Screening and Characterization of Microbially Produced Iron Binding Exobiopolymers

Dessertation

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IN

BIOTECHNOLOGY

Under the guidance of:

Dr. MOUSHUMI GHOSH
Associate Professor
Dept. of Biotechnology

Dr. SUDHIR KUMAR SINGH
Assistant Professor
Dept. of Chemical Engineering

Submitted By:

Divya Sharma

Roll No. 601204006

THAPAR UNIVERSITY, PATIALA (PUNJAB)-147004

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CANDIDATE DECLARATION

I, hereby declare that the work presented in this thesis entitled **"Screening and characterization of microbially produced extracellular polymeric substances (EPS) for metal removal''** in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology (DBT), Thapar university, Patiala, is an authentic record of my work during the period of one year from August 2013 to July 2014, under the guidance of **Dr. Moushumi Ghosh**, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

DIVYA SHARMA

(601204006)

CERTIFICATE

This is to certify that the thesis entitled **"Screening and characterization of microbially produced iron binding exobiopolymers"** submitted by Divya Sharma in partial fulfilment of the requirement for the award of Degree of Masters of Technology in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Dr. Moushumi Ghosh
Associate Professor, Supervisor
DBT, TU
Patiala

Dr. Dinesh Goyal
Professor & Head of department
DBT, TU
Patiala

Dr. Sudhir Kumar Singh
Assistant Professor, Co-supervisor
CHED, TU
Patiala

Dr. S.K. Mohapatra
Dean
Academic Affairs,
Thapar University
Patiala

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Date: **DIVYA SHARMA**

Place: **(601204006)**

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ABSTRACT

Human activities release large amounts of toxic elements such as iron in nature which can be harmful to human beings. Exobiopolymers can act as substitutes to other technologies for iron removal as they are more economical, effective and safe alternative to conventional processes. In the present study, removal of iron by exobiopolymers was investigated. Firstly, screening of iron binding by exobiopolymers extracted from eighteen different isolates was evaluated. Out of eighteen different exobiopolymers, five exobiopolymers viz. W1b, P2, Z3, X2 and W2b with high iron binding ability were selected for further studies. Characterization studies showed that the chemical composition of exobiopolymer consisted of sugars, proteins, uronic acids and amino sugars. SEM demonstrated the morphological changes of the exobiopolymers by the binding of metallic cations and EDS spectrum revealed the percentage weight of components present before and after iron binding by exobiopolymers. FTIR analysis indicated the presence of carboxyl, esters, hydroxyl, and sulphate groups of exobiopolymers involved in the metal binding processes. The explaination for adsorption of iron by exobiopolymers is the interaction between metal cations and negatively charged acidic functional groups present on exobiopolymers structure. Optimization studies revealed that the optimal binding efficiency was observed at optimum iron concentration and exobiopolymer concentration of 20 ppm and 5 ppm, respectively, in the case of P2, Z3, X2, W2b and iron concentration of 5 ppm in case of W1b. The optimized results were studied mathematically by Langmuir and Freundlich adsorption isotherms and the parameters were estimated using MATLab computational tool. The maximum adsorption capacities calculated according to isotherms were 3500 mgg^{-1}, 3240 mgg^{-1}, 3220 mgg^{-1}, 3500 mgg^{-1} and 2440 mgg^{-1} of exobiopolymers P2, Z3, X2, W2b and W1b, respectively. This finding could be the powerful step in the development of a low cost biological adsorbent i.e. exobiopolymers in bioremediation process.

Keywords: Exobiopolymers, Adsorption, Adsorption isotherms, Iron.

List of Abbreviations

APHA	American Public Health Association
ED	Electro-dialysis
EDS	Energy dispersive X-ray spectroscopy
EPA	Environmental Protection Agency
FTIR	Fourier Transform infrared Spectroscopy
OD	Optical density
PPM	Parts per million
RO	Reverse osmosis
SEM	Scanning electron microscopy
UF	Ultrafiltration
UV	Ultra violet

List of symbols

α	Alpha
L	Litre
°C	degree(s) Celsius
g	Gram
h	Hour
mL	Millilitre
$mgmL^{-1}$	milligram per millilitre
mgL^{-1}	milligram per litre
μgdm^{-3}	microgram per decimetre
$mgdm^{-3}$	milligram per decimetre
µg	Microgram
μgmL^{-1}	microgram per millilitre
µl	Microlitre
µM	Micromolar
Min	Minute
Mm	Millimetre
Nm	Nanometer
%	Percentage
Rpm	revolutions per minute
S	Second
U	Unit
v/v	Volume/volume
Wt	Weight

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1. INTRODUCTION

Metals are essential minerals for all aerobic and anaerobic organisms. Besides being essential, metals also cause pollution either directly by effluent outfalls from industries, refineries and waste treatment plants and indirectly by the contaminants that enter the water supply from soil/ground water systems (Vijayaraghavan *et al*., 2008). However, it has been proven that large amount of metals seriously affect human health. Some metals usually form compounds that can be toxic, carcinogenic or mutagenic, even in very low concentrations (Manriquez *et al*., 1977; Kazemian *et al*., 2008; Picardo *et al*., 2009). Iron is one of such metal which is commonly found in rocks and soil. Under proper conditions, iron leach into the water resources from rock and soil formations. Exceeding iron concentration than permissible limit i.e. 0.3 mgL^{-1} causes serious health disorders (Shokoohi *et al*., 2009). So, it is essential to remove iron from water to protect the health and environment.

Various conventional treatment processes such as chemical precipitation, ion exchange, and electrochemical removal are employed for metal removal but these processes have significant disadvantages, which are, for instance, incomplete removal, high-energy requirements, and production of toxic sludge (Barakat *et al.*, 2011). Considering these limitations of conventional methods make the alternative biological methods more appealing. Over the last few decades studies on the use of microorganisms for environmental restoration have been primarily focused. Various studies have been done towards exploiting microbial potential for removal of iron contamination in both terrestrial and aquatic systems (Tapai *et al.*, 2011; Llamas *et al*., 2010). Removal of iron from polluted sediments and waste stream employ living and / or non-living microbial biomass or isolated exobiopolymers as agents for adsorption. These relatively simple and inexpensive technologies try to exploit the metal-binding ability of exobiopolymers to form a stable, non-toxic complex. The electrostatic interactions between the metal ligands and negatively charged exobiopolymers lead to formation of stable complexes. Microbial exobiopolymers differs both in specificity and

metal-adsorption ability with only few functional groups potentially involved in metal-binding. In spite of constraint, the ease of application of purified exobiopolymers in water treatment has demonstrated efficiency in several studies which have led to a large number of reports favoring this approach.

Microbial exobiopolymers from diverse sources are chemically complex and comprised of a variety of high molecular weight organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids along with other non-polysaccharide constituents of low molecular weight. Due to their many interesting physical and chemical properties e.g. stabilizing, suspending, thickening, gelling, coagulating, film-forming and water retention capability, polysaccharides have found applications in many industrial sectors and removal of metals from industrial wastes (Moppert *et al*., 2009). The production of exobiopolymers is the general property of microorganism in natural environment and has been shown to occur in both prokaryotes (bacteria, archaea) and in eukaryotes (algae, fungi).

The present study aimed to explore the potential of exobiopolymers for iron binding. It is expected that the interaction of exobiopolymers, through active functional groups, with iron in solution will cause adsorption (Sandell *et al*., 2006). The adsorption and binding of iron onto exobiopolymer has been introduced as a cost-effective method in multiple studies (Boettcher *et al*., 1991; Shokoohi *et al*., 2009; Moppert *et al*., 2009; Tapia *et al*., 2011). Its use has carried the advantages of reducing dissolved metals related toxicity, improving operating conditions and cost-benefit utility maintenance including nutrient supply. The more selective adsorption performance of exobiopolymers can be studied by establishing the adsorption isotherms of the exobiopolymer with iron which are evaluated through MATLab.

Objectives and Scope of the study:

The objectives of this study are

(i) Screening and characterization of potential exobiopolymers on the basis of their nature,

(ii) Optimization and evaluation of the iron removal efficiency of exobiopolymers and

(iii) Study Langmuir and Freundlich isotherms parameters of the exobiopolymers by using MATLab.

The present study provides a promising approach for the reduction of iron from water. This could be proved as better means for the removal of iron from water based on binding efficiencies of exobiopolymers, instead of the conventional treatments and generates novel idea for water treatment.

2.1) Presence of metals in water

Water of high and acceptable quality is essential to human life for agriculture, industrial, domestic and commercial uses. Due to improper waste disposal, the water resources are becoming unfit for use. Metals are the natural component of water. These are essential minerals for all aerobic and most anaerobic organisms. There are varied sources of metals in water as through industries like mining operations, paper industries, fertilizer industries, batteries, tanneries and pesticides, etc. or by consumer waste or, even from acidic rain breaking down soils etc. releasing metals into streams, lakes, rivers, and groundwater (Sag *et al*., 1996; Selatnia *et al*., 2004). Unlike organic contaminants, metals are not biodegradable and tend to accumulate in living organisms termed as bioaccumulation. So compounds accumulate in living things, any time they are taken up and stored faster than they are broken down (metabolized) or excreted. The damage that they do is on the cellular level, and can cause cancer and many other diseases (Renge *et al.,* 2012**)**. There are certain elements or compound present in water include sodium, magnesium, nitrate, chloride, sulphate, iron, manganese, etc. Table 2.1 shows the advantages and limitations of these metals.

Iron is the second most abundant element in earth's crust and fourth most abundant of all elements. Typical concentration of iron-containing substances environmentally (and biological) system is given in Table 2.2.

Iron is an essential nutrient required for all living cells of plants and animals for their metabolism in trace quantities. It is vital for almost all living organisms due to the fact that it is present in a wide variety of metabolic processes, including, DNA synthesis, oxygen transport and electron transport (Lieu *et al*., 2001). Iron deficiency in human adults is manifested clinically by fatigue, palpitation on exertion and sometimes by a sore tongue, angular stomatitis and dysphasia.

Table 2.1: Advantages and limitations of metals present in water

Metals	**Advantages**	**Limitations**
Sodium	Regulate blood pressure, Helps nerves and muscles properly, Helps sustain a regular blood pH level.	Increased blood pressure can ultimately lead to heart disease, heart failure or stroke.
Magnesium	Maintain heart beating, Support a healthy immune system, Keeps bones strong.	Excess the limit amount cause irregular heartbeat, low blood pressure, slowed breathing, coma, and death.
Nitrate	Use as inorganic-fertilizers, Added in food to serve as source of nitrite	Cause methemoglobinemia also known as blue-baby syndrome
Iron	Help in variety of metabolic processes such as oxygen transport, DNA synthesis, and electron transport	Excess over the limit cause tissue damage a genetic disorder anorexia, diarrhoea

Table 2.2: Typical concentrations of iron containing substances in environmental and biological systems

Location	**Concentration**
Earth's crust (by weight)	5%
Sea water (by weight)	
Surface	0.01-0.1 μgdm^{-3}
Deep	0.1-0.4 μgdm^{-3}
Stream water (by weight)	0.01-0.1 μgdm^{-3}
Drinking water (by weight)	0.01-10.0 $mgdm^{-3}$
Human body(by weight)	
Blood	60 $mgdm^{-3}$
Average	450 $mgdm^{-3}$
Atmosphere	0.5 μgm^{-3}

Iron is normally found in steel mills, spent pickle and etch baths from plating shops, foundries, wire drawing operations and chemical milling (Tapia *et. al*, 2011). It is also found

in groundwater. Iron exists in two inorganic forms, unstable divalent Fe(II) and stable trivalent Fe(III) ions. Iron in water is normally found in the ferrous state (Fe(II)). Iron enters into water bodies in the form of Fe(II), which can be easily oxidized to Fe(III) ions by the oxygen dissolved in water and then hydrolyse with water. According to EPA, the acceptable value of iron in drinking water is 0.3 mgL^{-1} (EPA, 2000; Ncibi *et al*., 2007). Excessive iron concentration leads to tissue damage as a result of formation of free radicals (Burtis *et al.*,1994) and also it is associated with haemochromatosis, a genetic disorder of iron metabolism, characterized by a brown discoloration of the skin and other organs, life-threatening problems such as diabetes, anorexia, diarrhoea, diphasic shock, metabolic acidosis and death, vascular congestion of the gastrointestinal tract, brain, spleen and thymus, heart failure and poor growth (Namdeo *et al*., 2008; Yavuz *et al*., 2005). Due to all these ill effects, there is a need for removal of iron from water bodies.

2.2) Methods of iron removal from water

In recent years, widespread concern over the cumulative toxicity and environmental impact of metals has lead to extensive research into developing effective alternative technologies for the removal of these potentially damaging substances from effluent and industrial wastewater. Physicochemical and biological methods have been proposed and applied to remove metal ions from effluents.

2.2.1) Physiochemical Methods

Physical and chemical methods for the removal of metal ions from aqueous solution include chemical precipitation, ion-exchange, chemical oxidation/reduction, reverse osmosis, electrodialysis, ultra filtration, etc., which have their own advantages and limitations (Eccles *et al*., 1999).

2.2.1.1) Chemical Precipitation

Chemical precipitation is the most widely used method for removal of metals from inorganic effluent (Ku *et al.*, 2001). Lime and limestone are the most commonly employed precipitant agents due to their availability and low-cost in most countries (Mirbagherp *et al*., 2004; Aziz *et al*., 2008). The advantages of using chemical precipitation include the simplicity of the process, inexpensive equipment requirement, and convenient and safe operations. Apart from advantages this method has limitations also, as it requires a large amount of chemicals to reduce metals to an acceptable level for discharge. Other drawbacks are its excessive sludge production that requires further treatment, slow metal precipitation, poor settling, the aggregation of metal precipitates, and the long-term environmental impacts of sludge disposal (Wang *et al*., 2004; Kurniawan *et al*., 2006).

2.2.1.2) Ion Exchange

It is one of the methods used successfully in the industry for the removal of metals from effluent. Commonly used matrices for ion exchange are synthetic organic ion exchange resins (Kang *et al*., 2004). Ion-exchange processes have been widely used to remove metals from wastewater due to their many advantages, such as high treatment capacity, high removal efficiency and fast kinetics (Alyuz *et al.*, 2009). However, ion-exchange resins must be regenerated by chemical reagents when they are exhausted and the regeneration can cause serious secondary pollution. And it is expensive; especially when treating a large amount of wastewater containing metal in low concentration, so they cannot be used at large scale it cannot handle concentrated metal solution as the matrix gets easily fouled by organics and other solids in the wastewater. Moreover ion exchange is non-selective and is highly sensitive to the pH of the solution (Barakat *et al.*, 2011).

2.2.1.3) Ultra-filtration

Ultrafiltration (UF) is a membrane technique working at low transmembrane pressure for the removal of dissolved and colloidal material (Landaburu-Aguirre *et al*., 2009). The advantages include high removal efficiency, high binding selectivity and highly concentrated metal concentrates for reuse, etc. The limitations include the decrease in performance of UF due to membrane fouling which hindered it from a wider application in wastewater treatment as it cause adverse effects on the membrane system such as flux decline, an increase in transmembrane pressure and the biodegradation of the membrane materials. These effects result in high operational costs for the membrane system (Barakat *et al*., 2011).

2.2.1.4) Reverse osmosis

Reverse osmosis (RO) is one of the techniques able to remove a wide range of dissolved species from water. RO process uses a semi-permeable membrane, allowing the fluid that is being purified to pass through it, while rejecting the contaminants (Shahalam *et al*., 2002). It accounts for more than 20% of the world's desalination capacity. RO is an increasingly popular wastewater treatment option in chemical and environmental engineering (Abu-Qdaisa *et al*., 2004). The major drawback of RO is the high power consumption due to the pumping pressures, and the restoration of the membranes, high pressures membrane scaling and expensive (Fu *et al*., 2011).

2.2.1.5) Electrodialysis

Electrodialysis (ED) is another membrane process for the separation of ions across charged membranes from one solution to another using an electric field as the driving force. In most ED processes, ion exchange membranes are used. The membranes are actually of two basic types: cation-exchange and anion-exchange membranes (Chen *et al.*, 2004). This process has been widely used for the production of drinking water from brackish water and seawater, treatment of industrial effluents, recovery of useful materials from effluents and salt

production. The results showed that increasing voltage and temperature improved cell performance; however, the separation percentage decreased with an increasing flow rate. At concentrations of more than 500 mgL^{-1}, dependence of separation percentage on concentration diminished (Sadrzadeha *et al.*, 2009).

The benefits of physiochemical methods, however, are outweighed by a number of drawbacks as discussed above. The task of providing proper treatment facility for all water contamination sources is difficult and also expensive, hence demanding for innovative technologies which require low maintenance, affordable and are energy efficient. Therefore, search for alternate and innovative treatment techniques has focused attention on the use of biological materials for metals removal and has gained important credibility during recent years because of the good performance and low cost of this complexing materials.

2.2.2) Biological methods

Capability of microorganisms to bind metals in aqueous solutions has long been of scientific interest. According to research various biological materials like live and dead cells, DNA, outer-membrane of micro-organisms, microbial envelope and microbially derived exobiopolymers used for metal removal (Emtiazi *et al*., 2004). On the basis of biological material used, biological process for removal of metal ion from aqueous solutions can be divided into three general categories:

(a) Adsorption of metal ions onto the surfaces of microbially produced substances.

(b) Intracellular uptake of metal ions.

(c) Chemical transformation of metal ions by microorganisms.

The latter two processes require live organisms to proceed (Veglio *et al*., 1994; Mamisahebei *et al.*, 2007). In addition to direct use of microbial biomass, their by-products including exobiopolymers may be applied as a low cost method of metal-removal process. The use of exobiopolymers have carried the advantages of reducing dissolved metals related

toxicity, improving operating conditions, and cost-benefit utility maintenance including nutrient supply (Hua-Jun *et al.*, 2008) and adsorption of metals on these substances has been proposed as an efficient and potentially cost effective tool for metal enriched industrial effluents in multiple studies. The adsorption technique is economically favourable and technically easy to separate, as the requirement of the control system is limited and is being widely used by various researchers (Shokoohi *et al*., 2009). In present work exobiopolymers were used as efficient adsorbent for binding of metal.

2.3) Exobiopolymers

Exobiopolymers are the substances produced by microorganisms during cellular lysis and hydrolysis of macromolecules during growth. They are composed of a variety of organic substances. Exobiopolymers are mainly high-molecular-weight compounds. Carbohydrate has been identified as the predominant constituent in the exobiopolymers of many pure cultures (Cescutti *et al*., 1999; Sutherland and Kennedy, 1996), whereas protein was found in substantial quantities in the sludges of many wastewater treatment reactors (Fang and Jia, 1996; Veiga *et al*., 1997). Humic substance (Frolund *et al*., 1995), uronic acid and deoxyribonucleic acids (DNA) (Tsuneda *et al*., 2001; Zhang *et al*., 1999) were also detected in exobiopolymers; however, information about their concentration in exobiopolymers is scarce (Liu *et al*., 2002). Many exobiopolymer-producing microorganisms including bacteria, fungi, yeast, and algae have since been isolated from soil and wastewater. Generally, soil and activated sludge samples are the best sources for isolating exobiopolymers-producing microorganisms (Salehizadeh *et al*., 2001). Due to their many interesting physico-chemical and rheological properties with novel functionality, the microbial exobiopolymers act as new biomaterials and find wide range of applications in many industrial sectors like detergents, adhesives, microbial enhanced oil recovery (MEOR) wastewater treatment, brewing, downstream processing, cosmetology, adhesives, pharmacology, textiles and as food

additives (Kurane, *et al*., 1994). Research has demonstrated the adsorption ability of exobiopolymers which have been extracted from bacterial cultures. Adsorption process by exobiopolymers offers some interesting advantages such as:

(i) low operating cost,

(ii) high efficiency in removing metals even from very dilute solutions,

(iii) the possibility of recovering the valuable metals adsorbed by the biosorbent, and

(iv) a lower amount of metal containing biological sludges that has to be disposed of after treatment (Kratochvil *et al*.,1998).

These exobiopolymers contain ionizable functional groups such as carboxyl, amine, sulphate, acetate, and hydroxyl groups which enable these exobiopolymers to bind metals. Various authors investigated exobiopolymers to be effective in binding metals and two type of mechanism involved:

(i) Ion exchange due to high amount negatively charged functional groups like carboxyl, phosphate and sulphate groups in exobiopolymers (Sutherland *et al*., 1984),

(ii) Complexation with negatively charged functional groups such as hydroxyl group, amino group, carboxylic group, phosphoryl and sulphate group (Brown *et al*., 1982).

2.4) Mechanism of metal binding by exobiopolymers involving adsorption

The mechanism of bacterial exobiopolymer adsorption on metal surfaces including hydrophobic, electrostatic, covalent interactions has been studied by several soil and environmental chemists over the past years. Recent research on the binding of iron to exobiopolymers indicated that carboxylic groups are the dominant functional groups involved in the complex of Fe(III). The electrostatic interactions between the metal ligands and negatively charged exobiopolymers outside the cells lead to formation of stable complexes. Exobiopolymers were assumed to have multiple metal binding sites with different binding strengths (Rudd *et al*., 1984a) and have a statistical distribution of binding constants (Kellems

et al., 1989). These modeling approaches are likely necessitated by the fact that exobiopolymer posses multiple functional groups to which sequentially bind, first occupying high binding energy site configurations and subsequently weaker site configurations as the metal concentration in solution is increased. The factors being able to affect metal adsorption are the ones having an influence on the environment of binding sites or on their chemical nature (Artola *et al*., 1998). Metal content of wastewater, pH, ionic strength, and temperature of the solution, surface properties, exobiopolymers composition, metals speciation (Rosin *et al*., 1982) are referenced as affecting metal binding. The efficient metal binding ability of exobiopolymers has been focused on. Future reseaches may be foccused on the physio-chemical nature of exobiopolymers and their influential mechanism, to serve as theoritical and technical basis for the application of exobiopolymers.

2.5) Equilibrium Isotherms and Modeling

Adsorption modeling can be used to guide experimental research, optimize a given process and provide a basis for process control strategies. Computer simulations can replace numerous tedious tests. However, the simulations are only as good as the model behind them. Langmuir and Freundlich models parameters were determined by using MATLab software. This software includes mathematical modeling and computer simulation which is an extremely powerful tool for adsorption studies. It is essential for process design and optimization. It allows a combination of equilibrium and dynamic studies information which cannot be appropriately handled without modeling. When reaction kinetics is combined with mass transfer studies which in turn are dependent on fluid flow properties, modeling allows simple comprehension of the data. Improved knowledge of mathematical modeling and simulations can allow this area to be dependable (Volesky *et al*., 2001). The adsorptive metal uptake can be quantitated by experimental adsorption equilibrium isotherms. The isotherm graphical expression is a plot of the metal uptake (q_e) by the adsorbent (weight/weight or

mole/weight units) against the residual concentration (C_{eq}). This graph is mostly hyperbolic because the uptake increases gradually and then levels off as it nearly completes saturation at high concentration of the adsorbate (metal). Uptake of the metal (q_e) in milligrams of metal per gram of exobiopolymer is calculated by the formula:

$$q_e = \frac{(C_0 - C)V}{M} \quad \text{(i)}$$

Where: 'q_e' (mgg^{-1}) is the amount of iron adsorbed by biomass; 'C_0' and 'C' (mgL^{-1}) are the initial and equilibrium liquid-phase concentrations of iron, respectively; 'V' represent the initial volume of iron solution in litre, and 'M' the mass of the biomass in gram. The metal removal percentage (R%) was calculated as:

$$R(\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad \text{(ii)}$$

where (R%) is the ratio of difference in metal concentration before and after adsorption and 'C_t' is the iron concentration at time 't' (Xing *et al*., 2008).

There are two widely accepted and easily linearized adsorption isotherm models proposed by Langmuir and Freundlich, respectively. The assumptions of the Langmuir model are:

- The surface consists of adsorption sites.
- All adsorbed species interact only with the sites and not with each other.
- Adsorption is limited to a monolayer.
- Adsorption energy of all sites is identical and independent of the presence of adsorbed species on the other adsorption isotherm (Oremusova *et al*., 2007).

A general form of the Langmuir model equation is

$$q_e = \frac{bC_e\, q_{max}}{(1+bC_e)} \quad \text{(iii)}$$

where 'q_e' is the amount of solute (metal) adsorbed per unit mass of adsorbent, expressed as mgg^{-1}; 'q_{max}' is the amount of metal adsorbed at saturation, per unit mass of adsorbent; 'b' is an equilibrium constant, related to the energy of adsorption; and 'C_e' is the equilibrium (final) concentration of the metal in the solution, expressed as mgL^{-1} (Langmuir, 1918). Low values of 'b' are reflected in the steep initial slope of an adsorption isotherm, indicating a desirable high affinity. Thus for "good" adsorbents, a high 'q_{max}' and a steep initial isotherm slope is desired. The essential characteristics of the Langmuir isotherm can be expressed in terms of dimensionless constant separation factor or equilibrium parameter, 'R_L', which is defined by equation (Hall *et al*., 1996).

$$R_L = \frac{1}{1+bC_0} \qquad \text{(iv)}$$

where: 'C_0' (mgL^{-1}) is the highest initial iron concentration. According to the value of R_L the isotherm shape can be interpreted as presented in Table 2.3 (Langmuir, 1918).

Table 2.3: Langmuir isotherm constant parameter

R_L value	**Type of isotherms**
$R_L > 1$	Unfavorable
$R_L = 1$	Linear
$R_L < 1$	Irreversible
$0<R_L<1$	Favorable

Freundlich adsorption isotherm

$$q_e = K_f C_e^{1/n} \qquad \text{(v)}$$

which was linearized as

$$\ln q_e = \ln K_f + n^{-1} \ln C_e \qquad \text{(iv)}$$

where 'q_e' is the amount of metal adsorbed per unit mass of adsorbent, expressed as mgg^{-1}; 'K_f' is a constant, related to the adsorbent capacity; '*n*' is a constant related to the energy of

sorption; and 'C_e' is the equilibrium (final) concentration of the metal in the solution, expressed as mgL^{-1} (Freundlich, 1906). The value of '*n*' indicates the degree of nonlinearity between solution concentration and adsorption as follows: if $n=1$, then adsorption is linear; if $n<1$, then adsorption is a chemical process; if $n>1$, then adsorption is a physical process. Both of these models are able to describe many data effectively. Since the parameters of these models do not have a physical interpretation to them, their use is limited (Mark *et al.*, 2006; Volesky *et al*., 1990).

3.1) Chemicals and Media

All chemicals and reagents used for microbiological and chemical determination were of highest analytical grade and purchased from Sigma. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (USA) and Hi-media (Mumbai, India). Autoclaved the media solutions at 121℃ and 15 psi for 15 min and were allowed to cool below 50℃ before use. The screening medium referred as BPB medium (Biopolymer Producing broth) (Ghosh *et al*., 2009) (Annexure I).

3.2) Extraction and Purification of exobiopolymers

Eighteen previously isolated strains from various industrial and river sites were used in the present study. The selected strains for exobiopolymers production were inoculated in 1 L BPB media (Annexure I) and incubated in a rotary shaker (120 rpm/min) (Labcon, 5081U, USA) for 48 h at 37℃. The cells were removed from culture solution by high speed centrifugation (CF15RX11, Hitachi, Japan) at 10,000 rpm for 10 min at 4℃. The supernatant was collected and from the supernatant, exobiopolymer was precipitated by addition of two volumes of chilled ethanol (95% ethanol) and kept overnight at 4℃. The precipitate was separated from ethanol suspension by centrifugation at 12,000 rpm for 20 min at 4℃ and lyophilized to form powder.

3.3) Characterization of exobiopolymers

3.3.1) Biochemical Characterization

3.3.1.1) Determination of total Protein

The total protein content of the purified exobiopolymers was determined by the method of Folin-Lowry (Lowry *et al*., 1951) by using Bovine serum albumin (BSA) as standard. Different concentrations of BSA (0.1-0.5 $mgmL^{-1}$) and exobiopolymers (1 $mgmL^{-1}$) were prepared. 1 mL of freshly mixed complex-forming reagent C (Annexure I) was added to 0.2 mL of the exobiopolymer and BSA. The solution was left undisturbed for 10 min at room

temperature. Then 0.1 mL of Folin reagent was added and mixture left undisturbed at room temperature for 30 min. The absorbance was taken at 750 nm. The amount of total protein present in the sample was calculated from the calibration curve (Annexure II).

3.3.1.2) Determination of total Sugars

The total sugar or carbohydrate content of exobiopolymer was determined by the method of phenol sulphuric acid (Dubois *et al*., 1986) by using glucose as standard. Different concentrations of glucose (0-1 $mgmL^{-1}$) and exobiopolymer (1 $mgmL^{-1}$) were prepared. 200 µl of phenol reagent (5 % v/v in water) was added to 200 µl exobiopolymer as well as glucose. After the addition of phenol reagent, 1 mL of concentrated sulphuric acid was rapidly added to the surface of the solution without touching the sides of the test tube. The tubes were left undisturbed for 10 min at room temperature. After incubation, the tubes were shaken vigorously and absorbance was taken after 30 min at 490 nm. The amount of total sugar present in the sample was calculated from calibration curve (Annexure II).

3.3.1.3) Determination of Amino sugars

The total amino-sugars content of exobiopolymer was determined by the method described by Elson-Morgan, (1934) by using galactosamine as standard. Different concentrations of galactosamine (0-1 $mgmL^{-1}$) and exobiopolymers (1 $mgmL^{-1}$) were prepared. 50 µl of reagent A (Annexure I) was mixed with 250 µl of galactosamine and exobiopolymer. Then each mixture was heated to 100ºC for 3 min. After rapidly cooling to room temperature, 1.5 mL of reagent B (Annexure I) was added. The mixture was incubated at 37ºC for 20 min. After cooling to room temperature, the absorbance was determined at 585 nm. The aminosugars content present in the sample was calculated from calibration curve (Annexure II).

3.3.1.4) Determination of Uronic Acid

The uronic acid content of exobiopolymer was determined by method described by Haug and Larsen, (1962) by using D-glucuronic acid as standard. Different concentrations of D-

glucuronic acid (0-1 $mgmL^{-1}$) and exobiopolymer (1 $mgmL^{-1}$) were prepared. 1.5 mL of ice cold reagent A (Annexure I) was carefully added in 250 µl of exobiopolymer and D-glucuronic acid with mixing and cooling in ice bath. The mixture was heated at 100ºC for 10 min and rapidly cooled in the ice-bath. 50 µl of reagent B (Annexure I) was added and mixed well. Reheating was done at 100ºC for 15 min. The mixture was cooled rapidly to room temperature and the absorbance was determined at 525 nm. The uronic acid content present in the sample was calculated from calibration curve (Annexure II).

3.3.1.5) Determination of Pyruvic Acid

The pyruvic acid content of exobiopolymer was determined by method described by Friedman and Haugen, (1943) by using pyruvic acid as standard. Different concentrations of pyruvic acid (0-3 $mgmL^{-1}$) and exobiopolymer (1 $mgmL^{-1}$) were prepared. Initially, exobiopolymer was treated with perchloric acid (50%) for deproteinization and was kept at 30ºC for 30 min. Then 1 mL of DNP reagent (Annexure I) was shaken to the extract. Further, 4 mL of water and 10 mL 2.2 N NaOH were added. The tubes were shaken and absorbance was taken at 416 nm. The pyruvic acid content present in the sample was calculated from calibration curve (Annexure II).

3.3.2) Structural and functional characterization

3.3.2.1) X-Ray diffraction spectroscopy

X-ray diffraction analysis of powder exobiopolymer was carried out with X-ray diffractometer (Xpert pro, Panalytical, United States). Copper Kα radiation was used with a wavelength of 1.54Å. The scan rate was measured between 5° and 85°.

3.3.2.2) Scanning Electron Microscopy (SEM) and Microanalysis by Energy-Dispersive X-Ray (EDX)

SEM analysis was done to obtain surface properties and EDX was performed to evaluate the amount of iron bound by exobiopolymer. Samples of native exobipolymer and iron bound

exobiopolymers were coated with a conductive layer of gold and then analyzed by SEM (6510-LV, JOEL, Japan) at an accelerating voltage of 20.0 kV, complemented with an energy-dispersive X-ray microanalyzer (EDS) (INCA x_act, Oxford Instrument, United Kingdom).

3.3.2.3) Fourier Transform- Infrared spectroscopy

Fourier Transform–Infrared spectrometer (Synthesis monitoring system, Perkin Elmer, USA) was employed to examine the interaction between exobiopolymer and iron. The spectrum of native exobiopolymer and iron bound exobiopolymer was recorded on the spectrometer over a wave number range of 400- 4000 cm^{-1}.

3.4) Metal binding Assay

The metal binding ability of exobiopolymer was determined. Initially, stock solution (100 ppm) of iron was prepared from metal salt ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$). Working solutions of desired concentrations were prepared by diluting the stock solution. Exobiopolymer dilutions were prepared from 1000 ppm stock solution. The binding experiment was performed by mixing 1 mL iron solution with 1 mL of exobiopolymers solution in test tube. The mixture solution was incubated at room temperature until equilibrium. The suspensions were then filtered by using syringe filter apparatus. The concentration of ferrous ions in the filtrate was analyzed by phenothroline method (APHA, 2012). Reagents include 5.0×10^{-3} M phenthroline solution (0.1 mL), 1.2 M hydroxylamine hydrochloride (0.01 mL), and 2 M sodium acetate (0.08 mL) was added in filtrate (1 mL). The reaction between ferrous ion and 1,10-phenanthroline to form a red complex at pH 3.2 to 3.3 serves as a sensitive method for determining iron.

$$Fe^{2+} + 3\ phen \rightarrow Fe^{+2}(phen)_3$$

The molar absorptivity of the complex, $[(C_{12}H_8N_2)_3Fe]^{2+}$, is 11,100 at 510 nm. The intensity of the color is independent of pH in the range 2 to 9. The complex is very stable.

The colored solution obeys Beer's law and its intensity does not change appreciably over long periods of time. The iron must be in the ferrous state, and hence a reducing agent is added before the color is developed. Hydroxylamine, as its hydrochloride, can be used to reduce any ferric ion that is present:

$$2\ Fe^{3+} + 2\ NH_2OH + 2\ OH^- \rightarrow 2\ Fe^{2+} + N_2 + 4\ H_2O$$

The pH is adjusted to a value between 6 and 9 by addition of ammonia or sodium acetate (Sandell *et al*., 1959). Blank solutions (without exobiopolymer) were treated similarly and under control condition. The removal of iron by exobiopolymer in the supernatant was measured using a spectrophotometer (HITACHI, U-2900, Japan) at a 520 nm wavelength and computing from the calibration curves (Annexure II).

3.5) Optimization studies for maximum iron-exobiopolymer binding

To study the effective exobiopolymer concentration, experiments were carried out using different concentrations of exobiopolymer i.e. 1 ppm, 3 ppm, 5 ppm, 10 ppm and 25 ppm. Different concentrations of iron (0.1 ppm, 1 ppm, 5 ppm and 20 ppm) were prepared. Experiment was performed according to metal binding assay.

3.6) Optimization studies for contact time

This experiment was conducted to analyze how long exobiopolymer would take to adsorb maximum amount of iron. Exobiopolymer and iron (standard solution) were mixed, kept undisturbed at room temperature at varied time intervals. At the interval of 5, 10 15, 30 and 60 min, 1 mL sample was withdrawn and mixed with phenthroline, hydroxylamine hydrochloride and sodium acetate. Absorbance was taken at 520 nm.

3.7) Metal binding capacity

In order to successfully represent the kinetic behaviour of any species from the fluid to the solid phase (Passos *et al*., 2008) Langmuir and Freundlich adsorption isotherms were used to analyze the binding efficiency of exobiopolymer. Adsorption equilibrium (the ratio between

the adsorbed amount with the remaining in the solution) is established when an adsorbate has been contacted with the adsorbent for sufficient time (Kumar *et al.*, 2007*)*. Adsorption capacity of the exobiopolymer at equilibrium (q_e, $mgml^{-1}$) was calculated by using the following equation:

$$q_e = \frac{(C_o - C_e)V}{M}$$

where, 'C'_o and 'C_e' are initial and final sample absorbance (O.D) respectively, 'V' is the volume of the sample solution and 'M' is the weight of adsorbent added. Langmuir model is, two parameters isotherm. It was originally developed to describe gas-solid-phase adsorption onto activated carbon but now it is been used to quantify the performance of different biological adsorbent (Langmuir *et al*., 1965). This model assumes that the adsorbed layer is one molecule in thickness (monolayer adsorption), and adsorption can only occur at a finite (fixed) number of definite localized sites (Vijayaraghavan *et al*., 2006). Freundlich isotherm is an empirical equation. The Freundlich model is based on adsorption on a heterogeneous surface.

The linear form of Langmuir equation,

$$q_e = \frac{bC_e\, q_m}{(1+bC_e)}$$

where 'q_e' is the amount of solute (metal) adsorbed per unit mass of adsorbent, expressed as mgg^{-1}; 'q_{max}' is the maximum amount of metal adsorbed at saturation, per unit mass of adsorbent; 'b' is an equilibrium constant (Langmiur constant), related to the energy of adsorption; and 'C_e' is the equilibrium (final) concentration of the metal in the solution, expressed as mgL^{-1}.

Freundlich equation, $$q_e = K_f C_e^{\,1/n}$$

where 'q_e' is the amount of metal adsorbed per unit mass of adsorbent, expressed as mgg^{-1}; 'K_f' is a constant, related to the adsorbent capacity, larger K_F value reflects a larger overall

adsorption capacity. On the other hand; *n* is a constant related to the energy of sorption; and 'C_e' is the equilibrium (final) concentration of the metal in the solution, expressed as mgL^{-1}. The parameters of Langmuir and Freundlich isotherms were evaluated by employing computational software MATLab.

4. RESULTS AND DISCUSSION

Iron is present in environment in favourable concentration but the improper dispose either industrially or domestically, increases the concentration of iron in water which is responsible for causing serious health hazards. So it becomes necessary to eliminate iron from water bodies. Exobiopolymers have the ability to bind with variety of metals. The present study contributes to the iron binding efficiency of exobiopolymers produced by the microorganisms isolated from various industrial and environmental sites. Eighteen powdered anionic exobiopolymers were extracted from eighteen different isolates and screened for their iron binding capability. Anionic nature of exobiopolymers was due to the precipitation of acidic groups (pyruvate, succinate, urinate, acetate or sulphate) by cetyl pyridinium chloride (CPC). These acidic groups may play an important role in causing the anionic (or acidic) charge on the polysaccharide (Pace *et al*., 1980; Sutherland, 1997).

4.1) Screening of iron binding exobiopolymers

Table 4.1 shows the percentage of iron reduction. Screening of eighteen different exobiopolymers was done at initial iron and exobiopolymer concentration of 100 ppm and 1000 ppm, respectively. Results revealed that out of the total eighteen exobiopolymers, five exobiopolymers (W1b, P2, Z3, X2 and W2b) with high binding efficiency of >60% were selected for further studies.

4.2) Characterization of Exobiopolymers

4.2.1) Physical and Biochemical characterization of Exobiopolymer

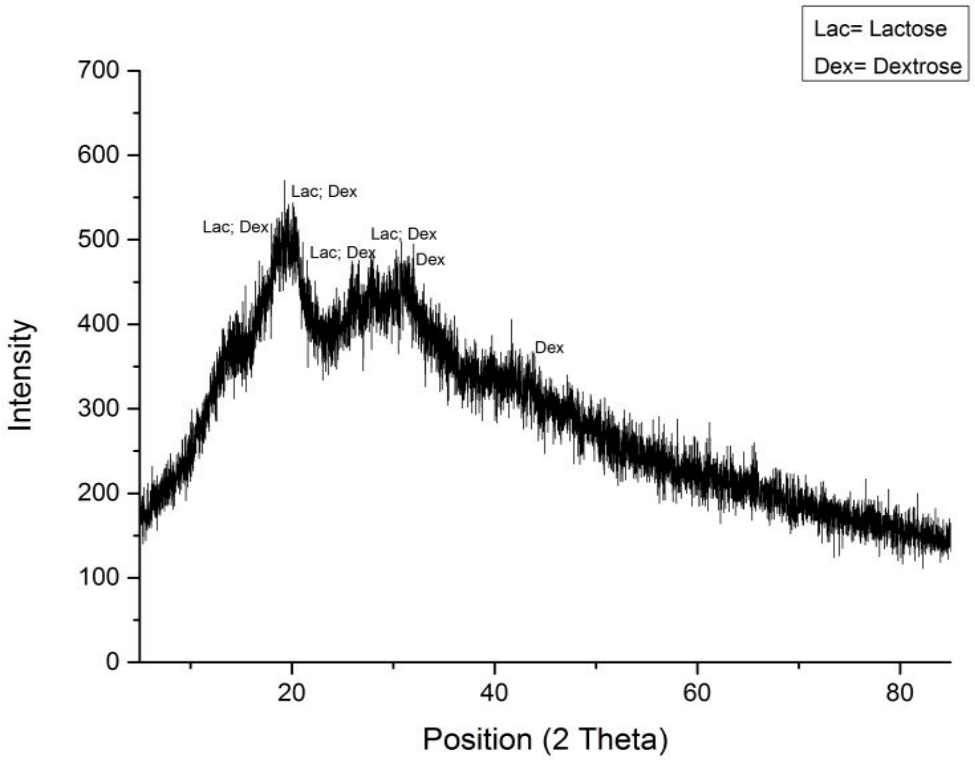
4.2.1.1) X-ray Diffraction

X-ray diffraction (XRD) is a very powerful analytical technique widely used for phase identification of materials. However, it is difficult to interpret broad amorphous peaks of several amorphous exobiopolymers in X-ray scattering profile (Shimazu *et al.,* 2000) while the interpretation of narrow crystalline exobiopolymers is easy.

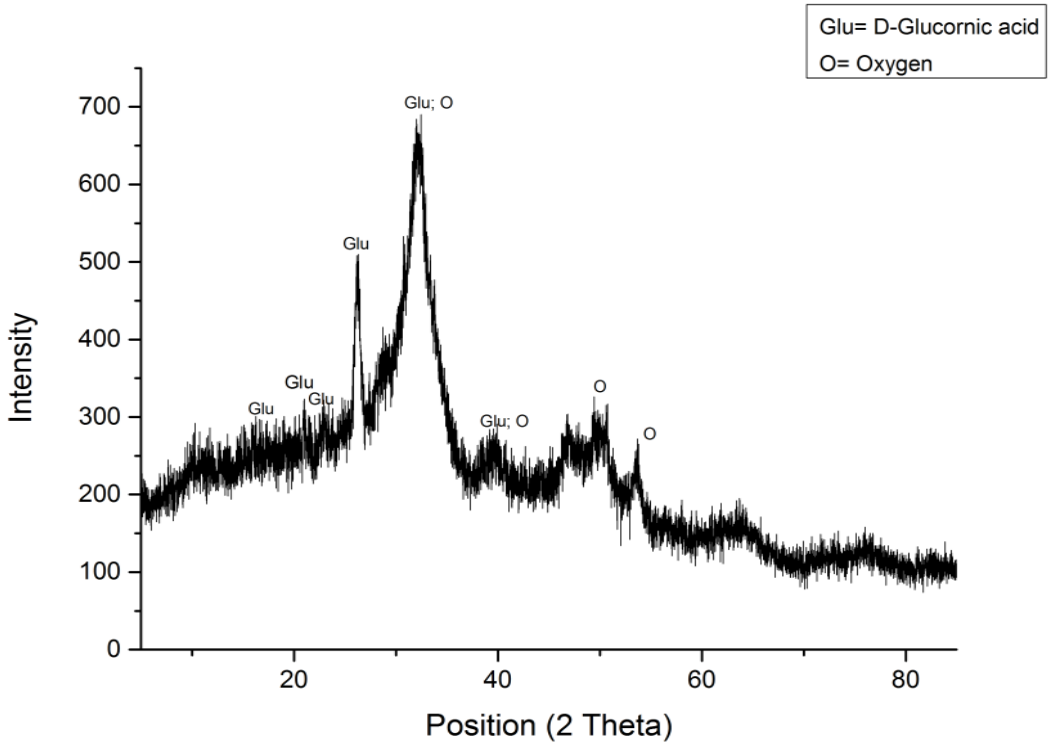
Table 4.1: Different exobiopolymers produced by the isolates with their percentage removal

Exobiopolymers	Iron removal (%)
W1b	59
P6a	40
P2	60
W1a	20
Z4	10
W3	22
O4	45
FM	25
Y1	50
R2MGa	19
Z3	57
X2	62
Y3	15
L2	47
W2b	75
L4	52
P8	51
W3	43

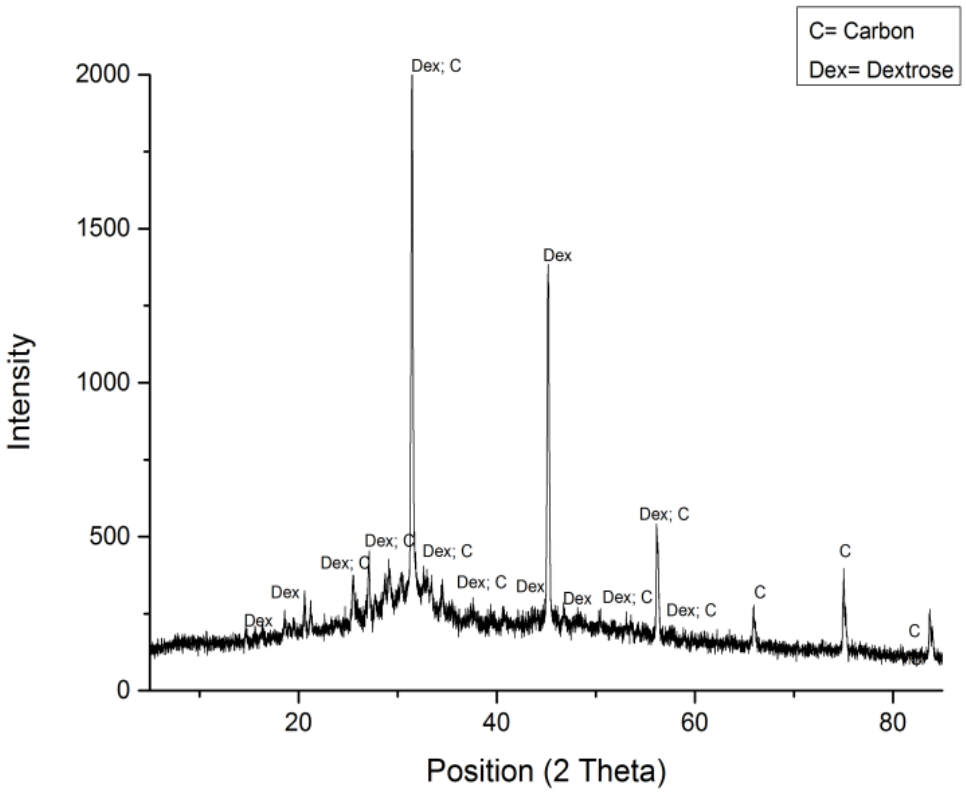
In the present study, the amorphous nature of W1b, P2, X2 and W2b was inferred from the intense peaks observed in the XRD pattern of the exobiopolymers (Figure 4.1). Results showed that Z3 was found to be crystalline. It was observed that different groups were present at different peak intensity in the XRD spectrum of all the five exobiopolymers. As in case of W1b and W2b, mainly lactose and dextrose were present in the range of 18^0 to 45^0. Similarly for P2, D-glucornic acid was found in same range. But in the case of X2 only carbon was present. Z3, which was showing crystalline nature constitute of carbon and dextrose in the range between 12^0 and 75^0.


Lac= Lactose
Dex= Dextrose
Intensity
Position (2 Theta)
Lac; Dex
Lac; Dex
Lac; Dex
Lac; Dex
Dex
Dex

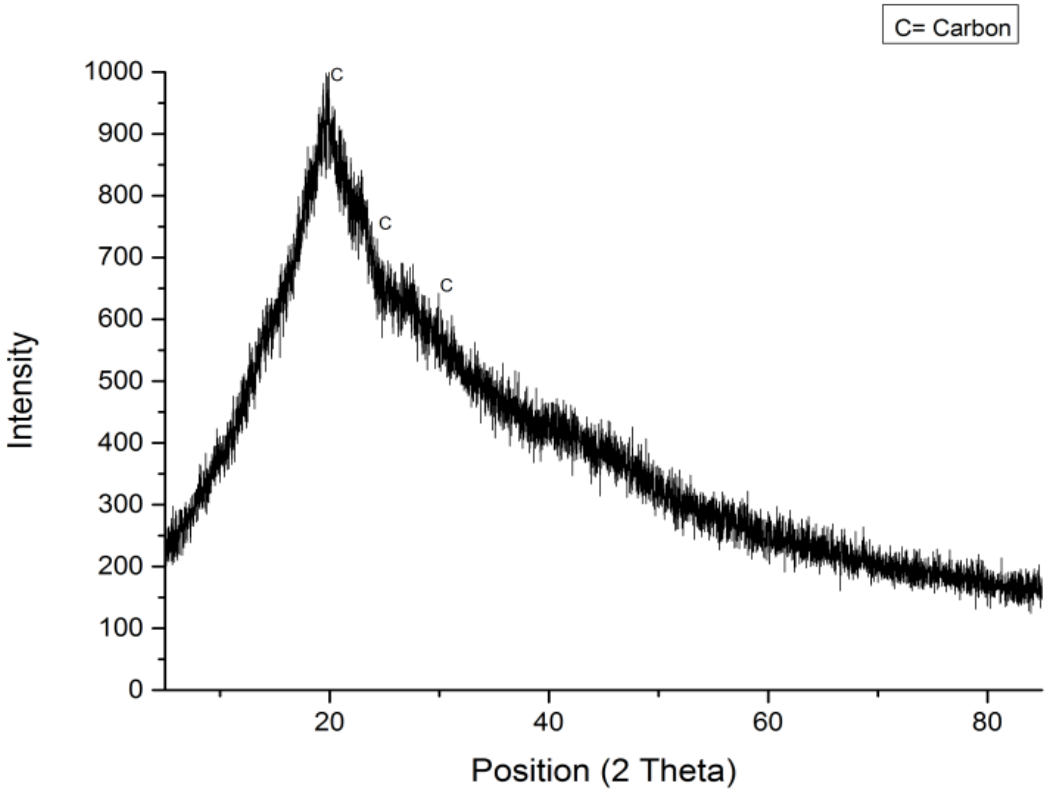
(a)


Glu= D-Glucornic acid
O= Oxygen
Intensity
Position (2 Theta)
Glu; O
Glu
Glu
Glu
Glu; O
O
O

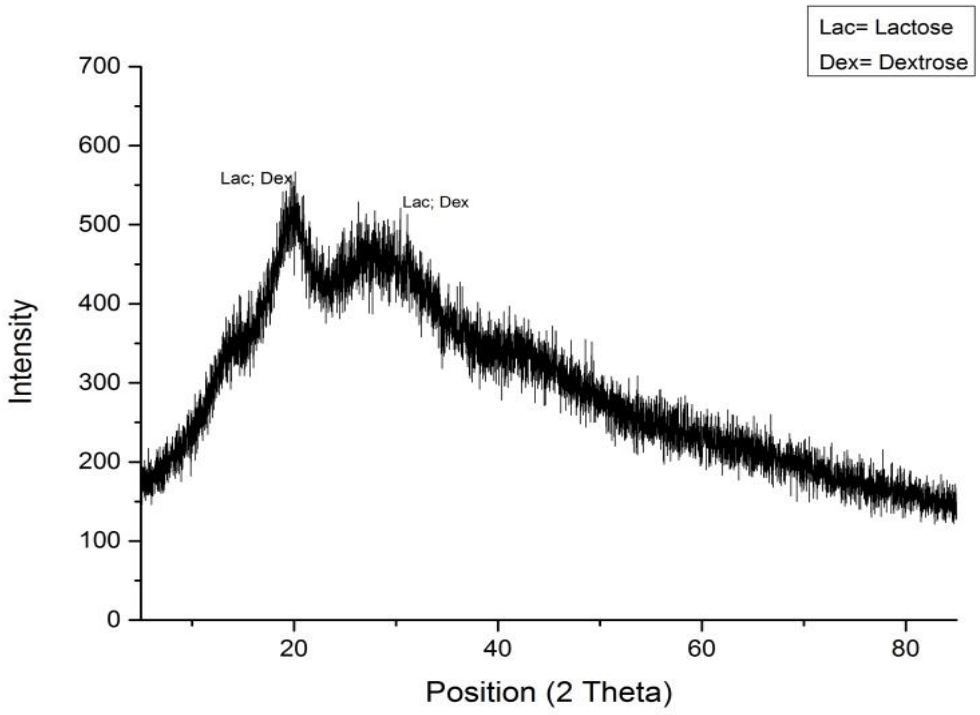
(b)


C= Carbon
Dex= Dextrose
Intensity
Position (2 Theta)
Dex; C
Dex
C

(c)


C= Carbon
Intensity
Position (2 Theta)
C

(d)



(e)

Figure 4.1: XRD spectra of (a) W1b; (b) P2; (c) Z3; (d) X2; (e) W2b.

4.2.1.2) Biochemical Charcterization

Most of the microbially produced exobiopolymers generally have heteropolysaccharide composition (Kumar *et al*., 2007). Several types of exobiopolymers have been reported with polysaccharides, proteins and lipids as their major components (Kurane *et al*., 1994). The knowledge about the structure and composition of the exobiopolymers is crucial for its effective binding ability.

Table 4.2: Composition of Exobiopolymers

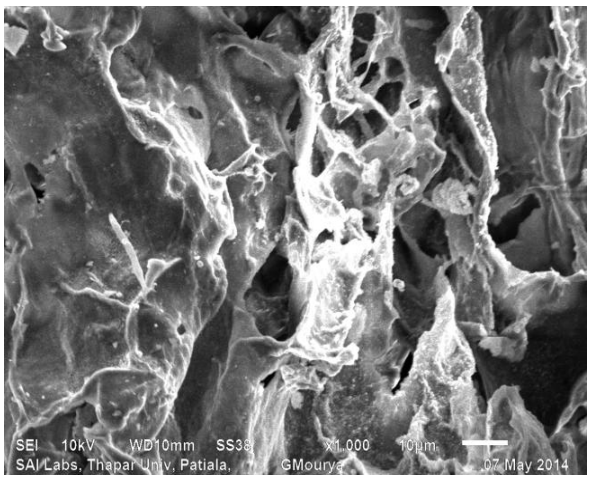
Exobiopolymers	Sugar (μgmL^{-1})	Protein (μgmL^{-1})	Amino sugars (μgmL^{-1})	Pyruvic acid (μgmL^{-1})	Uronic acid (μgmL^{-1})
W1b	74	40	20	28	14
P2	87	35	19	25	11
Z3	85	32	25	29	29
X2	80	30	21	30	25
W2b	74	29	22	37	10

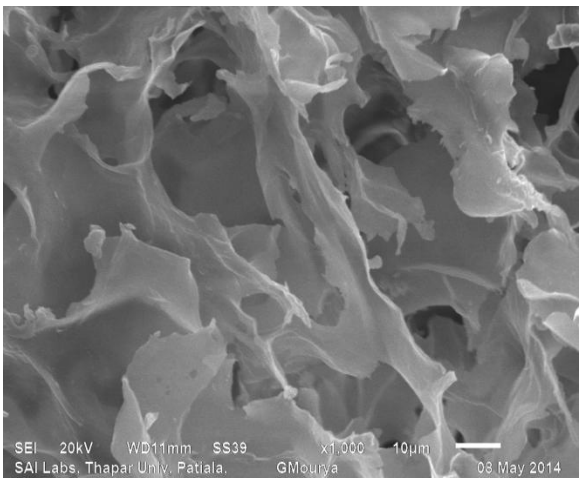
Table 4.2 illustrates the compositional characteristics of the five exobiopolymers. Biochemical analysis of all the five exobiopolymers revealed that they are polysaccharide in nature as they are composed 80-90% of sugar constituents. Apart from polysaccharides, exobiopolymers were also composed of other groups including uronic acid, amino sugars, protein and pyruvic acid.

4.2.2) Iron binding studies

4.2.2.1) Scanning electron microscopy (SEM) and Energy dispersive X-ray (EDS)

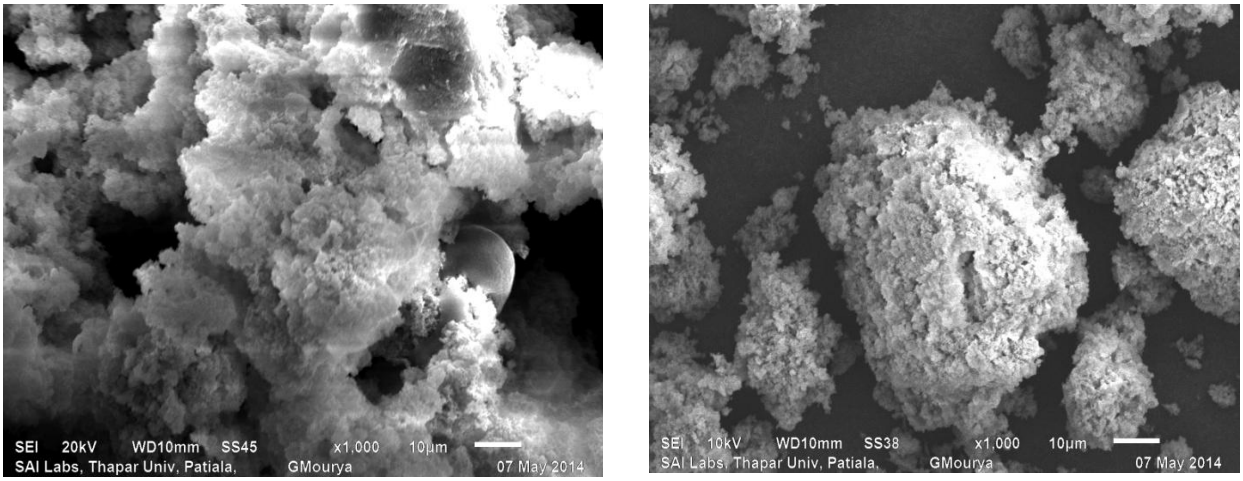
Scanning electron microscopic examination of exobiopolymers before and after metal adsorption (Fe^{2+}) was undertaken in order to locate the adsorptive sites of the exobiopolymers to form its metal complexes. SEM is a particularly useful tool for visual conformation of surface morphology and physical state of the surface. Figure 4.2- 4.6(a) showed the porous structure of the exobiopolymers and the presence of large number of linear grooves and projections on the surface of exobiopolymers indicated the high surface area of the exobiopolymers. The irregular particle size with porous structure on the surface was also observed. On the other side, binding of metal onto the surface of exobiopolymers is indicated in Figure 4.2-4.6(b) where the porous structure was not clearly visible because the pores were fully covered by iron.





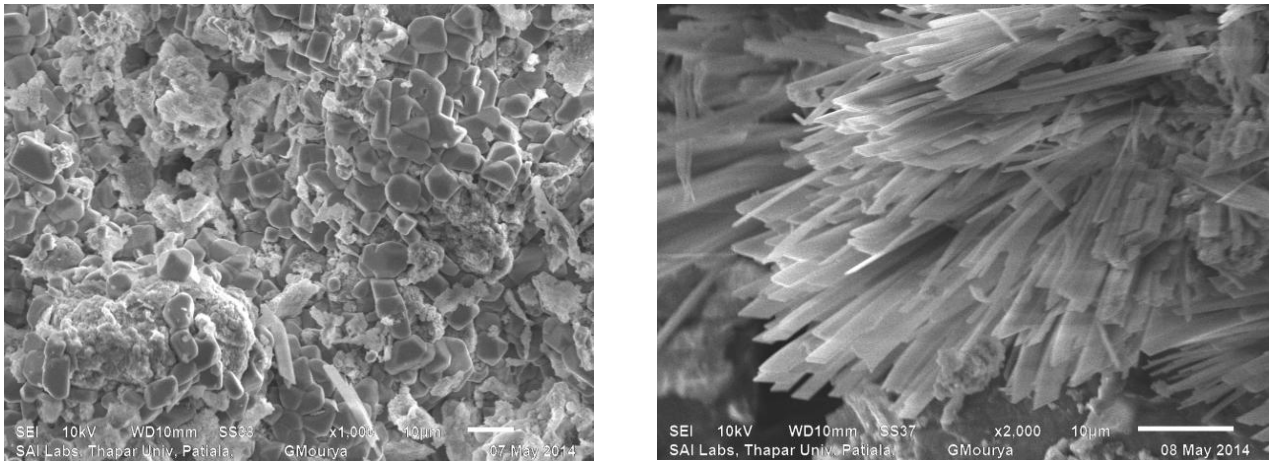
(a) **(b)**

Figure 4.2: Scanning electron micrograph of exobiopolymer W1b (a) before and (b) after binding with iron



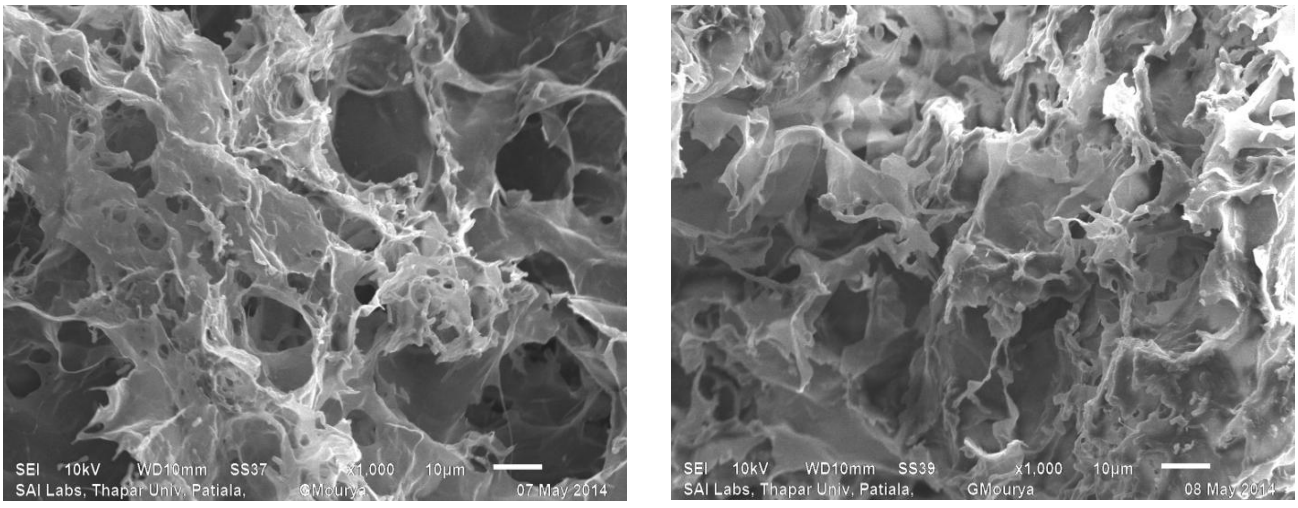
(a) **(b)**

Figure 4.3: Scanning electron micrograph of exobiopolymer P2 (a) before and (b) after binding with iron



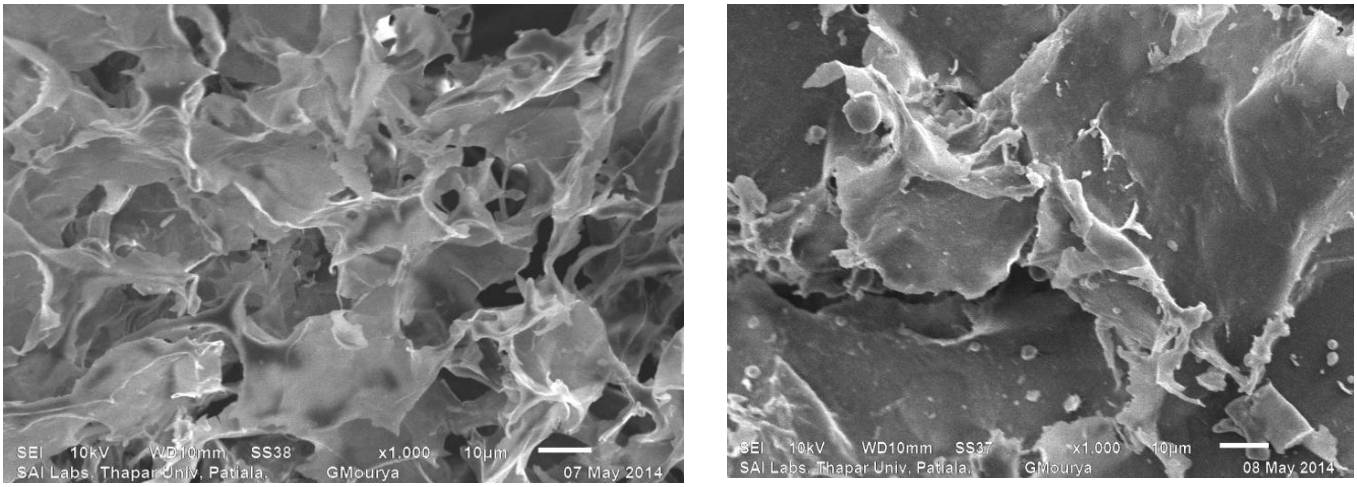
(a) **(b)**

Figure 4.4: Scanning electron micrograph of exobiopolymer Z3 (a) before and (b) after binding with iron



(a) **(b)**

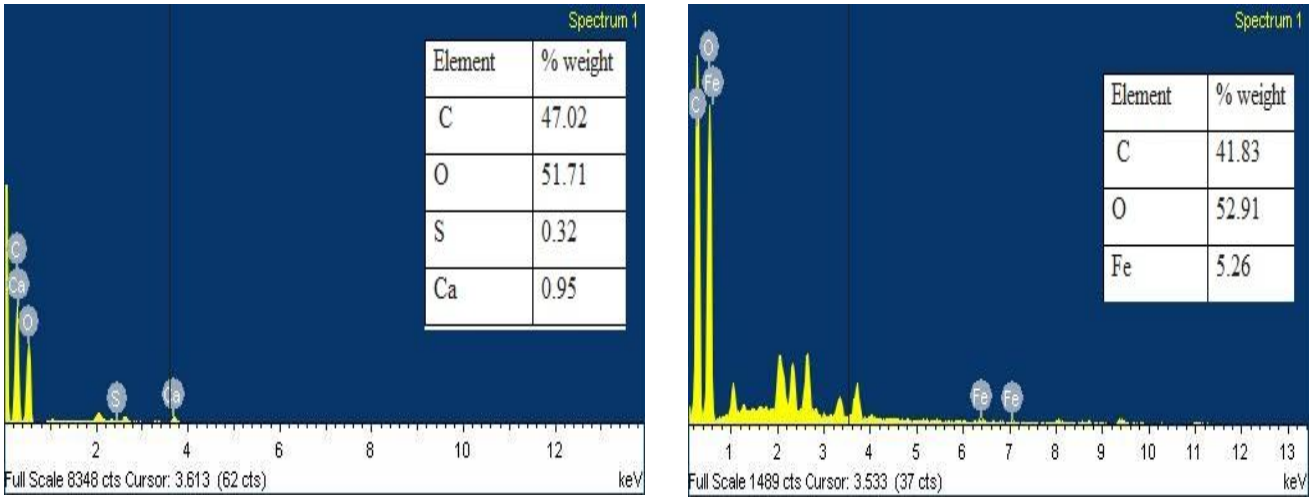
Figure 4.5: Scanning electron micrograph of exobiopolymer X2 (a) before and (b) after binding with iron.



(a) **(b)**

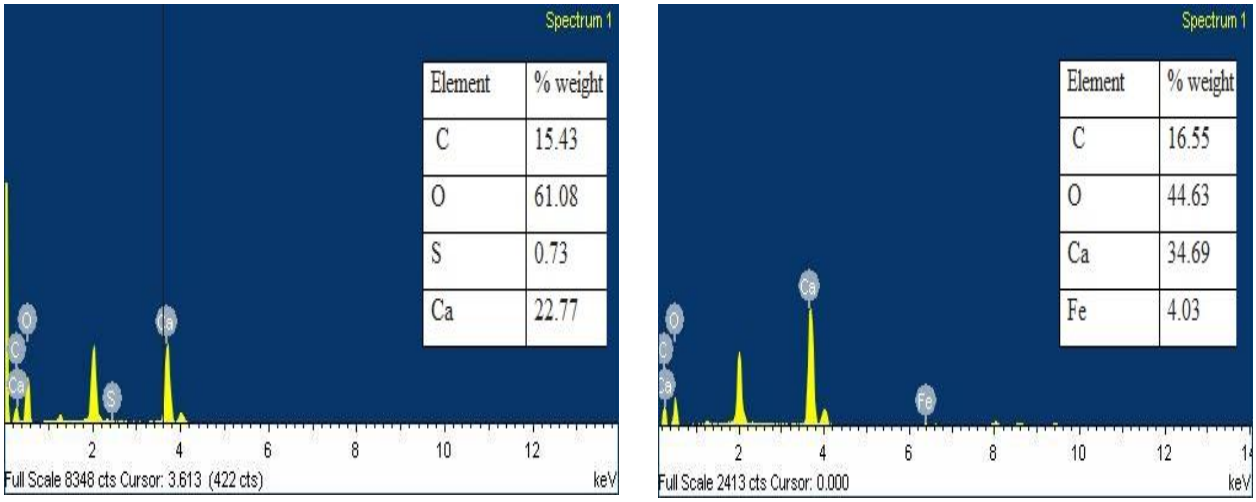
Figure 4.6: Scanning electron micrograph of exobiopolymer W2b (a) before and (b) after binding with iron.

Figure 4.7-4.11 presents the elemental quantitative analysis of all the five exobiopolymers before and after binding to iron ions. EDS spectrum of native exobiopolymers showed that the major elements present were C, O, S and Ca whereas after interaction between exobiopolymers and iron C, O and Fe were found as major elements. Figure 4.7-4.11 also depicting the percentage weight of the respective elements in all the five exobiopolymers is also presented with the EDS spectrum.



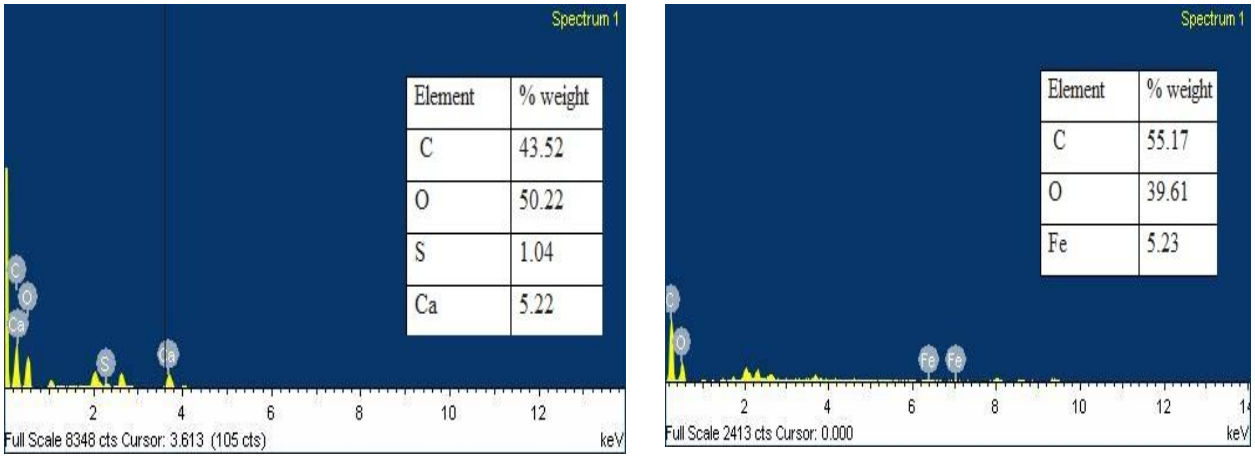
(a) (b)

Figure 4.7: EDS spectrum of exobiopolymer W1b (a) before and (b) after binding with iron



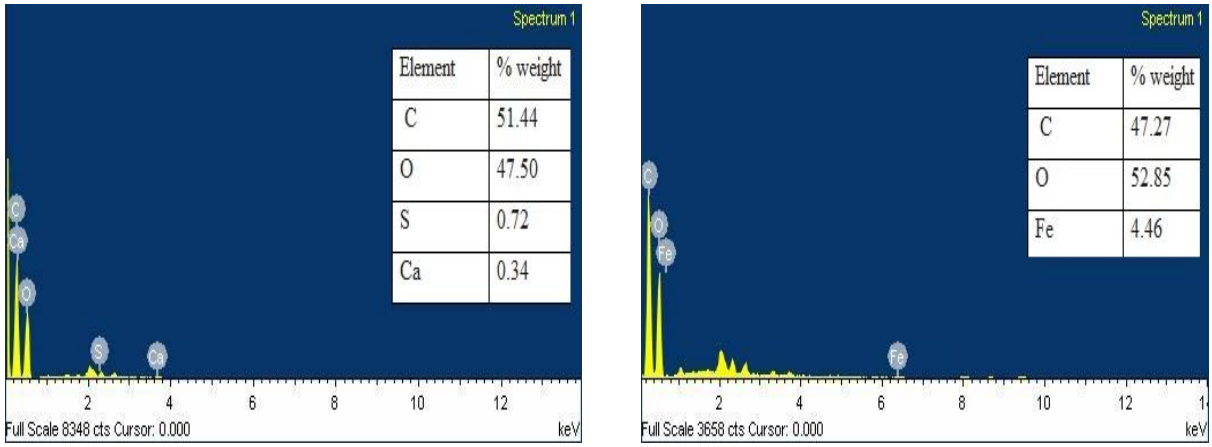
(a) **(b)**

Figure 4.8: EDS spectrum of exobiopolymer P2 (a) before and (b) after binding with iron



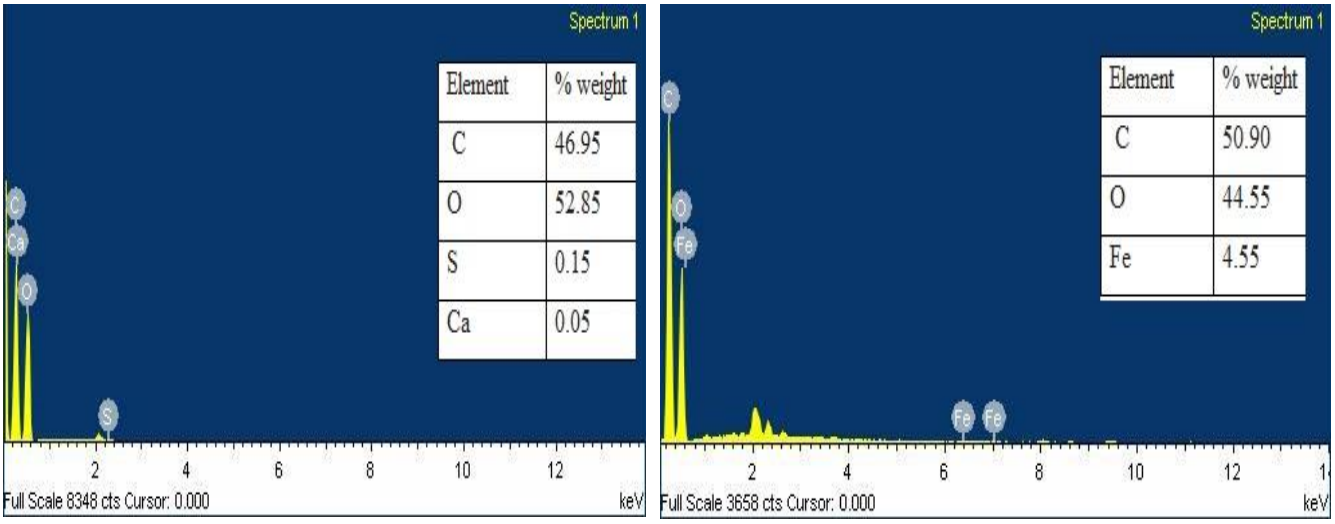
(a) **(b)**

Figure 4.9: EDS spectrum of exobiopolymer Z3 (a) before and (b) after binding with iron



(a) **(b)**

Figure 4.10: EDS spectrum of exobiopolymer X2 (a) before and (b) after binding with iron



(a) **(b)**

Figure 4.11: EDS spectrum of exobiopolymer W2b (a) before and (b) after binding with iron

4.2.2.2) Fourier Transform- Infrared Spectroscopy

Infrared spectroscopy has proven to be a powerful tool for studying biological molecules and for obtaining information about metal-exobiopolymer binding (Volesky, 1990). The identification of exobiopolymer active groups involved in the adsorption processes was achieved by infrared spectrum. The metal binding capacity of polysaccharides are usually attributed to the high hydrophilicity of the exobiopolymer due to the presence of hydroxyl groups, the presence of functional and reactional groups (sulphate, acetamido, primary amino and ester groups) and the flexible structure of the exobiopolymers chains (Moppert *et al.*, 2009).Figure 4.12- 4.16, presents the FT-IR spectrum which showed that some bands are coincident, some are specific and can be attributed to the interaction of functional groups of exobiopolymers with iron. For instance, the bands in spectrum of native exobiopolymers, at 1,722-1,738 cm^{-1} and 1,085-1,071cm^{-1}, was shifted to the right when iron is present (at 1,715-1,709 cm^{-1} and 1,064-1,058 cm^{-1}, respectively). The bands in these ranges correspond to the asymmetric stretching vibration of C=O bond in the carboxylic group (Schmitt & Flemming, 1998) and to the stretching vibration of the hydroxyl group (O-H) (Omoike & Chorover, 2004).

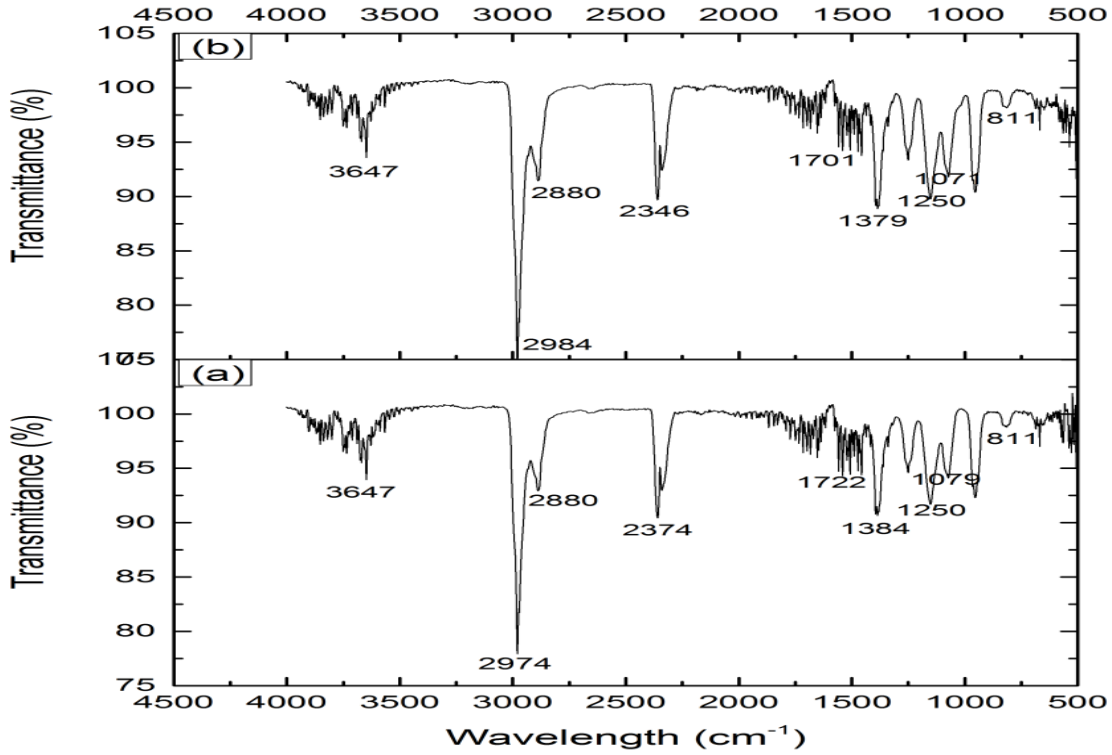


Figure 4.12: FTIR spectra of exobiopolymer W1b (a) before and (b) after binding with iron

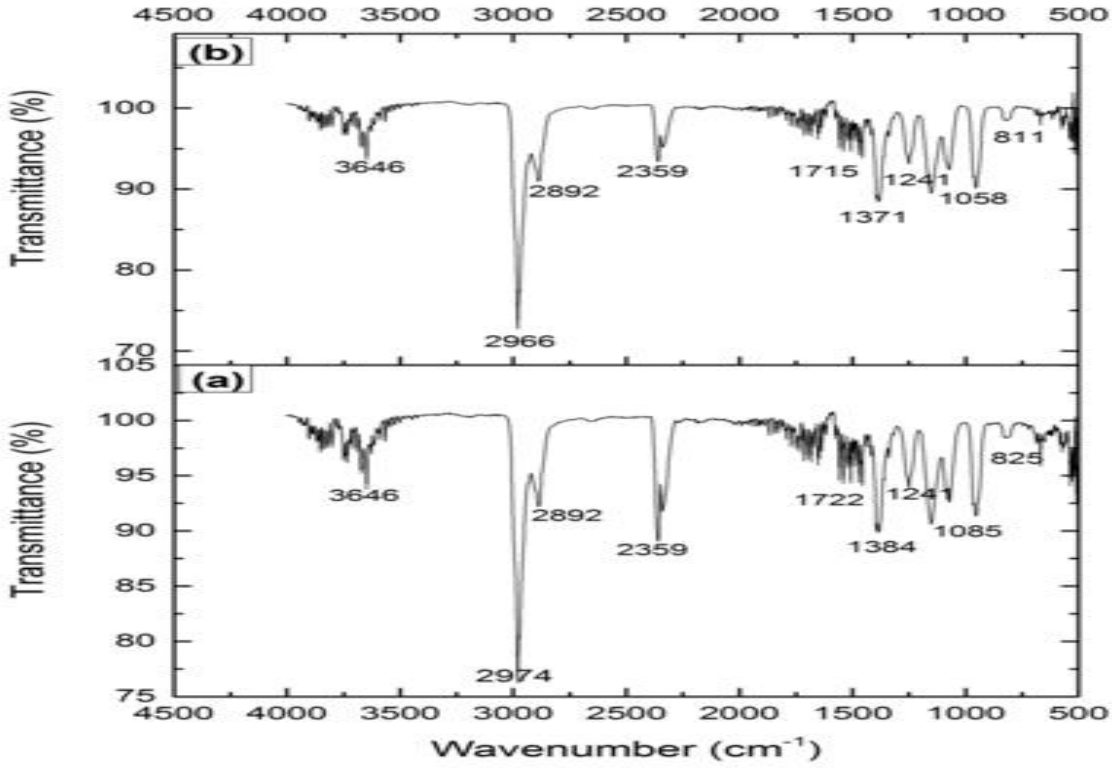


Figure 4.13: FTIR spectra of exobiopolymer P2 (a) before and (b) after binding with iron

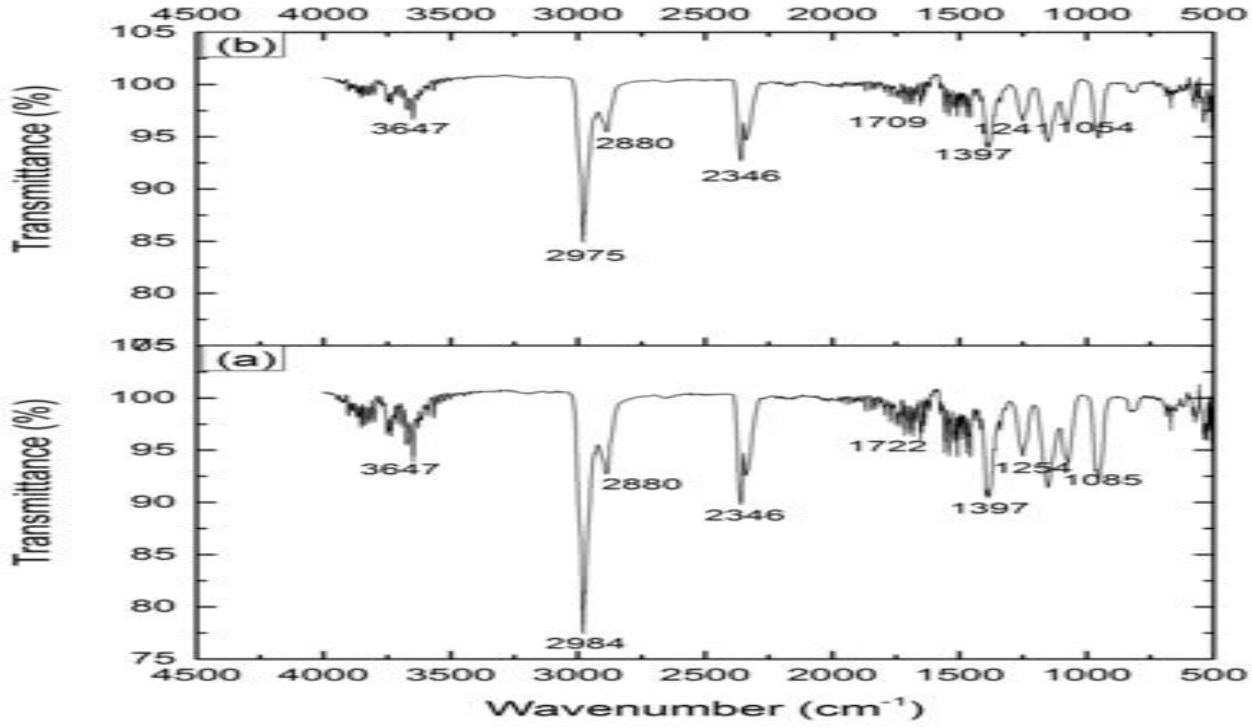


Figure 4.14: FTIR spectra of exobiopolymer Z3 (a) before and (b) after binding with iron

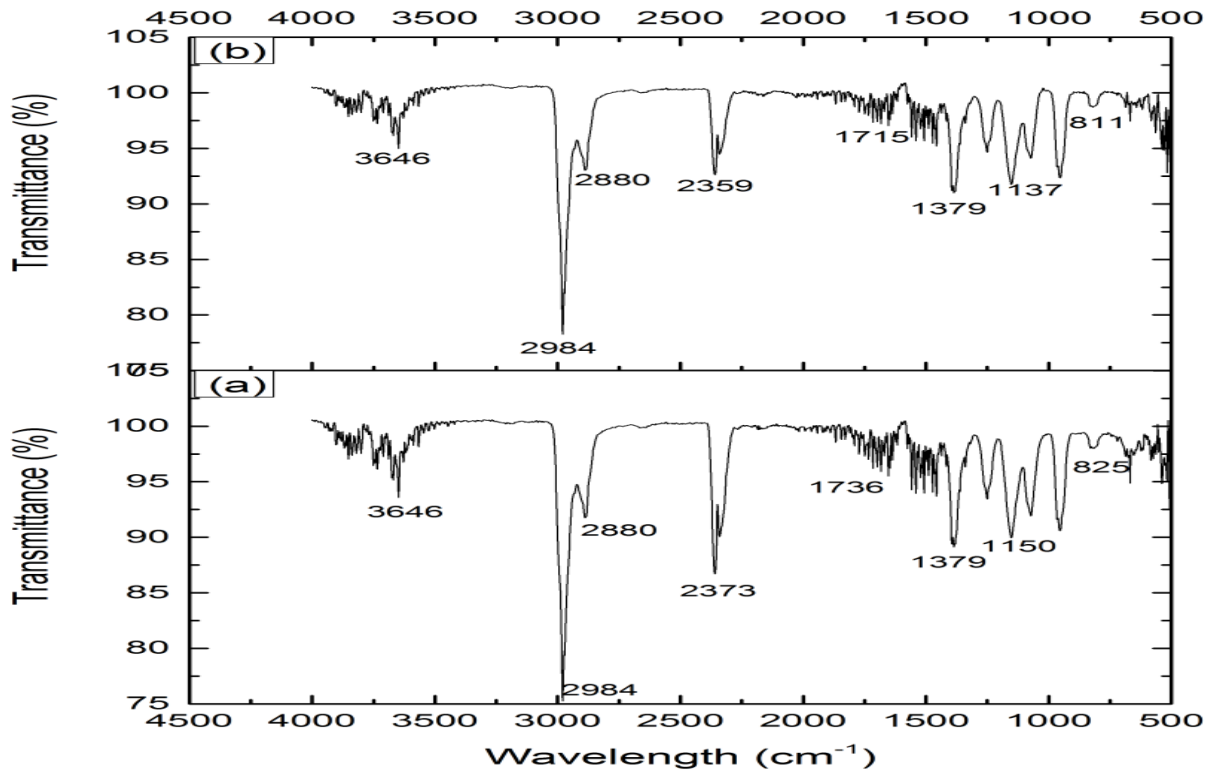


Figure 4.15: FTIR spectra of exobiopolymer X2 (a) before and (b) after binding with iron

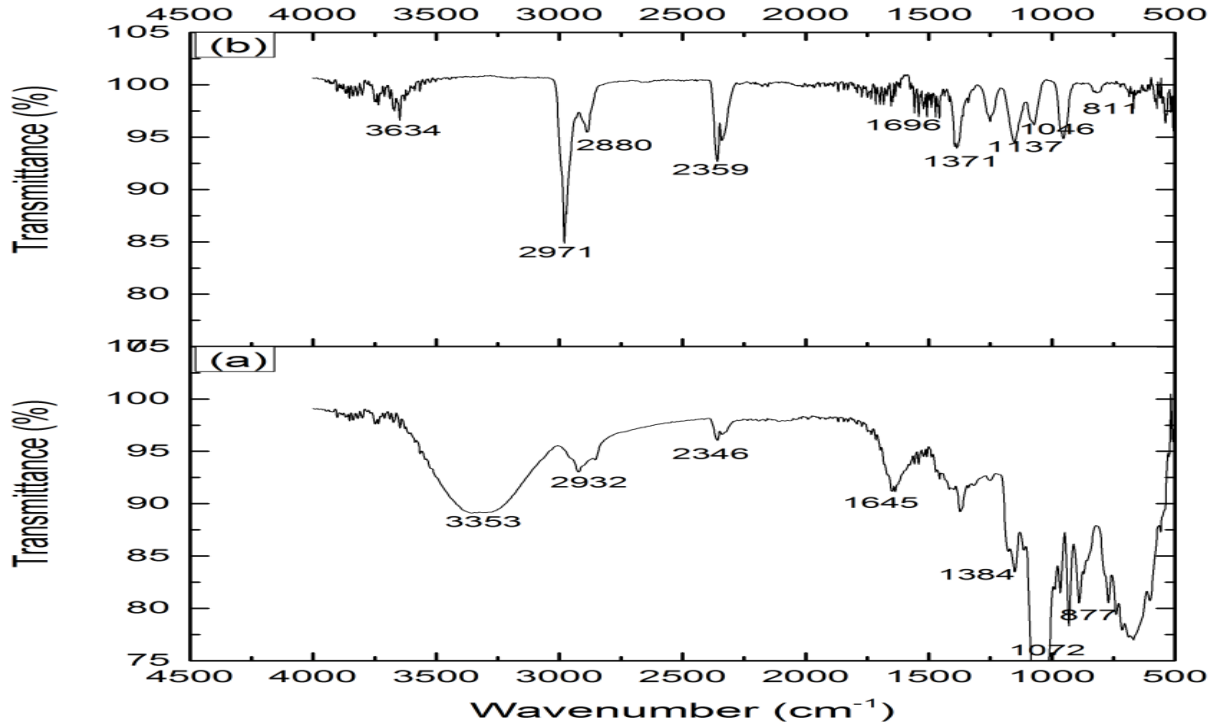


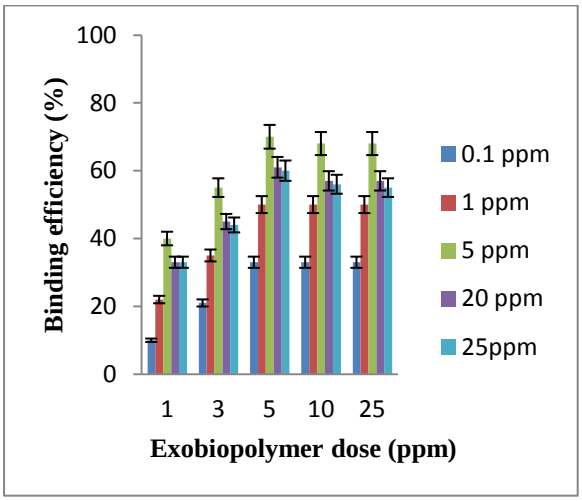
Figure 4.16: FTIR spectra of exobiopolymer W2b (a) before and (b) after binding with iron

As previously reported, these groups are the preferential participants in metal uptake by exobiopolymers (Ueshima *et al*., 2008) as presence of carboxyl group may serve as binding sites for divalent cations. The doublet at 1,250- 1,150cm^{-1} corresponds to the presence of the ester sulphate groups. The band around 815 cm^{-1} assigned to the interaction of iron with sulphonate group. Usually, the carboxylic group (-COOH), or its derivate carboxylate (-COO-), has two peaks in the IR spectrum: one in the range 1600–1800 cm^{-1}, corresponding to the C=O bond vibration and another around 1400 cm^{-1}, related to the deformation vibration of C–O bond in the carboxylate group. In this case, the peak intensity of the carboxyl group is higher in the spectrum of the native exobiopolymers than for exobiopolymer with iron. This suggests a preferential interaction between carboxyl groups and iron in solution. This interaction has also been reported in literature (Quintelas *et al*.2009).

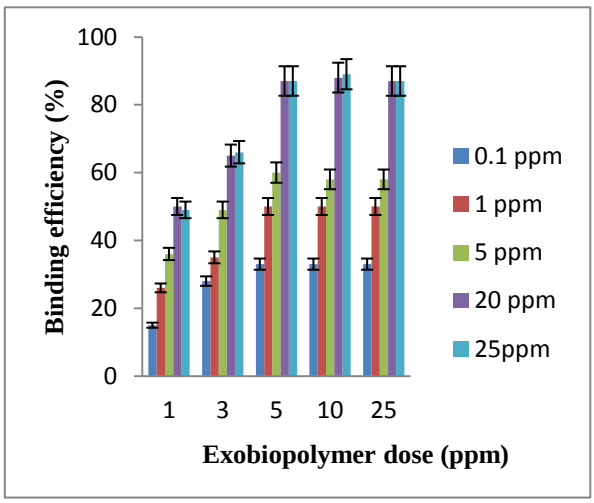
4.2.2.3) Effect of different parameters

4.2.2.3.1) Effect of polymer dose and iron concentration

Optimization of exobiopolymer and iron concentration is the most crucial step in determining the optimum conditions for the binding process. Optimum final concentration of exobiopolymer required for iron binding was evaluated by varying iron concentrations (0.1 ppm, 1 ppm, 5 ppm and 20 ppm) and exobiopolymers concentrations (1 ppm, 3 ppm, 5 ppm, 10 ppm and 25 ppm).



(a)



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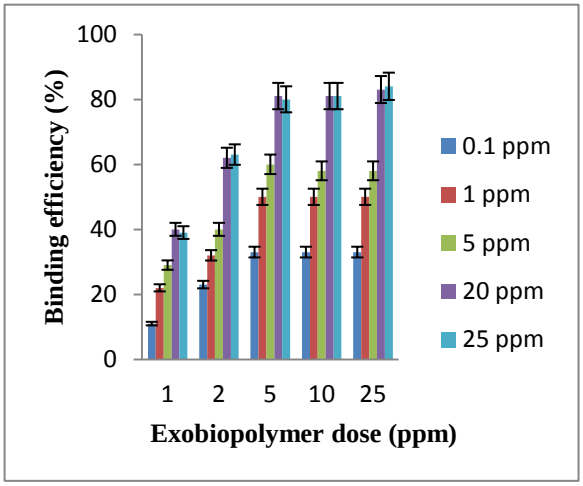

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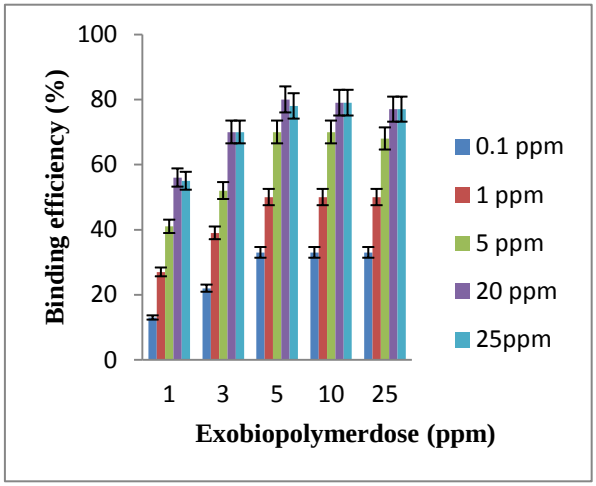
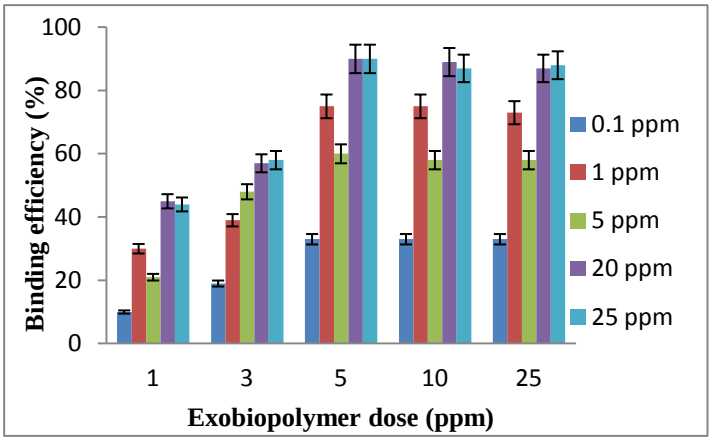


(d)



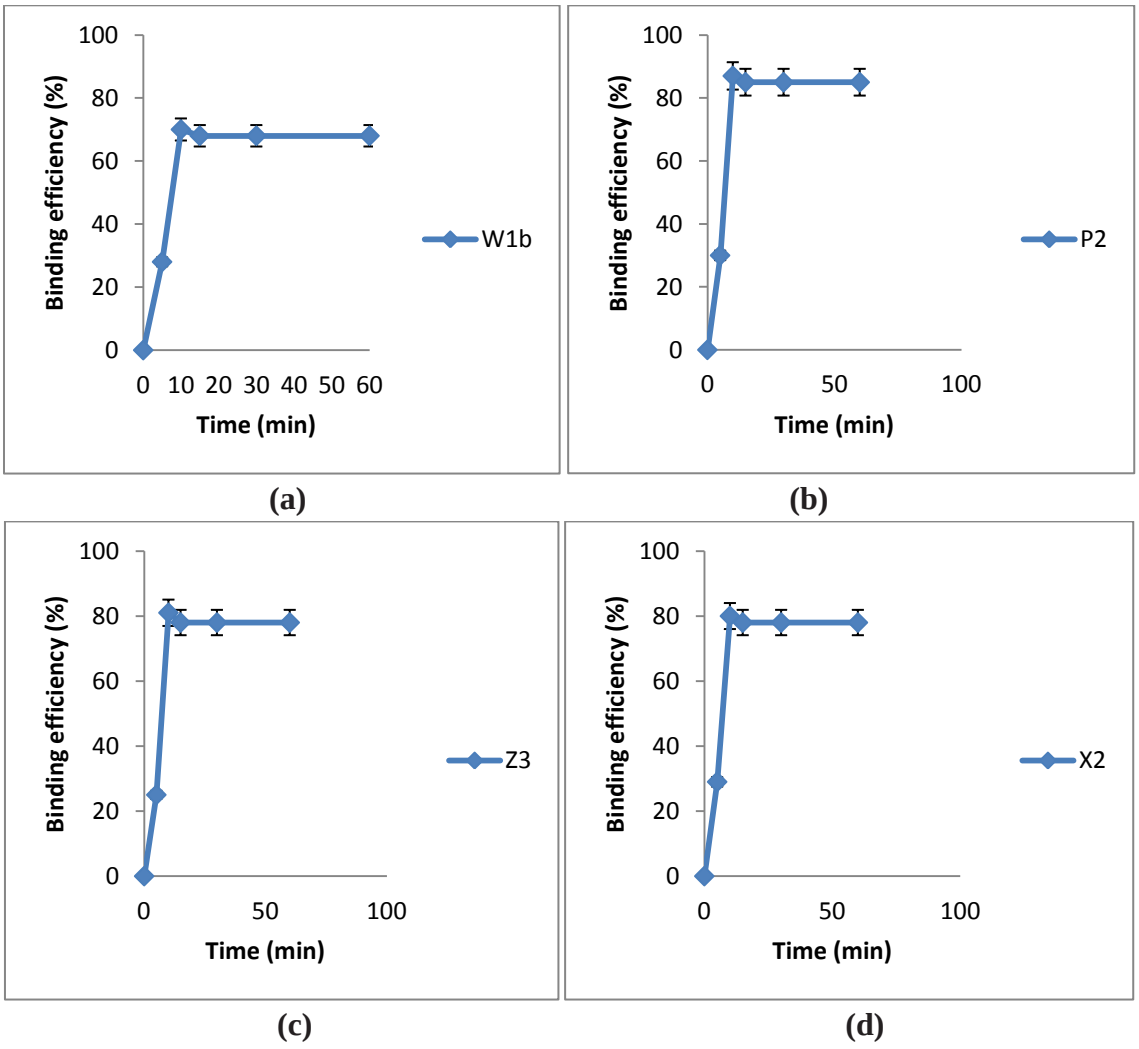
(e)

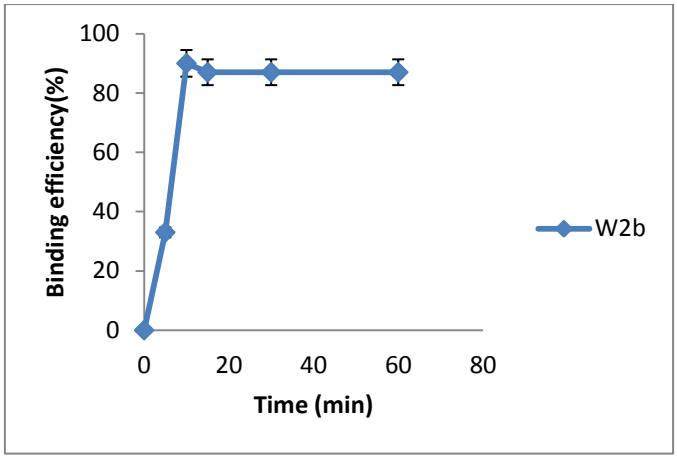
Figure 4.17: Percentage of binding efficiency by exobiopolymers a) W1b, b) P2, c) Z3, d) X2 and e) W2b at different iron and exobiopolymer concentrations

Figure 4.17(a) showed that the maximum iron binding to W1b was observed at iron concentration of 5 ppm. The significant decrease in iron binding was observed by further increasing the iron concentration. This decrease might be due to inhibition of the diffusion of iron ion to the unreacted functional groups (Wang *et al.*, 2006). Also it might be due to saturation of all the binding sites on exobiopolymer surface beyond a particular concentration (Salehizadeh *et al.*, 2003). However other four exobiopolymers (P2, Z3, X2 and W2b) indicated maximum binding at 20 ppm of iron concentration and 5 ppm of exobiopolymers concentration (Figure 4.17 (b, c, d and e)). Binding of iron by exobiopolymer increased with increasing the iron concentration from 0.1 ppm to 20 ppm. This might be due to availability of unsaturated binding sites which means the increase in the number of ions available for competing at the binding sites at higher metal concentrations (Moppert *et al*., 2009). Further increase in iron concentration to 25 ppm showed no significant effect. By increasing the exobiopolymer concentration from 1-5 ppm the binding of iron on exobiopolymer was increasing upto 70-90%. Exobiopolymer concentration was increased beyond 5 ppm showed no significant binding of iron. So it can be hypothesized that iron binding by P2, Z3, X2 and W2b is chemically equilibrated and saturated at iron concentration of 20 ppm whereas for W1b, the iron concentration was 5 ppm.

4.2.2.3.2) Effect of contact time

Besides the concentrations of iron and exobiopolymers, the time of contact also plays the important role in the iron binding studies. Figure 4.18 reveals the influence of contact time on the iron binding efficiency of the exobiopolymers. Removal efficiency of all the five exobiopolymers increased from 70% to 90% with increasing the time of contact from 5 to 10 min. The fast binding at the initial stage was probably due to the availability of more number of active sites on the exobiopolymers surface at the beginning (Onundi *et. al*., 2010). Further on increase the contact time showed no significant effect on iron binding. Hence 10 min was considered as optimum time, for maximum binding by all the five exobiopolymers.





(e)

Figure 4.18: Effect of contact time on iron binding efficiency of exobiopolymer (a) W1b, (b) P2, (c) Z3, (d)X2 and (e) W2b

Overall optimum study results the maximum binding of four exobiopolymers at concentration of 5 ppm to iron concentration of 20 ppm was achieved in 10 min of contact time. However, in the case of one of the exobiopolymers i.e. W1b (5 ppm), the effect was found maximum against 5 ppm concentration of iron ions. The results were further studied to evaluate the maximum binding capacity of the five exobiopolymers by adsorption isotherms i.e. Langmuir isotherm and Freundlich isotherm and the parameters were evaluated by computational tool, MATLab.

4.3) Modeling and validation of adsorption

Adsorption is a fundamental process in the physiochemical treatment of wastewaters, a treatment which can economically meet today's higher effluent standards. Adsorption is usually described through isotherms, that is, functions which connect the amount of adsorbate on the adsorbent. Distribution of metal ions between the liquid phase and the solid phase can be described by several isotherm empirical models such as Langmuir and Freundlich. Adsorption isotherm is important to describe how iron interacts with exobiopolymer. The Langmuir isotherm assumes monolayer adsorption onto a surface containing a finite number of adsorption sites of uniform strategies with no transmigration of adsorbate in the plane

surface (Hameed *et al.*, 2007) whereas Freundlich adsorption model is generally used to describe heterogeneous or multilayer adsorption.

Table 4.3 represents the Langmuir and Freundlich isotherms constants of different concentration of exobiopolymers at different iron concentrations. It shows the values of different parameters of isotherms i.e. q_m (amount of iron adsorbed at saturation per unit mass of adsorbent), b (equilibrium constant), R_L (equilibrium constant), K_f (adsorbent capacity), *n* (constant related to energy adsorbed) and R^2 (correlation coefficient) at five different concentrations (1 ppm, 3 ppm, 5 ppm, 10 ppm and 25 ppm) for all the five exobiopolymers (W1b, P2, Z3, X2 and W2b).The favourability of one model to another was estimated from the correlation coefficient (R^2). The essential characteristics of the Langmuir isotherm can be expressed in terms of dimensionless constant separation factor or equilibrium parameter, R_L, which is defined,

$$R_L = \frac{1}{1+bC_0}$$

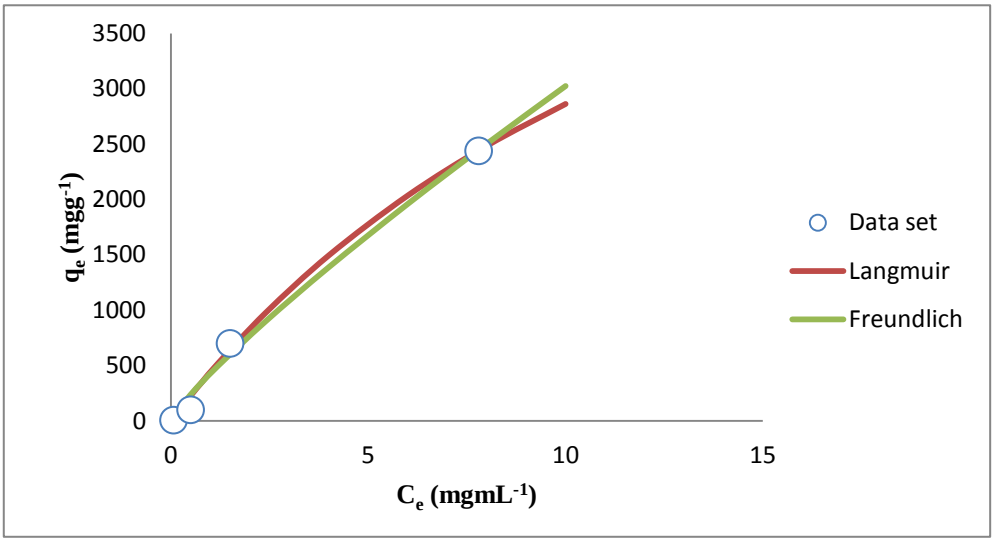
Where, 'C_0' (mgL^{-1}) is the highest initial iron concentration.

The Freundlich constant, *n*, indicates the degree of nonlinearity between solution concentration and adsorption as follows: if $n=1$, then adsorption is linear; if $n<1$, then adsorption is a chemical process; if $n>1$, then adsorption is a physical process. K_F is the heterogeneity factor about adsorption intensity and capacity. Larger K_F value reflects a larger overall adsorption capacity.

From the experiment data, the optimal concentration of exobiopolymers for all the five, was evaluated i.e. 5 ppm. So, here graph for optimum concentration was plotted between q_e (amount of iron adsorbed by exobiopolymers) versus C_e (equilibrium concentration) which explains the preference of one isotherm to another (Figure 4.19).

Table 4.3: Langmuir and Freundlich isotherms constant of different exobiopolymers at different concentrations

Exobiopolymers conc. (ppm)	Exobiopolymers	Langmuir $q_e = \frac{q_m b C_e}{1+bCe}$				Freundlich $q_e = k_f C_e^{1/n}$		
		q_m	B	R_L	R^2	K_f	*n*	R^2
1	W1b	7954	0.169	0.228	0.924	687	1.145	0.994
	P2	$1.15x10^4$	0.163	0.234	0.803	317.9	0.667	0.999
	Z3	$5.72x10^3$	0.324	0.134	0.639	249.5	0.716	1
	X2	$9.47x10^3$	0.307	0.140	0.683	385.1	0.645	1
	W2b	$2.77x10^4$	0.035	0.587	0.870	61.51	0.481	0.998
3	W1b	8469	0.499	0.501	0.995	407.9	1.199	0.992
	P2	$1.6x10^4$	0.045	0.234	0.916	317.9	0.667	0.999
	Z3	$1.34x10^4$	0.047	0.515	0.874	79.7	0.513	0.999
	X2	$1.39x10^4$	0.066	0.428	0.879	178.2	0.548	0.999
	W2b	$1.51x10^4$	0.035	0.582	0.965	231.8	0.769	1
5	W1b	7335	0.064	0.438	0.994	427.1	1.176	0.992
	P2	$1.15x10^4$	0.097	0.588	0.338	2.5	0.126	0.998
	Z3	$1.52x10^4$	0.054	0.477	0.815	99.5	0.383	0.999
	X2	$1.56x^4$	0.058	0.462	0.921	361.3	0.622	0.999
	W2b	$1.57x10^4$	0.070	0.416	0.614	2.5	0.126	0.997
10	W1b	2846	0.077	0.391	0.995	200.5	1.235	0.991
	P2	$1.62x10^4$	0.031	0.520	0.617	0.001	0.050	0.998
	Z3	$7.05x10^3$	0.058	0.079	0.789	34.3	0.346	0.998
	X2	$1.85x10^4$	0.020	0.707	0.956	187.9	0.673	0.999
	W2b	$1.70x10^4$	0.030	0.621	0.502	$8.23x10^{-4}$	0.025	0.997
25	W1b	1194	0.073	0.404	0.995	80.1	1.222	0.992
	P2	7675	0.026	0.428	0.651	173.1	0.847	0.425
	Z3	$1.26x10^4$	0.012	0.798	0.767	7.9	0.276	0.988
	X2	$1.34x10^4$	0.009	0.838	0.969	68.8	0.708	0.999
	W2b	$9.75x10^2$	0.351	0.124	0.442	218.5	1.149	0.434


3500
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q_e (mgg^{-1})
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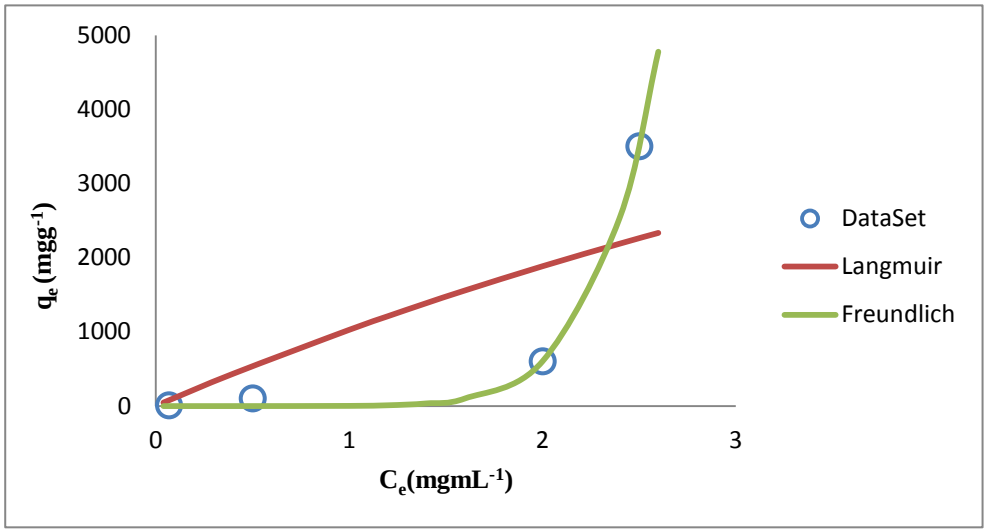
Data set
Langmuir
Freundlich

(a)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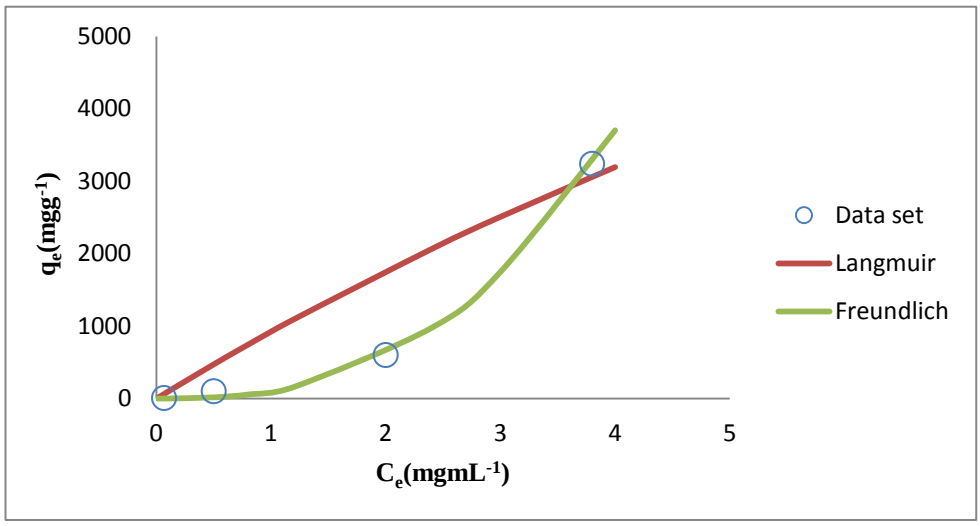
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Langmuir
Freundlich

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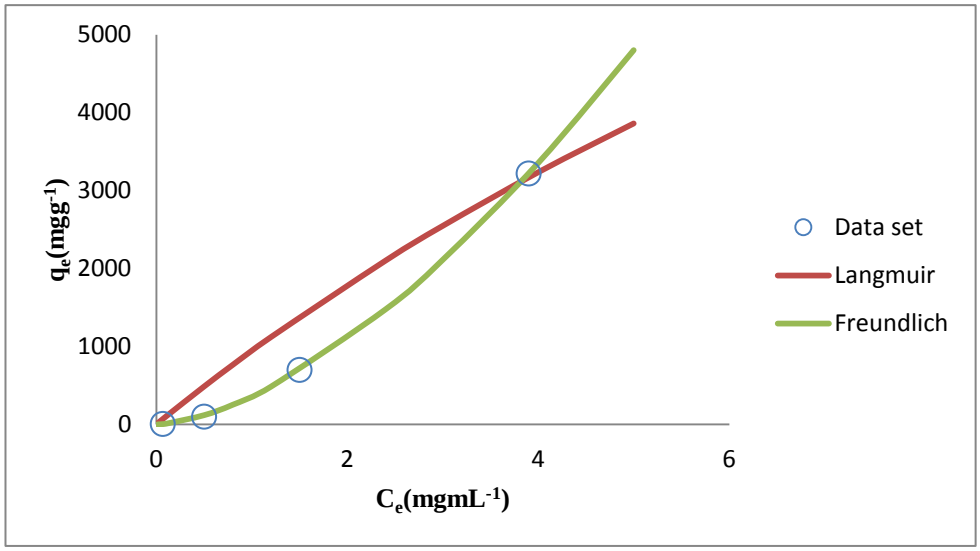

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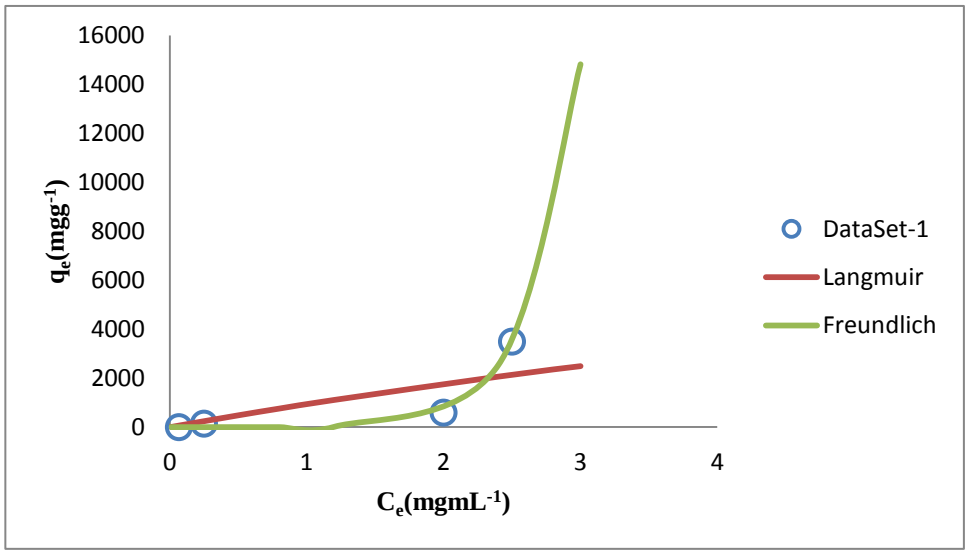

(e)

Figure 4.19: Plot of Langmuir and Freundlich isotherms for the adsorption of iron by exobiopolymers of (a) W1b; (b) P2; (c) Z3; (d) X2; (e) W2b.

In case of W1b, the Langmuir adsorption isotherm (R^2=0.995) was found to represent the equilibrium adsorption data more favourable as compared to Freundlich isotherm (R^2 =0.992). The R_L value calculated for Langmuir isotherm was 0.43, indicated that the binding of iron on exobiopolymer was favorable at the studied conditions. As for Freundlich isotherm, the value of *n* calculated was 1.176, which means that adsorption is a physical process. So it was resulted that both the isotherms were favourable for W1b. But in case of P2, the Freundlich isotherms (R^2=0.99) was found to better fit as compared to Langmuir

isotherm (R^2 =0.5881). The R_L value calculated for Langmuir isotherms was 0.3380. As for Freundlich isotherms, the value of *n* calculated was 0.1265, which means that it is chemical process. Similarly in case of Z3, the correlation coefficient for Freundlich isotherms (R^2=0.99) was much better fitted than Langmuir isotherm (R^2=0.8146). The R_L and *n* value calculated for Langmuir isotherms and for Freundlich isotherms was 0.4772 and 0.3833, respectively. In case of X2, R_L value of Langmuir isotherm and *n* value of Freundlich isotherms was calculated as 0.4615 and 0.6221, respectively. It was resulted that Freundlich isotherms (R^2=0.99) is favourable as compared to Langmuir isotherms (R^2=0.9212). Similar results were obtained in case of W2b, where R_L and *n* value was 0.4160 and 0.99 for Langmuir isotherm and Freundlich isotherm, respectively.

The maximum binding ability of the exobiopolymers was evaluated from the equation,

$$q_e = \frac{(C_0 - C)\, V}{M}$$

which were 2440 mgg^{-1}, 3500 mgg^{-1}, 3240mgg^{-1}, 3220 mgg^{-1} and 3500 mgg^{-1} for exobiopolymers W1b, P2, Z3, X2 and W2b, respectively. It is concluding from the results that the Langmuir model is better fitted for exobiopolymer W1b whereas for exobiopolymers P2, Z3, X2 and W2b Freundlich model is favourable and exobiopolymers P2 and W2b showing maximum binding ability for iron.

CONCLUSION

The exobiopolymers are of immense biotechnological and industrial importance. The aim of this work was to evaluate the efficacy of exobiopolymers to remove iron from the water. Salient findings of the study were:

- Five exobiopolymers (W1b, P2, Z3, X2 and W2b) obtained from five different bacteria were found to be endowed with the ability to bind iron.
- All the five exobiopolymers were characterized to gain the deeper insights for their ability to bind iron. XRD spectrum showed the amorphous nature of exopolysaccharides which means that exobiopolymers have no defined shape, so they can be easily altered for further applications. Biochemical characterization revealed that exobiopolymers were mainly composed of polysaccharides along with proteins.
- The morphological studies showed that the exobiopolymer has porous structure but in the presence of iron these pores were occupied and hence porosity was not clearly visible which revealed the binding behaviour of exobiopolymers. FT-IR spectrum revealed the presence of different functional groups such as hydroxyl, carboxyl and sulphate which are crucial in iron binding.
- The binding ability of the five exobiopolymers were in the order of W2b>X2>P2>W1b>Z3. The concentration of iron, dose of exobiopolymer and time of contact were optimized. The optimal conditions were found to be 5 ppm of exobiopolymers within a period of 10 min for 75-85% of removal of iron
- The results were validated by mathematical modelling. The Freundlich and Langmuir adsorption models were used for the mathematical description of the binding by using a computational tool, MATLab. Langmuir isotherm was favourable for exobiopolymers W1b only and Freundlich isotherms proved favourable for exobiopolymers P2, Z3, X2 and W2b. The maximum binding capacity of 3500 mgg^{-1} was attained by P2 and W2b.

It can be concluded from the results that the exobiopolymers which are produced by microorganism can be used as alternative to conventional methods (chemical precipitation, ion exchange, electrodialysis etc.) for iron reduction from waste water because of varied advantages of exobiopolymers i.e. high metal binding capacity, low production cost, and easily operated.

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ANNEXURE- I

1. Biopolymer Producing broth (BPB)

Distilled water	100mL
Peptone	0.5gm
Diammonium sulphate	0.2gm
$CaCl_2.2H_2O$	0.07gm
NaCl	0.01gm
$MgSO_4.7\ H_2O$	0.02gm
K_2HPO_4	0.1gm
Dextrose	0.1gm
Agar	0.2gm
Yeast extract	0.1gm

2. Reagents for Folin- Lowry assay

Reagent A (Alkaline solution 50 mL)

Na_2CO_3	2.0gm
NaOH	0.2gm

Reagent B (50 mL)

$CuSO_4$	0.5gm
Na-K-tartarate	1gm/100mL
Reagent C	50 mL of reagent A and 50 mL of reagent B were mixed

3. Reagent for Elson-Morgan assay

Reagent A (100 mL)

Dipotassium tetraborate tetrahydrate	6.1g

Reagent B

4-N, N-dimethyl-p-aminobenzaldehde	1 g
Glacial Acetic acid	50 mL
HCl (11.5 N)	1.5 mL
Standard solution (galactosamine)	0-1 $mgmL^{-1}$
	0-2

4. Carbazole Assay (Uronic Acid)

Reagent A (100 mL)

Sodium tetraborate decahydrate	0.9 g
Distilled water	10 mL
Concentrated H_2SO_4 (ice cold) (98%)	90 mL

Reagent B

Carbazole	100 mg
Absolute Ethanol	100 mL
Standard solution (glucuronic acid)	0-1 $mgmL^{-1}$

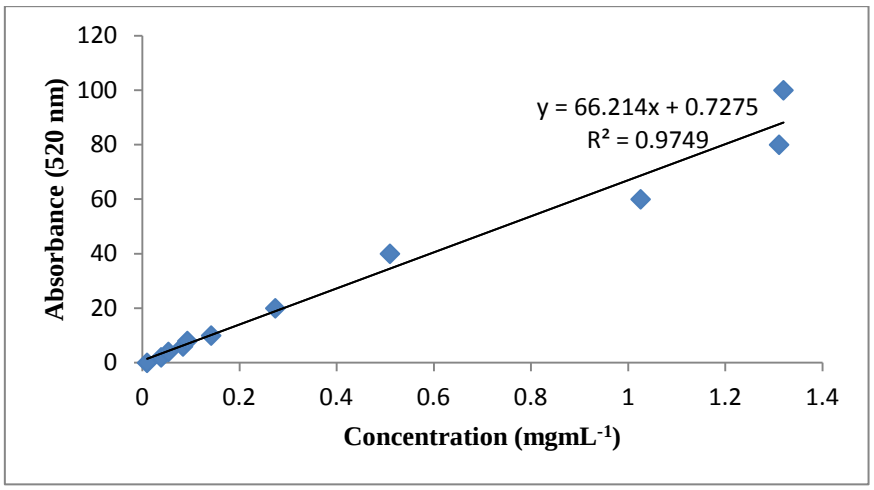
5. Friedman method (Pyruvic Acid)

Perchloric acid	50%
DNP reagent (2, 4-dinitrophenylhydrazine)	500 µM
NaOH (2.0 N)	10 mL
Sodium hydroxide	2.2 N
Standard solution	0-3 $mgmL^{-1}$

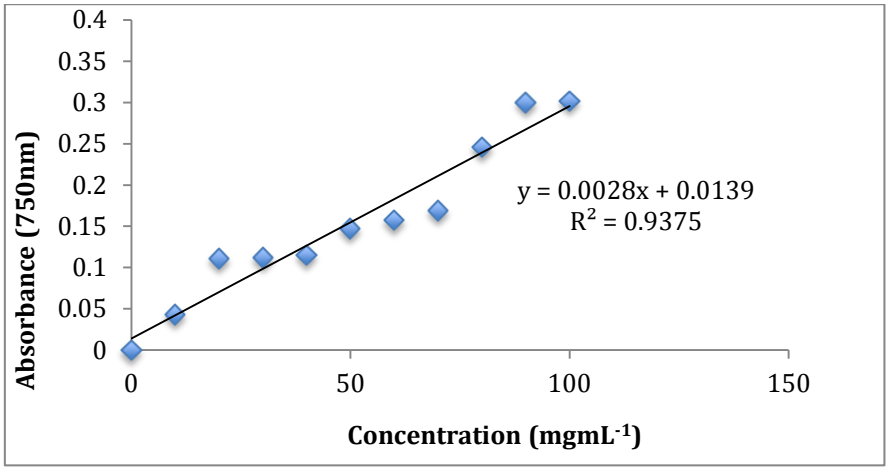
6. Total sugars

Phenol	5%
Sugar standards (glucose, galaxies, xylems, Maltose, mannose)	1 $mgmL^{-1}$

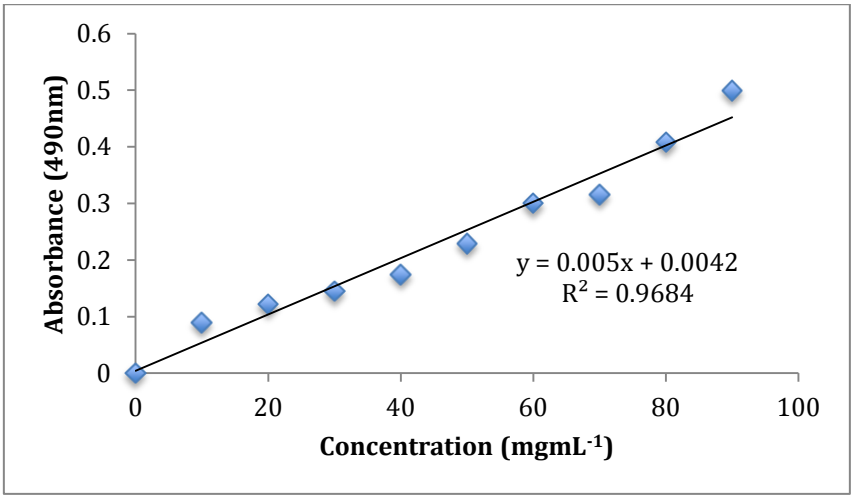
Annexure- II



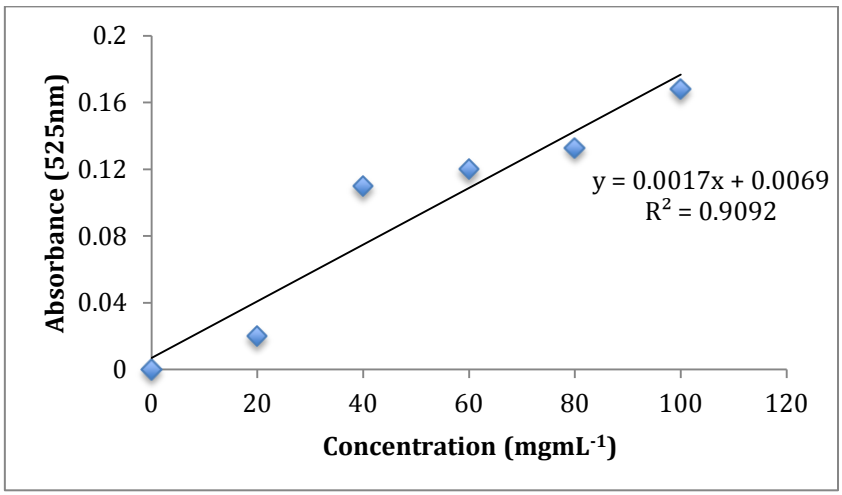
Calibration curve of iron concentrations



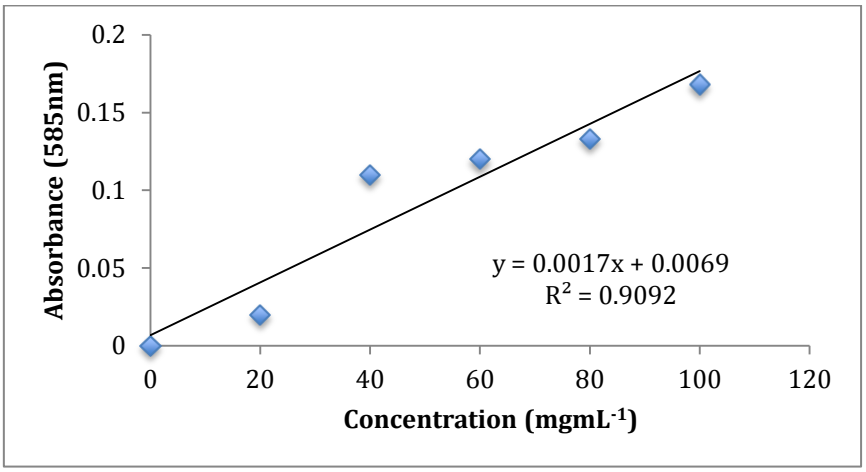
Calibration curve of protein using BSA as standard



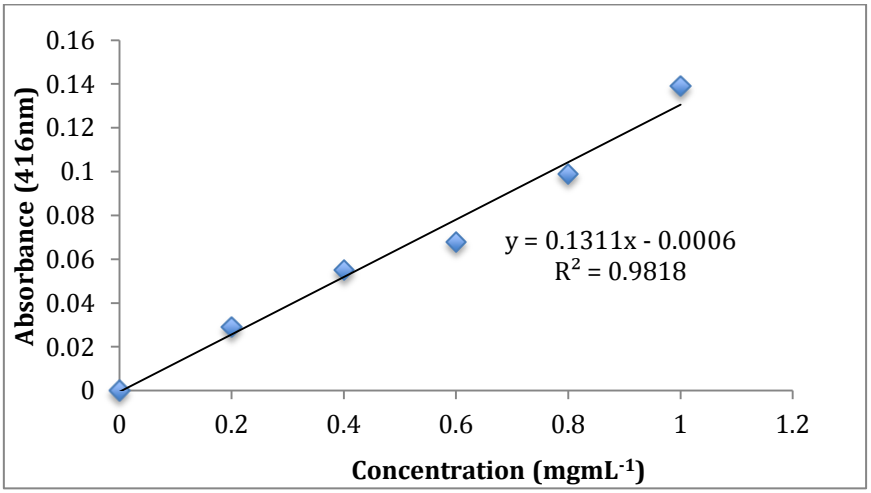
Calibration curve of sugar using glucose as standard



Calibration curve of uronic acid using D-glucuronic acid as standard



Calibration curve of amino sugars using Galactosamine as standard



Calibration curve of pyruvic acid using pyruvic acid as standard