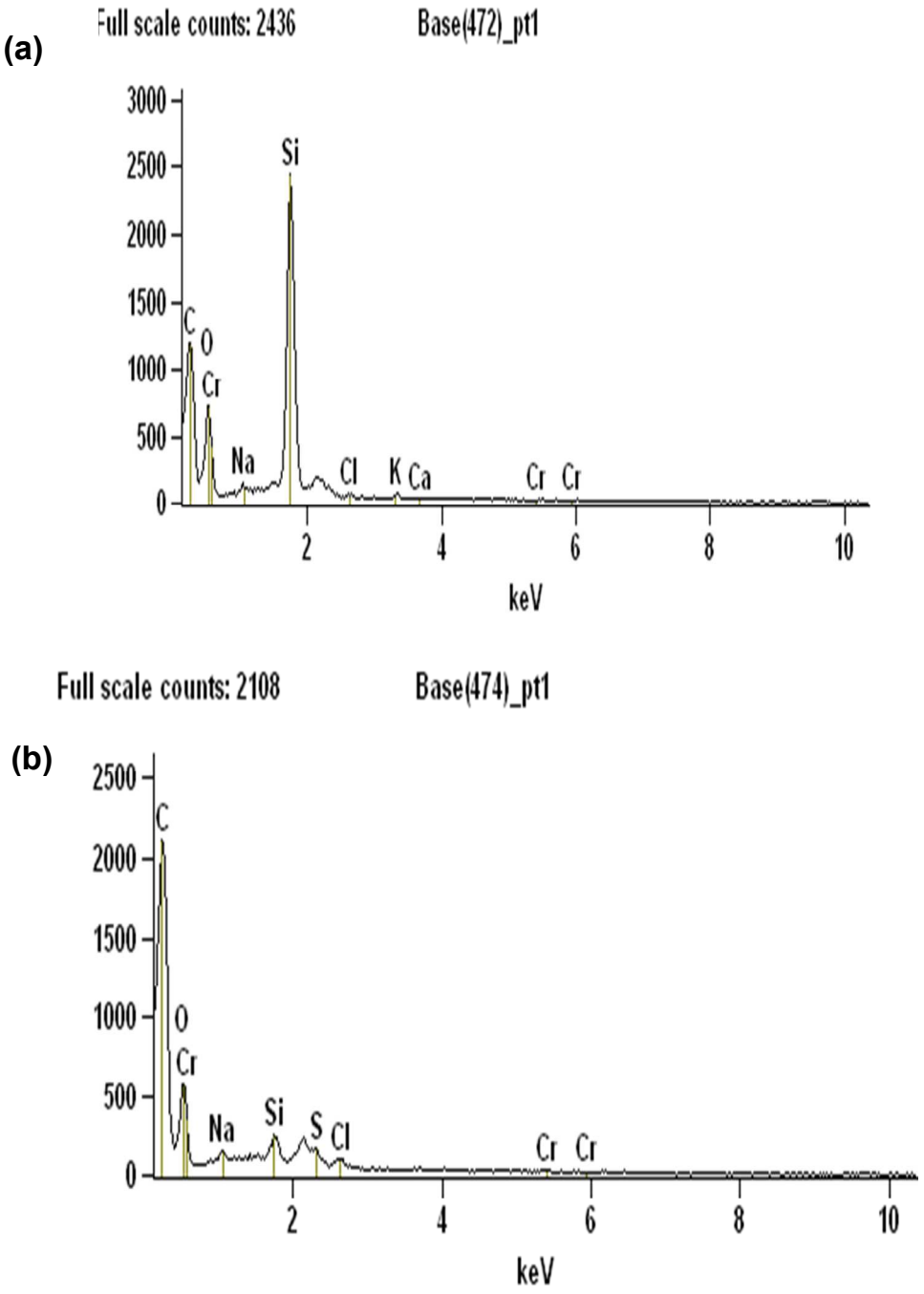

Fig. 13: EDX spectra of (a) Anionic BS with Cr(III) ions (b) Cationic BS with Cr(III) ions

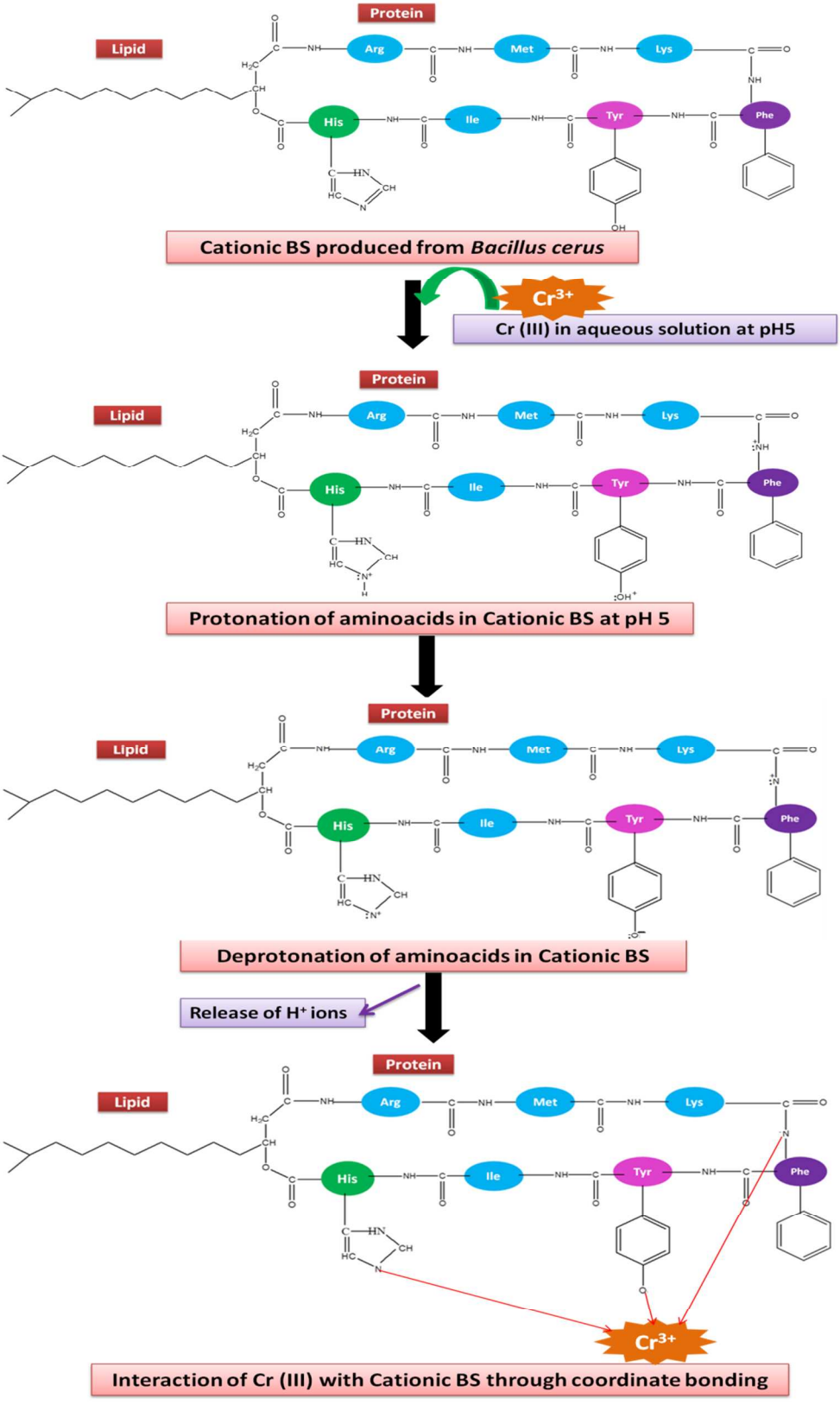

Fig.14: Mechanistic view for removal of Cr (III) by cationic BS in aqueous solution

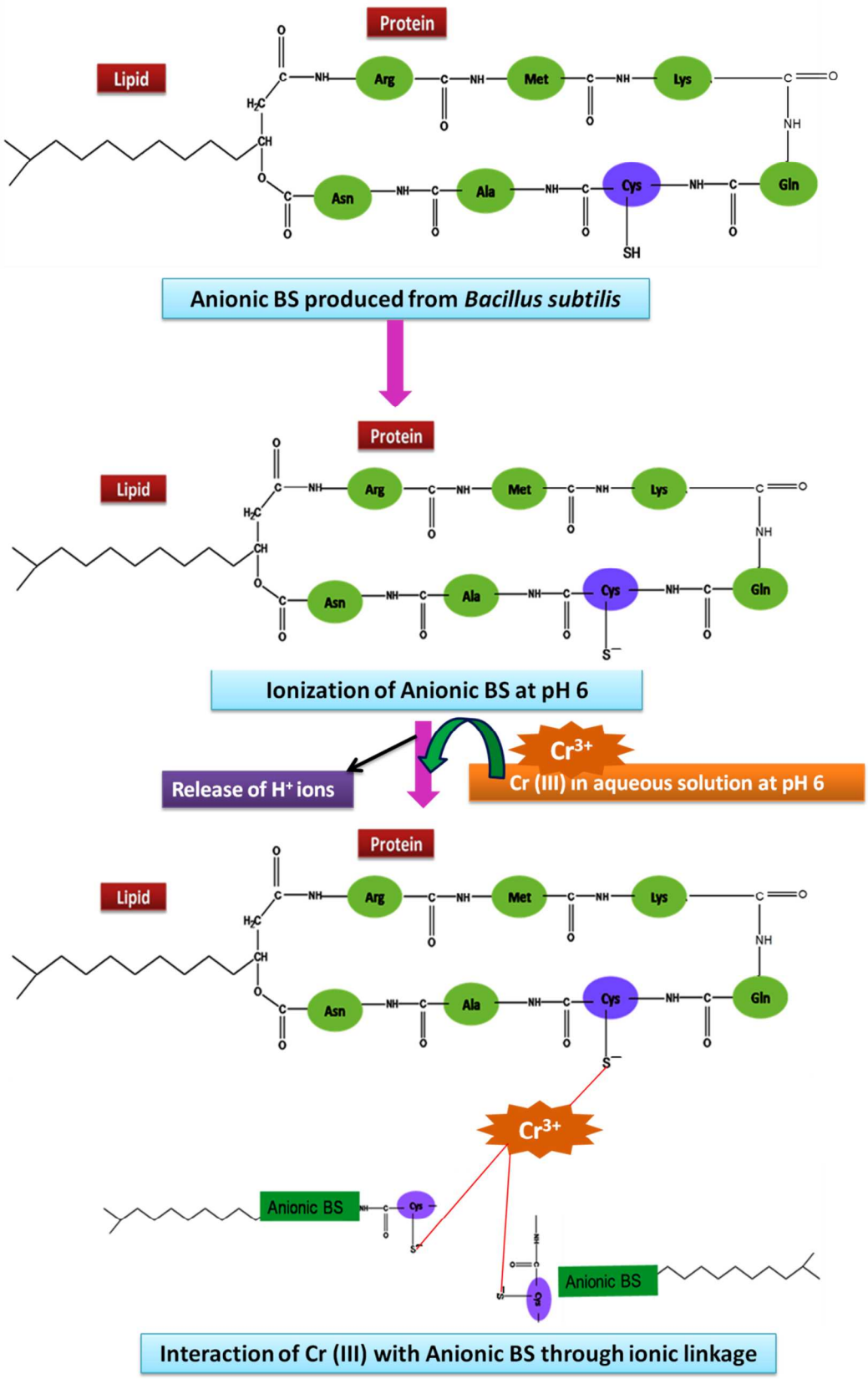

Fig.15: Mechanistic view for removal of Cr (III) by anionic BS in aqueous solution