Modelling of particulate adsorption efficacy of bioflocculants

Dissertation

submitted in the partial fulfillment of the requirement for

the award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

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JULY 2014

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled **"Modelling of particulate adsorption efficacy of bioflocculants**" in partial fulfilment of the requirement for the award of the degree of Masters of Technology 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 and **Dr. S.K Singh,** Assistant professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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This is to certify that the thesis entitled **"Modelling of particulate adsorption efficacy of bioflocculants"** submitted by Amanpreet Kaur 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.

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ACKNOWLEDGEMENT

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I take this opportunity to acknowledge the warmth, guidance and cooperation of my teachers, colleagues, friends and family members in this study. I am pleased to have the opportunity to express my gratitude to those precious people who have contributed to this thesis.

First and foremost, I would like to express my profound gratitude and great indebtedness to my supervisor, Associate Professor **Dr. Moushumi Ghosh**, for her benevolent supervision of my M.Tech Thesis. I am very grateful to her for giving me the opportunity to conduct this research project within her fascinating research group, for her continuous support and constructive criticism, and for encouraging and challenging me throughout the progress of the study; never accepting less than my best efforts.

I am deeply indebted to my co-supervisor, Assistant Professor **Dr. S.K Singh** from Chemical Department, he helped and encouraged me in all the time of research and in thesis writing.

I express my sincere thanks to **Dr. Dinesh Goyal**, Professor and Head, Department of Biotechnology (DBT), Thapar University, Patiala for providing the best laboratory facilities.

Special thanks to Ms. ***Gurpreet Kaur Khaira***, a research scholar, DBT, for her help, support and valuable suggestions throughout my project work. I also wish to express my thanks to Ms. ***Taranpreet Kaur*** and Ms**.** ***Seema Bhanwar***, research scholars, DBT for their constant encouragement and help. I am thankful to Ms. ***Parul and Mr. Arkadeep Mukherjee,*** research scholars, DBT for their valuable companionship and suggestions.

I was fortunate enough to avail the ungrudging help and assistance from all our laboratory staff, Mr. Babban, Mr. Mohinder, Mr. Surinder and Mrs. Lalita. I am duly acknowledge to SAI Labs, Thapar University and CSIR-Bhavnagar for the analysis of the samples.

Last but not least I am very thankful to my wonderful family and friends for their encouragement. I thank my friends **Divya, Sarbjot, Pargat singh, Aanchal** for their timely help and support. I owe to my **Parents** for their support and love.

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ABSTRACT

Microbial exopolymers are in widespread use as flocculants in several industries as they are potential alternatives to synthetic flocculants due to their biodegradable, non-toxic and eco-friendly nature. In most cases, flocculant adsorption is an essential prerequisite for flocculation process and also to understand the kinetic aspects. In the present study, out of total eighteen bioflocculants, three bioflocculants L2, X4 and W2B showed highest flocculating activity and turbidity removal efficiency against surrogates of two prevalent water borne pathogens, *Salmonella typhimurium* ATCC 23564 *and Shigella flexneri* Type 2a cells. Biochemical characterization revealed the polysaccharide nature of all the three bioflocculants and the porous structure of the bioflocculants was reflected in the scanning electron micrographs. Further, the presence of hydroxyl, amino, carbonyl and carboxyl functional groups were indicated by FTIR analysis of the bioflocculants. GPC analysis revealed the high molecular weight of the bioflocculants. The particle sizes of the L2, W2B and X4, were found as 528.2, 527.2 and 419 nm, respectively. Viscosity measurement studies in order to assess the effect of 0.1 mg/mL of bioflocculants on the viscosity of dispersed cells as a function of time, resulted in gradual increase of viscosity during 30 to 60 min of time. The effective orthokinetic and perikinetic flocculation models were developed which demonstrated the significant effect of particle size of the bioflocculants and number of dispersed particles on the adsorption mechanism. The order of bioflocculants leading to maximum adsorption rate against the dispersed particles was found as L2>W2B>X4. The study proves bioflocculants as effective adsorbents over synthetic flocculants with marketing advantages due to the public perception of the relative safety of natural compounds.

Keywords: Bioflocculants, water-borne pathogens, flocculating activity, adsorption rate, orthokinetic flocculation model, perikinetic flocculation.

List of Abbreviations

BHI	Brain Heart Infusion broth
CFU	Colony Forming Unit
CPC	Cetylpyridinium Chloride
DLS	Dynamic Light scattering
EDS	Energy dispersive X-ray microanalysis
EDTA	Etylenediamine-tetraacetic acid
et.al	Et alteri/ et alli(and others)
FIB	Flocculant Isolation broth
FT-IR	Fourier transform-infrared spectra
GPC	Gel Permeation Chromatography
KDa	Kilo Dalton
OD	Optical Density
SEM	Scanning Electron Microscopy

List of Symbols

L	litre
°C	degree(s) Celsius
g	gram
h	hour
mL	millilitre
mg/mL	milligram per millilitre
mg/L	milligram per litre
µg	microgram
µg/ml	microgram per millilitre
µL	microlitre
µM	micromolar
min	minute
Mm	millimeter
nm	nanometer
%	percentage
rpm	revolutions per minute
S	second
v/v	volume by volume
U	unit
Wt	weight

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1. INTRODUCTION

Particles in a raw water supply may be composed of inorganic materials, pathogens, or toxic materials. These particles may also provide sorbent sites for pesticides and other synthetic organic chemicals and heavy metals. Particles are undesirable not only for the cloudy appearance they impart to finished water, but because they also have the ability to shelter microorganisms from inactivation by disinfectants. Colloids are very fine particles, typically ranging from 10 nm to 10 μm. Colloidal particles in nature normally carry charges on their surface, which lead to the stabilisation of the suspension (Tripathy et al. 2006). In order for such particles to settle, they must be chemically destabilized or coagulated to neutralize the charge on them and to form larger flocs that can settle, thereby facilitating their removal from water.

Microbes present in water are also colloids, as most of the bacteria falls in the size range of 0.2 to 1 micron (Valeriani et al. 2014). Contamination of drinking-water by microbial pathogens can cause disease outbreaks and contribute to background rates of disease. Approximately 35% of the potential productivity of developing nations is lost due to waterborne diseases (WHO 2005). *Salmonella typhimurium, Escherichia coli O157:H7, Listeria monocytogenes, Shigella flexneri* Type 2a and *Yersinia enterocolitica* are among prevalent water borne pathogens among which, *Shigella* and *Salmonella* are known to cause serious diseases. Processes for removal of microbes from water include pretreatment, coagulation/ flocculation/sedimentation, and filtration. Flocculants are the substances which bring about solid-liquid separation by the process of destabilization either by bridging or charge neutralization. Mostly used flocculants are synthetic in nature and may lead to many environmental and health problems (Zouboulis et al 2003). However, their application is constrained with low flocculating efficiency. Hence, there is urgent need of natural alternatives to these chemical flocculants. Microbially produced flocculant can act as potential

alternative due to their biodegradability, non-toxicity and they are environmental friendly.

The interaction between the bioflocculant and colloidal particle depends on the nature of the bioflocculant and of the dispersed particles (Gregory et al. 2011). Bacterial particles easily get adsorbed on the surface of bioflocculant because of large surface area per unit volume, depending on particles size distribution and their binding capacity to the adsorbent (flocculant), which lead to flocculation (Brostow et al. 2007). Adsorption of bioflocculants with distinct structural properties onto dispersed particles need to be addressed (Vande 1994) as most of the earlier studies have focused on the flocculation mechanism of synthetic flocculants (Kislenko 2000). In general, the process of adsorption of bioflocculant chains on particles is an essential step and is influenced by the applied shear and various parameters such as viscosity, temperature, turbidity, effective radii of a particle, particle's initial concentration, adsorption rate constant and molecular weight of the flocculant. Although, various bioflocculants have been characterized for several applications but no systematic study on adsorption mechanism of bioflocculants under dynamic conditions have been reported.

In view of this, the present study has been designed to develop a systemic model for specific bioflocculant and dispersed particles system on the basis of orthokinetic and perikinetic flocculation and in exploring the suitable model for using low-cost eco-friendly alternatives to obtain biodegradable, abundant and readily available bioflocculants for the contaminated water treatment.

Objectives:

1. Screening of bioflocculants flocculating pathogens.
2. Characterization of bioflocculant and flocculation parameters.
3. Modeling of specific bioflocculant and related dispersed particles system.

2. REVIEW OF LITERATURE

2.1 Colloidal particles

Suspended particles, colloids and dissolved molecules are the three forms of solids, which are present in the raw water (Oliveira et al. 2012). Colloids are very fine particles ranging from 10 nm to 10 µm. Bacteria found in water can be considered as colloids, as most of the bacteria fall in the size range of 10 nm to 1µ. In general, the larger particles can be easily removed from water through sedimentation and filtrations. Since colloidal particles have similar negative electrical charges on their surfaces, which cause repulsion and in turn colloids remain suspended in water as small particles (Lamas et al. 2011). The removal of colloids is the most difficult aspect of the conventional water treatment system.

An adequate supply of safe drinking water is one of the major pre-requisite for a healthy life, but water borne diseases are one of the major causes of deaths worldwide. In 2006, waterborne diseases were estimated to cause 1.8 million deaths each year while about 1.1 billion people lacked proper drinking water (WHO 2005). Studies suggested that 35% of the potential productivity of developing nations is lost due to waterborne diseases.

2.2 Water –borne pathogens

Yersinia is a facultative anaerobe (Ryan et al. 2004). Some members of *Yersinia* are pathogenic in humans. For example, *Yersina pestis is* the causative agent of plague and *Yersinia enterecolitica* is a frequent cause of gastroenteritis in children and is bacterium commoly found in contaiminated water and soil.

Salmonella is facultative, non-spore forming, predominantly motile bacteria (Murugkar et al. 2005) with diameters around 0.7 to 1.5 µm. All strains of *Salmonella*

are pathogenic for human, causing enteric fever such as typhoid and paratyphoid fever, gastroenteristis, and septicemia. Over 2,000 antigenic type of *Salmonella* occurs. *Salomonella typhimurium* is known to cause *typhus abdonminals* in human and this is bacterium commonly found in contaiminated and infected food, water or fly excrement. Reports are available on the prevalence of salmonellosis in livestock and poultry in various regions (Ghosh et al. 2009)

Shigella is non-spore forming bacteria (Ryan et al. 2004). All strains of *Shigella* are pathogenic (Garrity et al. 2005), causing bacillary dysentery in humans that result in the destruction of epithelial cells of intestinal mucosa (Zychinsky 1992). *Shigella* infection is typically via ingestion (James et al. 2004). Some strains of *Shigella* are known to produce enterotoxin and shoga toxin, which are associated with causing haemolytic uremic syndrome (Smith et al. 1987). Human and the higher primates are the reservoir for this organism. The organism can be found in the faeces for the week after symptoms have caesed. It can survive in human faeces for days if the samples remain moist.

Escherichia coli is a facultative anaerobic (Ryan et al. 2004). The major species, *E.coli*, occur in the lower portion of the human and warm-blooded animals, where it is a part of normal flora. Some strains can cause gastroenteritis; other can cause urinary tract infections. *E.coli* is an enterohaemorrhagic strain and cause of water and food borne illness, result from swimming in or drinking contaiminated water, and eating contaminated vegetable.

Listeria is a gram-positive, non-spore forming, rod-shaped, aerobic or microaerophilic bacteria (Ramaswamy et al. 2007). *Listeria monocytogenes* is a parasite and pathogen of a wide variety of animals. It cause meningitis in adults and

prenatal and postnatal disease in infact and is bacterium commoly found in soil, stream water, sewage, plants and food.

2.3 Water treatment system for removal of colloidal particles: Flocculation

The goal of water purification process is to remove existing contaminants in the water, or reduce the concentration of such contaminants so that the water becomes fit for its desired end-use. To remove these microbial colloids from water, they have to be destabilized first, forming larger and heavier flocks. These flocs can be further removed by the process of sedimentation and filtration (Tripathy et al. 2007). Flocculation is an essential phenomenon, which is used in water treatment for separation of suspended particles from water. Flocculation is achieved with the help of flocculants, which are natural and synthetic substances that facilitate the agglomeration or aggregation of the particles (Liu et al. 2002).

2.3.1 Types of Flocculants

Flocculants are basically classified into three categories viz. 1) inorganic 2) organic and 3) natural flocculants. Inorganic and organic flocculants fall in the category of synthetic flocculants exemplified by aluminium sulfate, ferric sulfate and ferric chloride (Gurumoorthy et al. 2003). Polyaluminium Chloride and polyferric chloride are the polymerized flocculants that have been reported to be more efficient (Zouboulis et al. 2003). Organic flocculants have been widely used for the flocculation of suspended particles in wastewater treatment and for effluent (Jimoda et al. 2013). These flocculants possess high molecular weight, are effective over wide range of pathogens and have low charge density.

2.3.2 Limitations of synthetic flocculants

Although synthetic flocculants have high efficiency, cost effectiveness and easy availability, but their usage leads to environment burden and health hazards. Polyacrylamides are one of the most widely used flocculant, but the monomers of polyacrylamide have been reported to be carcinogenic and neurotoxic to humans and animals (Salehizadeh et al. 2011).

2.3.3 Natural Flocculants

Starch, chitosan, cellulose, quar gum and moringa oleifera are derived from the natural sources and have been extensively used in flocculation processes (jin et al. 2004). These natural flocculants can be polysaccharides, protein or lipids. Bridging is the mechanism of flocculation by the naturally produced flocculants that involves the flocculants binding to multiple particles leading to aggregate formation (Gao et al. 2006). Natural flocculants have several advantages of being environmental friendly, biodegradable, non-toxic and non- carcinogenic.

2.3.4 Microbial flocculants

The study of bioflocculants has attracted considerable attention over the years and the use of biodegradable flocculants produced by microorganisms has been extensively investigated. Many bioflocculant producing microorganisms including bacteria, fungi and yeast have been reported to produce protein (Takeda et al. 1991, 1992), polysaccharide (Kurane and Matsuyama 1994), and glycoprotein (Lee et al. 1995) flocculant. The presence of various side groups along the flocculant structure may promote intramolecular forces in individual molecules or intermolecular interactions between flocculant chains. They can produce large, strong aggregates, which can be easily separated by physical means (Kurane and Nohata 1991) (Yokoi et al. 1997).

Compared with conventional chemical and synthetic flocculants with well-established flocculating mechanisms (including bridging and charge neutralization), the flocculating mechanisms of bioflocculants appear to be less well investigated. Although most bioflocculants can be used to flocculate kaolin suspension, they show different flocculating ability for other colloids in aqueous solution (Mounir et al. 2014).

Almost all applications of bioflocculants are under conditions where a suspension is subjected to shear. When flocculation occurs by bridging or charge neutralization mechanisms, then adsorption of flocculant chains on particles is an essential step in the process and this may be considerably influenced by the applied shear (Valeriani et al. 2014).

2.4 Flocculation Process

Flocculation is the process of bringing together the particles to form large agglomerations by physically mixing or through the bridging action, such as long chain-flocculant (Lu et al. 2001). Destabilization by bridging occurs when a bioflocculant of a high molecular weight becomes attached at a number of adsorption sites to the surface of negatively charged particles along the bioflocculant chain (Moruzzi et al. 2012). The remainder of the bioflocculant may remain extended into the solution and may adsorb on available surface sites of other particulates, thus creating a bridge between the surfaces.

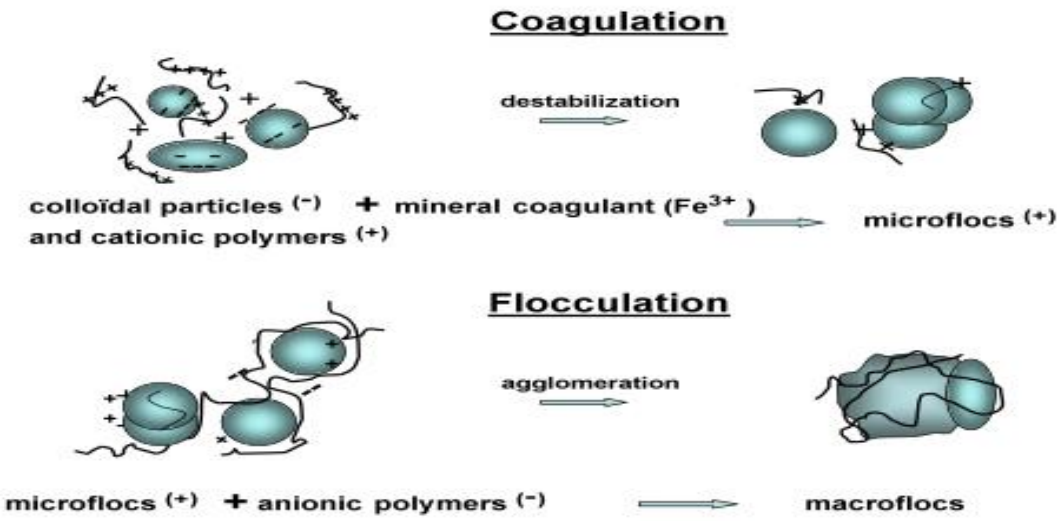


Figure 2.1 The process of coagulation and flocculation.

Due to its heterogeneous composition and complex structure, many factors may interact and influence the floc formation, which makes it almost impossible to evaluate the flocculation process by only a single mechanism (Jimoda et al. 2013). Several studies are presented in literature to identify and characterize the mechanisms involved in the flocculation process. Types of flocculation:

1) Bridging
2) Charge neutralization

2.4.1 Bridging

Bridging occurs when a flocculant forms threads or fibers which attach to several colloids, capturing and binding them together. Inorganic primary coagulants and organic polyelectrolytes both have the capability of bridging. Higher molecular weights mean longer molecules and more effective bridging. Bridging is often used in conjunction with charge neutralization to grow fast settling (Zhang et al. 2007). For instance, alum or a low molecular weight cationic flocculant is first added under rapid mixing conditions to lower the charge and allow microflocs to form. Then a slight amount of high molecular weight flocculant, often an anionic, can be added to bridge

between the microflocs (Zulkeflee et al. 2012). The fact that the bridging flocculant is negatively charged is not significant because the small colloids have already been captured as microflocs.

2.4.2 Charge neutralization

Colloid entrapment involves adding relatively large doses of flocculants, usually aluminum or iron salts which precipitate as hydrous metal oxides. The amount of flocculant used is far in excess of the amount needed to neutralize the charge on the colloid. Some charge neutralization may occur but most of the colloids are literally swept from the bulk of the water by becoming enmeshed in the settling hydrous oxide floc. This mechanism is often called sweep flocs (Han et al. 2003).

However, a clear distinctions between different mechanisms that may be responsible for floc formation is difficult, thus, one has to consider them separately (Dorota et al. 2011). Furthermore, there are not only the physico-chemical aspects that must be taken. The need for a general approach led to the conventional way to describe flocculation based on two discrete steps dealing with the transport and attachment of the particulate material into flocs, respectively (Thomas et al. 1999). In turn, the flocculation can be characterized by two distinct mechanisms: Perikinetic flocculation and Orthokinetic flocculation. The rate of flocculation is determined by the collision frequency induces by Brownian motion, is called perikinetic flocculation. The flocculation, which is caused by the velocity gradients, is called orthokinetic flocculation. The fundamentals of the flocculation mechanisms were presented by Smoluchowscki (1917), who described them on the bases of collision frequency functions and changes in particle size. From this concept, different approaches were taken in order to establish a good concenptual flocculation mechanism.

2.4.3 Perikinetic Flocculation Process

Perikinetic flocculation arises from thermal agitation (Brownian movement) and is a random process. Flocculation during this stage commences immediately after destabilization and is complete within seconds since there is a limiting floc size beyond which Brownian motion has no or little effect. Furthermore, although the potential energy barrier existing between colloidal particles may be overcome by the thermal kinetic energy of Brownian movement, as the particles progressively coalesce the magnitude of the energy barrier increases approximately proportional to the area of the floc, so that eventually Perikinetics flocculation of such potentially repellant particles must ceases (Kislenko 2000). The rate of flocculation or the rate of decrease in the number of particles of a suspension due to Perikinetic flocculation may be described by a second order rate law. Von Smoluchowski (1917) developed a model for Perikinetics flocculation where the frequency of collisions was obtained from the diffusional flux of particles towards a single stationary particle. The number of particles diffusing radially inwards through the surface of a sphere centered on the stationary particle is proportional to the Brownian diffusion coefficient of the particles, the surface area of the sphere and the particle concentration gradient in the radial direction. Since the diffusion is assumed to be radial to the number of particles diffusing radially inwards is equal to the number of collisions with the central particle (Gregory 1998).

2.4.4 Orthokinetic flocculation

The second stage in the flocculation process is given the name orthokinetic flocculation and arises from induced velocity gradients in the liquid. Setting the liquid

in motion may induce such velocity gradients by:

(a) Passage around baffles or mechanical agitation within a flocculation reactor.

(b) The tortuous path through interstices of a granular filter bed.

(c) Where flocs are sufficiently formed by sedimentation within a settling basin and so on.

The effect of velocity gradients within a body of liquid is to set up relative velocities between particles thereby providing opportunity for contact.

Perikinetics flocculation is complete the only way in which appreciable contact between particles can be promoted is to induce shear motion in the liquid. This is achieved by inducing velocity gradients whereby particles achieve mutual contact by movement with the surrounding liquid. The process of floc aggregation by this procedure is termed orthokinetic flocculation. The greater the velocity gradients induced in the liquid the more particle contacts there will be within a given time. However, the greater the velocity gradients the smaller will be the ultimate floc size due to a continuous breakdown of the larger floc. Hence, for a given velocity gradient there will be a limiting flocculation time beyond which floc particles will not grow further. The lower the velocity gradient the longer will be the time needed to reach the optimum floc size, but the larger will be the final floc size. Orthokinetic flocculation is imposed by velocity gradients created by mixing particles, which are moving faster to overtake slower-moving particles in a velocity field. Due to the collision between the particles, larger particles are formed which are easier to remove by gravity separation. The differential settling is determined by differences in settling velocity between different particles size. Larger particles overtake smaller particles, collide and stick together, forming larger particles that settle faster (Tripathi et al. 2006)

The majority of flocculants are able to flocculate colloidal particles due to adsorption. Their molecular weight plays a vital role in the adsorption. The higher molecular weight, the more adsorption sites and electric charges, thus the neutralizing capacity becomes stronger, and bridging effect develops (Swenson et al. 1998). Adsorption of a molecule (or ion) from solution on the surface of a solid involves a sequence of events involving a) Removal of the molecule from solution; b) Removal of solvent from the solid surface; c) Attachment of the molecule to the surface of the solid.

In case of continuous stirring, due to thermal agitation, molecules and ions in solution are in a continuous state of movement, colloidal particles are hazardly bombarded by molecules giving rise to a random motion. This continuous random movement of colloidal particles is known as Brownian motion, which results in contact between molecules and ions in solution and solid surfaces.

2.5 Adsorption Kinetics

When a flocculant solution is added to a stable suspension in an amount sufficient to de-stabilize the particles, several processes are initiated, the rates of which have significant effects on the overall flocculation process. The following steps, illustrated schematically in need to be considered:

(a) Mixing of the flocculant molecules among the particles.

(b) Adsorption of flocculant chains on the particles.

(c) Re-arrangement (re-conformation) of the adsorbed chains from their initial state to an eventual equilibrium configuration.

(d) Collisions between particles having adsorbed flocculant to form aggregates (flocs) , either by bridging or by charge effects.

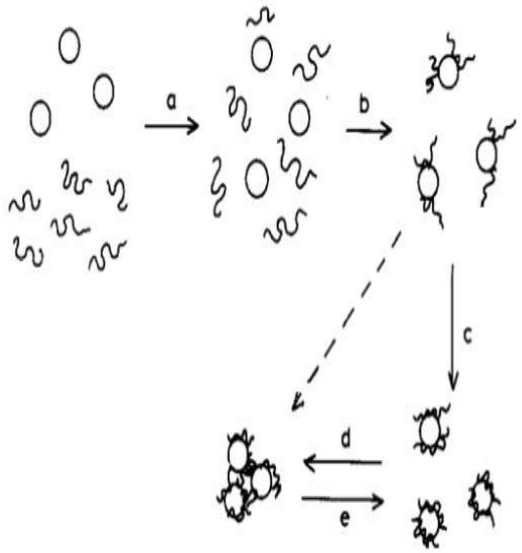


Figure 2.2 Schematic representation of adsorption kinetics.

Simultaneously, at rates, which depend on a number of factors, making the whole process difficult to analyze in a straightforward manner. In some studies, initial conditions have been arranged so that steps (a) - (c) above occur without flocculation, and then flocculation is initiated by changing the conditions (Gregory 1998). Flocculant adsorption is a second-order rate process, depending on the number concentrations of dispersed particles and flocculant molecules (Gregory 2011). We can find the rate of flocculant adsorption in terms of the rate of loss of flocculant molecules from solution:

$$- dn_2 / dn_1 = k_{ads} n_1 n_2 \qquad (1)$$

Where, ' n_1' and ' n_2' are the number concentrations of particles (used bacteria in present study) and flocculant molecules and k_{ads} is the adsorption rate coefficient. This expression gives the collision rate, but in the case of adsorbing flocculants it is reasonable to assume that every collision leads to attachment of one or more segments

to the particle surface (Moruzzi et al. 2013). The flocculant concentration required for effective flocculation is proportional to the particle concentration, because the adsorption is of the high affinity type and virtually the entire added flocculant is adsorbed (at least for fairly low surface coverage). This means that the rate of flocculant adsorption increases dramatically with increasing particle concentration. For concentrated dispersions (a few wt.%) flocculant adsorption is practically instantaneous, whereas for very dilute dispersions (a few mg/L), flocculant adsorption may take several minutes or even longer. By making some simplifying assumptions, it is possible to estimate the time, t_{ads} for a certain fraction of the added flocculant to be adsorbed on particles (Gregory 1998). Provided that the surface coverage achieved is quite low, and the particle number concentration remains constant (i.e. no flocculation occurs), the result is:

$$t_{ads} = -\ln(1-f)/k_{ads}n \qquad (2)$$

Where, ' f 'is the fraction of total flocculant adsorbed in time t_{ads}. It should be stressed that this approach is not suitable when adsorbed flocculant covers a significant fraction of the particle surface. In that case flocculant adsorption becomes hindered, since the available surface is reduced, and k_{ads} would decrease with time. Thus, actual adsorption times would be longer than those given by Eq. (1). Since flocculation by flocculants often occurs when the surface coverage is quite low, this simple approach may be adequate in many practical cases. The magnitude of the rate coefficient in Eq. (2) depends on number of factors, including the hydrodynamic sizes of the particles and flocculant molecules and the degree of agitation applied. One approach is to use the Smoluchowski expressions for hetero- aggregation as a result of collisions caused by Brownian diffusion and fluid shear.

Diffusion: $k_{ads} = 2kT/3\eta (a_1 + a_2)^2/a_1a_2$ (3)

Shear: $k_{ads} = 4/3\ G(a_1 + a_2)^3$ (4)

Where ' a_1' and ' a_2' are the effective radii of the particles and flocculant molecules (both assumed spherical), k is the Boltzmann constant, T the absolute temperature, η the viscosity and G the shear rate. (Note that in the Smoluchowski treatment of shear-induced collisions, the dispersion is assumed to be subject to uniform laminar shear (Gregory 2011).

The adsorption studies with bioflocculants and dispersed particles need to be addressed for industrial and biological processes or application.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Media

All chemicals used were of the highest analytical grade available. 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 121^0C and 15 psi for 15 min and were allowed to cool below 50^0C before use. The screening medium referred as FIB medium (flocculation isolation broth) (Ghosh et al. 2009) (Annexure I) and Brain-heart infusion medium (BHI), often containing agar, for culturing microorganisms.

3.1.2 Microorganisms

Surrogates of different bacterial strains (*Salmonella typhimurium* ATCC 23564 and *Shigella flexneri* Type 2a) were revived twice in a brain heart infusion (BHI) broth by incubating at 37°C for 12 hrs.

3.2 Extraction and purification of the bioflocculant

Eighteen previously isolated strains from various industrial and river sites were used in the present study. The selected strains for bioflocculant production were inoculated in 1 L FIB media (Annexure I) and incubated in a rotary shaker (120 rpm/min) at 30℃ (Labcon, 5081U, USA) for 48 h at 37°C. The cells were removed from culture solution by high speed centrifugation (CF15RX11, Hitachi, Japan) at 10,000 rpm for 10 min at 4°C. The supernatant was collected and from the supernatent bioflocculant was precipitated by addition of two volumes of chilled ethanol (95% ethanol) and kept overnight at 4°C. The precipitate was separated from ethanol suspension by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet obtained was dissolved in

de-ionized water and reprecipitated by drop wise addition of 10% cetyl prydinium chloride (CPC), which is cationic detergent. The precipitated bioflocculant complex was collected by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet was washed with 10% sodium chloride (NaCl). Further precipitation was done by adding double volume of chilled ethanol and the precipitates formed was washed with de-ionized water and resuspended in de-ionized water. Further dialyzed by using dialysis membrane (Cellulose membrane, cut off 12-14 kDa) against de-ionized water and purified bioflocculant was freeze-dried to obtain powder.

3.3 Flocculating activity

The flocculating activity was measured according to the method of Kurane et al. (1986) with minor modifications using a suspension of kaolin clay as test material. In a test tube, 4.5 mL of kaolin suspension (5 g/L) was added and mixed with 0.25 mL of $CaCl_2$ solution (90 mM). To this mixture, 100 uL of the test bioflocculant was added, vortexed for 30 sec and allowed to stand for 5 min at room temperature. The optical density of the upper phase at 550 nm (A) was measured with a spectrophotometer (HITACHI, U-2900, Japan). A control experiment was carried out in the same manner with 100 uL of distilled water instead of the bioflocculant addition and the optical density was measured (B). Flocculating activity (%) was defined and calculated as $\{(B-A)/ B\} \times 100$. The activity was expressed as the mean value from duplicate determinations.

3.4 Characterization

3.4.1 Biochemical characterization

3.4.1.1 Estimation of total Protein

The total protein content of the purified bioflocculant 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 mg/mL) and bioflocculant stock (1 mg/mL) were prepared. 1 mL of freshly mixed complex-forming reagent C (Annexure I) was added to 0.2 mL of the bioflocculant 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).

3.4.1.2 Determination of total sugars

The total sugar or carbohydrate content of bioflocculant was determined by the method of phenol sulphuric acid (Dubois et al. 1986) by using glucose as standard. Different concentrations of glucose (0-1 mg/mL) and bioflocculant (1mg/mL) were prepared. 200 µL of phenol reagent (5 % v/v in water) was added to 200 µL of bioflocculant and 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).

3.4.1.3 Determination of Amino sugars

The total amino-sugars content of bioflocculant was determined by the method described by Elson-Morgan, (1934) by using galactosamine as standard. Different concentrations of galactosamine (0-1 mg/mL$^{)}$ and bioflocculant (1mg/mL) were prepared. 50 µL of reagent A (Annexure I) was mixed with 250 µL of galactosamine and bioflocculant. 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 amino sugars content present in the sample was calculated from calibration curve (Annexure II).

3.4.1.4 Determination of Uronic Acid

The uronic acid content of bioflocculant was determined by method described by Haug and Larsen in 1962 by using D-glucuronic acid as standard. Different concentrations of D-glucuronic acid (0-1 mg/mL) and bioflocculant (1mg/mL) were prepared. 1.5 mL of ice cold reagent A (Annexure I) was carefully added in 250 µL of bioflocculant 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. Re-heating 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).

3.4.1.5 Determination of Pyruvic Acid

The pyruvic acid content of bioflocculant was determined by method described by Friedman and Haugen, (1943) by using pyruvic acid as standard. Different concentrations of pyruvic acid (0-3 mg/mL) and bioflocculant (1mg/mL) were

prepared. Initially, bioflocculants were 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).

3.4.2 Physical characterization

3.4.2.1 Fourier transform-infrared spectra (FT-IR)

Fourier Transform–infrared spectrometer (CARY 600, ALIGENT, California) was employed to determined the functional group of bioflocculants. The spectrum of pure bioflocculant was recorded on the spectrometer over a wave number range of 400-4000 cm^{-1}.

3.4.2.2 Dynamic light scattering (DLS)

The dynamic light scattering apparatus were performed at a scattering angle of 90Æ. A quartz sample cell was set in a silicon oil bath such that the refractive indices of the oil and the cell were nearly equal. Light scattering was measured at a regulated temperature of 25.0 ± 0.1°C.

3.4.2.3 Scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis (EDX)

SEM analysis was done to obtain surface properties and EDX was carried out for elemental analysis. Samples of pure bioflocculant were coated with a conductive layer of gold and then analyzed by SEM (JSM 541- V, JOEL, Japan) at an accelerating voltage of 20.0 kV, complemented with an energy-dispersive x-ray microanalyzer (INCAx_act, Oxford instrument, United Kingdom).

3.4.2.4 Gel Permeation Chromatography (GPC)

The chromatography experiment was conducted with 0.05 mol L^{-1} Nacl as the eluent, and 3 mL eluent fraction were collected and analysed by phenol-sulphuric acid method. Approximate molecular weight values were estimated by calibrating the same column using aqueous solution of monodisperse standards. Standard Dextran T-70 and Dextran T-40 were analysed on the column. The bioflocculant samples were monitored using refractive index detector. Based on these experiments a standard curve between elution volume and the logarithm of molecular weight was acquired, and a regressed equation was acquired:

$$\text{Log(MW)} = K1Ve + K2$$

Where MW and *Ve*(mL) are the molecular weight and elution, respectively, K1 and K2 are constants.

3.5 Preparation of dilutions

Bacterial strains of *Salmonella* and *Shigella* cells were inoculated in BHI and dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were prepared using saline water. 100 µL of suspension from the test tubes having 10^{-3}, 10^{-4}, and 10^{-5} were spread on BHI agar in petriplates and incubated at 37ºC for 24 hrs. To obtain the pure culture different colonies were streaked on the petriplate containing BHI agar and incubated at 37ºC for 24 hrs and then CFU was calculated. The BHI agar plates were prepared for taking the CFU count. 50 µL of the culture was taken from the inoculated tube of bacteria *(Salmonella* and *Shigella*) of dilution 10^{-3}, 10^{-4}, and 10^{-5} was plated. The plates were then incubated at 37°C for 24 hrs. After the period of incubation, the plates were observed and the numbers of colonies were counted.

3.6 Turbidity removal assay

The single colonies of *Salmonella* and *Shigella* cells were spiked in distilled water and 100 µL of the bioflocculant was added, vortexed for 30 second and allowed to stand for 5 min at room temperature. The optical density of the upper phase was measured with a spectrophotometer at 600 nm. A control experiment was carried out with 100 µL of distilled water instead of the bioflocculant. The turbidity removal efficiency was calculated as follows:

Removal efficiency (%) = $[C0 - C/C_0] \times 100$

Where, C_0 is the initial value and C is the value after the flocculation treatment

3.7 Viscosity measurement

Viscosity of samples was measured by viscometer (DV-11+ Pro, BROOKFIELD, USA) at 50 rpm a at time intervals of 10 minutes, at 37°C.

3.8 Adsorption kinetics

Adsorption rate constant was calculated through two processes.

3.8.1 For diffusion – controlled transport process (Perikinetic)

It is determined by the following equation

Diffusion: $k_{ads} = 2kT/3\eta (a_1 + a_2)^2/a_1a_2$

where ‘ a_1’ and ‘a_2’ are the effective radii of the particles and bioflocculant molecules (both assumed spherical), k is the Boltzmann constant, T the absolute temperature, η the viscosity.

3.8.2 For shear induced (Orthokinetic)

Strirring is induced in this process so, it is determined by the following equation

Shear: $k_{ads} = 4/3\ G(a_1 + a_2)^3$

Where 'a_1' and 'a_2' are the effective radii of the particles and bioflocculant molecules (both assumed spherical), k is the Boltzmann constant, T the absolute temperature, η the viscosity, G shear rate.

4.1 Screening of bioflocculants

A total of sixty bioflocculants were extracted, purified and lyophilized to powder form and preliminary screening for adsorption studies was done on the basis of their flocculating activity against kaolin clay suspension. A total of eighteen purified bioflocculants with more than 50% flocculating activity were selected to assess their pathogen binding abilities (Table 4.1).

Table 4.1 Flocculating activity of purified bioflocculants

S.no.	Bioflocculant	Flocculating activity (%)
1	L2	93
2	P6a	68
3	X2	76
4	Y5	59
5	P2	73
6	Z3	69
7	W2b	89
8	X4	91
9	W1b	63
10	Z4	78
11	P8	84
12	P1	59
13	W3	63
14	O4	76
15	R2MGa	59
16	Y3	74
17	W1a	71
18	L4	67

Further, the flocculating activity and turbidity removal efficiency of screened bioflocculants was assessed against the surrogates of two major water borne pathogens *(Salmonella typhimurium* ATCC 23564 and *Shigella flexneri* Type 2a). Results revealed that out of eighteen bioflocculants, three flocculants i.e L2, W2B and X4 showed the higher flocculating activity as well as turbidity removal efficiency against two pathogens, *Salmonella typhimurium* ATCC 23564 and *Shigella flexneri* Type 2a cells. It was observed that the turbidity removal efficiency of L2, W2B, and X4 was 95%, 57%, and 80%, respectively, whereas flocculating activity was 90%, 69%, and 82% against *Shigella* Cells. In the case of *Salmonella* cells, turbidity removal efficiency of L2, W2B, and X4 was 72%, 84%, and 80%, respectively, whereas their flocculating activity was 78%, 85%, and 87%, respectively.

Bioflocculants cause aggregation of particles and cells by bridging, it is a process of bringing together the particles to form large agglomerates by physically mixing. In the present study destabilization occurred when the high molecular weight bioflocculants became attached at a number of adsorption sites to the surface of negatively charged bacterial particles along the flocculant chain. The remainder of the flocculant may remain extended into the solution and may adsorb on available surface sites of other particulates, thus creating a bridge between the surface (Salehizadeh and Shojaosadati 2001).

Table 4.2 shows the binding ability of all the three bioflocculants against the viable and non viable cells of *Shigella* and *Salmonella*, results were compared to the binding capacity of bioflocculants for the kaolin particles. Results revealed no significant change in the binding capacity of the bioflocculants was observed against viable and non-viable cells.

Table 4.2 Binding ability of bioflocculants on viable and non-viable bacterial cells and kaolin particles

Bioflocculant	***Shigella* cells**		***Salmonella* cells**		**Kaolin particles**
	Binding capacity (%)		Binding capacity (%)		Binding capacity (%)
	Viable	Non-viable	Viable	Non-viable	Dry weight
L2	92	89	91	88	93
W2B	88	87	87	86	89
X4	90	88	88	86	91

4.2 Characterization

4.2.1 Biochemical characterization

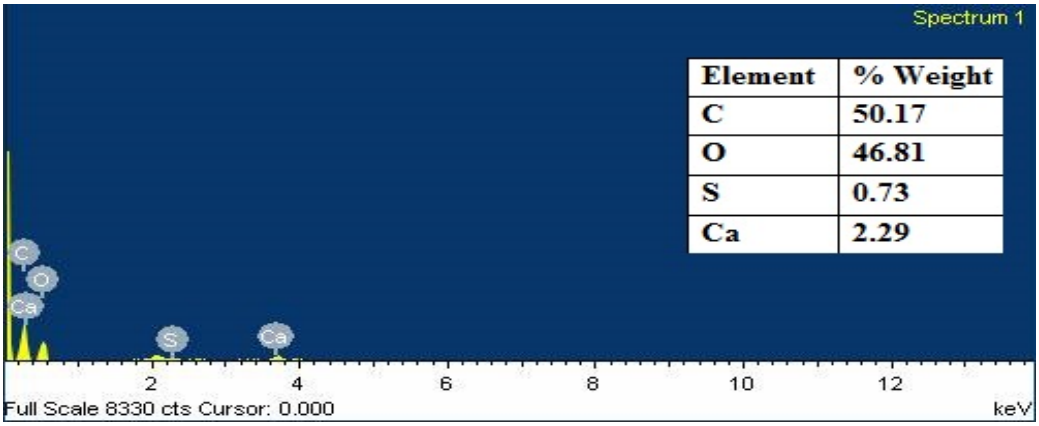
Composition of the bioflocculants L2, X4 and W2B is presented in the Table 4.3.

Table 4.3 Compositional characterization of bioflocculants

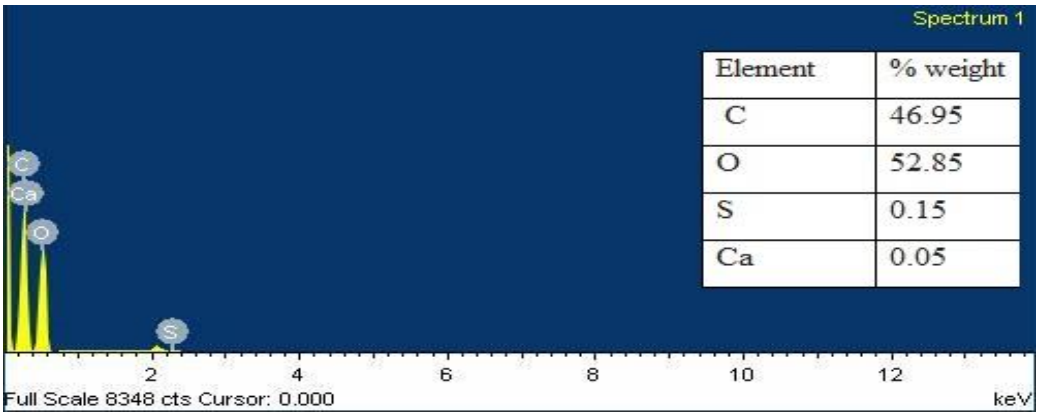
Bioflocculant	Total Sugar (%)	Total Protein (%)	Total Hexoamine (%)	Total Pyruvic acid (%)	Total Uronic acid (%)
L2	9.4	7.2	2.7	1.1	10.1
X4	6.9	5.4	2.3	0.9	11.2
W2b	6.3	5.0	2.1	0.7	12.1

All the three bioflocculants were mainly composed of carbohydrate, hexoamine, protien, pyruvic acid and uronic acid. The polysaccharide nature of all the three bioflocculants was depicted from the presence of high sugar content.

Figure 4.1 shows the EDX analysis of the three bioflocculants. The bioflocculants contained carbon, oxygen, sulphur and calcium elements and their percentage weight is also presented in the tables within the EDX spectrum.



(a)



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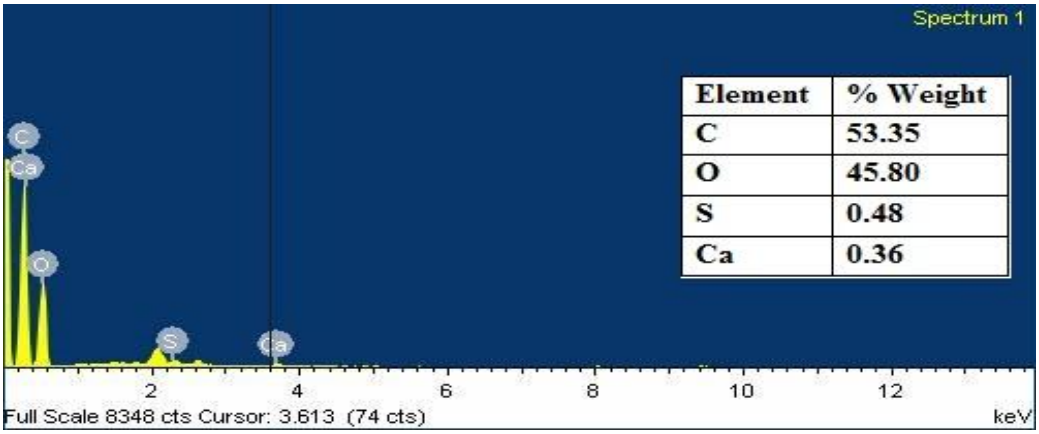
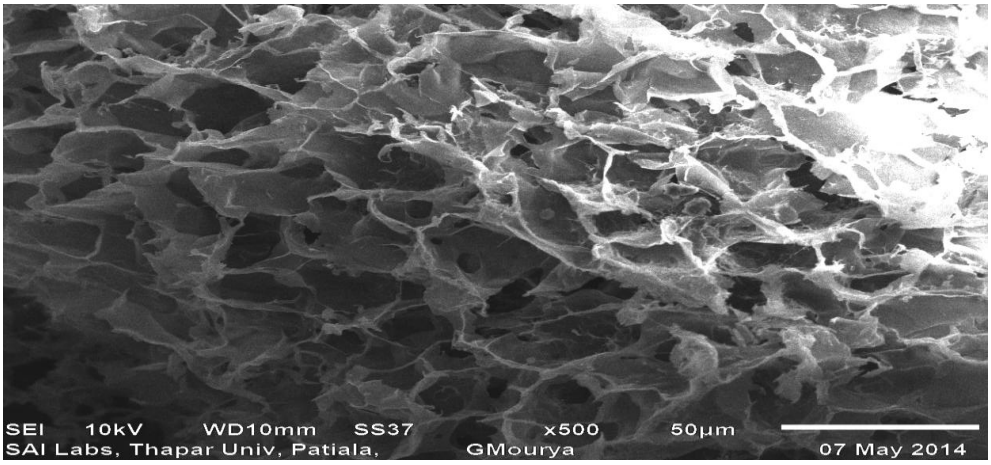


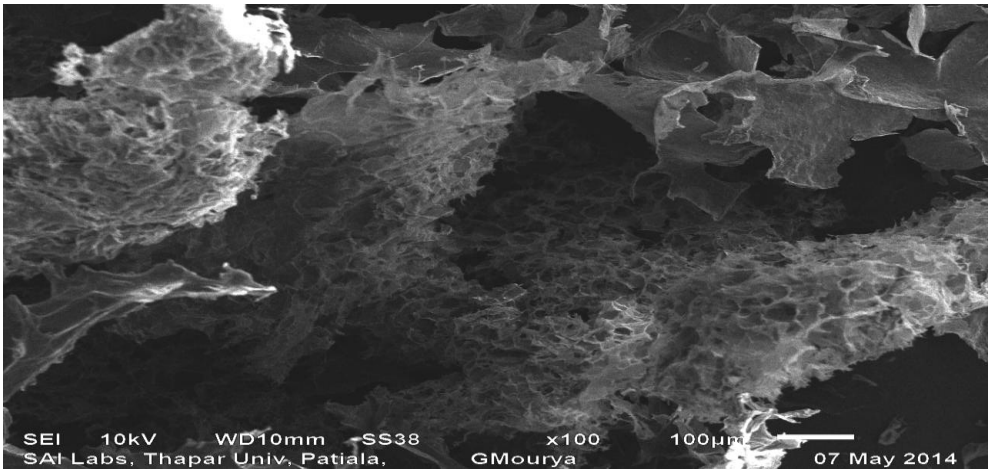
(c)

Figure 4.1 EDX spectrum of bioflocculants (a) L2 , (b) W2B and (c) X4

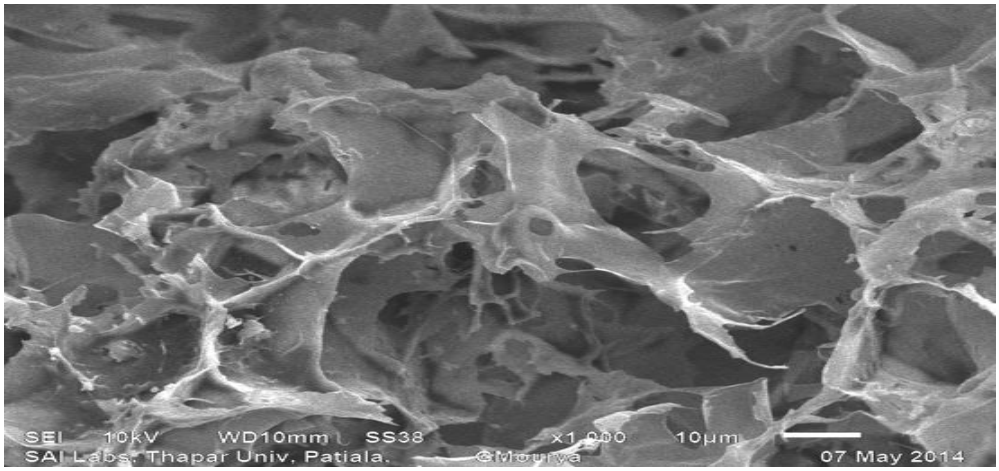
4.2.2 Surface properties of bioflocculants



(a)



(b)



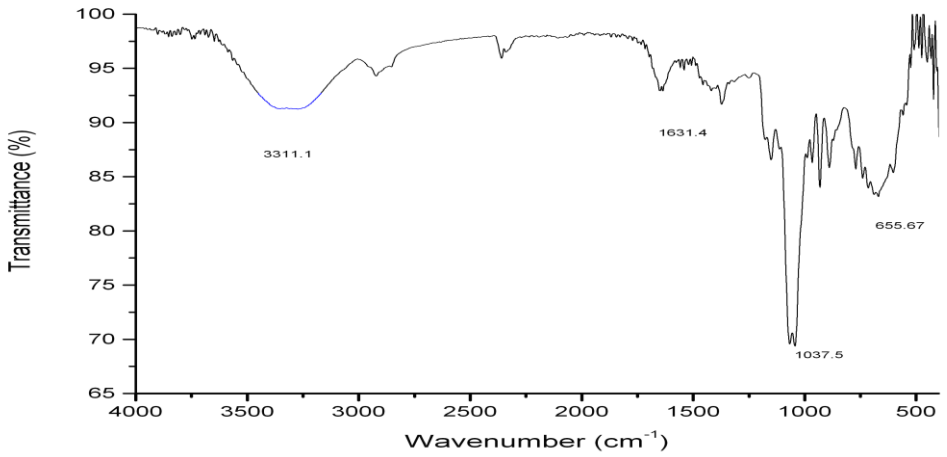
(c)

Figure 4.2 SEM micrograph of bioflocculants (a) L2 , (b) W2B and (c) X4

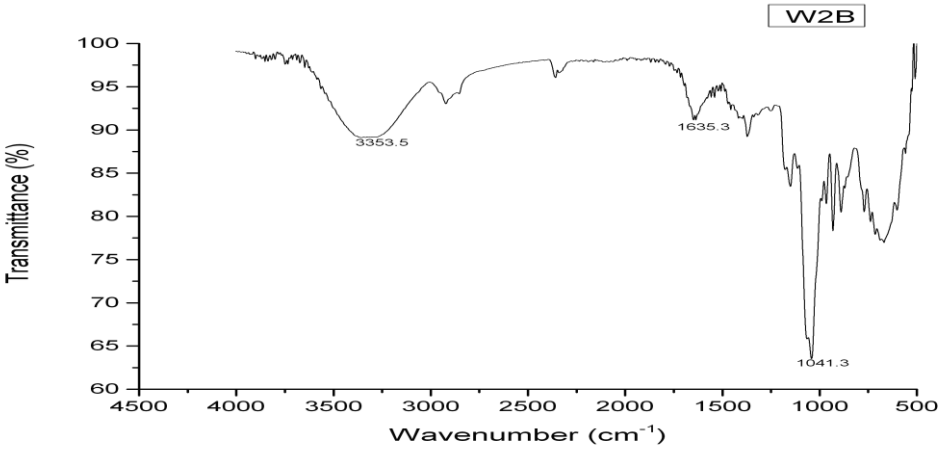
Scanning electron microscopy is one of the most widely used technique to study the morphological as well as structural changes on the surfaces. Figure 4.2 presents the SEM micrographs of the three bioflocculant L2, W2B, and X4. Results reflected the porous structure of the bioflocculants with different pore size distribution. The small pore size leads to compactness of the bioflocculants, which in turn may be found responsible for the high flocculating activity of the bioflocculants. Mao et al. in 2001 reported that the porous struture of the flocculant is responsible for the stability of the structure to the external forces and maintenance of the textural properties during storage.

4.2.3 Functional group analysis

Functional groups present in all the three bioflocculants are important determinants for the flocculating activity (Kumar et al. 2004). The FTIR spectrum recorded for the three purified bioflocculants is given in Figure 4.3 which displayed more complex pattern of peaks from 4500 to 500 cm^{-1}. The Infrared spectra revealed characteristic functional groups such as a hydroxy stretching group at 3311 cm^{-1} (W2B) and 3353.5 cm^{-1}(X4), the spectra also displayed an asymmetrical stretching at 1631cm^{-1} (W2B) and 1635.3cm^{-1}(X4). The absorption peak at 1037 cm^{-1} (W2B), 1041.3 cm^{-1} (L2) is known to be characteristic for all sugar derivates. A weak symmetrical stretching band near 1064.5 cm^{-1} shows the presence of uronate and alkene group at 2364.2 in case of L2. Polysaccharides contain a significant number of hydroxyl groups, which exhibits a broad absorption band above wave number 3000 cm^{-1}. In general a band of stretch of C-O-C, C-O at 1021-1200 cm^{-1} correspond to presence of carbohydrates, the absorbtion band at 1056 cm^{-1} is attributed to the substance is polysaccharide.



(a)



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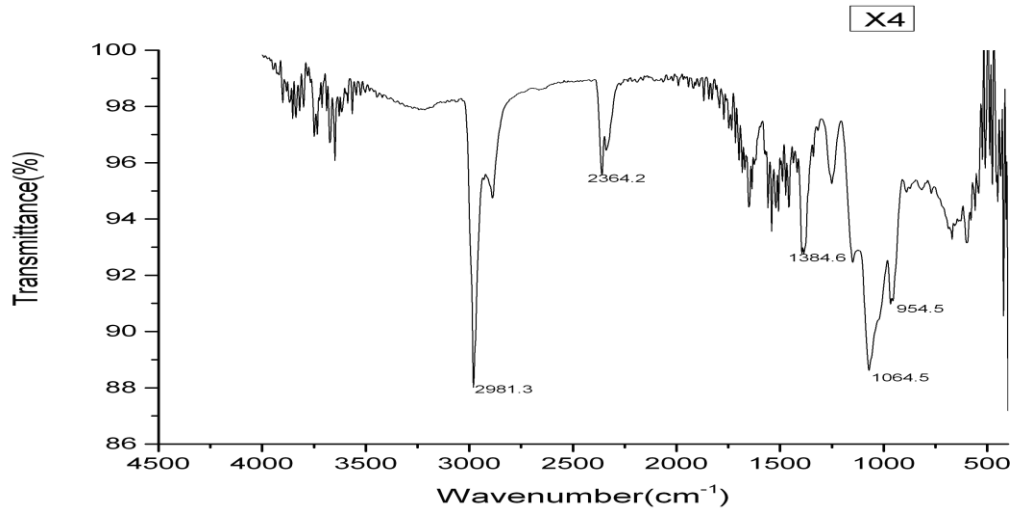
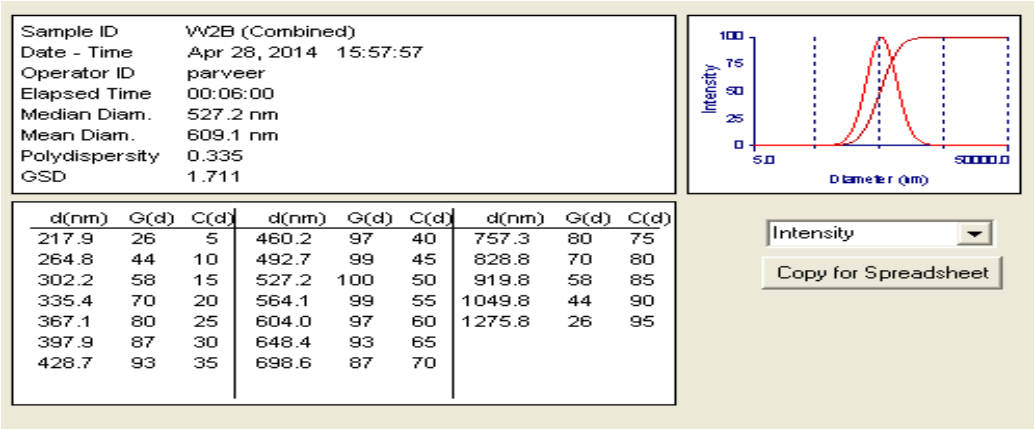


(c)

Figure 4.3 FTIR spectra from 4000-500 cm^{-1} of bioflocculants (a) L2 , (b) W2B and (c) X4

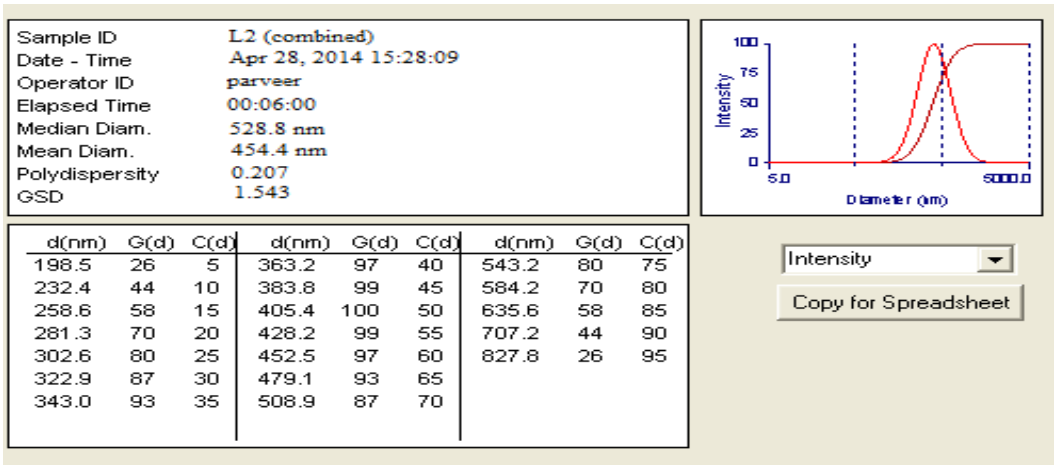
4.2.4 Particle size and molecular weight determination



Sample ID	W2B (Combined)
Date - Time	Apr 28, 2014 15:57:57
Operator ID	parveer
Elapsed Time	00:06:00
Median Diam.	527.2 nm
Mean Diam.	609.1 nm
Polydispersity	0.335
GSD	1.711

d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)
217.9	26	5	460.2	97	40	757.3	80	75
264.8	44	10	492.7	99	45	828.8	70	80
302.2	58	15	527.2	100	50	919.8	58	85
335.4	70	20	564.1	99	55	1049.8	44	90
367.1	80	25	604.0	97	60	1275.8	26	95
397.9	87	30	648.4	93	65			
428.7	93	35	698.6	87	70			

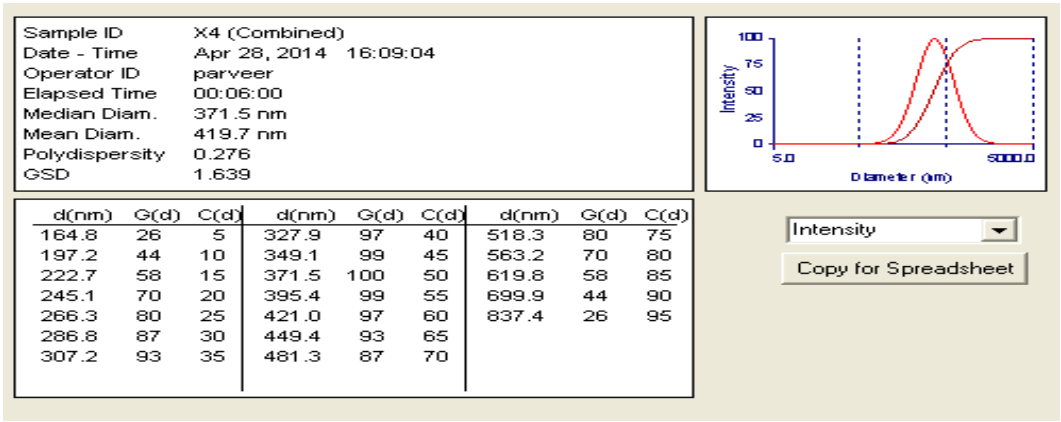
(a)



Sample ID	L2 (combined)
Date - Time	Apr 28, 2014 15:28:09
Operator ID	parveer
Elapsed Time	00:06:00
Median Diam.	528.8 nm
Mean Diam.	454.4 nm
Polydispersity	0.207
GSD	1.543

d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)
198.5	26	5	363.2	97	40	543.2	80	75
232.4	44	10	383.8	99	45	584.2	70	80
258.6	58	15	405.4	100	50	635.6	58	85
281.3	70	20	428.2	99	55	707.2	44	90
302.6	80	25	452.5	97	60	827.8	26	95
322.9	87	30	479.1	93	65			
343.0	93	35	508.9	87	70			

(b)



Sample ID	X4 (Combined)
Date - Time	Apr 28, 2014 16:09:04
Operator ID	parveer
Elapsed Time	00:06:00
Median Diam.	371.5 nm
Mean Diam.	419.7 nm
Polydispersity	0.276
GSD	1.639

d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)
164.8	26	5	327.9	97	40	518.3	80	75
197.2	44	10	349.1	99	45	563.2	70	80
222.7	58	15	371.5	100	50	619.8	58	85
245.1	70	20	395.4	99	55	699.9	44	90
266.3	80	25	421.0	97	60	837.4	26	95
286.8	87	30	449.4	93	65			
307.2	93	35	481.3	87	70			

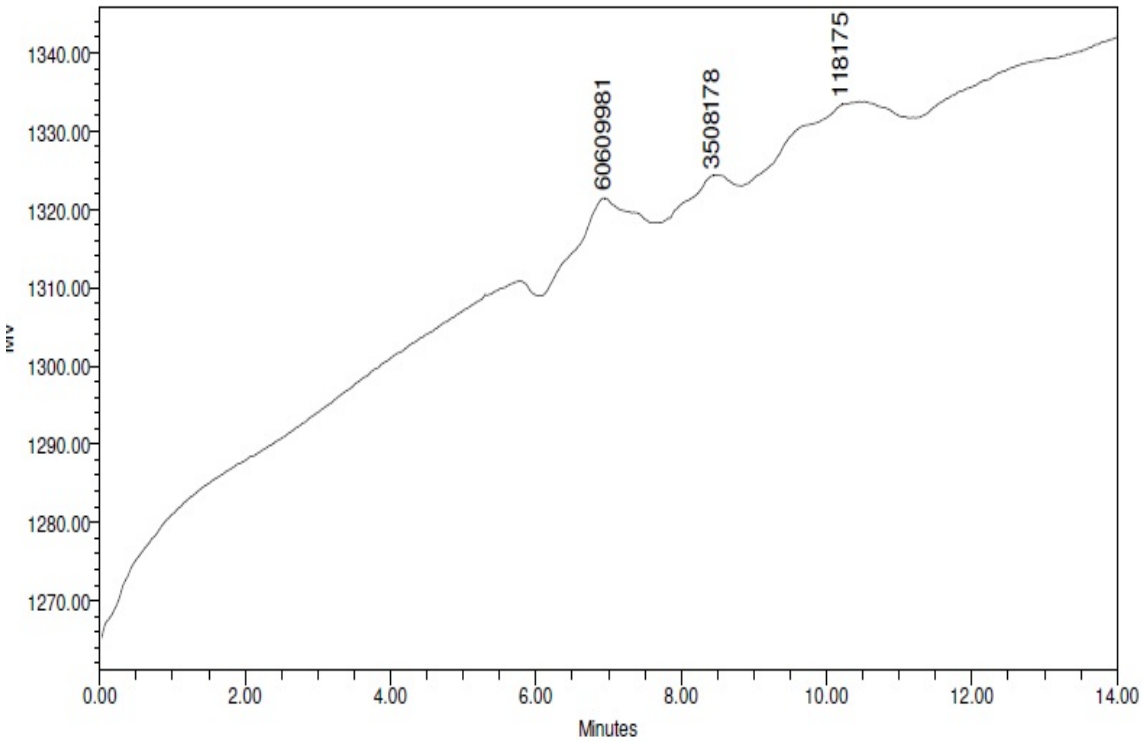
(c)

Figure 4.4 Particle size analysis of bioflocculants (a) W2B (b) L2 and (c) X4

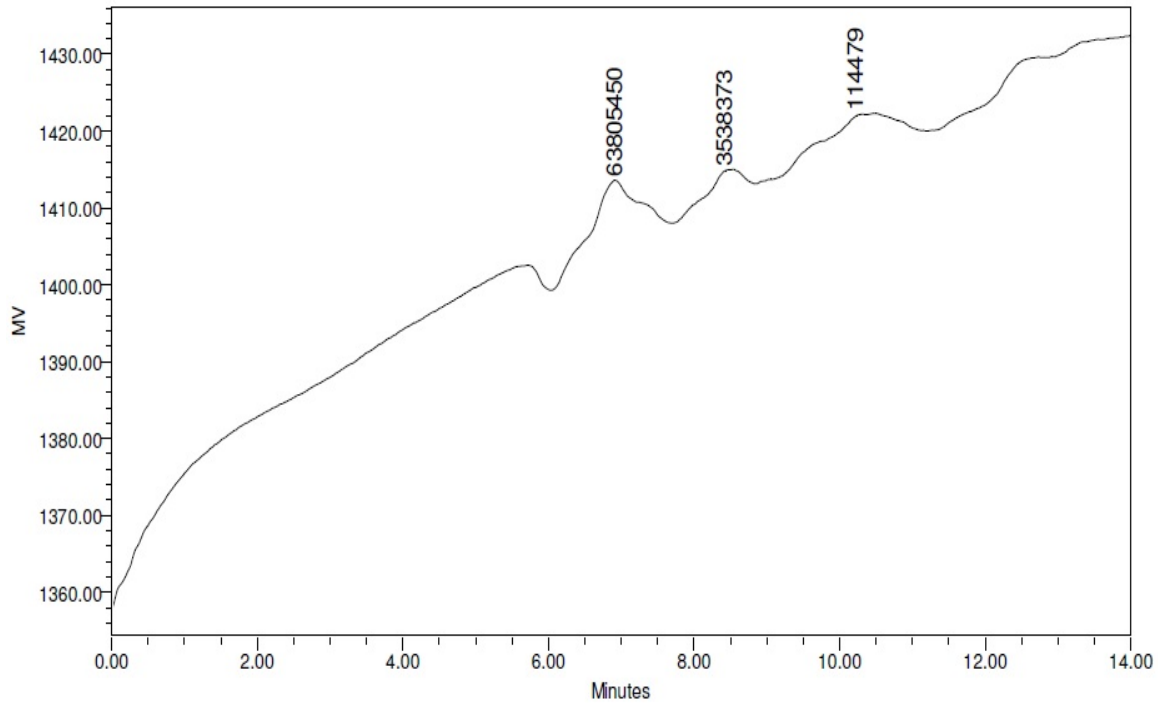
The particle size of bioflocculants and dispersed particles plays an important role in adsorption kinetic study, hence Dynamic Light Scattering (DLS) was used to measure the size of the bioflocculants. DLS measures the light scattered from a laser that passes through a colloidal solution and by analyzing the modulation of the scattered light intensity as a function of time, the hydrodynamic size of particles and particle agglomerates can be determined. Larger particles will diffuse slower than smaller particles and it measures the time dependence of the scattered light to generate a correlation function that can be mathematically linked to a particle size. The mean diameter of the bioflocculants W2B, L2 and X4 was determined as 527.2, 528.2 and 419 nm as shown in Figure 4.4.

The molecular weight of two of the bioflocculants L2 and X4 was determined by Gel Permeation Chromatography (GPC) using blue dextran as standard. Molecular weight of L2 and X4 was determined as 60609981 kDa and 63805450 kDa, respectively, as shown in Figure 4.5. The size was estimated by the comparision to the elution times of blue dextran standards of known size.

The correlation between particle size and flocculant molecular weight has been reported in terms of floc formation forces provided by flocculant bridging and floc breaking forces encountered in an agitated system (Moudgil et al. 1994), also the settling rate of suspensions decreased with decrease in particle size (Mishra et al. 1996).



(a)



(b)

Figure 4.5 Gel Permeation Chromatography of bioflocculant (a) L2 and (b) X4

4.3 Effect of bioflocculant on viscosity of dispersed particles

Flocculants are often characterized for their properties such as rheology, viscosity, solubility and molecular weight. In general, the viscosity of a flocculant is a measure of its thickness. The viscosity enhancing power of a bioflocculant is related to the size and extension of the flocculant molecule in a particular aqueous solution. It is the main parameter for the calculation of adsorption rate in the adsorption kinetics study.

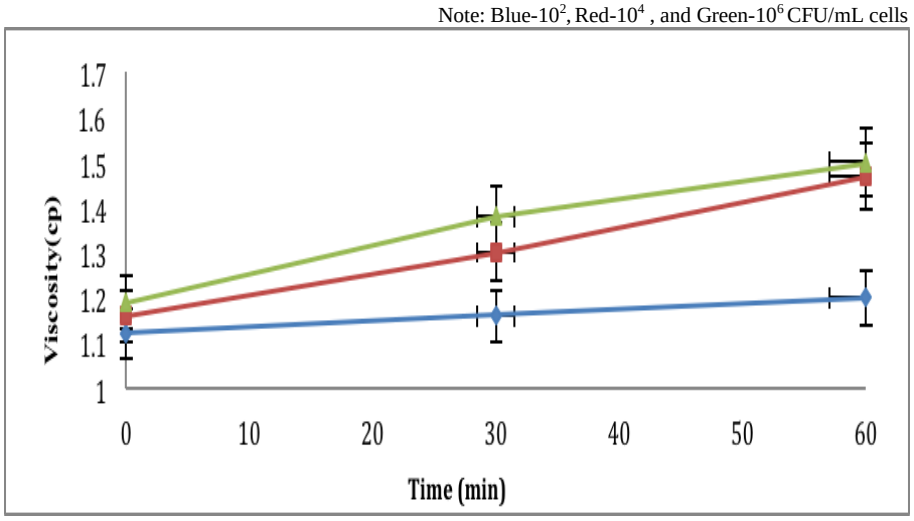
As 0.1 mg/mL concentration of all three bioflocculants resulted in highest flocculating activity against *Shigella* and *Salmonella* bacterial particles in the previous experiments, hence, concentration of 0.1 mg/mL was considered for the subsequent viscosity measurement experiments.

Figure 4.6, 4.7 and 4.8 exhibit plots of changes in viscosity versus time interval for sample containing *Shigella* and *Salmonella* cells as dispersed particles on addition of bioflocculants L2, X4 and W2B. Results revealed that there is a gradual increase in the viscosity of the solution containing 10^2, 10^4 and 10^6 CFU/mL of the dispersed particle cells when bioflocculants L2, X4 and W2B was added to the solution. This increase in the viscosity starts after 30 min of bioflocculant addition and increase was observed for 60 min of time, after which the viscosity remained constant. The possible explanation behind the increasing viscosity for the first 30 minutes even after addition of bioflocculants might be the increase in the number of the bacterial cells in the solution due to normal growth of the bacteria. The constant viscosity was observed after 60 min in case of all the bioflocculants and bacteria, indicates that all bioflocculant molecules get adsorbed on the surface of bacterial cells and bioflocculant has arrested the further growth of bacteria in the system (Gregory 2011).

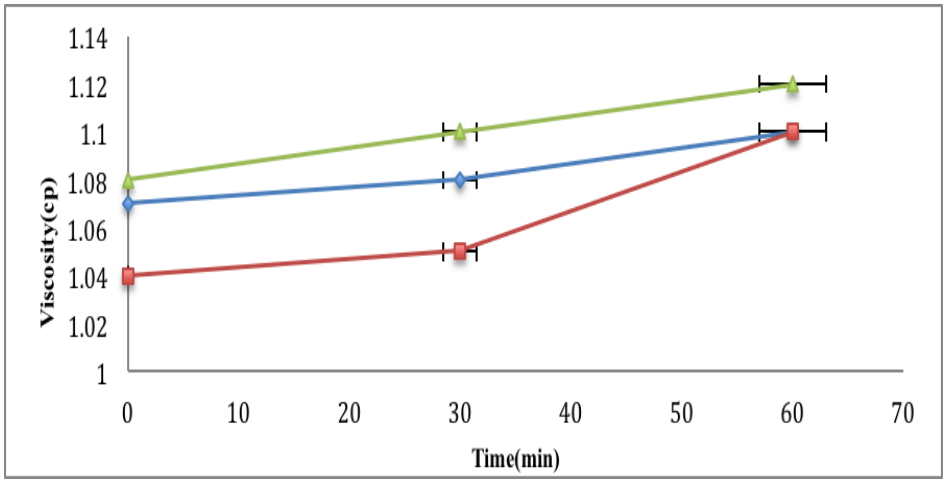
Table 4.4 Viscosity (cp) of dispersed cells with bioflocculant L2 at different time intervals

Bacterial count (cfu/mL)	Viscosity (cp) (*Salmonella* cells)				Viscosity (cp) (*Shigella* cells)		
	0 min	30 min	60 min		0 min	30 min	60 min
10^2	1.12	1.16	1.20		1.06	1.08	1.10
10^4	1.16	1.30	1.47		1.07	1.09	1.12
10^6	1.19	1.38	1.50		1.08	1.10	1.12

Note: Blue-10^2, Red-10^4 , and Green-10^6 CFU/mL cells



(a)

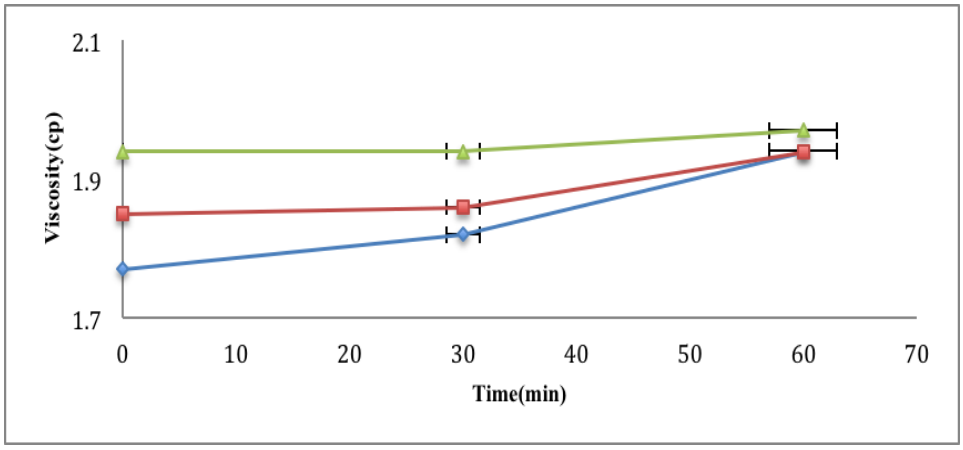


(b)

Figure 4.6 Viscosity (cp) of, a) *Salmonella* and b) *Shigella* cells, with bioflocculant L2 at different time intervals

Table 4.5 Viscosity (cp) of dispersed cells with bioflocculant X4 at differant intervals

Bacterial count (cfu/mL)	Viscosity (cp) (*Salmonella* cells)				Viscosity (cp) (*Shigella* cells)		
	0 min	30 min	60 min		0 min	30 min	60 min
10^2	1.77	1.82	1.92		1.30	1.36	1.42
10^4	1.85	1.86	1.94		1.49	1.44	1.50
10^6	1.94	1.94	1.97		1.74	1.68	1.55



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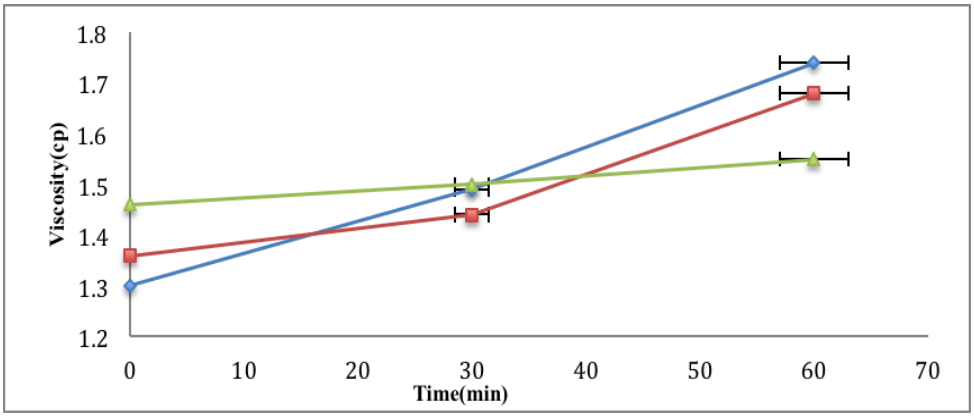
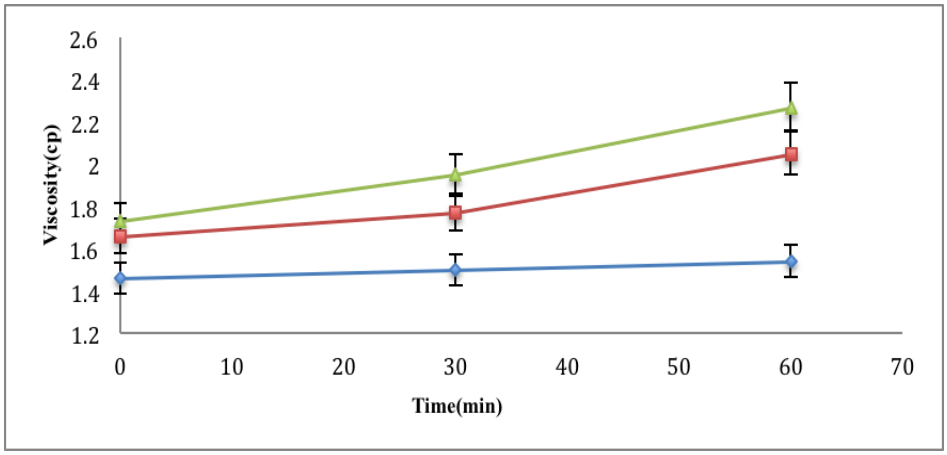


(b)

Figure 4.7 Viscosity (cp) of a) *Salmonella* and b) *Shigella* cells, with bioflocculant X4 at different time intervals

Table 4.6 Viscosity (cp) of dispersed cells with bioflocculant W2B at different time intervals

Bacterial count (cfu/mL)	Viscosity (cp) (*Salmonella* cells)				Viscosity (cp) (*Shigella* cells)		
	0 min	30 min	60 min		0 min	30 min	60 min
10^2	1.46	1.50	1.54		1.49	1.84	1.83
10^4	1.66	1.77	2.05		1.78	1.85	1.87
10^6	1.73	1.95	2.27		1.70	1.76	2.07



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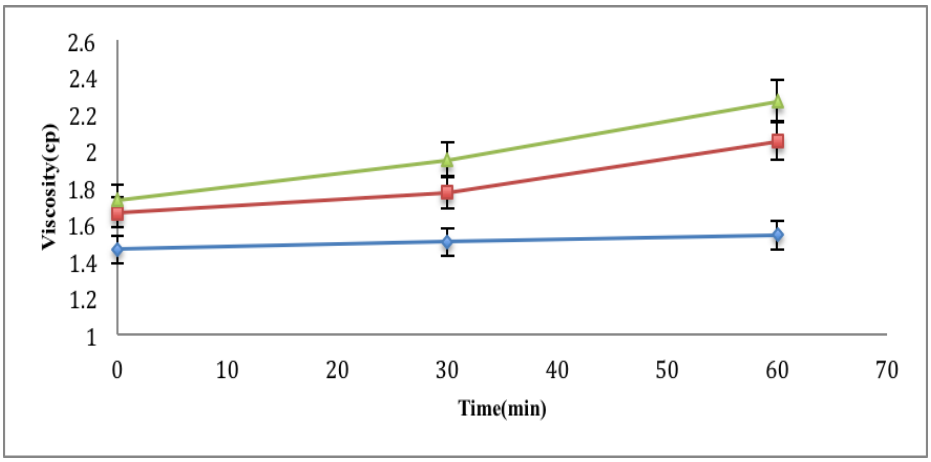


(b)

Figure 4.8 Viscosity (cp) of a) *Salmonella* and b) *Shigella* cells, with bioflocculant W2B at different time intervals

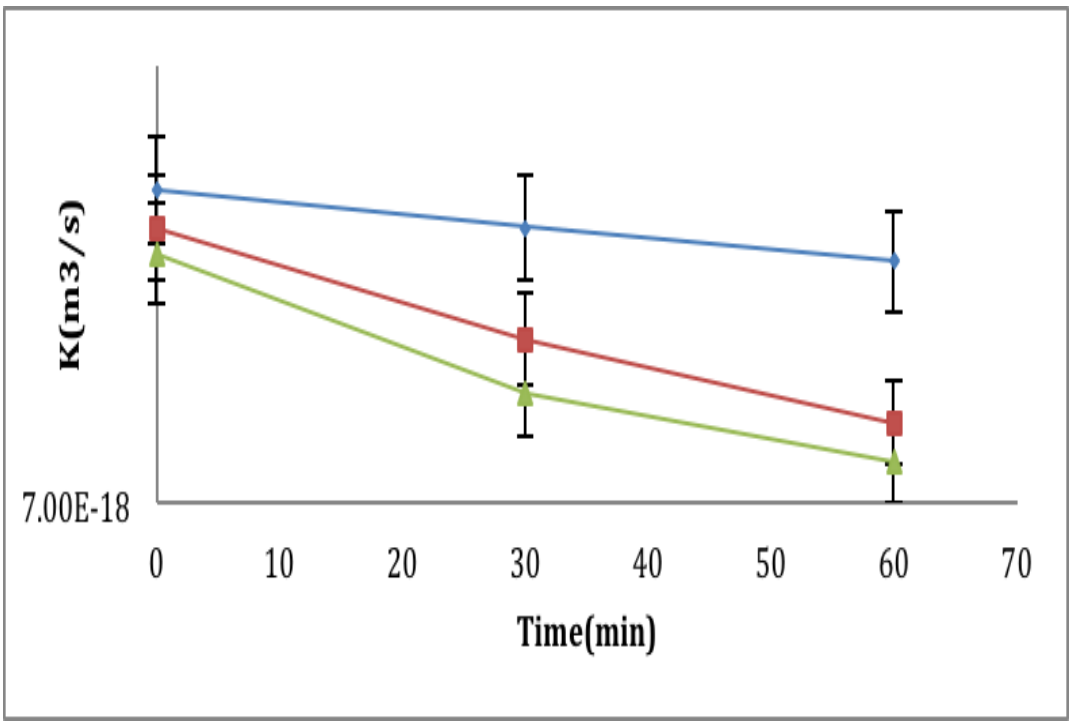
4.4 Adsorption kinetic model

Flocculant adsorption is a second-order rate process, depending on the concentrations of dispersed particles and flocculant molecules (Gregory 2011). Rate of bioflocculant adsorption can be measured in terms of the rate of loss of bioflocculant molecules from solution. Although, the kinetic of bioflocculant adsorption depends upon the dispersed particles, the adsorption rate also depends upon the rate of arrival of bioflocculant molecule at a particle surface. As particles surfaces become more fully covered by adsorbent bioflocculant, then the rate of futher adsorption will be reduced since there are fewer adsorption sites.

Figure 4.9, 4.10 and 4.11, shows adsorption rate constant as determined with *Salmonella* and *Shigella* cells, respectively, with the three bioflocculants (X4, L2, W2B). Results revealed that the adsorption rate constant was least in the case of highest bacterial count (10^6 CFU/mL). The same pattern of adsorption rate was observed in the case of all the three bioflocculants (L2, X4 and W2B), i.e with increase in the bacterial count and time, there was a significant decreases in the adsorption rate. This behaviour probably indicates that with increasing time the available sites for adsorption of bioflocculant on the surface of bacteria decreases, which might have resulted in lesser adsorption rate constant.

Adsorption is the process of collecting soluble adsorbate that are in solution, on the suitable adsorbent. The rate of adsorbent depends upon rate at which molecule moves in a solution by diffusion in solution. When a bioflocculant solution was added to a stable suspension in an amount sufficient to de-stabilize the particles, several processes were initiated, the rates of which could have significantly affected the overall flocculation process.

Note: Blue-10^2, Red-10^4 , and Green-10^6 CFU/mL cells



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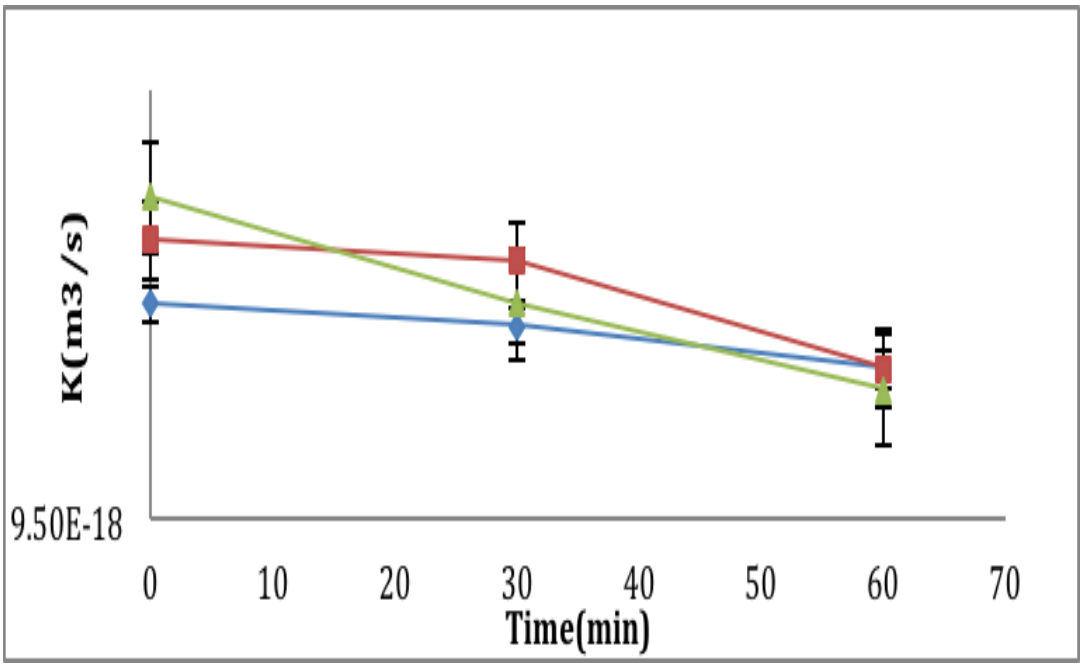
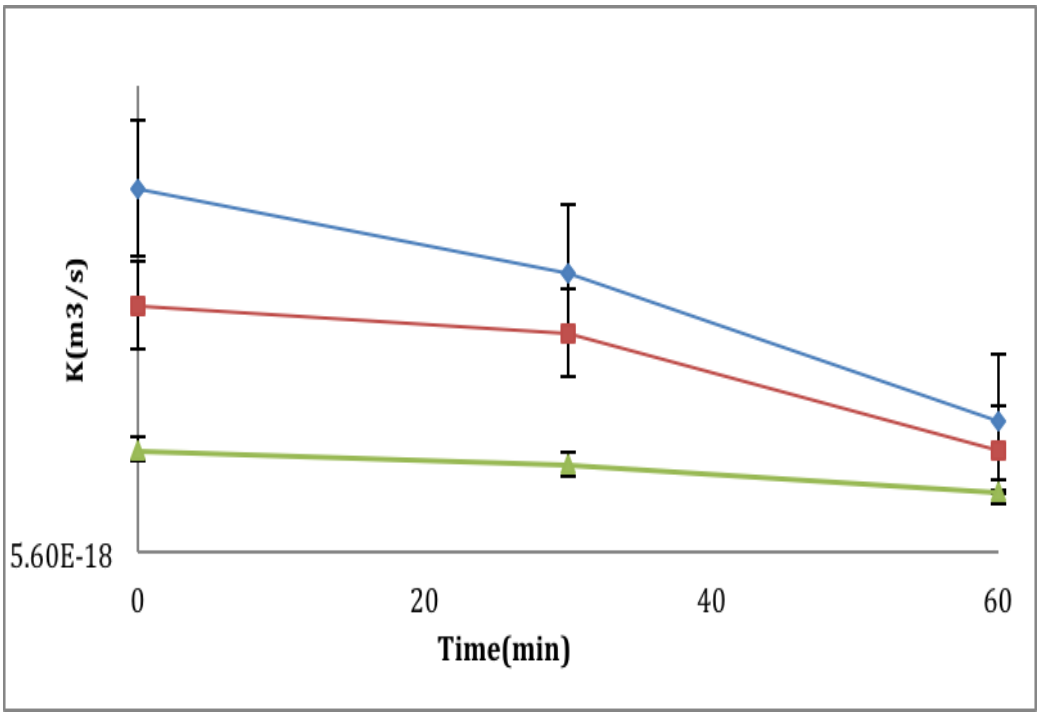


(b)

Figure 4.9 Adsorption rate of bioflocculant L2 with cells (a) *Salmonella* and (b) *Shigella*



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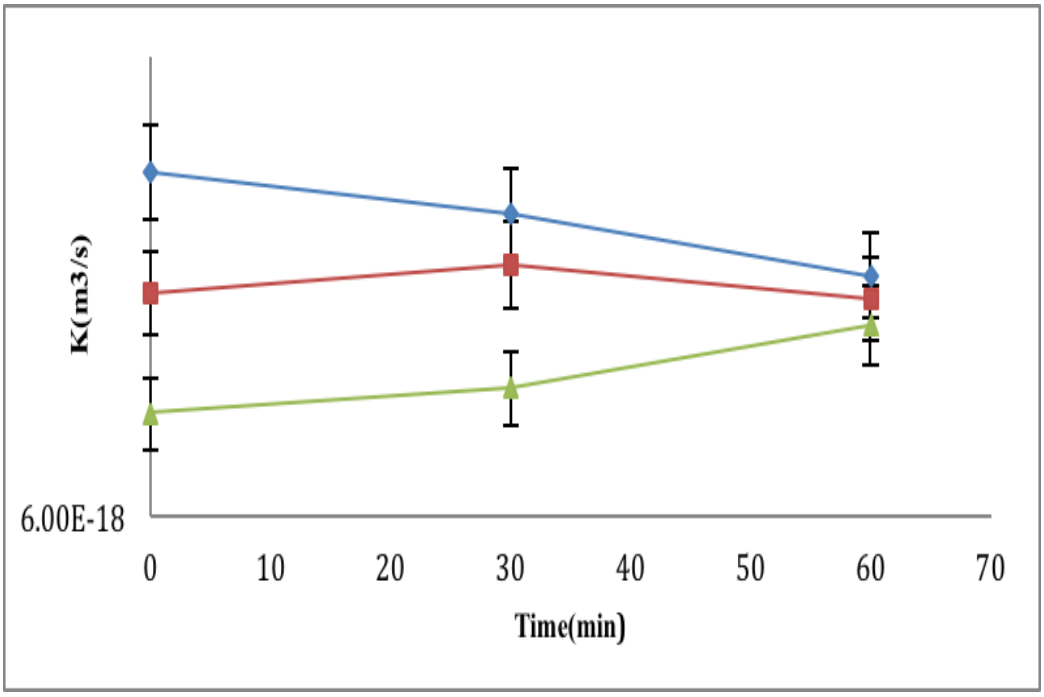
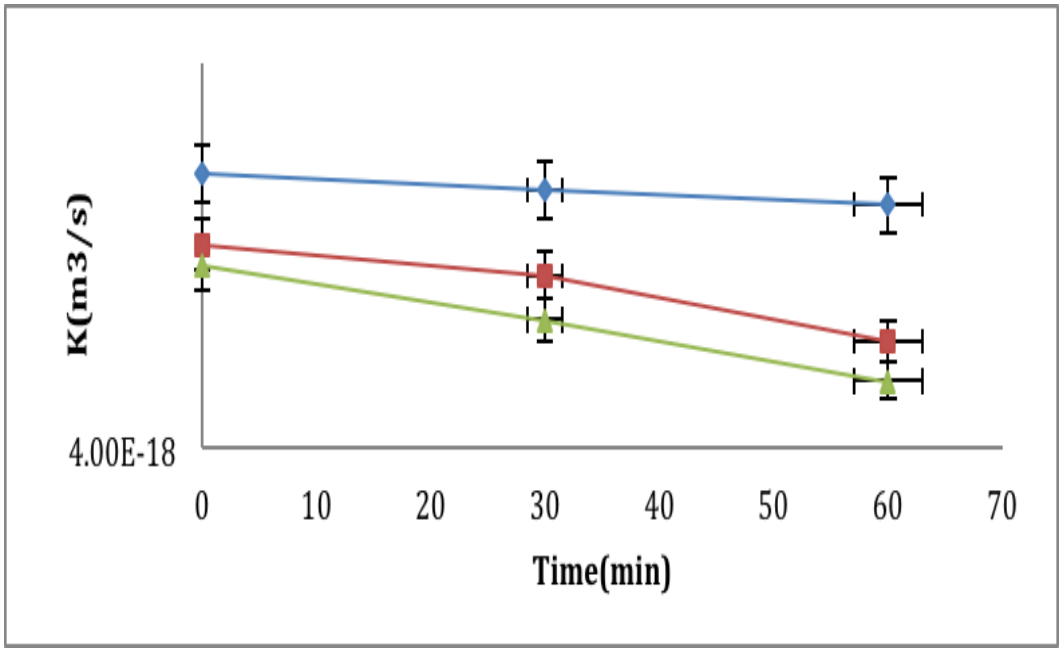


(b)

Figure 4.10 Adsorption rate of bioflocculant X4 with cells (a) *Salmonella* and (b) *Shigella*



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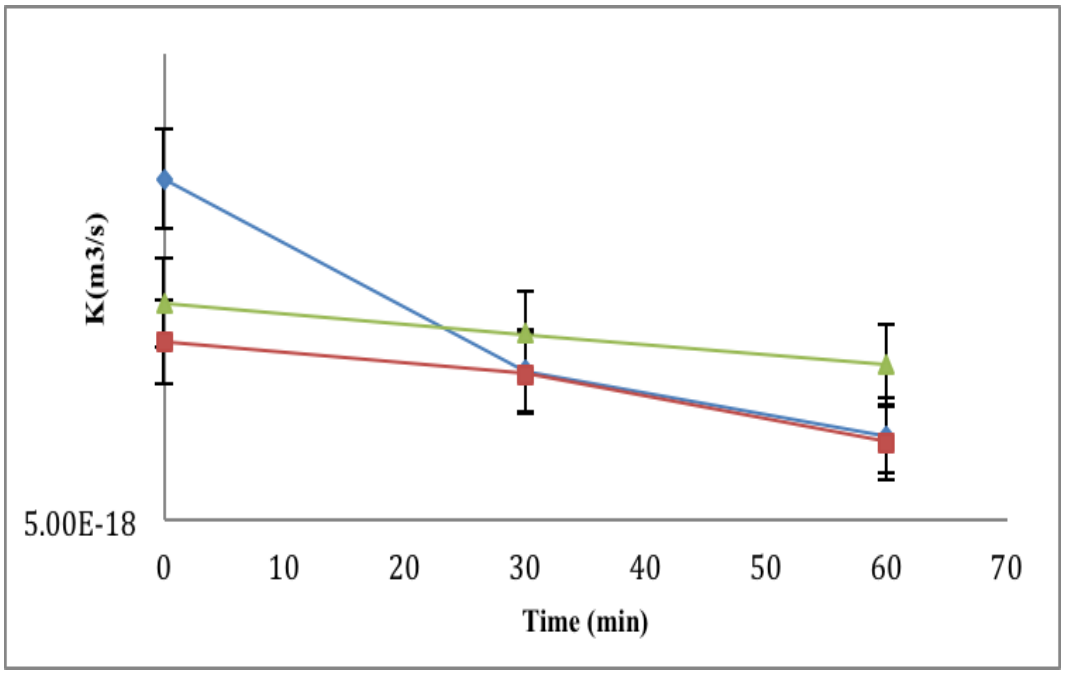

(b)

Figure 4.11 Adsorption rate of bioflocculant W2B with cells (a) *Salmonella* and (b) *Shigella*

4.4.1 Orthokinetic model

Orthokinetic flocculation refers to the collisions of colloidal particles resulting from bulk fluid motion, such as stirring. In this system of stirring, the velocity of the fluid varies both from point to point and from time to time. Larger the size of adsorbant more will be the adsorption rate constant. In Table 4.7 and 4.8, adsorption rate constant of all the three bioflocculants against *Salmonella* and *Shigella* cells, respectively, for orthokinetic system is shown. The effect of size of bioflocculant on adsorption rate was seen with both the dispered particles for orthokinetic model. Results indicated that adsorption rate constant was dependent on the size of bioflocculants as constant increase of around 1.5 to 1.7 times in the case X4 (185.75 nm) to L2 (264.4 nm) was observed.

Table 4.8 Adsorption rate constant of bioflocculants on *Salmonella* cells for orthokinetic system

Shear rate (S^{-1})	**Radius of *Shigella* cells (nm)**	**Radius of bioflocculant (nm)**	**Adsorption rate constant (m^3/s)**
2.1	250	185.75 (X4)	2.32^{-19}
2.1	250	263.6 (W2b)	3.78^{-19}
2.1	250	264.4(L2)	3.80^{-19}

Table 4.9 Adsorption rate constant of bioflocculants on *Shigella* for orthokinetic system

Shear rate (S^{-1})	**Radius of *Shigella* cells (nm)**	**Radius of bioflocculant (nm)**	**Adsorption rate constant (m^3/s)**
2.1	300	185.75(X4)	4.31^{-19}
2.1	300	263.6 (W2b)	6.45^{-19}
2.1	300	264.4 (L2)	6.48^{-19}

Note: Red- *Shigella* , Blue- *Salmonella* cells

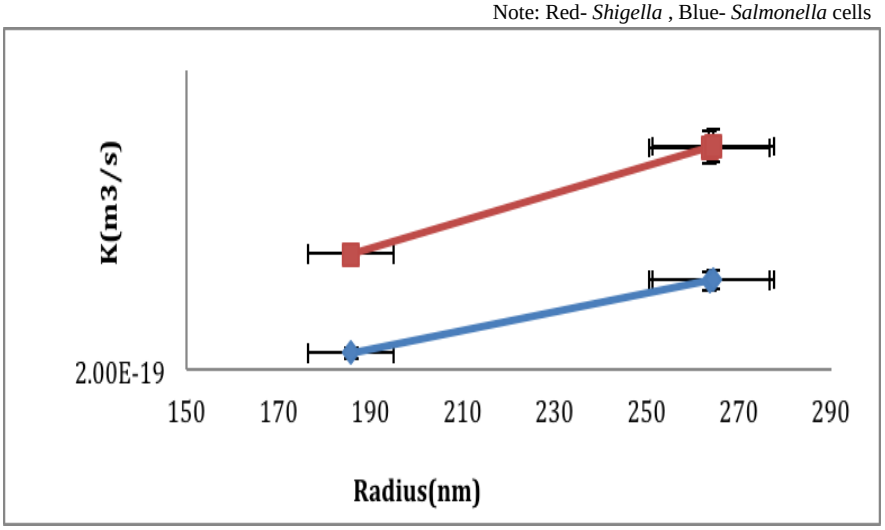

Figure 4.12 Plot of adsorption rate constant of *Salmonella* and *Shigella* cells vs radius

For flocculation, the bioflocculant was typically added with intense mixing and it was revealed that rate of adsorption increased with high molecular weight of bioflocculants (L2>W2B>X4) (Figure 4.12). Moudgil et al. in 1897 reported that there exists a strong correlation between aggregation of a given size and the molecular weight of the flocculant. They have explained the correlation between particle size and flocculant molecular weight in terms of floc formation forces provided by flocculant bridging and floc breaking forces encountered in an agitated system. Orthokinetic flocculation was imposed by velocity gradients created by mixing particles which, are moving faster to overtake slower-moving particles in a velocity field. Due to the collision between the particles, larger particles are formed which are easier to remove by gravity separation. The differential settling is determined by differences in settling velocity between different particles size. Larger particles overtake smaller particles, collide and stick together, forming larger particles that settle faster. Mishra et al. in 1987 have reported the decreasing settling rate of suspensions with decreased particle size. This has been attributed to an increase in

surface area that increased the charge neutralization capacity of the surface by the flocculant.

4.4.2 Perikinetic model

Perikinetic flocculation refers to flocculation due to Brownian motion of colloidal particles. The random motion of colloidal particles results from their rapid and random bombardment by the molecules of the fluid. The effect of size of bioflocculant was determined with both the dispered particles for perikinetic model.

In Table 4.9 it was seen that in perikinetic (without stirring) model the bioflocculant L2, which has larger size, have more adsorption rate constant as compared to other two bioflocculants for *Shigella* cells. The greater the velocity gradient in the liquid induces the more contact between the particles. However, greater the velocity gradient the smaller will be the ultimate floc size due to a continuous breakdown of the larger flocs. Hence, for a given velocity gradient there is a limiting flocculation time beyond which floc particles does not grow further. The lower the velocity gradient the longer will be the time needed to reach the optimum floc size, but the larger will be the final floc size.

In Table 4.10 it was observed that in case of *Shigella* more absorption rate was seen as compared to *Salmonella* with all the three bioflocculants, indicating increase in the rate of bioflocculant adsorption with increasing particle concentration. Both the models follow same sequence of bioflocculant adsorption rate which was L2 > W2B >X4 due to their particle size variations. Results revealed that for concentrated dispersions, bioflocculant adsorption was practically instantaneous, whereas for very dilute dispersions (a few mg/L), bioflocculant adsorption may take several minutes or even longer.

Table 4.9 Adsorption rate constant of bioflocculants on *Salmonella cells* for perikinetic system

Radius of *Salmonella* (nm)	Radius of Bioflocculant (nm)	Number of particles (cm^{-3})	Time (min)	Viscosity (Ns/m^2)	Adsorption rate constant(m^3/s)
250	185.75	10^2	0	0.00177	6.38^{-18}
250	185.75	10^2	30	0.00182	6.20^{-18}
250	185.75	10^2	60	0.00192	5.88^{-18}
250	185.75	10^4	0	0.00184	6.13^{-18}
250	185.75	10^4	30	0.00186	6.07^{-18}
250	185.75	10^4	60	0.00194	5.82^{-18}
250	185.75	10^6	0	0.00194	5.82^{-18}
250	185.75	10^6	30	0.00195	5.79^{-18}
250	185.75	10^6	60	0.00194	5.73^{-18}
250	263.6	10^2	0	0.00146	7.57^{-18}
250	263.6	10^2	30	0.0015	7.36^{-18}
250	263.6	10^2	60	0.00154	7.17^{-18}
250	263.6	10^4	0	0.00166	6.65^{-18}
250	263.6	10^4	30	0.00177	6.24^{-18}
250	263.6	10^4	60	0.00205	5.39^{-18}
250	263.6	10^6	0	0.00173	6.39^{-18}
250	263.6	10^6	30	0.00195	5.67^{-18}
250	263.6	10^6	60	0.00227	4.87^{-18}
250	264.4	10^2	0	0.00112	9.86^{-18}
250	264.4	10^2	30	0.00116	9.52^{-18}
250	264.4	10^2	60	0.0012	9.21^{-18}
250	264.4	10^4	0	0.00116	9.52^{-18}
250	264.4	10^4	30	0.0013	8.50^{-18}
250	264.4	10^4	60	0.00143	7.73^{-18}
250	264.4	10^6	0	0.00119	9.28^{-18}
250	264.4	10^6	30	0.00138	8.01^{-18}
250	264.4	10^6	60	0.0015	7.37^{-18}

Note Radius of L2: 264.4, W2B: 263.6 and X4: 185.75

Table 4.10 Adsorption rate constant of bioflocculant on *Shigella* cells for perikinetic system

Radius of *Salmonella* cells(nm)	Radius of Bioflocculant (nm)	Number of particles (cm^{-3})	Time (min)	Viscosity (Ns/m^2)	Adsorption rate constant(m^3/s)
300	185.75	10^2	0	0.0013	9.37^{-18}
300	185.75	10^2	30	0.00136	8.96^{-18}
300	185.75	10^2	60	0.00146	8.35^{-18}
300	185.75	10^4	0	0.00149	8.18^{-18}
300	185.75	10^4	30	0.00144	8.46^{-18}
300	185.75	10^4	60	0.0015	8.12^{-18}
300	185.75	10^6	0	0.00149	7.00^{-18}
300	185.75	10^6	30	0.00168	7.25^{-18}
300	185.75	10^6	60	0.00155	7.86^{-18}
300	263.6	10^2	0	0.00149	7.56^{-18}
300	263.6	10^2	30	0.00184	6.12^{-18}
300	263.6	10^2	60	0.002	5.63^{-18}
300	263.6	10^4	0	0.00178	6.33^{-18}
300	263.6	10^4	30	0.00185	6.09^{-18}
300	263.6	10^4	60	0.00202	5.58^{-18}
300	263.6	10^6	0	0.0017	6.63^{-18}
300	263.6	10^6	30	0.00176	6.40^{-18}
300	263.6	10^6	60	0.00183	6.16^{-18}
300	264.4	10^2	0	0.00107	1.05^{-17}
300	264.4	10^2	30	0.00108	1.04^{-17}
300	264.4	10^2	60	0.0011	1.02^{-17}
300	264.4	10^4	0	0.00104	1.08^{-17}
300	264.4	10^4	30	0.00105	1.07^{-17}
300	264.4	10^4	60	0.0011	1.02^{-17}
300	264.4	10^6	0	0.00102	1.10^{-17}
300	264.4	10^6	30	0.00107	1.05^{-17}
300	264.4	10^6	60	0.00112	1.01^{-17}

Although anionic charge on bioflocculant can impede adsorption onto a negative surface (bacteria), it serves to promote extension of bioflocculant chain through mutual charge repulsion, enhancing its approachability. It was observed that beyond an optimum molecular weight, flocculation efficiency decreases which is attributed to steric repulsion between biflocculant molecules.

Salient finding of this study are:

- A total of eighteen purified bioflocculants with more than 50% flocculating activity were selected to assess their pathogen binding abilities. Three bioflocculants named as L2, W2B and X4 showed highest flocculating activity as well as turbidity removal efficiency against the surrogates of *Salmonella typhimurium* ATCC 23564 and *Shigella flexneri* Type 2a.
- The characterization was done on the basis of biochemical and biophysical properties of the bioflocculants, results revealed that all the three bioflocculants were mainly composed of carbohydrate, hexoamine, protien, pyruvic acid and uronic acid. The high sugar content depicted the polysaccharide nature of the bioflocculants. SEM studies reflected the porous structure of the bioflocculants with different pore size distribution and functional group analysis by FTIR showed the presence of hydroxyl, amino and carbonyl groups.
- The viscosity measurements of all the three bioflocculants against the dispersed particles showed a linear increase in viscosity during 30 to 60 minutes of bioflocculant addition. Further, the orthokinetic and perikinetic flocculation models were developed by calculating adsorption rate constant of three bioflocculants against *Salmonella* and *Shigella* cells, and it was observed that particle size of bioflocculant and number of dispersed particles plays a major role in the adsorption kinetics. The 1.5 to 1.7 folds increase in the adsorption rate was found as the particle size of bioflocculant increased from 419 to 528.2 nm in the case of X4 and L2. The order of adsorption rate with the bioflocculant was L2 > W2B > X4 and in the case of dispersed particles, the adsorption rate of *Shigella* cells was more as compared to *Salmonella* cells.

- The molecular weight of two of the bioflocculants L2 as 60609981 kDa and X4 63805450 kDa, was determined by Gel Permeation Chromatography (GPC). The particle size and high molecular weight of bioflocculants resulted in floc formation forces provided by flocculant bridging, which plays a major role in the effective flocculation process.

In conclusion, a systemic mathematical model was developed for specific bioflocculant and dispersed particles system on the basis of orthokinetic and perikinetic flocculation process. The study was aimed in exploring the suitable model for using low-cost eco-friendly alternatives to obtain biodegradable, abundant and readily available bioflocculants for the contaminated water treatment.

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Annexure 1

1 Flocculant isolation broth (FIB)

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.Brain-heart infusion medium Agar (BHI)

Distilled water	1000mL
Calf brains	12.5gm
Proteose peptone	10.0gm
Sodium chloride (NaCl)	5 .0gm
Na_2HPO_4	2.5gm
Dextrose	2.0gm
Dextrose	0.1gm
Beef extrct	0.5gm

3 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

4 Reagent for Elson-Morgsn assay

Reagent A (100 mL)

Dipotassium tetraborate tetrahydrate	6.1g

Reagent B

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

5 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 $mgmL^{-1}$

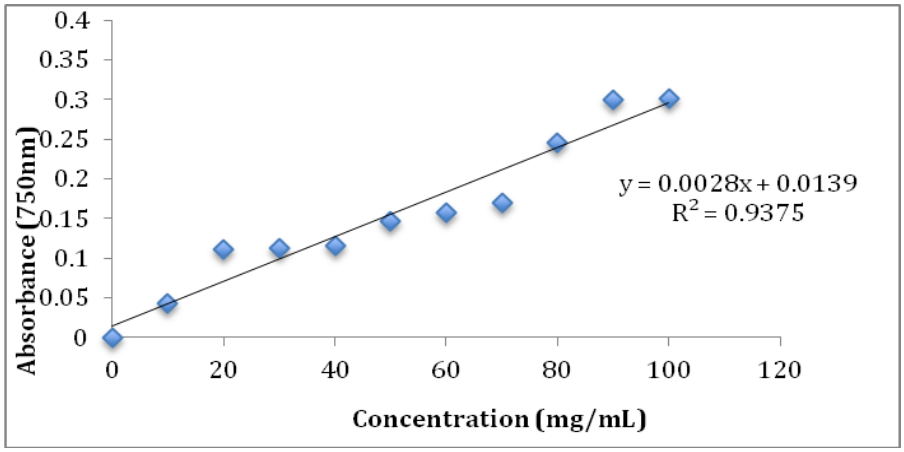
6 Friedman method (Pyruvic Acid)

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 $mgmL^{-1}$

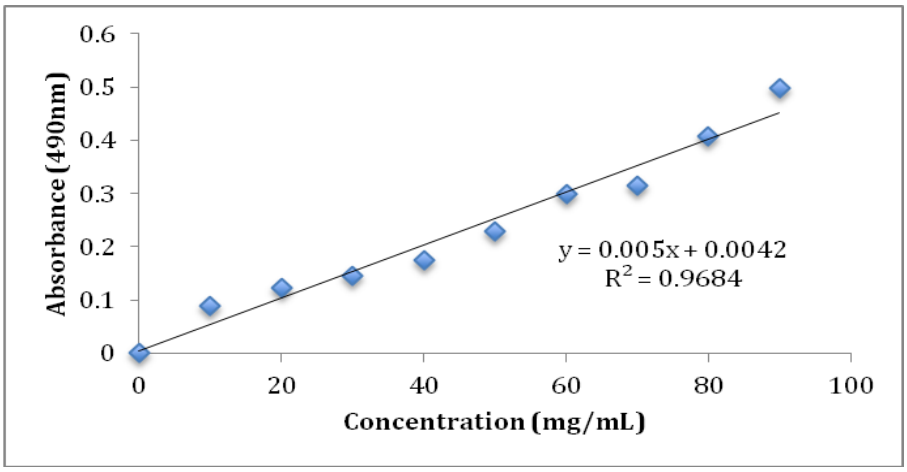
7 Total sugars

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

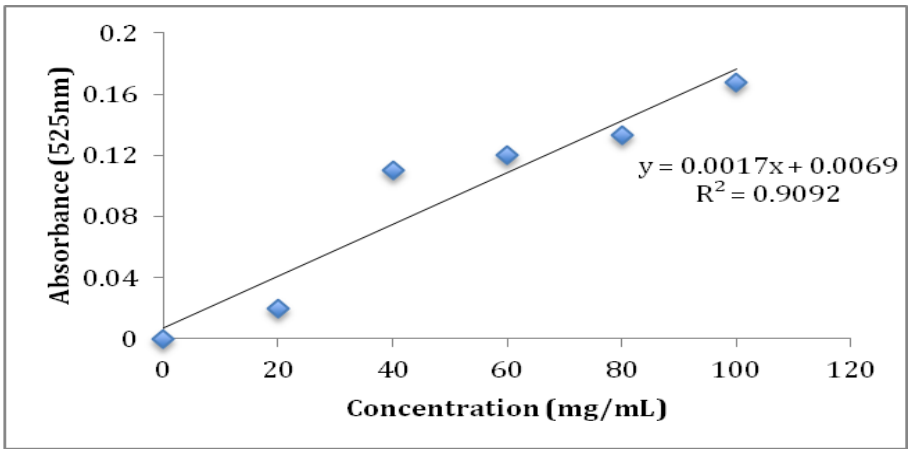
Annexure- II



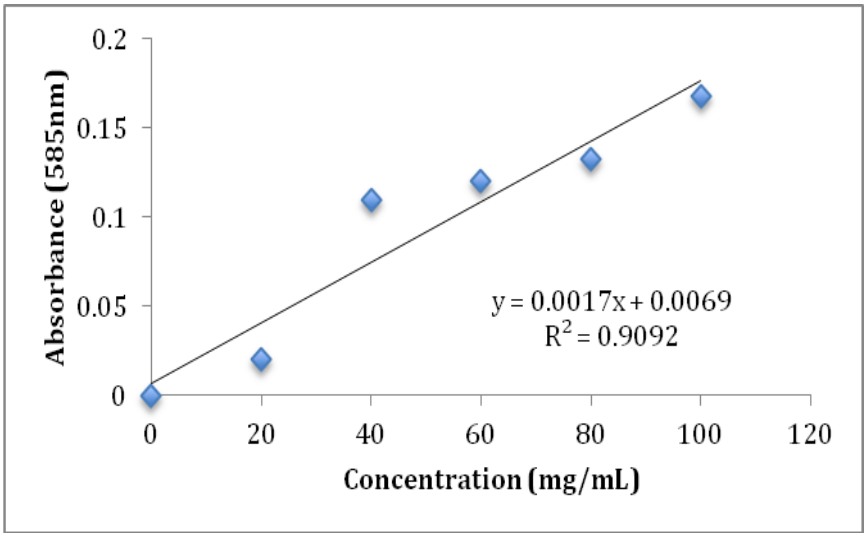
Calibration curve of protein using BSA



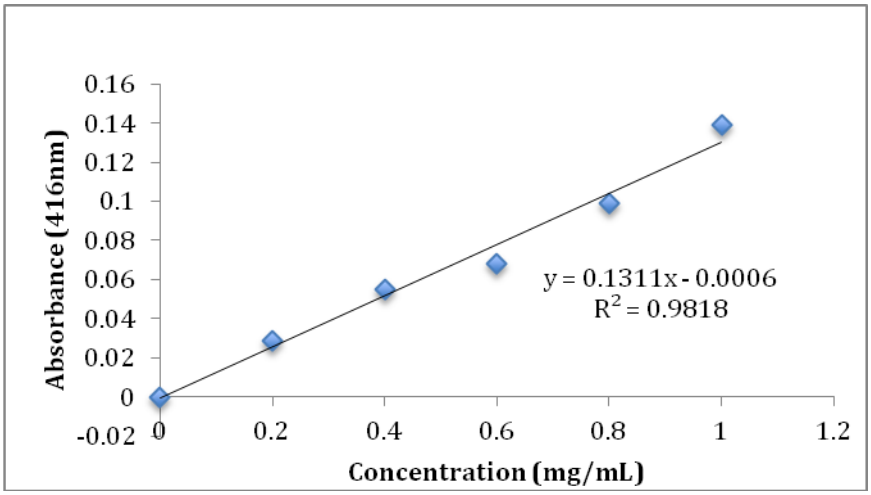
Calibration curve of sugar using glucose



Calibration curve of uronic acid using D-glucuronic acid



Calibration curve of amino sugars using Galactosamine



Calibration curve of pyruvic acid using pyruvic acid