Studies on microbial prospecting for exobiopolymeric flocculants

A thesis
submitted in fulfillment of the requirement
for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

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I hereby declare that the work which is being presented in this thesis **"Studies on microbial prospecting for exobiopolymeric flocculants"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Moushumi Ghosh**, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

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Acknowledgement

I pay my reverence and gratitude to the Almighty for his everlasting blessings.

This thesis arose out of years of research that has been done since I joined Thapar University. I have known a great number of people during these many years, whose contribution in assorted ways to the research and the making of the thesis deserved special mention. It is a pleasure to convey my gratitude to them all in my humble acknowledgment.

Firstly, I offer my gratitude to my esteemed supervisor **Dr. Moushumi Ghosh**, Associate Professor, Thapar University, Patiala, for her supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, she provided me unflinching encouragement and support in various ways. Her truly scientific intuition has made her as a constant oasis of ideas and passions in Science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be. I am indebted to her insightful comments, and for putting so much energy for this work.

I take this opportunity to thank **Dr. Abhijit Mukherjee, Director**, Thapar University, for all the facilities which have been immensely helpful for the completion of my work.

I express my gratitude to **Dr. P. K. Bajpai,** Dean (Research and Sponsored Projects), and **Dr. Susheel Mittal,** former Dean, Thapar University, Patiala, for their encouragement and support during my research work in the University.

I am extremely thankful to **Dr. M Sudhakara Reddy,** Professor and Head Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, for providing me all the help and support during the progress of my work at the Institute. I would also like to thank **Dr. Niranjan Das,** former Head and Associate Professor, for his encouragement and suggestions during the course of my work.

I am highly thankful to the members of my doctoral committee **Dr. N. Tejo Prakash,** Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University and **Dr. Haripada Bhunia,** Associate Professor, Department of Chemical Engineering, for their comments and suggestions during the course of my PhD.

It is my privilege to thank **Dr. Abhijit Ganguli,** Asssociate Professor, Department of

Biotechnology and Environmental Sciences for providing necessary guidance, help and suggestions, for my work and at personal level.

I would like to thank my labmates cum friends ***Meenakshi Malik, Richu Singla, Mukesh Kumar, Seema Bhanwar, Gurpreet Kaur Khaira, Taranpreet*** *and* ***Gatha Sharma*** *for their cooperation in the hours of need. I thank them for their affection and concern at all times.*

I am thankful for the help rendered by colleagues namely ***Pankaj Kumar, Varenyam Achal, Nirankar Singh, Ravi Shukla, Vishal, Dinesh, Jasminder*** *and all the research scholars who stood by me and supported me.*

I am thankful to the office and laboratory staff of Department of Biotechnology and Environmental for all the cooperation and help. I would also like to thank ***Mr. Anirudh Handa*** *for his help whenever required.*

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. I owe sincere thank to them for their unconditional love and care. I would like to thank my sisters ***Rekha*** *and* ***Rakhi*** *and brother* ***Vicky*** *for being supportive and caring siblings. I would also like to thank* ***Vijay****, an important member of my family for his everlasting help.*

Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally one by one.

Date: 14/3/2011

Place: Patiala

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List of Publications[1]

Publications in peer reviewed journals

1. Ghosh, M., Ganguli, A. and **Pathak, S**., 2009. Application of a novel biopolymer for removal of *Salmonella* from poultry wastewater. *Environ. Technol*., 30: 337–344.

2. Ghosh, M., **Pathak, S**. and Ganguli, A., 2009. Effective removal of *Cryptosporidium* by a novel bioflocculant. *Water Environ. Res*., 81: 160-164.

3. Ghosh, M. and **Pathak, S**., 2009. Biopolymer based intervention for *Salmonella in* water. *Ind. J. Biotechnol*., 8: 298-303.

4. Ghosh, M., and **Pathak, S**., 2010. Application of microbial exopolymers for removal of protozoan parasites from potable water. *Res. J. Biotechnol.*, 5: 58-62.

Conference Proceedings

Oral Presentations

1. **Pathak, S**., Ghosh, M., and Ganguli. A. Physicochemical properties of a novel polymeric flocculant material from microbial source at National Conference on Emerging Trends in Engineering Materials, Thapar Institute of Engineering & Technology (February 1-3, 2006).

2. Ghosh, M., **Pathak**, S., and Ganguli, A. Biotechnological Evaluation of a novel Exopolymeric Flocculant produced by *Klebsiella terrigena* for removal of *Cryptosporidium parvum* – an Emerging Water Borne Pathogen at ICBioE"07 in Kuala Lumpur , Malaysia (May 8-10, 2007).

3. **Pathak, S.** and Ghosh, M. Targeted removal of *Salmonella* spp. by a novel biopolymer – a futuristic biodefense tool at XIth Punjab Science Congress, Thapar University (7-10 February, 2008).

4. **Pathak, S**., Ghosh, M. and Ganguli, A. Application of microbial exopolymers for removal of pathogens from potable water. First International Biotechnology Conference, SMIT, Sikkim (28-30 December, 2008).

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[1]Related to present thesis work

Poster presentations

1. **Pathak, S**. and Ghosh, M. Removal of high risk pathogens in waste water by novel biopolymeric flocculants. 49th Annual conference of Association of Microbiologists of India, University of Delhi, Delhi, November 18-20, 2008.

2. **Pathak**, S., Ghosh, M., and Ganguli. A. Removal of *Cryptosporidium parvum* from water samples by a novel exopolymeric bioflocculant produced by *Klebsiella terrigena* at 47th Annual Conference of Association of Microbiologists of India (AMI), Barkatullah
University, Bhopal (MP), during December 6-8, 2006.

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PUBLICATIONS

Abstract

There are currently few effective bioflocculants as alternatives to chemical flocculants used extensively in solid-liquid separation processes. The present study was carried out with an objective of bioprospecting efficient and novel flocculant producing bacterial strains. A potential strain (isolated from activated sludge) exhibiting high flocculating activity, metal and pathogen removal ability was identified as *Klebsiella terrigena*. The bioflocculant purified from culture supernatants exhibited flocculating ability over a wide range of colloid particles (0.5 to 100µm) at low dosage, its flocculating ability being better than or equivalent to both natural and synthetic flocculants. Highest flocculating activity was observed in the pH range of 6-8, temperature 30°C and in presence of $CaCl_2$ (5mM). Thermal gravimetric (TGA) and rheological analysis of the bioflocculant demonstrated it to be thermostable (100°C, 5min) with temperature dependent viscosity and elasticity changes. The purified flocculant had a high molecular weight (~2.6×10^{6} Da) as determined by gel permeation chromatography; the ultrastructural pattern of purified bioflocculant was visualized by SEM as a porous structure with randomly distributed small pores interconnected with channels. HPLC of hydrolyzed flocculant could establish it to be a polysaccharide comprising of D-Glc, D-Man, D-Gal and D-GlcA monomers with linkage pattern resembling galactoglucans. FTIR of the biopolymer showed abundance of hydroxyl, carboxyl, and methoxyl groups as well as uronic acid residues.

To elucidate the role of cellular mechanisms in flocculation, mutants defective in glucose utilization were generated and their flocculant analyzed for functionality as well as composition. High Performance liquid Chromatograms of flocculants obtained from mutant indicated a complete absence of galactose residues and a loss of flocculating activity, suggesting the crucial role of galactose for functionality of the flocculant. The effect of Water

activity (a_W) and oxidative stress were studied to understand their involvement in flocculant production by *K.terrigena*. Though growth was observed at oxidative stress and low water activity, the flocculant production could not be ascribed to protective mechanism by the cells. The purified flocculant could remove *Salmonella* significantly (80.3%) both under simulated conditions and contaminated poultry waste water. Fluorescent *in situ* hybridization (FISH) with genus specific *Sal3* probe of the flocculated samples confirmed complete removal of *Salmonella* enmeshed in the bioflocculant matrix. The flocculant could remove (62.3%) *Cryptosporidium* oocysts as well from tap water at low dosage of 2mg/L, incorporation of $CaCl_2$ (5mM) enhanced removal efficacy. The purified bioflocculant was also capable of

removing Cadmium, Copper, Zinc and Lead within a wide range of temperature (28-45°C)

and pH (5-8). Metal removal however varied with both the type and concentration of the metal (57-84%) and was completed within 2 hours of contact time. Overall the result of the current study suggests the flocculant to be of considerable potential, suitable for further

commercial applications.

List of Abbreviations

ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic Local Alignment Search Tool
CLSM	Confocal laser scanning micrograph
CPC	Cetylpyridinium chloride
DNA	Deoxyribonucleic acid
dNTP	2“-deoxynucleoside-5“-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FIB	Flocculant isolation broth
FISH	Fluorescent *in situ* hybridization
FTIR	Fourier transform infrared spectroscopy
GPC	Gas permeation chromatography
GSH	L-glutamyl-L-cysteinyl glycine
GTP	Glutathione-S-transferases
HPLC	High performance liquid chromatography
KBF	*Klebsiella* bioflocculants
LB	Luria Bertani
MRD	Maximum recovery diluents
NCC	Nebauer cell counter
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
NTU	Nephelometric Turbidity Units
OD	Optical Density
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
SEM	Scanning electron micrography
SOD	Superoxide dismutase
TAL	Thin Agar Layer
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UV	Ultraviolet
VDW	*van der Waals* forces

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Chapter 1

Introduction

Introduction

Microorganisms represent the largest pool of untapped genetic and biochemical diversity on earth. It is prospected that microbial products may be as diverse as the microbes. This enormous diversity can translate to multitudes of products like novel extracellular polymers with unique characteristics. Thus, microbial biopolymers have been viewed upon as potentially useful for new industrial applications where sources of unique material for industrial use and technological innovation are sought. An important and challenging area of current biotechnological applications is effective separation of solids from liquid phase and is normally achieved through sedimentation aids. Commonly known as flocculants, sedimentation aids are cationic, anionic or nonionic in nature and functions by causing colloids and other suspended particles in aqueous solution to aggregate, thereby forming a floc.During the last decade a variety of flocculating agents comprising of inorganic (Polyaluminium chloride, Aluminum sulfate) organic (polyacrylamide, polyethylene amine) and natural bioflocculants (gelatin, chitosan, guar gum etc) have found extensive applications in pharmaceutical, fermentation, food industries, dredging, downstream processing heavy metal removal, oil water separation, mining and milling operations and several other industrial and wastewater treatment processes (Kurane, 1986; Dugan, 1987; Takeda et al 1991; Bender et al., 1989, 1991, 1994; Salehizadeh and Shojaosadati, 2001; Kaewchai and Prasertsan, 2002; Vijayalakshmi and Raichur, 2003).

The current interest in microbial biopolymers stems from their inherent advantages over other natural biopolymers, principally regarding the space and time needed to grow microorganisms, the cost of their cultivation, and the ratio between growth rate and production.

Among the microbial polymeric molecules applied in industrial processes, exopolysaccharides (EPS) stand out by virtue of its singular importance. EPS are produced by several microbial isolates and are believed to be crucial in ascribing tolerance to nutrient and other environmental stressors. In fact, the role of EPS for cell aggregation is one of the important ecological functions that microbes adopt as a viable strategy for survival. The widely divergent structure and a complex composition of EPS are important in the extensive gamut of functions they perform including high value biotechnological applications. A primary reason being their non toxicity and degradability in contrast to their synthetic polymeric counterparts. Other important characteristics which impart prominence to microbial flocculants include chemical stability, high reactivity and excellent selectivity towards colloidal particles. The structural variability, rheological behaviour (reflected by the ordered conformation and consequent intermolecular interactions usually adapted in aqueous solutions), pseudoplasticity and a high degree of dispersion are considered to be important physical properties. Additionally, the short generation time and the ease of metabolic accessibility of the producer microbial strains further widen the scope of their application.

Although the mechanism of flocculation remains to be clearly understood it is postulated that upon mixing with colloidal particles, synthetic polymeric flocculants (like polyacrylamide and its derivatives). The sequence of flocculation proceeds with an initial polymer adsorption onto the surface of the particle, which is energetically favourable. The polymer, may "bridge" the particles and an aggregate is formed. Low concentrations are typically effective by virtue of their length, they are able to span the two electric layers, thereby alleviating the need for particles to approach closely to flocculate. An alternative mechanism suggested is charge neutralization. A polyelectrolyte adsorbed onto a surface by electrostatic attraction creates a region on the surface

differently charged from the remaining surface. The difference in charge is sufficient to allow other particles to bind into agglomerate. The flocculating mechanisms of exopolymeric flocculants are poorly understood (Esser and Kues, 1983) but aggregation of particles and cells by bridging and charge neutralization (Salehizadeh et al., 2001) is an accepted mechanism. The biopolymers adsorb to other particles to form flocs. Charge neutralization occurs when the biopolymer flocculant is oppositely charged as compared to the particles. However, since most exopolymeric flocculants and particles are negatively charged, charge neutralization seldom occurs in the bioflocculation process. Cations such as Ca^{2+}, Fe^{2+}, Al^{3+} are known to stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups and by forming bridges between particles. Bivalent and trivalent cations help in the initial adsorption of biopolymers on suspended particles by decreasing the negative charge on both the polymer and the particle (Levin and Friesen, 1987; Levy et al., 1992).

Colloidal particles abound in water and require to be separated for human consumption. Important amongst these are heavy metals, or their salts, toxic to humans. These metals cannot be separated by ordinary physical means of separation. Chemical precipitation, oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion exchange, and membrane technologies have been widely used to remove heavy metal ions. Such processes may be ineffective or expensive, especially when the heavy metal ions are in solutions in the order of 1-100 mg dissolved ions/L. Adsoprtion of heavy metals using microbial biomass is generally recognized as an effective, sustainable option for removal of heavy metals. The living or dead biomass with multiple charged surface groups have been recognized as excellent binding sites for metals. Although a similar mode of binding may be envisaged for exopolysaccharides as well, the structural diversity, fluidity and ability to reorient itself in solutions may endow a

superior capability to these materials; it is tempting therefore to explore EPS as viable options for heavy metal removal.

Microbial pathogens represent yet another important colloidal contamination to water. In fact, waterborne transmission is regarded as a highly effective means for disseminating infectious agents to a vast proportion of human population. Conventional pathogen treatment is approached in two ways: removal processes and/or inactivation (disinfection) processes. These processes ideally form part of an over-arching “multiple-barrier” treatment strategy that ensures water source protection (using water of the highest initial quality possible), followed by appropriate pathogen removal, subsequent disinfection, and final contamination protection strategies for the water distribution system. However, polymeric synthetic flocculants have rarely been successful in removal of key water borne pathogens, apparently on account of their relatively low variability in structure.

Given the vast scope of application, microbial exopolymeric flocculants has met with little practical success. Low flocculating capability, large dosage requirement and consequently high cost has been a major impediment in commercial feasibility. Consequently, prospecting microorganisms that are not only able to produce novel high yielding flocculants but also with high flocculating activity may partially address this problem. Innovative approaches towards efficient, cost effective production and recovery may subsequently enable a rational commercial strategy. Besides, a clearer understanding of the mechanism involved behind bioflocculation may help in a better usage of exopolymeric flocculants at various levels. The current paucity of scientific information indicates that systematic research emphasizing these facts was not carried out. This study was therefore, carried with an aim to address the gaps in knowledge and develop

deeper insights, with an approach encompassing microbial prospecting for commercial exploitation of novel exopolymeric flocculants.

Scope of the present study

To date attempts have focused on isolation, identification and characterization of exobiopolymeric flocculant producing bacteria for the production of novel and unique exobiopolymeric flocculant suited for specific purposes. It is still necessary to bioprospect new producers of bioflocculants with useful chemical and physical properties that can have varied industrial applications; arguably, removal of toxic metals as well as microbial pathogens remain one of the biggest application areas for bioflocculants. Low flocculating capability, large dosage requirement and high cost has been an impeding factor for application of bacterial exopolymeric flocculants. Despite these facts, there is a considerable interest for novel exopolymeric flocculants, presumably because of two important reasons:- the structural plasticity, which allows scope of further structural amendments (polymer engineering) and exploration of selective and unique functional attributes. It is undeniable that extraordinary benefit may be derived from bioprospecting in finding potential beneficial biological substances that can be used for mankind. The paucity of scientific information necessitates an approach encompassing microbial prospecting, optimization of production parameters and application of newly characterized exopolymers for specific purposes. The use of modern methods used for the characterization of bioflocculants like FTIR, SEM, TGA, HPLC etc. can help to have a better insight and understanding of the functional aspects of the bioflocculants. In an attempt to address the afore mentioned knowledge gaps, potential exopolymer and the producer bacterial strain(s) have been characterized; the optimal production conditions established and their functionality evaluated in areas most relevant to environment.

Thus the following underlying objectives were derived for the present study:

1. Isolation and characterization of flocculant producing microorganisms and their exopolymeric flocculant
2. Optimization for maximal bioflocculant production and elucidation of cellular mechanisms
3. Evaluation of the exopolymeric flocculants for removal of pathogens and some heavy metals

Chapter 2

Review of Literature

Review of Literature

The term “coagulation” and “flocculation” have been used indiscriminately to describe the process of removal of turbidity from water. The term “coagulation” comes from the Latin *coagulare,* meaning to drive together (Faust and Aly, 1998; Nester et al., 2001). This process describes the effect produced by the addition of chemicals to a colloidal dispersion resulting in particle destabilization by a reduction of the forces tending to keep the particles apart. Under these conditions, particle growth does not occur and Brownian motion keeps the particles in suspension. Operationally, coagulation is achieved by adding appropriate chemicals, which causes particles to stick together when contact is made. Rapid mixing is important at this stage to obtain uniform dispersion of the chemical and to increase the opportunity for particle-to-particle contact. The entire process occurs in a very short time, probably less than a second, and initially results in particles submicroscopic in size (Cohen et al., 1972; Prescott et al., 1996).

2.1 Flocculation dynamics

The settling velocities of finely divided and colloidal particles under gravity alone are so small that ordinary sedimentation is not practical. It is therefore necessary to use procedures, which agglomerate the small particles into larger aggregates, which then have the settling velocities required for various applications. Formation of larger particles from smaller ones is also required for their removal by filtration (Nester et al., 2001). The second stage of the formation of settleable particles from destabilized colloidal-sized particles is termed flocculation. This term also has its derivation from Latin, *flocculare*, meaning to form a floc. Flocculation is operationally obtained by gentle and prolonged mixing which converts the submicroscopic coagulated particles into discrete, visible, suspended particles. At this stage, the particles are

large enough to settle rapidly under gravity and may be removed from suspension by filtration. The more usual practice has been to physically separate the unit processes into coagulation-flocculation, sedimentation, and filtration (Faust and Aly, 1998).

Thus the dynamics of flocculation is as follows: Initially, aggregation is controlling, resulting in a rapid increase in floc size. Breakage becomes more dominant as the floc size increases. Eventually, equilibrium is reached between aggregation and breakage and a steady-state floc size is maintained. As flocculation occurs, the size distribution shifts into the larger sizes and the spread of the distribution narrows. The steady-state aggregate size is interpreted as the result of a dynamic equilibrium between floc growth and floc breakup.

The rate of flocculation is determined by the collision frequency induced by the relative motion. Because Brownian movement causes this, it is called perikinetic flocculation. That which is caused by velocity gradients is called orthokinetic flocculation. If there is no surface, repulsion between the particles, then every collision leads to aggregation and the process is called rapid flocculation. If a significant repulsion exists, then only a fraction of the collisions results in aggregation. This is called slow flocculation. The floc blanket clarifier provides a special case of orthokinetic flocculation. In addition to the fluidized bed giving rise to velocity gradients, the fluidized particles are participating in the process of agglomeration. If particles are settling at different velocities, then the faster settling particles may collide with slower settling particles, leading to aggregation. The aggregates will then settle faster due to their increased mass, and possibly experience further collisions and aggregations (Yan et al., 2004).

2.1.1 Particle Charge

The key to effective flocculation is an understanding of how individual colloids interact with each other. Turbidity particles range from about .01 to 100 microns in size. The larger fraction is

relatively easy to settle or filter. The smaller, colloidal fraction, (from .01 to 5 microns), presents the real challenge. Their settling times are intolerably slow and they easily escape filtration. The behavior of colloids in water is strongly influenced by their electrokinetic charge. Each colloidal particle carries a like charge, which in nature is usually negative. This *like* charge causes adjacent particles to repel each other and prevents effective agglomeration and flocculation. As a result, charged colloids tend to remain discrete, dispersed, and in suspension. On the other hand if the charge is significantly reduced or eliminated, then the colloids will gather together. First forming small groups, then larger aggregates and finally into visible floc particles which settle rapidly and filter easily.

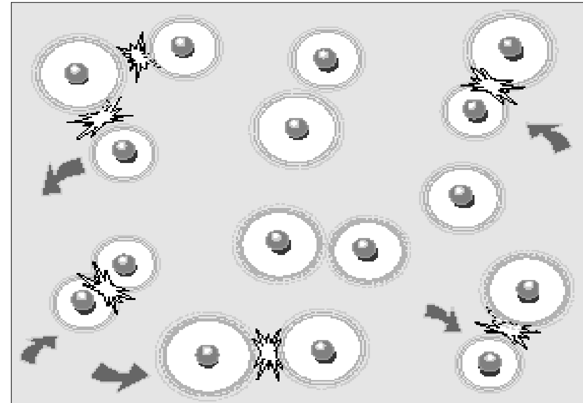


Fig . 2.3 Charged Particles repel each other

2.2 Types of flocculation

2.2.1 Charge neutralization

In charge neutralization adsorption of a positively charged flocculant on the surface of the colloid takes place. This charged surface coating neutralizes the negative charge of the colloid, allows Van der Waals forces to reduce the energy barrier resulting in the formation of stable flocs. : If charged (+) counterions have a specific merely electrostatic attraction) then adsorption of the counter-ion will reduce the primary charge of the colloid. Counter-ions can be adsorbed by ion exchange, coordination bonding, van der Waals forces and repulsion of the flocculants by the

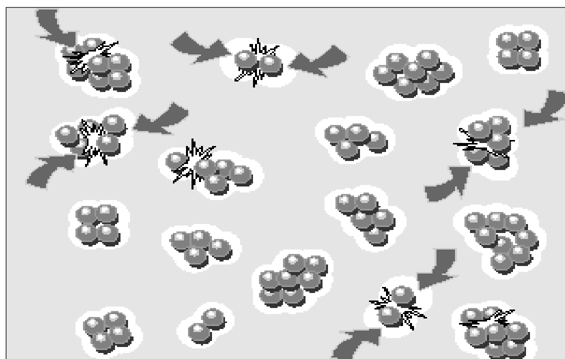


Fig.2.4 Uncharged Particles are free to collide and aggregate

aqueous phase. Inorganic flocculants and cationic polymers often work through charge neutralization.

2.2.2 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 and/or shear resistant flocs. For instance, alum or a low molecular weight cationic polymer is first added under rapid mixing conditions to lower the charge and allow microflocs to form. Then a slight amount of high molecular weight polymer, often an anionic, can be added to bridge between the microflocs. The fact that the bridging polymer is negatively charged is not significant because the small colloids have already been captured as microflocs.

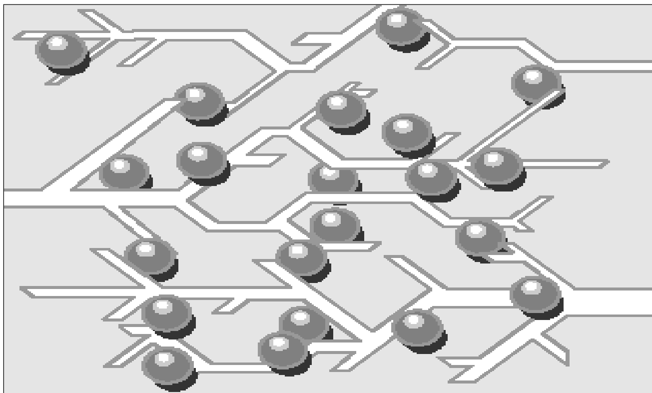


Fig. 2.1 Each polymer chain attaches to many colloids

2.2.3Colloid Entrapment

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

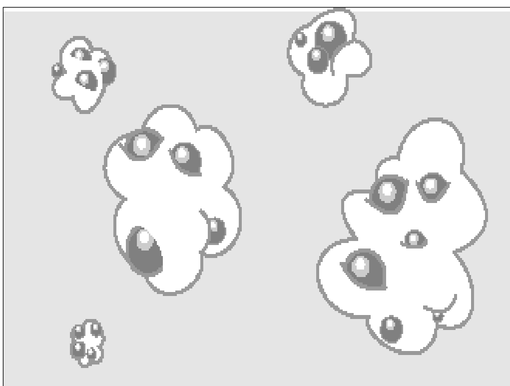


Fig. 2.2 Colloids become enmeshed in the growing precipitate

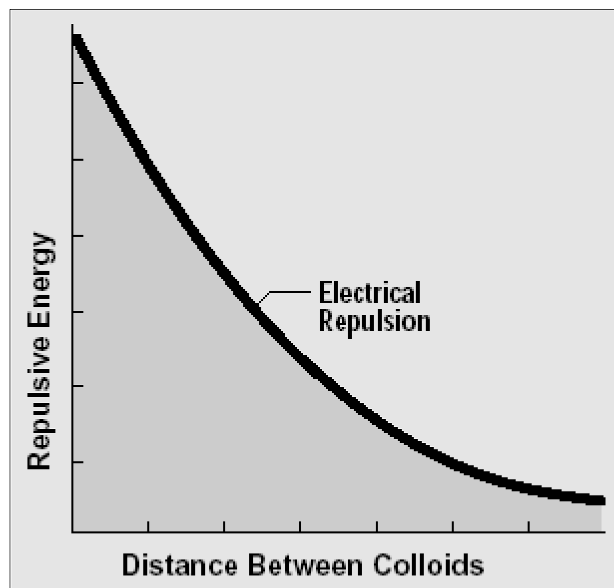
literally swept from the bulk of the water by becoming enmeshed in the settling hydrous oxide floc. This mechanism is often called *sweep floc.*

2.3 Mechanisms involved in flocculation

A significant amount of research has been performed to better understand the mechanisms of bioflocculation and solid/liquid separation processes

2.3.1 Balancing Opposing Forces

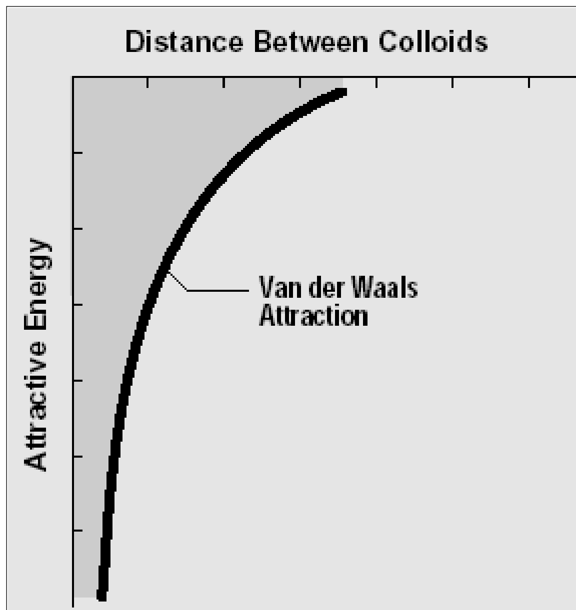
The DLVO Theory (named after Derjaguin,Landau, Verwery and Overbeek) is the classic explanation of how particles interact.It looks at the balance between two opposing forces - electrostatic repulsion and van der Waals attraction - to explain why some colloids agglomerate and flocculate while others will not.



Repulsion*:* Electrostatic repulsion becomes significant when two particles approach each other and their electrical double layers begin to overlap. Energy is required to overcome this repulsion and force the particles together. The level of energy required increases dramatically as the particles are driven closer and closer together. An electrostatic repulsion curve is used to indicate the energy that must be overcome if the particles are to be forced together. The maximum height of the curve is related to the surface potential.

Attraction: Van der Waals attraction between two colloids is the result of forces between

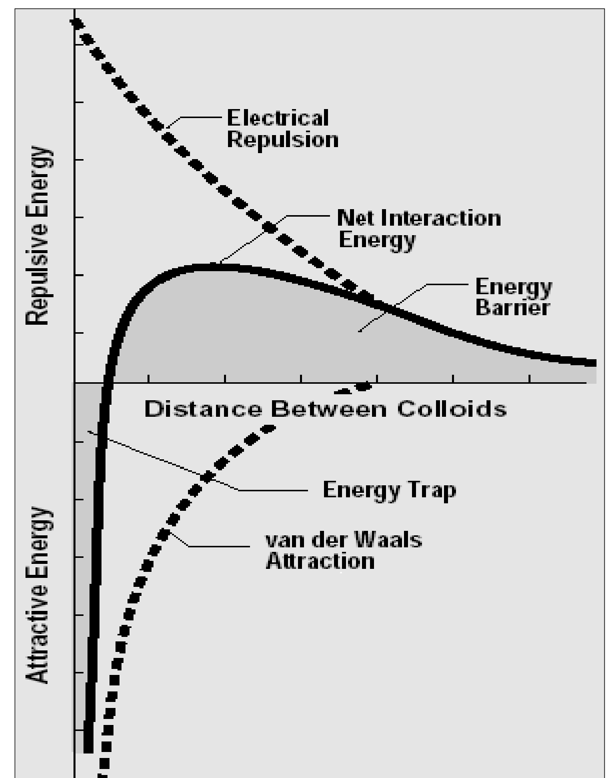
individual molecules in each colloid. The effect is additive; that is, one molecule of the first



colloid has a van der Waals attraction to each molecule in them second colloid. This is repeated for each molecule in the first colloid and the total force is the sum of all of these. An attractive energy curve is used to indicate the variation in attractive force with distance between particles.

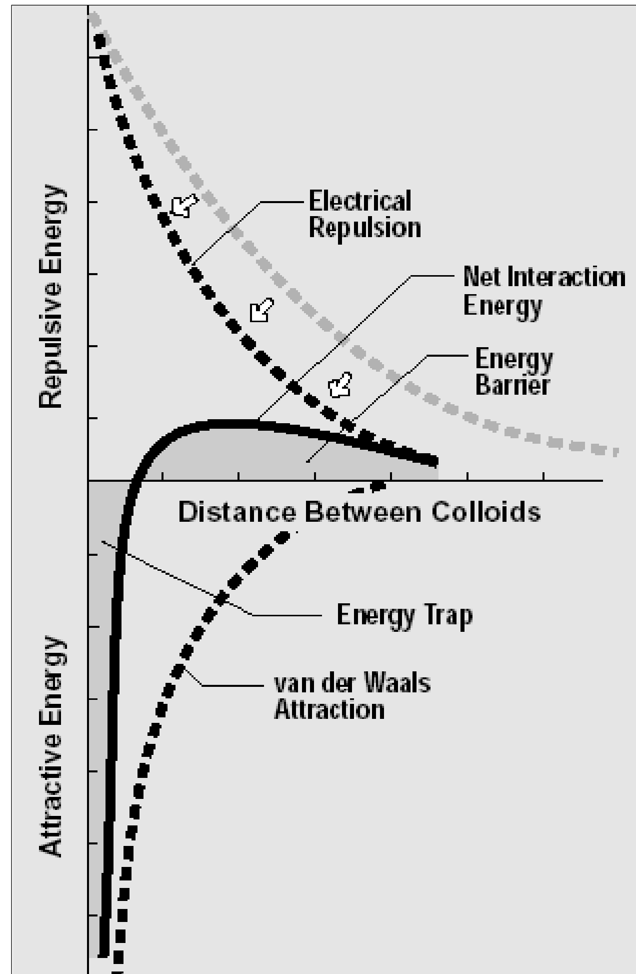
2.3.2 **The Energy Barrier**

The DLVO theory combines the van der Waals attraction curve and the electrostatic repulsion curve to explain the tendency of colloids to either remain discrete or to flocculate. The combined curve is called the *net interaction energy*. At each distance, the smaller energy is subtracted from the larger to get the net interaction energy. The net value is then plotted -above if repulsive, below if attractive – and the curve is formed. The net interaction curve can shift from attraction to repulsion and back to attraction with increasing distance between particles. If there is a repulsive section, then this region is called the *energy barrier* and its maximum height indicates how resistant the system is to effective coagulation. In order to agglomerate, two particles on a collision course must have sufficient kinetic energy (due to their speed and mass) to *jump over* this barrier. Once the energy barrier is cleared, the net



interaction energy is all attractive. No further repulsive areas are encountered and as a result the particles agglomerate. This attractive region is often referred to as an *energy trap* since the colloids can be considered to be trapped together by the van der Waals forces.

Lowering the Energy Barrier



For really effective coagulation, the energy barrier should be lowered or completely removed so that the net interaction is always attractive. This can be accomplished by either compressing the double layer or reducing the surface charge.

Compress the Double Layer

Double layer compression involves adding salts to the system. As the ionic concentration increases, the double layer and the repulsion energy curves are compressed until there is no longer an energy barrier. Particle agglomeration occurs rapidly under these conditions because the colloids can just about fall into the van der Waals "trap" without having to surmount an energy barrier.

2.4 **Types of flocculants**

A number of chemicals are used either as aids to coagulation or as flocculants. Colloidal particles generally have a net negative surface charge. The size of colloids is such that the

attractive forces between particles are considerably less than the repelling forces of the electrical charge. In order for a polymer molecule to be an effective flocculant, it must contain constituents that can interact with sites on the colloidal particle (Singh et al., 2000). In addition, molecular weight and degree of branching of the polymer play a mechanistic role in their ability to flocculate (Bitton, 1994; Faust and Aly, 1998).

Flocculants can be divided into three distinct categories: Inorganic, organic and natural (Kurane *et al.*1994).The most commonly used are the inorganic flocculants. The latter are a type of synthetic flocculants exemplified by aluminium sulfate, ferric sulfate and ferric chloride. Prepolymerised flocculants are used such are polyaluminium chloride (PAC) (Dampsey *et. al;* 1984) and polyferric chloride (PFC) (Leprince *et.al;* 1984) are more efficient. Both PAC and PFC are prepared by undertaking a partial hydrolysis of acid aluminium chloride or ferric chloride solution using a specific reactor. Another inorganic flocculant polyaluminium silicate sulfate (Smith *et. al;* 1997) is commercially available and it has been used in drinking water in full scale. An iron based flocculant-poly ferric sulfate or PFS (Jiang *et.al;*. 1993) has been developed. PFS is prepared by technique involving the oxidation of ferrous state iron solution under the conditions of high temperature and high pressure.

Organic flocculants are used extensively to enhance the flocculation of suspended solids in the treatment of process water, waste water and effluents (Boulton; 1997) Important among this type are polyacrylamide and polyacrylic acid. (Kurane et. al; 1994). Organic flocculants are especially recommended for flocculating suspended solids and in non potable raw water clarification, primary and secondary effluent clarification and oil waste clarification. Organic flocculants are also used in effluent, pulp

and paper process and are important since they increase retention of fibers, fillars additives and improve drainage. Organic flocculants have high molecular weight, can work over wide range of pH, and has a low charge density and a very high molecular weight. The synthetic organic flocculants are commercially marketed in the form of dry powder, granules, beads, aqueous solutions, aqueous gels and oil in water emulsions.

If the monomer contains an ionisable group, such as carboxyl, amino, or sulfonic, then the polymer is called a polyelectrolyte. There are cationic, anionic, or ampholytic (has both positive and negative) groups which, of course, depends on the nature of the functional groups within the monomer. Non-ionic flocculants without any ionisable groups are also available. These polymers and polyelectrolyte are able to flocculate colloidal particles due to adsorption. Polyacrylamide (PAM) and poly (ethylene oxide) (PEO) are non-ionic.

The majority of the cationic groups of polyelectrolytes are derived by introducing quaternary ammonium groups on to the polymer backbone, although polymers containing sulfonium and phosphonium groups are used to a limited extent. The most commonly used cationic polyelectrolytes are poly (diallyl dimethyl ammonium chloride) (polyDADMAC). In the anionic group of polyelectrolytes, mainly two types of polymers are used; one type is polymers containing carboxyl (eg:- poly(acrylic acid) and its derivatives) functional groups and the other containing sulfonic acid groups (eg:- poly(styrene sulfonic acid) (PSSA)). Flocculants are also available in wide range of molecular weights, charge density, and degree of branching. Among the inorganic flocculants, salts of multivalent metals like aluminum and iron are applied most often - at high concentrations. Inorganic flocculants are used in very large quantities; they leave large amounts of sludge and are strongly affected by pH changes. Organic flocculants which are

typically polymeric in nature by contrast to inorganic ones are effective already in low ppm concentrations.

2.5 Limitation of synthetic flocculants

Inorganic and organic synthetic polymer flocculants are frequently used in water and wastewater treatment because they are economical and highly effective. However, their use often gives rise to environmental and health problems. Some of them are not readily biodegradable and some of their degraded monomers, such as acrylamide, are neurotoxic and even strong human carcinogens. Residual alum concentration in treated water can also impose health problems apart from the production of high amount of sludge (Letterman and Driscoll, 1988). There is also a problem of reaction of alum with natural alkalinity present in water leading to a reduction of pH and a low efficiency in coagulation of cold waters (Ndabigengesere and Narasiah, 1998). Thus, the development of safe biodegradable flocculants that will minimize environmental and health risks is urgently required (Shih et al., 2001). Aluminum salts are by far the most widely used coagulants in water and wastewater treatment. However, studies have pointed out that there are several serious disadvantages of using aluminum salts including Alzheimer's disease and similar health related problems associated with residual aluminum in treated waters (Yokoi et al., 1995). A significant economic factor is that many developing countries can hardly afford the high costs of imported chemicals for water and wastewater treatment. Therefore, it is desirable that other cost effective and more environmentally acceptable alternative coagulants be developed to supplement if not replace alum, ferric salts, and synthetic polymers (Ndabigengesere and Narasiah, 1998).

The heightened concerns posed by the synthetic chemical flocculants have prompted research for alternative, environment friendly, nontoxic alternatives from other sources. Flocculants obtained from microbial sources have been found to possess several advantages such as diversity, biodegradability and harmlessness to humans and to environment and hence can be potentially applied as a substitute for synthetic flocculants (Salehizadeh and Shojaosadati, 2001; Oh et al., 2001; Gao et al., 2006). Although synthetic flocculants are frequently used, flocculants from microbial systems have gained popularity in various industrial applications.

2.6 Microbial flocculants

Flocculation in microbial systems was first reported by Louis Pasteur in 1876 (Salehizadeh and Shojaosadati, 2001) for the yeast *Levure casseeuse*. Two years later, this phenomenon was observed in bacterial cultures (Salehizadeh and Shojaosadati, 2001). A few years later the phenomenon was observed and confirmed in several other bacterial cultures by Bordet (1899). Butterfield (1935) isolated *Zoogloea*-forming bacteria from activated sludge in 1935. Later, bioflocculation was investigated extensively and a correlation was established between the accumulation of extracellular biopolymeric flocculants (EBFs) and cell aggregation (McKinney, 1956; Tenny and Stumm 1965). Many EBF-producing microorganisms including bacteria, fungi, yeast, and algae have since been isolated from soil and wastewater (Bar-or and Shilo, 1987; Bender et al., 1989; Huang, 1990; Kakii et al., 1990; Morgan et al., 1990; Fumio, 1991; Hantula and Bamford, 1991a, b; Dube, 1992; Guirand, 1992; Sousa et al., 1992; Kim, 1993; Seo, 1993; Kurane et al., 1995; Yokoi et al., 1995; Suh et al., 1997; Salehizadeh et al ., 1998; Tong et al., 1999; Misra, 2002)

Many microorganisms synthesize exopolysaccharides (EPS) or EBFs, which either remain attached to the cell surface or are found in the extracellular medium in the form of

amorphous slime. In the natural environments in which the microorganisms are found, such polymers may either be associated with virulence, as in the case of plant or animal pathogens, with plant microbial interactions or even protect the microbial cell against desiccation or attack by bacteriophages and protozoa. Several such microbial polysaccharides are now widely accepted products of biotechnology, while others are in various stages of development. The uses of such polymers vary widely; some are employed because of their unique or superior physical properties relative to traditional plant polysaccharides. In this category are xanthans, from *Xanthomonas campestris* pv. *campestris* and gellan (gelrite) from *Sphingomonas paucimobilis* strains. These two polysaccharides have found various applications. The microbial products always have to compete against other natural or synthetic polymers, which may be inferior in their physical or ecological properties but are much cheaper to produce and market. According to Sutherland, 1998, alteration of the chemical properties of the original exopolysaccharide may also greatly enhance their value and extend their range of applications, as exemplified by the dextran-derived Sephadex products.

Activated sludge and soil are considered as potential sources for screening bioflocculant producing microorganisms. *Rhodococcus erythropolis* (Kurane et al., 1994), *Zoogloea* MP6 (Farrah and Unz, 1976), *Zoogloea ramigera* (Norberg and Enfors 1982), *Pseudomonas* C120 (Sakka et al., 1981) and *Bacillus* sp As101 (Salehizadeh et al., 1998) were isolated from activated sludge. Bioflocculant producing organisms such as *Bacillus mucilaginosus* (Deng et al., 2003), *Bacillus firmus* (Salehizadeh and Shojaosadati, 2002), *Pseudomonas* sp. A-99 (Yokoi et al., 1998), *Enterobacter* sp. BY-29 (Yokoi et al., 1997), *Bacillus* sp. DP-152 (Suh et al., 1997), and *Alcaligenes latus* B-16 (Kurane et al., 1994) were all isolated from soil samples. Recently, bioflocculant producing bacteria from some other sources such as *Lactobacillus plantarum* EP56

from corn silage (Tallon et al., 2003), *Myxobacterium* NU-2 from salt soil sample (Zhang et al., 2002), *Vagococcus* sp W31 (Gao et al., 2006) from river water have been isolated.

2.7 Production and characterization of bacterial bioflocculants

Microbial EPS or EBFs are either associated with, and often covalently bound to, the cell surface in the form of capsules, or secreted into the environment in the form of slime. They are referred to as capsular (CPS) or slime exopolysaccharides, respectively. Cell wall polysaccharides (WPS) are another type that, in contrast to EPS, are not released into the medium and are associated with the cell envelope, and may again be either covalently bound to the peptidoglycan layer or loosely associated with it. Polysaccharides, which constitute the outermost layer of cells, are thought to mediate interaction between cells or the adherence of cells to surfaces. Some polysaccharides are liberated outside of the cell, and some are bound to the cell envelope, but under certain physiological conditions, extracellular polysaccharide molecules may remain bound and associated with the cell surface without detectable membrane anchoring. The function of the extracellular polysaccharide in flocculation and adhesion is an important theme as is the function of envelope bound polysaccharides (Nakata and Kurane, 1999).

The correlation between cell growth and secretion of extracellular biopolymeric flocculants (EBFs) was originally reported by McKinney (1956). Fujita et al. (2000) noted that the biological flocculation does not occur until the microorganisms have entered into an endogenous phase. In a study done by Norberg and Enfors (1982) on *Z. ramigera*, the flocculant production ended after 90 hours in the stationary phase. The production of the flocculant by *S.*

griseus was not growth-related. For *S. griseus*, the flocculating activity increased rapidly with increasing time of cultivation after the third day and reached a maximum value after 4 days (Shimofuruya et al., 1996). *Flavobacterium* sp. showed flocculating activity at the end of exponential growth phase and the beginning of the stationary phase (Hantula and Bamford, 1991a; b). In contrast to those observations, the flocculant production in *R. erythropolis* (Kurane et al., 1991), *A. sojae* (Nakamura et al., 1976b), *Zoogloea* MP6 (Unz and Farrah, 1976), and *Alcaligenes latus* (Kurane and Nohata, 1991) have been found to be parallel to cell growth.

Many factors influence the production of extracellular bioflocculants (EBFs) and the bioflocculation process. These include genotypic, physiological and environmental aspects. The environmental aspects involve physical, chemical and biological factors. The carbon and nitrogen concentration (C/N ratio), culture pH, temperature, and agitation speed used in the fermentor need to be optimized for efficient production (Salehizadeh and Shojaosadati, 2001). This optimization is essential because productivity and distribution of EBFs depend on the culture conditions. The pH of the culture medium can influence the production of EBFs. In the case of *C. xerosis*, the flocculant was produced at relatively low pH whereas the optimum pH for production of EBF by *Proteus mirabilis* TJ-1 (Xia et al., 2008) was in the neutral range and for *Serratia ficaria* is at a pH range of 6-8 (Gong et al., 2008). Temperature is another physical factor that affects the production of EBFs. The best production of EBFs by *Enterobacter* sp. BY-29 was obtained at 37°C (Yokoi et al., 2001) for *Sphingomonas paucimobilis* (Sanayei et al., 2010) is at 35 ^{o}C and *Bacillus* sp. DP-152 is 30°C (Suh et al. 1998). The interaction between different microorganisms in a mixed culture is another biological parameter that can have a positive effect on aggregation of cells and the production of EBFs (Nakamura et al*.,* 1976c). By adjusting the growth conditions, the adsorption of the flocculant F-1 on the cell's surface could

be raised by 5% of the concentration in the filtrate (Nakamura et al., 1976c). The importance of carbon and nitrogen sources and the C/N ratio has been emphasized for EBFs production (Nakamura et al., 1976c; Kurane et al., 1994a). Ethanol is a good carbon source for flocculant production on an industrial scale (Nakata and Kurane, 1999). The canning factories wastes and spillage from distilleries are alternative inexpensive carbon sources. Urea, ammonium sulphate, yeast extract, and casamino acids are good nitrogen sources for EBF production and growth of *R. erythropolis* (Tong et al., 1999). The application potential of EBF is determined by their physical and rheological properties. Factors influencing these properties are molecular mass, stiffness of the polymer, presence of side chains, and presence of monsaccharide components, such as organic (e.g., acetyl, pyruvyl, or succinyl groups) or inorganic (e.g., sulphate or phosphate groups) substituents. Genetic engineering may be applied as a tool to direct the EPS synthesis and introduce desired properties by altering the composition or chain length. This requires a proper understanding of the genetics and biochemistry of EPS biosynthesis (Van-Kranenburg et al., 1999).

To date, many studies on the microbial production of flocculating substances have been reported. Microbiologically-produced bioflocculants are generally high molecular weight polymers, and have been identified or presumed to be proteins (Takeda et al., 1991; Takeda et al., 1992), glycoproteins (Kurane et al., 1986), polysaccharides (Kurane and Nohata, 1991; Toeda and Kurane, 1991), glycolipids (Kurane et al., 1994a), cellulose (Napoli et al., 1975), DNA (Sakka and Takahashi, 1981) or complex heteropolymers (Nakamura et al., 1976a). The primary structure of proteins and polysaccharides describes the arrangement of the different building blocks, amino acids and monosaccharides, respectively, along the polymer chain. The possible structural variability due to available units and connecting patterns is estimated to be

about three orders of magnitude larger for polysaccharides than for proteins (Sletmoen et al., 2003). The uronic acid content of the extracellular polymers can be measured by the formation of the ester and the reduction of the carboxylic acid moiety to an alcohol. This process eliminates the problems of resistance to hydrolysis and of quantitative recovery in separation from the neutral carbohydrates (Fazio et al., 1982). They are also found in the polysaccharide polymers of higher plant cell walls, in gram-positive microbes under conditions of phosphate limitation (Elwood and Tempest, 1972), and in some gram-negative microbial lipopolysaccharides. Polymers containing uronic acid are resistant to quantitative hydrolysis, and the uronic acid, once released, form lactones irreproducibly and are difficult to separate from the neutral sugars. Uronic acids are often estimated by their acid catalyzed decarboxylation under controlled conditions (Kiss, 1974). The known microbial exopolysaccharides contain D-glucuronic acid, D-galacturonic acid, D-mannuronic acid, and L-gulonic acid (Dudman, 1977). Amino sugars are important structural components of bacterial cell walls, and neutral carbohydrates make up a basic unit of plant cells (Cheng and Kaplan, 2003). Generally, amino sugars are present in very small quantities in bacterial cell walls. Fujita et al. (2000) reported that bioflocculant produced by *Citrobacter* sp. TKF04 was composed of 29.4% of hexosamine. In this study, bacterial bioflocculants were produced and characterized. They were found to be composed of carbohydrate, protein, uronic acid, and hexosamine in varying quantities.

The basic carbohydrate structure of most exopolysaccharides does not change with growth conditions, but the content of groups attached to the basic carbohydrate structure, can vary widely and may have a dramatic effect on the properties of the polymer and hence their effectiveness in various applications (Lopez et al., 2003). For the production of these biopolymer flocculants, sugars such as glucose, fructose or sucrose (Takagi and Kodowaki, 1985; Kurane et

al., 1986; Kurane and Nohata, 1991; Toeda and Kurane, 1991), casein, L-glutamate or citrate are usually required as the main substrate(s).

2.8 Flocculant attributes

Bioflocculants are generally anionic in nature. The anionic nature of the bioflocculants might be due to the negative charge of functional groups present on the biopolymer (Bar-Or and Shilo, 1987). These acid groups help in the formation of precipitates when Cetyl pyridinium chloride (CPC) is added to the bioflocculant solution. The quaternary ammonium ion (QN+) of the CPC forms a bioflocculant - CPC complex (Dermlim et al., 1999). The infrared spectrum of the bioflocculants generally shows the presence of carboxyl, hydroxyl, amino and sugar derivative groups, which are the preferred groups for the bioflocculants. Trace amounts of uronic acid, glucuronic acid or galacturonic acid are also found in most acidic polymers. The components and structures of bioflocculants are complex and different bioflocculants produced by different bacteria can have different properties (Deng et al., 2005). Biopolymeric flocculants consisting of polysaccharides, proteins, lipids, glycolipids and glycoproteins have been reported (Kurane et al., 1994b).

Most bioflocculants are reported to comprise of polysaccharides and proteins. For example, *Bacillus subtillis* (Yokoi et al., 1996), *Bacillus licheniformis* (Shih et al., 2001), *Paecilomyces* sp. (Shubo et al., 2005) and *Nocardia amarae* YK1 (Takeda et al., 1992) produce proteinaceous bioflocculants. *Alcaligenes latus* KT201 (Toeda and Kurane, 1991) and *Enterobacter* sp. (Yokoi et al., 1997) produce polysaccharide bioflocculants, while glycoprotein bioflocculants are produced by *Arcuadendron* sp. TS-4 (Lee et al., 1995). The backbone of

polysaccharide bioflocculants generally consist of uronic acids, hexosamines and neutral sugars such as glucose, galactose, and mannose (Bar-or and Shilo, 1987; Dermlim et al., 1999; Kumar et al., 2004; Gao et al., 2006). In proteinaceous bioflocculants, the amino and carboxyl groups are the effective groups for flocculation, and their molecular weights are usually low (Kurane et al., 1994a). In contrast, polysaccharide bioflocculants have high molecular weights and many functional groups (Kurane et al., 1991). The molecular weight and functional groups in the molecular chains are important factors in determining the flocculating activity of bioflocculants. The molecular weight of most bioflocculants is in the range of 10^5 to 2.5×10^6 Da (Salehizadeh et al., 2000). The molecular weight of protein bioflocculants is usually low as compared to polysaccharide bioflocculants (Kurane et al., 1994). Infrared spectroscopic studies of various bioflocculants show the presence of carboxyl, hydroxyl, methoxyl, amino and phosphate groups in the structure (Zajic and Knetting, 1970; Kurane et al., 1994; Gao et al., 2006). Although most researchers agree that carboxyl groups play an important role in flocculation, others emphasize the importance of amino and phosphate groups (Sousa et al., 1992). Surface structure is another important factor that determines the flocculating ability of a bioflocculant (Kumar et al., 2004). Scanning Electron micrographs of purified biofloccuants have indicated a generally porous surface morphology of these exopolymers (Kumar et al., 2004). The flocculating activity of *Bacillus* sp PY 90 showed optimum activity in acidic pH (pH 3-5), whereas for *Rhodococcus erythropolis* optimum activity was observed at neutral pH (Kurane et al., 1994; Yokoi et al., 1995).

2.9 Factors affecting flocculant activity

2.9.1 Molecular Structure

According to the bridging theory, all the acting macromolecules are linear molecules, and they should be long enough, the bridging effect won't be created if the length is not enough. If the molecule has a cross-linked structure or a branched structure, the flocculating effect will not be so obvious. Yin et al. (2003) found that the major contents of JMBF-25 is high molecular polysaccharide, the acting force (such as van der Waals force, hydrogen bond force, and the attractive force among the different electric charges, etc.) between MBF molecules is large when the temperature is low, the molecular chain is curled, and the effective length of the chain is short; the acting force among the molecules is broken when the temperature rises, so the molecule chain smooth out, and the effective length of the chain increases, this is propitious to the adsorbing and bridging process between microbial flocculants macromolecules and colloid particles, so the peak value of microbial flocculants is attained when the temperature is high.

2.9.2 Molecular Weight

The molecular weight of microbial flocculant is vital to the flocculating activity of microbial flocculants. The bigger the molecular weight, the more the adsorption sites and electric charges, thus the neutralizing capacity becomes stronger, and the bridging effect and the sweeping effect will become obvious. Currently, except a few microbial flocculants which have been separated and purified, most of the microbial bioflocculants has a molecular weight of $10^5 \sim 2.5\times10^6$ (Wang et al., 2001). The reduction of the molecular weight decrease the flocculating activity of microbial flocculants, e.g. after protease treatment, the flocculating activity of MBF produced by *Aspergillus Sojae* AJ7002 was decreased, this is as a result of

hydrolysis of the protein components, which lead to the molecular weight reduction of the polymer (Nakamura et al., 1976).

2.9.3 Temperature

The flocculating ability of various flocculants is affected by the temperature. Protein bioflocculants are usually heat labile as proteins are temperature sensitive and can be destroyed upon heating. In flocculants which contains proteins as the main component, the protein or the polypeptide skeleton of the flocculant gets easily denatured under the condition of high temperature hence causing decrease in flocculation rate. Since the protein plays the high molecular weight role in the flocculation process, the protein is damaged as the temperature increases, resulting in the decrease of the flocculation activity (Salehizadeh and Shojaosadati, 2001). However, flocculants being composed of polysaccharide are not much influenced by temperature and are usually functional at a wide temperature range (Deng et al., 2003). In microbial flocculant WF-1, the sugar proportion occupies 86.1% and the protein occupies 10.2%, therefore, the polysaccharides is the main component, whose flocculation activity will reduce by 22% only being heated up for 15 min under the condition of 100°C, showing the good thermal stability (Lu et al., 2005). The flocculant generated by *Bacillus* sp.DP-152 (Zhang et al., 2002) when heated at 100°C up for 15 min, the flocculation activity gets reduced by 50% and flocculant generated by *Paecilomyces* sp. is when incubated at 0～100°C, the flocculation activity is not much effected (Takagi and Kadowaki, 1985). If the major component of a bioflocculant is a glycoprotein, its stability depends on the relative contents of protein and polysaccharide.

2.9.4 pH

The flocculating activity of bioflocculants varies with pH (Salehizadeh et al., 2000). It's value is able to change the surface charge, the electrification situation, charge neutralization capacity of the large molecule of flocculant and colloid particle, thus by changing pH value interaction force among the particles, influencing the form of bridge work is also changed (Shih et al., 2001). As the flocculation is in a certain scope of pH value, the surface charge of the colloid particle gets reduced, weakening the mutual exclusive force among the particles, hence to make for the bridging role between the flocculants and the particles, and the pH value is the best acid-base environment of the flocculants. The microbial flocculants REA-11 can sustain the stronger flocculation activity in the scope of 3.0 to 6.0 pH value and the flocculation rate is more than 80% (He et al., 2004). Some flocculants have different flocculation activity for the same flocculate under the condition of different pH value. The flocculant generated by the *Paecilomyces* sp. shows high flocculating activity in the scope of 4 to 7.5 pH value. However, at pH 3 or 8, the flocculation capacity gets reduced sharply (Takagi and Kadowaki, 1985).

2.9.5 Inorganic metal ions

The ions in the system, especially the high valence foreign ions are able to change the electric potential of the colloid obviously, reducing its surface charge and promoting the adsorption and bridging of the large molecule and the colloid particle. The bivalent cations may promote the generation of flocculation with the ionic bond between the flocculant molecule and the suspended particles. The monovalent cations, such as Na^{+} generally cannot bind with the suspended particles very efficiently. The trivalent cation, such as Al^{3+}, Fe^{3+} and others may result into high ion degree and a lot of ions occupy the active site of the bioflocculant and the suspended particles are separated for restraining the flocculation. Yao and Xu (2004) found that

the flocculating activity of γ-PGA was influenced to different degree on adding the metal ions, among which, Ca^{2+} has the maximum promotion for the flocculating activity, thus reducing the amount of usage and the cost of the flocculants, a similar observation was reported by Takeda et al., (1992).

2.9.6 Dose

Bioflocculant dose is another important parameter that influences the flocculating activity of bioflocculants. An important hindrance in the industrial application of bioflocculant is their large dosage requirement. However the bioflocculants thus reported have varied optimum dose (Table 2.1).

Table 2.1 Microbial Bioflocculants, their dose and flocculating activity

Microrganisms	Optimum Dose (ppm)	Flocculating activity	References
Bacillus sp. DYU1	40	97[a]	Wu and Ye, 2007
B. licheniformis CCRC 12826	3.7	8.5[b]	Shih et al , 2001
Bacillus sp. PY-90	20	33[b]	Yokoi et al 1995
Bacillus coagulants AS101	30	92[a]	Salehizadeh et al., 2000
B. Subtilis	20	21.3[b]	Yokoi et al., 1996
Gyrodinium impudicum KG03	1	90[a]	Yim et al., 2007
Aeromonas sp.	1	92.4[a]	Li et al., 2007
Bacillus subtilis	10	14.53[b]	Bajaj and Singhal, 2009
Bacillus circulans X3	2	99[a]	Zhong, et al., 2009
Vagococcus sp.	25	93[a]	Gao, et al., 2006
Klebsiella terrigena	2	93.6[a]	Ghosh et al., 2009
Bacillus mucilaginosus	1	99.6[a]	Deng et al., 2003

a Flocculating activity was calculated according to the following equation:
Flocculating activity = $1/(OD_{550}) - 1/(OD_{550})_C$
Where OD_{550} is the optical density of sample at 550 nm and $(OD_{550})c$ is the optical density of control at 550 nm.

b Flocculating activity was calculated according to the following equation:
Flocculating activity = $[(B-A)/B]\times 100\%$, in which A and B were the absorbances at 550 nm for sample and control, respectively.

2.10 Application of bioflocculants

One of the most important applications of bioflocculants is in brewing and fermentation industries where they are used for removal of cell debris from culture broths (Salehizadeh et al., 2000). Bioflocculants can play an important role in drinking water treatment (Zhong et al., 2009) as well as used for wastewater treatment (Gao et al., 2006). Besides studies have shown that bioflocculants can play a vital role in colour removal from wastewater (Kurane, 1986). Bioflocculants have found application in treatment of indigotin printing and dyeing wastewater, jean dying wastewater and starch wastewater (Zhang et al., 2007; Liu et al., 2009, Sanayei et al., 2010, Ge et al., 2011; Deng et al., 2003). The dewatering and settling characteristics of activated sludge can be improved significantly using bioflocculants (Salehizadeh and Shojaosadati, 2001). Bioflocculants have been found to play a key role in the removal of suspended solids (Kurane et al 1991). Bioflocculant has also found application for the removal of humic acids from stabilized landfill leachates (Zouboulis et al., 2003). Bioflocculants can also be used for several other industrial applications such as heavy metal removal, oil water separation and mining and milling operations (Kurane, 1986; Dugan, 1987; Takeda et al., 1991; Bender et al., 1989, 1991, 1994; Vijayalakshmi and Raichur, 2003). An enhanced Bioflocculant can enhance anaerobic granulation in a UASB reactor (Wang et al., 2005) by bioflocculants can also be used for treatment of palm oil mill effluent was reported (Kaewchai and Prasertsan, 2002).

2.11 Constraints of bioflocculant application

Although it is possible to produce microbial flocculants in large scale and recover easily from fermentation broth, low flocculating capability and large dosage requirement has been a major problem in bioflocculant development for their commercial application. Another important hindrance in large commercial application of bioflocculants is the high production cost. The lack

of economical production limits their use and consequently they represent a small fraction of the current biopolymer market. Efficient production and reduction in recovery cost requires knowledge of biosynthesis and adoption of appropriate bioprocess technologies (De Vuyst et al., 2001). Therefore, to utilize bioflocculants widely in industrial fields, it is desirable to screen various microorganisms with high bioflocculant ability, improved flocculating efficiency with reduced production cost. There is a need to understand the mechanism involved behind bioflocculation in a better way for better usage of bioflocculants at industrial level. Understanding and controlling the important environmental variables affecting polymer synthesis can be advantageous in the design of an economic process.

2.12 Heavy-metal Contamination and Toxicity

Heavy-metals occur naturally in the environment in rocks and ores and cycle through the environment by geological and biological means. The geological cycle begins when water slowly wears away rocks and dissolves the heavy-metals. The heavy-metals are carried into streams, rivers, lakes and oceans and may be deposited in sediments at the bottom of the water body or they may evaporate and be carried elsewhere as rainwater. The biological cycle includes accumulation in plants and animals and entry into the food web (Young, 2000). Some heavy-metals are not available to the living cell in the usual ecosystems. They may be present in the earth's crust only in very low amounts or the ion of the particular heavy-metal may not be soluble (Nies, 1999). As they are non-biodegradable, their threat is multiplied by their accumulation in the environment through the food chain.

It has been realized that sometimes the natural cycles can pose a hazard to human health because the level of heavy-metals exceed the body's ability to cope with them. Heavy metals are

toxic even at low concentrations. The situation becomes worst by the addition of heavy-metals to the environment as a result of both the rapidly expanding industrial and domestic activities. The metals are introduced into the environment during mining, refining of ores, combustion of fossil fuels, industrial processes and the disposal of industrial and domestic wastes (Xie et al., 1996). Human activities also create situations in which the heavy-metals are incorporated into new compounds and may be spread worldwide (Young, 2000).

The effects of metals in water and wastewater range from beneficial through troublesome to dangerously toxic. The presence of heavy metals in aquatic environments is known to cause severe damage to aquatic life, beside the fact that these metals kill microorganisms during biological treatment of wastewater with a consequent delay of the process of water purification. Some metals may be either beneficial or toxic, depending on concentration.

Heavy metals especially Lead (Pb) is a serious body cumulative poison. Natural waters seldom contain more than 5 μgL^{-1}. Lead is generated in the effluents from the production of television picture tube, storage batteries, pigments, petroleum fuel, photographic materials, mining, smelting, painting, etc. Tap waters that are soft, acidic, and not suitably treated may contain lead resulting from an attack on lead service pipes or solder pipe joints (Clesceri et al., 1990). Another important heavy metal that cause a lot of health problems is cadmium (Cd). Major industrial releases of cadmium are due to waste streams and leaching of landfills, and from a variety of operations that involve cadmium or zinc. In particular, cadmium can be released to water from the corrosion of some galvanized plumbing and water main pipe materials. It can have a major impact on human health. Short term exposure of cadmium can lead to nausea, vomiting, diarrhea, muscle cramps, salivation, sensory disturbances, liver injury, convulsions, shock and renal failure whereas long term exposure can cause kidney, liver, bone

and blood damage. One of the most commercially important metals, copper (Cu) is necessary for good health. However, very large single or long-term intakes of copper may harm human health. Because copper can find its way into virtually any product that we put inside our body, it's important to try to limit how much copper we are exposed to. Traces of copper in regular drinking water is generally low, but can be intensified in areas around industrial plants, mining sites, or areas where copper pipes are corroded. The corroding of copper pipes is the biggest cause of copper water and the most potentially harmful. Another important heavy metal Zinc (Zn) is present in drinking water in small amounts and is essential to health. Zinc concentration of more than 5 mgL^{-1} gives water a bitter astringent taste. Zinc contamination can result from corrosion of galvanized pipes by soft, acidic water. Zinc is used in fertilizers and may be found in landfill leachates or in industrial wastes.

The efficient removal of toxic metals from wastewater is an important matter and is a widely-studied area. A number of technologies have been developed over the years to remove toxic metals from wastewater. Physical treatment can also be used to remove small concentrations of hazardous substances dissolved in water that would never settle out. One of the most commonly used techniques involves the process of adsorption, which is the physical adhesion of chemicals onto the surface of a solid. The effectiveness of the adsorbent is directly related to the amount of surface area available to attract the molecules or particles of contaminant (Master, 1991). Most of the heavy metal salts are soluble in water and form aqueous solutions and consequently cannot be separated by ordinary physical means of separation. Physico-chemical methods, such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion exchange, and membrane technologies have been widely used to remove heavy metal ions from industrial wastewater.

These processes may be ineffective or expensive, especially when the heavy metal ions are in solutions containing in the order of 1-100 mg dissolved heavy metal ionsL^{-1} (Volesky, 1990a; Volesky, 1990b). The development and implementation of cost-effective process for removal/recovery of metals is essential in order to improve the competitiveness of industrial processing operations and to minimize the environmental hazard of toxic metal-containing effluents. In recent years, there has been a significant effort to search for new methods of heavy metal removal from contaminated sites.

Many biopolymers are known to bind metals strongly, and the use of biopolymers as adsorbents for the recovery of valuable metals or the removal of toxic metal contaminants has been studied in recent years (Chen et al., 1993; Deans and Dixon., 1992; Jang et al., 1990a,b, 1991; Seki et al. 1990 and Seki and Suzuki, 1995). Biopolymers are industrially attractive for a number of reasons. Biopolymers are capable of lowering heavy metal ion concentrations to part per billion concentrations (Deans and Dixon, 1992). Moreover, they are widely available and are ecologically acceptable. Such materials include cellulosics, alginates, carrageenans, lignins, proteins, and chitin derivatives. The application of alginic acid to the aqueous-phase separation of heavy metals, and the possibility of alginic acid for the adsorbent material has been suggested by several researchers (Chen et al. 1993; Deans and Dixon, 1992 and Jang et al., 1990a, b, 1991). Various polysaccharides and other biopolymers exhibit metal-binding properties (Kim et al. 1996; Loaec et al. 1997; Salehizadeh & Shojaosadati 2003). For electrostatic interactions, the binding of cations to bacterial biopolymers generally occurs through interaction with negatively charged functional groups. In addition, there may also be cation-binding by positively charged polymers or coordination with hydroxyl groups (Gutnick & Bach 2000). So, an increase on the availability of possible cation-binding groups would be expected as the biopolymer is dispersing

in the contaminated solution. Thus, biological methods that present many potential advantages and provide an attractive alternative to physico-chemical methods can be used to remove metals from water and wastewater (Kapoor and Viraraghavan, 1995).

2.13 Water contamination by pathogens

Pollutants have been transported by wind and rain to every place on earth. Today, in most industrialized countries, drinking water is ranked as food, and high standards are set for its quality and safety. The strict requirements for microbiological factors specify that bacterial content should be very low and that no pathogenic microorganisms should be detectable. These strict demands for the absence of pathogens, however, are meaningful only for the classical pathogens like *Vibrio cholerae* and *Salmonella typhi* (USEPA, 1991). In recent years, several so-called "new or emerging pathogens" have arisen as problems in drinking-water production and distribution. These include, on the one hand, newly recognized pathogens from faecal sources like *Campylobacter jejuni*, pathogenic *Escherichia coli, Yersinia enterocolitica*, new enteric viruses like rotavirus, calicivirus, small round-structured virus, astrovirus, and the parasites *Giardia lambia, Cryptosporidium parvum*, and microsporidia. On the other hand, some new pathogens comprise species of environmental bacteria that are able to grow in water distribution systems and only recently were recognized as relevant pathogens, such as *Legionella* spp., *Aeromonas* sp., *Mycobacterium* spp., and *Pseudomonas aeruginosa* (Brugha et al., 1999). The discovery of new pathogens and new insights into the microbiology of drinking water require a more detailed investigation toward the occurrence of potentially pathogenic bacteria, viruses, and parasites.

Faecal coliforms and enterococci have been widely used as indicators of faecal pollution (Sinton et al., 1998). Both microbial groups can be determined by their enumeration. Different

agents can determine the proportion of faecal coliforms/enterococci, or their inactivation. Both bacterial groups include several species. For example, the genus *Enterococcus* contains 19 recognized species (Manero and Blanch, 1999). Any determination of their diversity in the environment should consider this aspect. Urban or rural wastewaters normally contain many bacterial species, each with a large number of strains. Biological treatment processes at sewage treatment plants could produce selective elimination and/or changes of proportion, in the bacterial populations (Mezrioui and Baleux, 1994). Moreover, the sewage effluent, as well as urban or industrial waste, could modify some microbial populations in the reception waters, such as rivers, lakes, or lagoons (Sinton and Donnison, 1994). This effect could become more important where policies of water re-utilization are applied in regions with poor water resources. The determination of the origin of faecal pollution in waters is important for the management and quality control of water resources.

Enteric *Salmonella* infection is a global problem both in humans and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide (McCormick et al., 2003). The natural habitat of *Salmonella* is the gastrointestinal tract of animals, including birds and human beings (Spector et al., 1998). This organism finds its way into the river water, coastland estuarine sediments through fecal contamination. Aquatic environments are the major reservoirs of *Salmonella* and aid its transmission between the hosts (Spector et al., 1998). The survival rate of *Salmonella* in such aquatic environments is very high, outliving even *Vibrio cholerae* in highly eutrophic river water. Besides, *Salmonella* spp. are easily disseminated, causing moderate morbidity and low mortality, and require enhancements of diagnostic and surveillance capability. For these reasons, this pathogen has been categorized currently as category B biowarfare agent by CDC (Centers for Disease Control, Atlanta, USA).

Since bioterrorism is difficult to predict or prevent, reliable detection as well as highly specific platforms are especially important to minimize dissemination of biothreat agents and to protect the public health.

Waterborne cryptosporidiosis presents a serious threat to human health on account of the ubiquitous distribution of *Cryptosporidium* spp. in humans, animals, and water. Among the five *Cryptosporidium* spp. considered to be human pathogens, *Cryptosporidium parvum* have been linked to numerous waterborne outbreaks across the world (Craun et al., 1998; Smith and Rose, 1998; Barwick et al., 2000). Infected hosts discharge *Cryptosporidium parvum* oocysts (approximately 5 μm diameter), which are nonreproductive, into the environment through municipal wastewater treatment facilities, agricultural operations, and wildlife populations (Searcy et al. 2006). *Cryptosporidium parvum* oocysts are resistant to removal and inactivation by conventional water treatment including coagulation, sedimentation, filtration, and chlorine disinfection. Resistance of oocysts to typical environmental stressors has prompted research to optimize treatment processes and develop new technologies to reduce concentrations of viable/infectious oocysts to a level that prevents disease. To remove *Cryptosporidium* oocysts from potable water, the addition of flocculants, like aluminium sulfate, have been proposed. The low surface charge of oocysts promotes clumping with other negatively charged particles (American Water Works Association, 1995; Bustamante et al., 2001).

Chapter 3

Materials &Methods

Materials and Methods

3.1 Isolation and characterization of flocculant producing microorganism

3.1.1 Collection of sludge samples

For isolation of exopolymeric flocculant producing microorganisms activated sludge samples were collected from 19 industrial units. Sampling was carried out from 7-8 sites of each industrial unit (Table 4.1). The samples were collected in sterile screw capped containers and transported to the laboratory on ice. The samples were stored at $4^{o}C$ until their use.

3.1.2 Chemicals and Media

All chemicals and reagents used for microbiological and chemical determinations were of the highest analytical grade and purchased from Sigma unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (USA) and Hi-Media (Mumbai India). Enzymes, Restriction enzymes, and other chemicals of molecular biology grade were procured from New England Biolabs, UK. Media solutions were sterilized by autoclaving at 121°C and 15 psi for 20 mins. and were allowed to cool below 50°C before use. Luria-Bertani (LB) agar (10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl, 15 g agar per liter, pH 7.0 ± 0.2) were made by the addition of 17 gL^{-1} of agar prior to autoclave sterilization. The screening medium referred as FIB medium (flocculant isolation broth) was composed of peptone 5 gL^{-1}, ammonium sulphate 1 gL^{-1}, KH_2PO_4 1 gL^{-1}, $CaCl_2.2H_2O$ 0.7 gL^{-1}, NaCl 0.1 gL^{-1}, $MgSO_4.7H_2O$ 0.3 gL^{-1}, K_2HPO_4 1 gL^{-1}, glucose 1 gL^{-1} and agar 3 gL^{-1}, pH 7.0 ± 0.2. Unless otherwise noted, the carbon source for all experiments was 1 g of glucose in a liter of media.

3.1.3 Screening of bioflocculant producing bacteria

Bioflocculant producing bacteria were isolated from sludge samples collected from different sites of industrial units located across Punjab and Haryana region in India. Isolates were obtained by serial dilution plating on Luria-Bertani (LB) agar. The plates were incubated at 30°C for 24 to 48 hours. A total of about 957 colonies were isolated and the polymer producing bacteria were screened for their ability to produce biopolymer based on colony morphology (mucoid). The isolated strains were grown in 100 mL screening medium referred as FIB medium in 500-mL flasks on a rotary shaker at 37°C for 4 days, the resultant fully grown bacteria were examined for their flocculating activity. Stocks of the isolated colonies were made by growing the isolates in LB broth to a final volume of 5 ml and were grown to mid log phase. The cultures were centrifuged and the pellet were washed with 0.85% saline and resuspended in fresh LB and stored in cryovial containing 40% glycerol at –80°C. For the screening of bioflocculant producing bacteria 5 µl of glycerol stock was inoculated to 5 ml of LB broth and was grown to mid log phase. 2% (v/v) of the grown culture was then inoculated to autoclave sterile 50 ml FIB contained in erlenmeyer flasks with shaking was then incubated at 30°C. Among the screened 43 isolates, one strain that was isolated from activated sludge sample of a textile industrial unit named as Rainbow Denim Ltd. [located at village Chaundheri, district Mohali (Punjab), India] was selected by virtue of its biopolymer's highest removal efficiency of bacterial suspension and some heavy metals like (Cu, Cd, Pb and Zn). This strain was designated as RD4.

3.1.4 Bacteria and Culture conditions

The biochemical characterization of RD4 strain was carried out at microbial type culture collection (MTCC), Chandigarh, India. The strain was identified according to *Bergey's Manual of Systematic Bacteriology* (Holt, 1994) as *Klebsiella terrigena* (MTCC 7805). *Klebsiella*

terrigena RD4 strain was grown at 30°C and pH 7.0±0.2 unless otherwise noted, in either LB or FIB media.

3.1.5 DNA Isolation and PCR amplification

All molecular biology techniques were performed as outlined in Sambrook et al. unless otherwise noted (Sambrook, 1989). All enzymatic reactions and routine purification of nucleic acid involving kits were performed as indicated in the manufacturer instructions (Promega, Madison, WI, USA). All DNA modifying enzymes were obtained from Promega or Fisher Scientific (USA). All PCR reactions were conducted using universal primers P0 (5' GAGAGTTTGATCCTGGCTCAG-3') and P6 (5' CTACGGCTACCTTGTTACGA-3'). The PCR mixture contained 1 µl *Taq* (10 X) commercial buffer, 5 µl purified DNA (50-100 ng), 150 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase. Total reaction volume was 100 µl. The program for 16S rDNA was as follows: The reaction mixtures were first incubated for 5 min at 95°C, and then cycled for 36 cycles according to the following temperature profiles: 1 min at 94°C, 1 min at 55°C 2 min at 72°C, followed by a final extension for 10 min at 72°C, unless otherwise specified, on a Bio Rad thermal cycler (Bio Rad, MyCycler, USA).

3.1.6 DNA sequencing and analysis of sequence data

All DNA sequencing was performed by the Geneii Molecular Biology Resource Facility at Bangalore India, using an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with Big Dye Terminator Cycle Sequencing v 2.0 (PE Applied Biosystems). The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTALW (Thompson et al., 1997). The resulting multiple alignments were optimized visually and the evolutionary distance was calculated by Kimura 2 parameter (Table 4.1). Phylogenetic dendogram was constructed by neighbor-joining method using MEGA

4 software (Tamura et al., 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses.

3.1.7 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Because of the lack of readily distinguishable phenotypic characteristics of RD4 and the risk of cross contamination with other bacteria, ARDRA was used to verify the authenticity of the strain. When separated on an agarose gel, the characteristic pattern of the multiple bands produced in the PCR reaction could be used to differentiate between RD4 or contaminating bacteria. Following amplification as stated above, restriction digestion of the PCR products was performed. Restriction was performed separately with each enzyme by incubation with 5U endonuclease in a final volume of 25 µl. Digest reactions included: 16 µL PCR reaction product, 1.0 µL either *Alu*I or *Sau*3A and 2.0 µL Buffer C (Promega). All restriction enzyme concentrations were 5 U/µL, and reactions were allowed to incubate at 37°C for 1 hour. The restriction patterns were obtained by electrophoresis run on 1.4% (w/v) agarose gel at 2.5 V/cm for ~ 2h.

3.2 Growth kinetics and flocculating activity of *Klebsiella terrigena*, and purification of its bioflocculant

3.2.1 Growth kinetics of *Klebsiella terrigena* RD4 strain

The test organism was grown on FIB medium with glucose as carbon source. The cells were washed twice and resuspended in 0.85% saline. 1% inoculum was used and the culture was incubated at 30°C with shaking at 150 rpm. 1 ml of aliquots in triplicate samples were withdrawn aseptically at regular intervals of 1 hr, centrifuged at 10,000 rpm for 3 min and resuspended the cells in 1ml of 0.85% saline. Cell density was estimated by measuring the optical density of cell suspension at 600 nm in spectrophotometer (U-2800, Hitachi, Japan). Growth curves and

doubling times were determined based on the increase in optical density at 600 nm (OD 600) over time of liquid cultures grown in FIB media**.** Specific growth rate was calculated from the slope of the natural log of OD 600 measurements corresponding to the linear portion of the curve during exponential growth phase, typically between 12 and 18 hours. All experiments were conducted in triplicate. The average slope was used for the mathematical calculation of generation time and the standard deviation for the replicates is reported**.**

3.2.2 Determination of flocculating activity

The flocculating activity of the culture was determined by the method described by Kurane et al., (1986). Kaolin clay was used as suspension material for estimating the flocculating activity in all the experiments, unless otherwise noted. A volume of 0.05 mL culture supernatant (cell free extract) or purified lyophilized bioflocculant powder dissolved in water (concentration as specified in different experiments) and 0.95 mL of distilled water were added to 8 mL of kaolin suspension (5 gL^{-1}) with 1 mL $CaCl_2$ (10 mM) in a tube. The pH was adjusted to 7 with 1 N NaOH and 1 N HCl and solution mixed gently; 0.05 ml of distilled water was added in place of culture to the reference tube. The solution was thoroughly vortexed for 30 sec and allowed to stand at room temperature for 5 min. 2 ml of aliquots were withdrawn from the upper phase and O.D. was recorded at 550 nm with a spectrophotometer (U-2800, Hitachi, Japan). The Flocculant activity was calculated in percent as follows:

Flocculant activity (%) = (B-A) *100 / B

Where A – Optical density of sample at 550 nm

B – Optical density of reference at 550 nm

3.2.3 Extraction and partial purification of bioflocculant (KBF)

One litre cultures of *Klebsiella terrigena* RD4 strain in FIB media, pH 7 ± 0.2 was incubated in a rotary shaker (120 rpm/min) at 30°C (Labcon, 5081U, USA) for 48 hours and were centrifuged at 12,000 g for 30 min at 4°C (Sigma 2-16KC, Germany). The supernatant fraction was then separated from the cellular debris and then concentrated to one-tenth its original volume by freezing at -80°C and lyophilizing (Modulyod-230, Thermo, USA). The retentate, which contained the putative bioflocculant, was then diluted back to 1 L with ultrapure water (Millipore, Germany) and the process was repeated for a total of three times. The bioflocculant was further purified by the addition of two volumes of ethanol to 100 mL of concentrated supernatant and allowed to precipitate at 4°C for 24 hr. The precipitated polymer was collected by filtration (Whatman GF Filter) and dialyzed extensively, against deionized water. The bioflocculant was reprecipitated by addition of a 10% solution of cetylpyridinium chloride (Yim et al., 2007). The precipitated polymer complex was collected by centrifugation at 10,000 g for 20 min at 4°C and redissolved in 10% NaCl solution. Fractionation and purification were achieved using gel chromatography on a Sepharose 4B column followed by elution with a 0.4 M NaCl buffer. Three volumes of ethanol were added to recover the purified bioflocculant, which was further dialyzed and lyophilized to powder. Weight of the bioflocculant was measured using a weighing balance (Sartorius CPA 225D, Germany).

3.2.4 Homogeneity of bioflocculant

The homogeneity of purified bioflocculant (1mgmL^{-1}) was checked by paper electrophoresis. The sample was electrophoresed on cellulose acetate strip at a constant current (2.5 mA, 60 V) for 30 min using 1 M pyridine acetate as electrolyte. The strip was then stained

using 3% (v/v) acetic acid solution containing 0.5% toluidine blue in distilled water for 10 min and followed by destaining and clearing by washing with water for 10 min.

3.2.5 Stability and storage of bioflocculant

Purified bioflocculants were stored in bottles capped with glass stoppers at 25°C in the form of powder. The stablity of purified powder in solution (1 mgL^{-1}) was determined by measuring its flocculating activity each day for five days.

3.2.6 Determination of alcian blue binding activity

The alcian blue binding assay was performed with a minor modification by the method of Bar-Or and Shilo, (1988). Alcian blue (Sigma) was dissolved in 0.5 M acetic acid to give 1mgL^{-1}. 200 µl of bioflocculant sample was dissolved in 700 µl 0.5 M acetic acid. To this 100 µl alcian blue solution was added and left for 2 hour at room temperature. The mixture was centrifuged at 10,000 rpm for 5 min. The decrease in the absorbance compared to the control (without bioflocculant) was recorded at 610 nm.

3.3 Analysis of structure, composition and properties of bioflocculant

3.3.1 Enzymatic and EDTA treatment of Bioflocculant

To analyse the role of nucleic acids, proteins and divalent cations in the flocculation, the bioflocculant was treated separately with the protease (Proteinase K) and nucleases (DNase, RNase) and EDTA. The bioflocculant solution of 1 mgL^{-1} was treated with 1 µl of Proteinase K (10 U), DNase (1 U) and RNase A (1 U) and 10 µL 0.5 M EDTA vortexed briefly and incubated for 1 hour at 55°C 30 min at 37°C, 5 min at room temperature and 30 min at 37°C repectively. Subsequently the ability of the treated samples to flocculate kaolin was evaluated.

3.3.2 Molecular mass determination of bioflocculant

The chromatography experiments were conducted on a Sephadex G-150 column (2.5 cm × 40 cm) with 0.05 mol L^{-1} NaCl as the eluent, and 3 mL eluent fractions were collected and analysed by the phenol–sulphuric acid method. Approximate molecular weight values were estimated by calibrating the same column using aqueous solutions of monodisperse standards. Standard Dextran T-70 and Dextran T-40 were analysed on the above sephadex column. The EPS samples were monitored using a Varian Pro Star 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:

$$\text{Log (MW)} = \text{K1}\,Ve + \text{K2}$$

where MW and *V*e (ml) are the molecular weight and elution volume, respectively, K1 and K2 are constants.

3.3.3 Scanning electron microscopy

Dried purified polymer samples were fixed on double sided tape attached to SEM stubs and were gold-coated (∼10 nm). Their morphology was examined under a scanning electron microscope (JSM 840A, JEOL, Tokyo, Japan) at an accelerating voltage of 20.0 kV.

3.3.4 Compositional Analysis

3.3.4.1 Determination of total Sugar

The total carbohydrate content of the flocculant was determined by phenol sulphuric acid method as described by Dubois et al. (1986). The reagent was prepared by dissolving phenol in water (5% w/v). 200 µL of samples, standards and controls (containing up to 100 µg carbohydrates) were mixed with 200 µL of phenol reagent (Appendix 1). 1.0 mL of concentrated

sulphuric acid was rapidly and directly added to the solution surface without allowing it to touch the sides of the tube. The solutions were left undisturbed for 10 min before shaking vigorously. The absorbance was determined at 490 nm after a further 30 min. All experiments were conducted in triplicate. The amount of total carbohydrates present in the sample solution was calculated from standard curve (prepared using glucose, 0-1 $mgmL^{-1}$), fig. 3.1(AppendixII).

3.3.4.2 Determination of protein

The protein contents were estimated by method described by Lowry et al., (1951). Bovine Serum Albumin (BSA) was used as standard in different concentrations (0.2, 0.4, 0.6, 0.8 and 1 $mgmL^{-1}$) and bioflocculant was taken in the range of 1 $mgmL^{-1}$. 50 ml solution of (2 g of Na_2CO_3 + 0.1M NaOH) and 50 ml solution of (0.5 g per 100 mL of $CuSO_4$ + 1 g per 100 mL of Na-K-tartarate) were mixed to make Reagent. To 0.2 mL of sample or standard 1 mL of freshly mixed complex-forming reagent A was added. The solution was left standing at room temperature for 10 min. Then 0.1 mL of diluted Folin reagent was added using a vortex mixer, and the mixture was left standing at room temperature for 30 min. The absorbance was read at 750 nm. All experiments were conducted in triplicate. The amount of total protein present in sample was calculated from standard curve prepared using pure BSA as standard (Fig. 3.2) (AppendixII).

3.3.4.3 Determination of hexosamine

Hexosamine content of the bioflocculant was determined by Elson-Morgan method (1934). 6.1 gm of dipotassium tetraborate tetrahydrate in 80 mL of water was taken and the total volume was made to 100 mL with water (Reagent A). In a separate flask 1.5 mL of water was added to 11 mL of concentrated HCl. A further 87.5 mL of glacial acetic acid was added to it and 10 gm of 4-(N, N-dimethylamino) benzaldehyde was further added. 10 mL of this mixture was

diluted to 100 mL with glacial acetic acid prior to use (Reagent B). 250 µL of standards (0-1 $mgmL^{-1}$, glucosamine), controls and samples were added to 50 µL of reagent A. Then each mixture was heated at 100°C for 3 min. After cooling rapidly to room temperature 1.5 mL of reagent B was added, washing down any condensate formed. The mixtures were incubated at 37°C for 20 min. After cooling to room temperature, the absorbance was determined at 585 nm. All experiments were conducted in triplicate. A standard curve for hexosamine is shown in fig. 3.3 (Appendix II).

3.3.4.4 Determination of uronic acid

The uronic acid content of the bioflocculant was determined by the method described by Haug and Larsen, (1962). 0.9 gm sodium tetraborate decahydrate was dissolved in 10 mL of water and 90 ml of ice-cold concentrated sulfuric acid (98%) was carefully added to form a layer. The mixture was left undisturbed overnight without excessive heat production (Reagent A). In a separate flask 100 mg of carbazole (recrystallized from ethanol) was mixed in 100 mL of absolute ethanol (Reagent B); 250 µL of samples, standards (0-1 $mgmL^{-1}$, D glucuronic acid) and controls were cooled in an ice-bath; 1.5 mL of ice-cold reagent A was carefully added with mixing and cooling in the ice-bath. The mixture was heated at 100°C for 10 min. The mixture was rapidly cooled in the ice-bath. 50 µL of reagent B was added and mixed well. Reheating was done at 100°C for 15 min. The mixture was cooled rapidly to room temperature and the absorbance was determined at 525 nm. All experiments were conducted in triplicate. A standard curve for uronic acid is shown in fig. 3.4 (AppendixII).

3.3.4.5 Determination of pyruvic acid

Pyruvic acid content of the bioflocculant was determined by the method described by ***Friedman*** and Haugen, (1943). For the determination of the pyruvic acid content of the

bioflocculant, the sample (5 mg dissolved in 5 mL dH_2O) was deproteinized with dilute perchloric acid (50%) and was incubated at 30°C for 30 min. Then 1 mL of DNP reagent (500 µmoles of DNP was dissolved in 100 mL 2.0 N hydrochloric acid at 40°C) was added to the extract. Further, 4 mL of water and 10 mL 2.2 N NaOH were added. The tubes were shaken and optical density was taken at 416 nm. A standard curve was made by using pyruvic acid (0-3 μgmL^{-1}). All experiments were conducted in triplicate. A standard curve of pyruvic acid is shown in fig. 3.5 (appendix II).

3.3.4.6 Elemental analysis

The powdered bioflocculant was analysed for C, H, N on a 2400 II elemental analyser (Perkin Elmer Company, Bedford, Massachusetts, USA).

3.3.5 Thin Layer Chromatography

Hydrolysis of the bioflocculant (1 $mgmL^{-1}$) was done by treating KBF (1 $mgmL^{-1}$) with 2 M TFA (Trifluoroacetic acid) at 120°C for 2 hours. Then, TFA was removed at 40°C using a rotary evaporator apparatus. After hydrolysis the solution was neutralized with a 1.0 N NaOH solution and lyophilized.

Silica gel G plates were prepared in 0.02 M Sodium acetate. 10 µL hydrolysate 1% (w/v) was applied on the silica gel G TLC plates. Carbohydrate standard solution 1% (w/v) was prepared in water and 10 µL each were spotted. The chromatogram was developed in a solvent system of water, ethyl acetate and butanol in a ratio of 3:2:10. The chromatogram was visualised using a spray reagent consisting of diphenylamine, aniline and phosphoric acid. Spray reagent was prepared by dissolving 4 g of diphenylamine in 80 ml of acetone and the total volume was made upto 100 mL with more acetone (A). 4 mL of aniline was added in 96 mL of acetone and mixed well (B). Solution A and B were mixed with 20 mL of 85% orthophosphoric acid just

prior to use. The plates was sprayed with spray reagent, air dried and then heated at 100°C for a few min. The chromatographic spots appeared within 2-4 min.

3.3.6 High Performance Liquid Chromatography

Monosaccharide composition of KBF was analysed by HPLC analysis of the hydrolyzed sample with a HPLC system (Waters 600 controller), RI detector (Waters 410), Reodyne injector at room temperature. The mobile phase cosisted of acetonitrile and water in a ratio of 70:30. The flow rate was set at 0.8 mL/min. The column used was NH_2 column (Emerck, 4.6x250 mm). The chromatogram was recorded by using millennium 32 software.

3.3.7 Fourier Transform Infrared Spectroscopy

Infrared spectrum of the bioflocculant was measured using a FTIR spectrophotometer (Perkin Elmer Spectrum RX1 FTIR system). The dried bioflocculant (~5 mg) was blended with KBr and pressed into a disc for FTIR analysis. The spectrum of the sample was recorded on the spectrophotometer over a wave number range of 4000-400 cm^{-1} under ambient conditions.

3.3.8 Thermal stablity

The bioflocculant solution was mantained at temperature ranging from 30°C to 100°C for different length of time (5 min to 30 min). The stablity of the bioflocculant was assessed by loss in the bioflocculant activity. Degradation of the bioflocculant was carried out by thermogravimetric analysis (TGA) using a TGA apparatus (Mettler Toledo). The accurately weighed sample of the bioflocculant (6.11 mg) was heated from 25 to 900°C under a constant flow of nitrogen gas with an increase of 15°C per sec. The degradation temperature was determined from the TGA curve for the bioflocculant**.**

3.3.9 Rheological measurements of the bioflocculant

Thermorheological measurements were performed with a Physica MCR 100 Rheometer (Anton Paar, Ostfildern, Germany). 2 mL of bioflocculant sample (1 mg/mL) at neutral pH was put on the ram of the rheometer and the shear rate and shear stress was set at a frequency of 1 Hz with a gap of 0.2 mm between the sensor and ram. The sample was loaded between parallel plates, the excess was wiped off with a spatula and a thin layer of glycerol was applied to the edge of the exposed sample to prevent moisture loss during measurements. The effects of temperature and heat treatment were investigated over ranges of 20–85°C and 85–25°C. Rheological parameters (storage modulus, G'; loss modulus, G''; tan δ, as the ratio between G'' and G') were obtained from the Physica Universal Software US200. All the rheological measurements were performed at least in duplicate.

3.4 Optimization of reaction system conditions

3.4.1 Bioflocculant Dosage

To determine the critical dose, 200 mL of kaolin solution (5 gL^{-1}) in a beaker was kept on a magnetic stirrer. Bioflocculant solution at a concentration of 1 mgL^{-1} was prepared and were added in small increments at neutral pH and room temperature. After each addition, one minute rapid mixing at 100 rpm followed by a 3-minute slow mixing at 30 rpm was done. This addition was continued till a visible floc was formed. At this time further addition of flocculant was stopped and the doses were noted and the determined critical doses were used for further study.

3.4.2 Medium pH

The optimum pH of the reaction was determined by adjusting the pH of the bioflocculant solution to a range from 4 to 9 by 1 N NaOH and 1 N HCl solutions. The optimised value of the pH was used throughout the study unless otherwise stated.

3.4.3 Cations

Sodium chloride (Na^{+}), Potassium chloride (K^{+}), Magnesium sulphate (Mg^{2+}), Calcium chloride (Ca^{2+}), Ferrous sulphate (Fe^{2+}), Cobaltous chloride Co^{3+} and Aluminium chloride (Al^{3+}) were used in the reaction mixture at a concentration range of 0-10 mM to analyze their effect on the flocculant activity. The cation showing highest flocculant activity was further used in the present study.

3.5 Evaluation of bioflocculant efficiency

3.5.1 Comparision of efficiency of bioflocculant (KBF) with other synthetic and natural flocculants

The comparative study of the bioflocculant KBF was done with chemical flocculants like polyacrylamide (6.00×10^{7} Da) and CHEMFLOC 985W (2.5×10^{6}) (Chemical Systems, New Delhi, India) and the natural flocculants xanthan (2.5×10^{5}), chitosan (2×10^{4}) and polyglutamic acid (5×10^{5}). The flocculant activity of the various flocculants was checked by flocculation of kaolin suspension and their flocculating activity was calculated as described earlier. A stock solution of these flocculants was prepared ($10 mgL^{-1}$). The dose optimization for these flocculants was done as described earlier for KBF. Flocculation of kaolin clay was carried at a pH range of 4-9 to determine their optimum pH. The effect of various cations was studied by replacing Ca^{2+} with other cations (Cu^{2+}, Fe^{2+}, Al^{3+}, Mg^{2+} and Co^{2+}, Fe^{3+}) in the kaolin suspension.

3.5.2 Flocculation of various suspended solids in aqueous solution

The bioflocculant was evaluated for its ability to flocculate suspensed solids of varied size. A standard solution of suspended solids of 2000 NTU was made by suspending active carbon, silica, magnesium hydroxide, cellulose and yeast in 100 mL water. A bacterial suspension of 2000 NTU was also used for this study. A suspension of desired turbidity was prepared by diluting the suspension form stock to desired turbidity with a turbiditimeter (CyberScan TBDIR1000 Meter, Eutech, Netherlands). An assay was performed by adding 10 mL $CaCl_2$ (5mM), 0.5 mL bioflocculant (2 mgL^{-1}) and 9.5 mL of distilled water to 80 mL of these solutions. The pH was adjusted to 7±0.2. The solutions were allowed to stand at room temperature for 5 min. 20 mL of aliquots were withdrawn from the upper phase and its turbidity was measured. Its flocculating activity was calculated by recording its optical density with a spectrophotometer at 550 nm. The Flocculant activity was calculated as explained earlier.

3.6 Effect of nutritional factors and culture conditions of *Klebsiella terrigena* RD4 on bioflocculant production growth and bioflocculant activity

3.6.1 Carbon-sources

Six carbon sources, glucose, galactose, rhamnose, fructose, sucrose and maltose were examined for their suitability for maximal activity or biopolymeric flocculant production maximally by *Klebsiella terrigena* RD4 strain when individually used as sole carbon source. Prior to batch experiments, a single well isolated colony of *K.terrigena* RD4 was transferred to 5 mL of FIB medium and incubated with shaking at 37°C overnight. Batch experiments were conducted by inoculating 1 mL of the freshly grown culture to 100 mL FIB medium containing various carbon sources at concentrations of 0.5 to 2.5 gL^{-1} in 250 mL Erlenmeyer flasks. The flasks were incubated at 30°C with shaking. Thereafter, overnight cultures grown in FIB medium were pelleted by centrifugation, washed two times, and resuspended in 0.85% Saline. Growth of

the culture was determined by recording absorbance (optical density) at 600 nm using a spectrophotometer. To determine the flocculating activity, assay of kaolin clay was carried out as described above. Flocculating activity and yield of the purified bioflocculant was determined as described above.

3.6.2 Nitrogen-sources

Peptone, yeast extract, beef extract and tryptone were chosen as representative organic nitrogen sources whereas ammonium sulphate, ammonium chloride and ammonium phosphate were selected as inorganic nitrogen source were tested individually to determine either maximal activity or maximal biopolymeric flocculant production by *Klebsiella terrigena* RD4 strain. Batch experiments were conducted as described above with 100 mL cultures in 250 mL Erlenmeyer flasks in which various organic nitrogen compounds (2.5-10 gL^{-1}) and inorganic nitrogen compounds (0.5-2 gL^{-1}) were added to the FIB medium. All experiments were conducted under aerobic conditions. Overnight cultures grown in FIB medium were pelleted by centrifugation, washed twice, and resuspended in 0.85% Saline. Growth of the culture was determined by recording absorbance (optical density) at 600 nm using a spectrophotometer. Flocculating activity and yield of the purified bioflocculant was determined as described above.

3.6.3 Metal ions

Copper sulphate, ferrous sulphate, aluminium chloride, magnesium sulphate and cobaltous sulphate as metal ion source were examined for their ability to either produce maximal activity or biopolymeric flocculant maximally by *Klebsiella terrigena* RD4 when individually used as metal ion source respectively. For batch experiments overnight grown cultures as described before, were used to inoculate 100 mL FIB medium contained in 250 mL Erlenmeyer flasks in which various metal ions (0.1-0.5 gL^{-1}) were added. All combinations were incubated

with shaking at 37°C. Overnight cultures grown in FIB medium were pelleted by centrifugation, washed two times, and resuspended in 0.85% Saline. Growth of the culture was determined by recording absorbance (optical density) spectrophotometrically at 600 nm. Flocculating activity and yield of the purified bioflocculant was determined as described above.

3.6.4 Culture pH

To determine the optimum pH responsible for either maximal activity or biopolymeric flocculant production by *Klebsiella terrigena* RD4, an overnight grown culture was inoculated in 100 mL FIB media contained in 250 mL Erlenmeyer flasks. The pH of the media was adjusted prior to inoculation, to 4-9 by using 1 N NaOH or 1 N HCl solutions respectively. All combinations were incubated with shaking at 37°C. Flocculating activity and yield of the purified bioflocculant was determined as described above.

3.6.5 Incubation temperature

For determining the optimum temperature necessary for either maximal activity or biopolymeric flocculant production by *Klebsiella terrigena* RD4, the strain was pregrown overnight as described before in FIB media. Batch experiments were conducted using overnight grown cultures which were inoculated in 100 mL FIB media in 250 mL Erlenmeyer flasks; all flasks were incubated in rotary shaker at a temperature range of 28-42°C. Flocculating activity and yield of the purified bioflocculant were determined as described above.

3.6.6 Inoculum size

The optimum inoculum size for maximal activity or biopolymeric flocculant production by *Klebsiella terrigena* RD4 strain was determined by using overnight grown culture in FIB media as described before. Batch experiments were carried out by inoculating 100 mL FIB in 250 mL Erlenmeyer flasks with inoculum sizes ranging from 3 log – 10 log CFU/mL. All

combinations were incubated with shaking at 37°C. Flocculating activity and yield of the purified bioflocculant was determined as described above.

3.6.7 Agitation

The effect of agitation on either maximal activity or biopolymeric flocculant production by *Klebsiella terrigena* RD4 strain was determined using overnight grown culture in FIB medium as described above. For batch experiments, overnight grown cultures were inoculated in 100 mL FIB medium contained in 250 mL Erlenmeyer flasks. The flasks were incubated at 37°C at following shaking speeds: 0, 50, 100, 150 and 200 rpm/min. Flocculating activity and yield of the purified bioflocculant were determined thereafter, as described above.

3.7 Cellular response of *K. terrigena* RD4 to environmental modulators and its impact on flocculant exopolysaccharides

3.7.1 Mutagenesis

Chemical mutagenesis on ampicillin resistant *Klebsiella Terrigena* RD4 strain was carried out using *N*methyl- *N'*-nitro-*N*-nitrosoguanidine (NTG) (Sigma Mo, USA) in a manner similar to that outlined by Coschigano and Young (1997). *Klebsiella terrigena* RD4 strain was grown in LB at 30°C at 150 rpm to log phase. 6 mL of the culture was harvested by centrifugation (1200 rpm, 4°C, 4 min). Pellet was washed twice by centrifugation with 0.1 M citrate buffer (1 ml), pH 5.5. The pellet was resuspended in 5 mL of citrate buffer. NTG was added to achieve a final concentration of 50 μgmL^{-1}. The NTG-treated *Klebsiella terrigena* RD4 kill curve is given in fig. 3.1. The culture was then incubated for 30 min in dark with shaking. The cells were harvested by centrifugation. The pellet was then washed with 0.1 M Phosphate buffer. The pellet was resuspended in 5 mL of FIB minimal media containing glucose as sole carbon source. The cells were allowed to grow for 45 min. Ampicillin was then added at a final concentration of 200μgmL^{-1} from a sterile stock solution of 100 $mgmL^{-1}$. The culture was then

incubated for 30 min. The culture was again harvested by centrifugation and the pellet was washed with 0.1 M Phosphate buffer. The pellet was resuspended in FIB minimal media containing malate as a sole carbon source. The culture was then incubated for 1 hr. The process was repeated for 4-5 times to obtain a mutant which was unable to metabolize glucose. The mutant so obtained was maintained in FIB minimal media plates containing malate as carbon source. It was intermittently checked for its frequency of reversion by growing it in glucose.

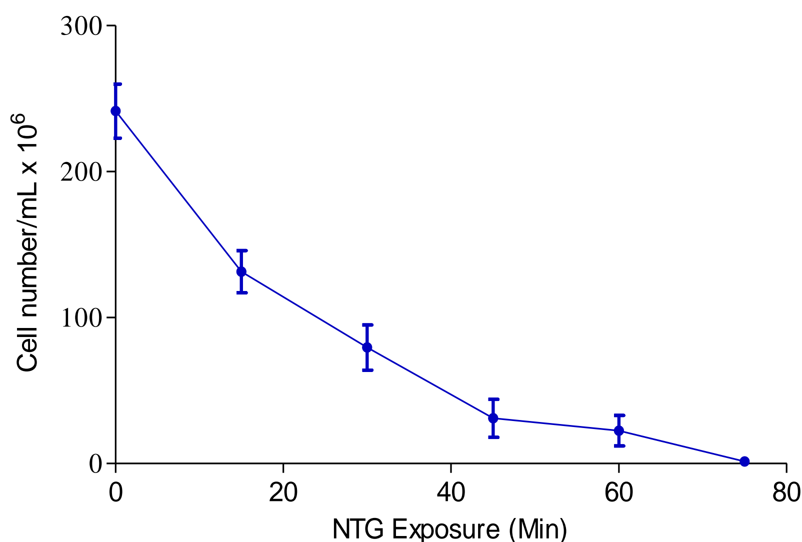


Fig. 3.1 NTG-treated *Klebsiella terrigena* RD4 kill curve. Semi-log plot of cell number when exposed to 50 µgmL^{-1} NTG. Samples were taken at 15 min intervals, washed two times with 0.1 M potassium phosphate (pH 7.0), serially diluted in PBS, and plated on luria agar plates. Cell number values after 75 min were too few to count (<20 colonies/plate without dilution)

3.7.2 Screening and selection of mutants

The selection mutants were based on the use of the lipophillic dye Sudan Black B. A 0.02% solution of Sudan Black B (Sigma, St. Louis, Mo. USA) dissolved in 96% ethanol was used for the staining and screen for mutants defective in glucose utilization. Mutants were grown as single colonies on plates containing Luria-Bertani (LB) agar. They were then replica plated onto FIB medium with either malate or glucose as carbon source. Approximately 8 mL of the

0.02% Sudan Black B solution was applied to each replica plate and allowed to remain undisturbed for about 10 min. The dye was then decanted, and the plates were gently rinsed by adding 10 mL of 100% ethanol and swirling for a few min. Colonies unable to incorporate the Sudan Black B stain appeared white, while colonies able to incorporate the dye appeared bluish black was selected for further study.

3.7.3 Oxidative stress

In order to determine whether oxidative stress was important for maximal activity or biopolymeric flocculant production by *Klebsiella terrigena* RD4 strain, the cells were grown overnight in FIB. 100 mL of FIB medium contained in screw capped Erlenmeyer flasks were inoculated with the fresh overnight culture. Prior to inoculation H_2O_2 solution was added to the FIB medium at a final concentration ranging from 10-40 mM. Following incubation, aliquots of cultures was withdrawn, centrifuged and the supernatant examined for flocculating activity.

3.7.4 Water activity

For determining the effect of water activity on either maximum flocculating activity or bioflocculant production by *K.terrigena* RD4, the culture was grown overnight in FIB. 100 mL of FIB medium (contained in 250 mL Erlenmeyer flasks) was adjusted to water activity values ranging from 0.98 to 0.60 using various concentrations of NaCl and glycerol respectively. All flasks were inoculated with the overnight grown culture and incubated with shaking. Prior to incubation aliquots of FIB medium was used to determine the water activity at 25°C by a water activity meter (Decagon, USA). Flocculating activity and yield of the purified bioflocculant was determined thereafter as described above.

3.8 Simulated and real time evaluation of pathogen removal efficacy by bioflocculant

3.8.1 *Salmonella* removal assays

Cultures of *Salmonella typhimurium* ATCC 23564, *Staphylococcus aureus* ATCC 9144, *Escherichia coli,* DH5α and *Enterococcus faecalis* ATCC 35550 were grown overnight in BHI broth (HiMedia, Mumbai), harvested by centrifugation, cell pellets of all the cultures were washed thrice with sterile tap water, mixed, resuspended in sterile tap water, mixed in equal proportions and spiked in 1 L of sterile tap water held in Erlenmeyer flasks (3 replicates). Purified biopolymeric material in different doses was then added to each replicate, mixed thoroughly by hand rotation and allowed to stand at ambient temperature for 1 hr. Aliquots of each sample were carefully removed by a vacuum pump, diluted in maximum recovery diluent (MRD), aliquots from each combination were plated onto the following respective selective media, viz XLD (Xylose Lysine Desoxycholate Agar), BPA (Baird Parker Agar), VRBA (Violet Red Bile Agar) and BEA (Bile Esculin Azide Agar). All the plates were incubated at 37°C for 18-24 h; counts were expressed as log cfu/mL. Control experiments comprised of similar combinations, but lacked biopolymers**.** The agglomerated biopolymer in sediment was used for Fluorescent *in situ* hybridization (FISH).

3.8.2 Fluorescent *in situ* hybridization (FISH) experiments with pathogen and Salmonella removal

The first step of the FISH technique is the fixation of the biopolymer flocculated sample. Two equal proportions (from 10 mL), 5 mL each were disposed into 50 mL sterile Falcon tubes. The first aliquot was fixed by mixing with 300 µL of 4% paraformaldehyde in PBS, and incubated at 4°C for 16 h. Fixed samples were washed twice with PBS, resuspended in 50% ethanol in PBS, and stored at −20°C. The remaining 5 mL of the samples was centrifuged (8000 rpm, 20 min) and pellets were resuspended in 5 mL of a 96% ethanol/PBS solution (1:1, v/v) and

stored at −20°C until used for FISH experiments; 1 μL of a solution of DNA interacting dye (DAPI) (200 ngμL^{-1}) at 42°C for 2 hr; 2 μL of the fixed cell solution was added to a teflon poly-lysin coated slide (Merck, Darmstadt, Germany). The sample was allowed to dry on the slide at 42°C for 20 min and subsequently dehydrated by passing through successive washes of 50, 80, and 96% ethanol for 3 min each. Hybridizations were performed in 9 μL of hybridization buffer (0.9 M NaCl, 20 m*M* Tris/HCl, 5 mM EDTA, 0.01% SDS; *p*H 7.2) in the presence of 10% *Sal* 3, 25% ENF191, 30% Eub338 formamide, 1 μL of *Sal*3-Cy3 (indocarbocyanine) labeled oligonucleotide probe or EUB338 labeled with indodicarbocyanine(Cy5). ENF191 labeled with FLUOS [5(6) carboxyfluorescein-N-hydroxysuccinimide ester].

These oligonucleotide probes were 5'-labeled and unlabeled competitor oligonucleotides were obtained from MWG (Ebersberg, Germany). The sequences of oligonucleotide probes used in this study bound to nearly all members of the domain bacteria, and were used to verify that all the cells present were permeable for probes and thus accessible for *in situ* hybridization with more specific probes. After hybridization, the slides were gently washed with distilled water and immersed in buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM EDTA, 0.01% SDS and 440 mM NaCl for 15 min at 43°C. Slides were removed from buffer, rinsed with distilled water and air-dried. Fixed cells of *Salmonella typhimurium* ATCC 23564 and *Escherichia coli* DH5α were always used as positive and negative controls, respectively.

3.8.3 Microscopy and digital image analysis

The slides were mounted with Citifluor AF1 immersion oil solution (Citifluor Ltd., London, UK) and examined with a Nikon microscope (Nikon, Lewisville, TX) fitted for epifluorescence microscopy with a high-pressure metal halide lamp and filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, D460/50, for DAPI detection). The

probe positive signals were visualized and pictures of fluorescent cells were recorded using a confocal laser scanning microscope (CLSM 510, Zeiss, Oberkochen, Germany). For the detection of Cy5 (Helium –Neon 633 nm) Fluos-labeled cells, an argon laser (450 to 514 nm) was used and for Cy3, helium-neon laser (543 nm) was used to visualize probe positive signals. For each microscope field, fluorescence conferred by the different probes was recorded in separate images. For each hybridization experiment, 30 microscope fields at random positions and in random focal planes were recorded using a Zeiss Plan-Neofluar 40×/1.3 oil objective. This procedure (30 images at low magnification) allowed us to record a high number of probe-target cells and thus to accurately determine the relative abundance of heterogeneously distributed probe-target cells. All the acquired pictures corresponded to optical sections obtained by adjusting the pinhole diameter of the CLSM were accordingly recorded as 8-bit images of 512/512 pixels with a resolution of 1.6/1.6 pixels per µm.

All probes consist of single-stranded DNA covalently linked to fluorescein at 5’ end:

Probe	Sequence	Organism	Location	Reference
EUB 16S rRNA	GCTGCCTCCC GTAGGAGT	*E. coli*	338-355 Bacterial kingdom	Amann et al., 1990(a)
Non-EUB	ACTCCTACGG GAGGCAGC	–	Negative control	Amann et al., 1990(b)
GAM42a 23S rRNA	GCCTTCCCAC ATCGTTT	*E. coli*	1027-1043 g-proteobacteria	Manz et al., 1992
Sal3 23S rRNA	AATCACTTCA CCTACGTG	*S. enterica*	1713-1730 *Salmonella enteric*	Nordentoft et al., 1997
ENF 191 16S rRNA	GAAAGCGCCT TTCACTCTTAT GC	*E. faecalis*	191-233 *Enterococcus faecalis*	Wellinghaus en et al., 2007

3.8.4 *Cryptosporidium parvum* removal assays

Replicates of 1 L, 1 X 10^6 oocysts were added to tap water (pH7.1; hardness: 171 mgL^{-1}; total organic carbon: 1510±21; dissolved oxygen: 6.9 ppm; alkalinity: 125 ppm as $CaCO_3$) by seeding a certain volume from the stock solution (approximately 100 µL) with gentle mixing using a micropipette. The stock solution was counted using the NCC, and oocyst concentration was determined by averaging five independent counts just before the seeding procedure. Contaminated tap-water samples were then subjected to the flocculation procedure as described by Kimura et al. (2002) as follows. Approximately 1 X 10^6 oocysts were spiked into 1 L tap water replicates. *K. terrigena* bioflocculant (2mgL^{-1}) was added to a 1-L glass flask and the pH was adjusted to 5.4 to 5.8. Samples were left overnight at room temperature to complete floc precipitation. The supernatant fluids were carefully removed the next day by vacuum pump without disturbing the sediment; resultant pellets were transferred to 50 mL sterile polypropylene tubes and centrifuged at 2000 g for 10 min at 48°C.

After discarding the supernatant fluids, the 1 mL pellets from the same trial were combined and recentrifuged. The supernatant fluids were discarded and the final pellets were resuspended in 1 mL of citric acid buffer (8.4 g citric acid monohydrate, 17.64 g trisodium-citrate-dihydrate, distilled H_2O up to 100 mL; pH 4.7). The pellets were left to settle with the buffer for 1 hr with intermittent vortexing. Following settlement, the samples were washed twice by centrifugation (2000 g for 10 min) with distilled water. Oocysts in the final pellets were then counted by the Nebauer Cell Counter. In a similar fashion, aluminum sulphate (10mgL^{-1}) flocculation was carried out along with each removal assay with *K. terrigena* flocculant.

3.9 Optimization studies on heavy metals removal

The metal-binding capacity of KBF was determined. Initially, stock solutions (1000 mg/L) of lead, cadmium, Zinc and Copper were prepared from their respective metal salts lead (II) nitrate ($Pb(NO_3)_2$) and cadmium(II) sulfate ($3CdSO_4.8H_2O$), Cuprous chloride($CuCl_2 2H_2O$)and Zinc nitrate $Zn(NO_3)_{2.}6H_2O$. Suitable dilutions were made with deionized water to obtain working solutions of desired concentration. 10 ml of polymer solutions (0–2.5%) (w/v) containing different concentration of heavy metals, Cu (1.5-3.5 ppm), Cd (0.01-.2 ppm), Pb (0.01-.05 ppm) and Zn (0.5-2.5 ppm) The binding experiments were carried out by taking 50 mL of metal solutions in 125 mL volumetric flasks and adjusting to the desired pH using a pH meter. A weighed amount of purified bioflocculant was then added and the mixture was shaken at 120 rpm in a water bath shaker at 37 °C until equilibrium or defined time intervals. The solutions were then filtered through a 0.2µm membrane filter and the filtrates were analyzed for metal concentration. Control experiments were also performed to monitor any metal adsorption on volumetric flask and/or membrane filter. Initial and final metal concentrations were determined using Atomic Absorption Spectrometer (Perkin Elmer Atomic Absorption Spectrophotometer Model 4100, USA) with the analytical conditions as given in Table 3.1.

Table 3.1 Analytical conditions for determination of metals in solution by AAS

Metals	Analytical conditions
Copper (Cu)	Air flow 12 lpm, Acetylene 1.8 lpm, Wavelength 324.8 nm, Slit 0.5 nm, Relative sensitivity 1
Zinc (Zn)	Air flow 12 lpm, Acetylene 1.8 lpm, Wavelength 213.9 nm, Slit 0.5 nm, Relative sensitivity 1
Cadmium (Cd)	Air flow 12 lpm, Acetylene 1.8 lpm, Wavelength 228.8 nm, Slit 0.5 nm, Relative sensitivity 1

Lead (Pb)	Air flow 12 lpm, Acetylene 1.8 lpm, Wavelength 217.0 nm, Slit 0.5 nm, Relative sensitivity 1

3.10 Statistical Evalulation

The statistics software package SPSS (version 11.5) was used to carry out the statistical tests (Kinnear, 1999). The aim of the statistical treatment was to establish that a result is robust to repetition (or replication) of the study. For every experiment, the average and standard deviation (SD) were calculated as measures of central tendency and dispersion of the values acquired with the experiment. However, the screening and rejection of atypical values (outliers) was always done. For confirmatory statistical analysis was used the paired samples Student *t*-test, in order to ascertain the significance of a difference between two means. The model underlying a *t*-test assumes that the data have been derived from normal distributions with equal variance, being considered a parametric test. The homogeneity of variance was assessed by the Levene´s test for equality of variance. The output of the statistical test is the *P* - value. When the *P* - value of a statistic was less than the significance level, the assumption of the statistic was said to be significant. If the *P*- value was larger than the significance level, the assumption was accepted. The statistical calculations were based on a significance level equal or higher than 95 %. The quantitative characters were statistically analyzed. The mean, standard deviation (SD) and standard error (SE) were calculated for each of the characters using the following formula.

Mean, $\bar{x} = \Sigma x/N$

Where x= measured characters

N= number of observations

Standard error (SE)= $SD/\sqrt{N}$

Where N= number of observations and SD = Standard deviation

Each bar in the graph represents mean ± SD of triplicate data

Chapter 4

Results

Results

4.1 Isolation and screening of exopolymeric flocculant producing potential strain

The bioprospecting of flocculant producing bacteria was carried out from activated sludge samples collected from 19 industrial units. All 632 bacterial isolates were selected on the basis of the mucoidy appearance of the colonies for flocculant producing ability out of a total of 957 isolates. An initial screening procedure, based on the ability of the culture to sediment kaolin suspension was applied to confirm flocculant production (Kurane et al., 1986; Deng et al., 2003), resulted in 42 positive bacterial strains with varying degree of flocculant activity as shown in (Table 4.1). It was also observed that the flocculating rate of the culture of each bioflocculant producing microorganism was not consistent with the degree of mucosity. We found that the flocculating activity that was observed in all of the 42 strains was mainly distributed in the cell free supernatant indicating the bioflocculant produced to be the extracellular in nature. A variation in the flocculating rate was observed in different strains when their cell free culture supernatants were treated with different temperature and pH. It was observed that especially at very high and low pH values and at temperature of 100°C (incubated for 20 min) the activity decreased to different degrees. Amongst these isolates, 11 were able to flocculate pathogenic bacteria and 6 exhibited binding to selected heavy metals. The only potential strain that had not only exceedingly high (>80%) flocculating activity, was stable at wide range of temperature and pH but also could flocculate pathogens and bind to some of the tested heavy metals (Cu, Cd, Pb and Zn) was selected for the subsequent studies. This isolate was sourced from activated sludge sample of a textile industrial unit named as Rainbow Denim Ltd [located at village Chaundheri, district Mohali (Punjab), India]. This strain was designated as RD4.

Table 4.1: 43 Bacterial isolates from industrial units with more than 50 % of flocculant activity

Industrial units	Isolate(s)	Flocculant activity (%)	Pathogen removal (%)	Average metal removal (%)
Pepsico Panipat Plant, Panipat	PPP7	54.8±3.8	32.6±2.4	12.8±1.3
	PPP12	64.4±4.2	24.8±3.1	28.6±2.2
Bus stand pond, Patiala	BSP6	73.6±2.7	14.9±2.3	22.4±1.7
JCT, Hoshiarpur	JCT6	57.8±3.2	50.2±3.3	19.4±1.9
	JCT10	78.6±3.6	55.6±3.1	22.6±2.8
	JCT13	69.6±3.9	18.4±2.5	57.7±2.4
Bhakhra Canal, Patiala	BCL3	54.2±4.7	31.6±2.7	33.7±1.7
	BCL14	59.6±4.4	58.9±3.4	28.3±2.6
Hindustan Lever Ltd., Rajpura	HLL12	62.3±3.3	52.6±2.6	42.9±1.8
	HLL17	65.4±3.9	14.7±2.2	18.4±2.1
Milk Plant Ltd., Bahadurgarh	MPL7	71.3±3.6	26.7±3.9	53.7±3.2
	MPL13	65.8±4.8	21.8±3.8	24.4±2.2
ACME Life Sciences, Baddi	ALS2	73.5±4.7	51.7±3.2	16.9±2.7
Chemical Factory, Patiala	CF11	55.3±2.9	23.9±2.7	33.2±2.9
	CF15	69.4±2.7	14.8±2.9	22.4±3.2
Golden Textiles, Baddi	GT3	61.2±3.9	54.6±3.7	16.7±2.7
Rainbow Denim Ltd., Lalru	RD1	79.3±2.9	16.6±2.8	18.8±2.2
	RD4	80.4±4.4	62.3±3.8	68.7±2.9
	RD8	62.7±3.8	12.3±1.9	24.6±1.8
	RD12	69.6±3.2	6.4±1.2	28.3±2.3
	RD19	57.8±4.1	22.9±2.1	13.9±2.1
Ajanta Food Products Company, Parwanoo	AFP3	69.7±2.6	28.3±2.7	42.7±2.6
Patiala Distillers, Patiala	PDA17	55.6±2.9	59.2±3.3	23.9±2.1
	PDA23	75.3±3.8	17.4±1.9	56.2±2.3
AFI Food Pvt Ltd., Nalagarh	AFI6	72.9±3.1	12.8±1.2	22.6±3.1
	AFI14	54.2±4.6	18.6±1.7	8.3±1.5
SYL Canal, Rajpura	SYL3	59.6±2.8	19.4±2.7	52.3±2.9
	SYL9	71.2±3.7	27.8±3.1	28.9±2.4
	SYL15	56.9±4.3	30.2±2.7	48.4±3.4
	SYL19	63.7±4.7	51.4±3.6	13.7±2.3
	SYL21	71.6±3.8	40.2±3.1	23.3±2.3
	SYL23	63.4±4.2	21.6±1.4	37.8±2.7
Cadbury India Ltd., Baddi	CIL11	59.8±3.5	15.3±1.9	31.4±1.6
	CIL13	57.6±4.1	27.4±2.7	33.2±2.9
Rana Sugars Ltd., Amritsar	RSL2	63.4±4.7	42.8±2.4	50.6±2.5
	RSL7	67.8±2.8	14.7±3.2	22.7±2.2
Amrit Banaspati Compant Ltd., Rajpura	ABC5	71.6±2.6	56.2±2.4	29.4±3.1
	ABC9	70.2±3.9	24.8±2.9	10.2±1.2
Gopal Oil & General Mill, Baddi	GOM1	64.3±4.6	28.3±3.1	36.2±2.6
	GOM5	69.2±3.8	16.5±1.9	20.2±2.5
Dhiman Udyog, Jalandhar	DUJ2	58.9±4.1	60.2±2.4	33.7±1.8
	DUJ6	56.4±2.7	22.3±1.7	28.3±2.6

4.2 Characterization and Identification of the strain RD4

4.2.1 Morphological and biochemical characterization

Morphological and biochemical properties of the isolated colonies of RD4 were assayed and identified according to based on the keys described in Bergeys manual of determinative bacteriology (Holt et al., 1994). Morphologically the bacterial colony appeared to be cream colored circular mucoid. The strain was aerobic, Gram negative, rod shaped, non motile catalase positive and non endospore forming. And the other physiological and biochemical properties are summarized in Table 4.2.

4.2.2 Molecular characterization and identification of the bacterial isolate RD4

The 16S rDNA sequence of 1467 bp was obtained by sequencing the 16S rDNA amplicon with the P0 and P6 primers (Table 4.4). This sequence was deposited in the GenBank database (accession number EU082029). The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTALW (Thompson et al., 1997). The resulting multiple alignments were optimized visually and the evolutionary distance was calculated by Kimura 2 parameter (Table 4.3). Phylogenetic dendogram was constructed by neighbor-joining method using MEGA 4 software (Tamura et al., 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. The phylogenetic tree revealed that strain RD4 formed evolutionary lineage within the radiation cluster comprising the *Klebsiella* spp. and the strain type RD4 phylogenetically was most closely related to the type strain *Klebsiella terrigena* (98.6%) (Fig.4.1). Thus, based on its morphological, physiological, biochemical properties and 16S rDNA sequence results, the isolated strain RD4 was assigned as *Klebsiella terrigena.*

Table 4.2 Morphological and biochemical tests of the bacterial isolate RD4

Morphological tests		Biochemical tests	
Configuration	Circular	Indole test	-
Size	1-3	Methyl red test	-
Margin	Entire	Voges Proskauer test	+ (w)
Elevation	Convex	Citrate utilization	+
Surface	Smooth	Casein hydrolysis	-
Pigment	Cream	Esculin hydrolysis	+
Opacity	Opaque	Gelatin hydrolysis	-
Gram's reaction	-ve	Starch hydrolysis	+
Cell shape	Rod	Urea hydrolysis	-
Size	0.8-1.5	Gas production from glucose	+
Arrangement	-	H_2S production	-
Spore(s)	-ve	Catalase test	+
Endospore	-	Oxidase test	+ (w)
Motility	-	Tween 20 hydrolysis	-
		Tween 40 hydrolysis	-
Fluorescence (UV)	-	Ornithine decarboxylase	-
		Growth on MacConkey agar	LF

Physiological tests		Utilization of sugar	
Growth on NaCl (%)		Adonitol	+
2.0	+	D-alanine	+
4.0	+	i-erythritol	-
6.0	+	D-galacturonate	+
Anaerobic growth		Lactulose	+
		Malonic acid	-
Acid production from sugar		L-rhamnose	+
Glucose	+	D-sorbitol	+
Lactose	+	Sucrose	+
Sucrose	+	Turanose	+
		D-xylitol	-

***Remarks = +(positive), - (negative)**

Table 4.3 Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter)

Distance Matrix												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
AF129448	1	---	0.999	0.999	0.999	0.999	0.999	0.999	0.996	0.997	0.999	0.997
Y17658	2	0.001	---	1	1	1	0.999	1	0.997	0.998	1	0.997
EU304253	3	0.001	0.000	---	1	1	0.999	1	0.997	0.998	1	0.997
AB244467	4	0.001	0.000	0.000	---	1	0.999	1	0.997	0.998	1	0.997
AJ871856	5	0.001	0.000	0.000	0.000	---	0.999	1	0.997	0.998	1	0.997
Y17670	6	0.001	0.001	0.001	0.001	0.001	---	0.999	0.996	0.997	0.999	0.997
EU888474	7	0.001	0.000	0.000	0.000	0.000	0.001	---	0.997	0.998	1	0.997
AF129442	8	0.004	0.004	0.004	0.004	0.004	0.004	0.004	---	0.994	0.997	0.994
NR 037085	9	0.003	0.002	0.002	0.002	0.002	0.003	0.002	0.006	---	0.998	0.995
AB244467	10	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.004	0.002	---	0.997
RD4	11	0.004	0.003	0.003	0.003	0.003	0.004	0.003	0.006	0.005	0.003	---

Table 4.4 Aligned sequence data of *Klebsiella terrigena* RD4

Aligned Sequence of RD4: (1467 bp)
GGGATGACGCTGGCGGCAGGCCTAACACATGCACTTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGC GGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC ATAATGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTA GGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGAC ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATGAGGTTAATAACCTTCGTCG ATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGG GAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGG CTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTATTTCGATGCAACGCGAaGAACCTTACCTGGTCTTGACATCCACTTT CCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGC CAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTA CAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGA GTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGG CCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGAGC TTACCACTTTGTGATTCATGACTGGGGTGAAG

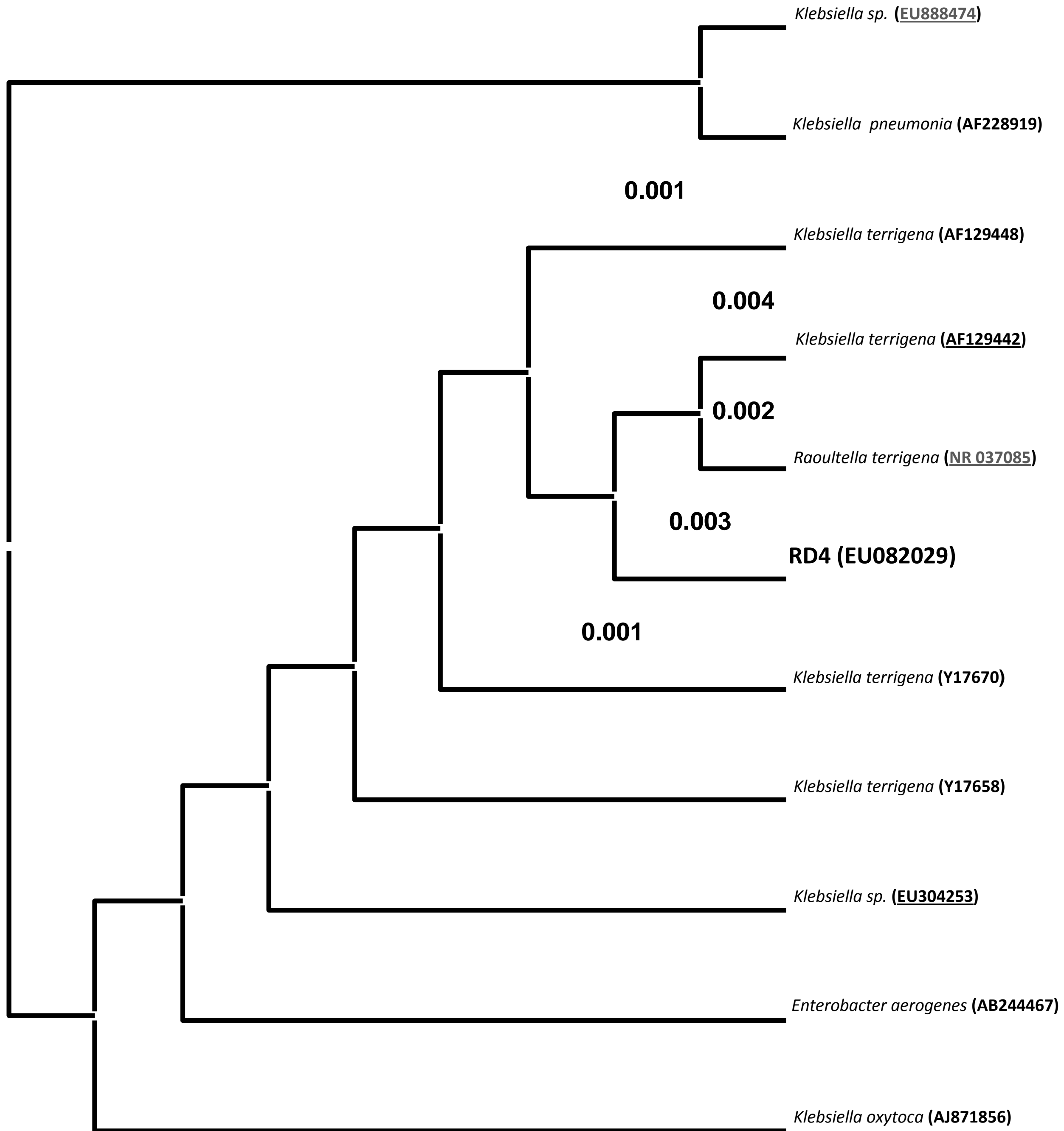


Fig 4.1 Neighbour-joining tree of the isolate based on bacterial 16S rDNA sequence. A neighbor-joining tree was constructed by aligning the sequences with other selected members from the prokaryotic domain. Listed beside each organism or strain name is the GenBank accession number (in Parenthesis). Numbers at branch-points represents confidence values obtained after boots strap analysis of the neighbor joining tree

4.2.3 Genetic fingerprinting of *Klebsiella terrigena* RD4 strain by ARDRA

The amplified ribosomal DNA restriction analysis (ARDRA), a PCR-based genomic fingerprinting method (Arturo et al., 1995) was used to obtain the molecular signature of *Klebsiella terrigena* RD4 strain. The 1.5 kb amplicon of 16S rDNA in the test strain was digested with the tetra nucleotide cutting restriction endonucleases *Alu*I and *Sau*3A to generate different restriction fragment pattern depending on the strain specific restriction sites on 16S rDNA. It produced 4 bands with *Alu*1 and 5 bands with *Sau*3A digestion (Fig. 4.2). These two strain specific restriction fragment patterned were designated to be ARDRA type I and ARDRA type II Both the ARDRA types were observed to produce the same restriction fragment pattern when the test strain was analyzed for ten generations. Thus both the ARDRA types served as a molecular signature for confirming the purity and authenticity of the *K. terrigena* RD4 strain during the entire study.

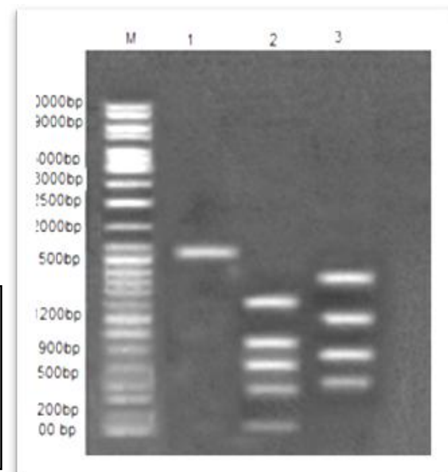


Fig 4.2 16S rDNA restrictional analysis (ARDRA) profile of *Klebsiella terrigena* RD4 strain. Lane M: Molecular weight DNA marker; Lane 1: Undigested 1.5 kb 16S DNA amplified product, Lane 2: Amplicon digested with *Sau* 3a, lane 3: Amplicon digested with *Alu1*

4.3 Kinetics of growth and biofloculant production

In order to establish the bioflocculant production kinetics by *K.terrigena* RD4, the strain was cultured using the screening medium FIB (Fig. 4.3). The flocculating activity as well as bioflocculant synthesis was measured at various growth phases during the growth in

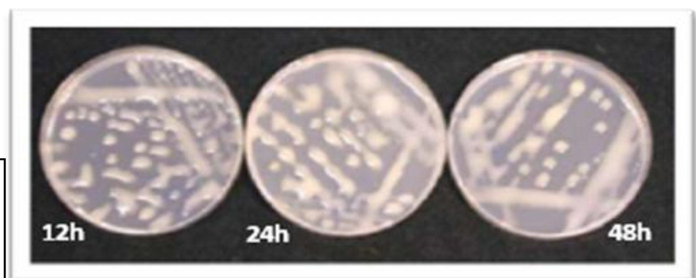


Fig. 4.3 Culture of *Klebsiella terrigena* strain RD4 showing production of biopolymeric flocculant

batch culture. As shown in table 4.5, the bioflocculant production was observed to increase during the stationary phase of growth, and its concentration showed a decline after 60 hours of growth.

Table 4.5 Growth kinetics of *Klebsiella terrigena* with bioflocculant production and flocculant activity

S.No.	Time (hr)	Cell count (CFU/ml)*	Flocculant amount (ppm)	Flocculant activity (%)
1.	0	1.2×10^2	N.D	N.D
2.	6	3.2×10^3	N.D	N.D
3.	12	2.1×10^4	N.D	N.D
4.	18	6.9×10^5	0.3±0.01	11.2±2.1
5.	24	1.6×10^7	0.86±0.04	34.6±3.6
6.	30	7.9×10^7	1±0.04	46.3±3.2
7.	36	6.7×10^7	1.7±0.07	76±4.7
8.	42	6.4×10^7	2.2±0.12	80.8±3.8
9.	48	6.0×10	15.6±0.67	81.3±4.9
10.	54	4×10^6	48±2.1	78±4.1
11.	60	2×10^6	82±3.7	71.8±3.1
12.	72	7.7×10^6	79±4.2	70.1±4.8

± SD are average of three readings

Initially with increase in the production of the polymeric flocculant the flocculating rate also increased. The highest flocculant activity (81.3%±4.9) was observed in 48 hours. The amount of the polymeric flocculant was approximately 15.6 ppm. This polymer was produced when the cell count per mL was approximately 7 log cells in the stationary phase. The culture reached death phase in 54 hours of incubation. A closer analysis of the data shown in the table 4.5 revealed that there was no significant increase in the flocculant activity when the yield was increased from 2.2 ppm in 42 hours to 15.6 ppm in 48 hours. Therefore a dose of 2.2 ppm could be considered for achieving maximal flocculant activity. A steady decline in flocculating

activity was observed thereafter although the yield of the flocculant was maximum. The increased concentration of the biopolymer might be responsible for the observed decline in flocculating activity after 48 hours. According to the results, biopolymer accumulated was more than 3 times in 54 hours as compared to the polymer amount obtained in 48 hours and a decrease in the amount of polymeric flocculant was observed after the culture was incubated beyond 60 hours.

4.4 Nature of *K. terrigena* bioflocculant (KBF)

The crude polymer was dissolved in deionized water and treated with a solution of cationic salt cetylpyridinium chloride (CPC). Formation of precipitates with CPC indicated the possibility of anionic nature of bioflocculant. The *bioflocculant* was purified to homogeneity by ethanol and cetylpyridinium chloride precipitation. The homogeneity of KBF was confirmed with cellulose acetate electrophoresis on cellulose acetate strip. A single spot on the cellulose acetate strip confirmed the electrophoretic homogeneity of KBF (Fig. 4.4).

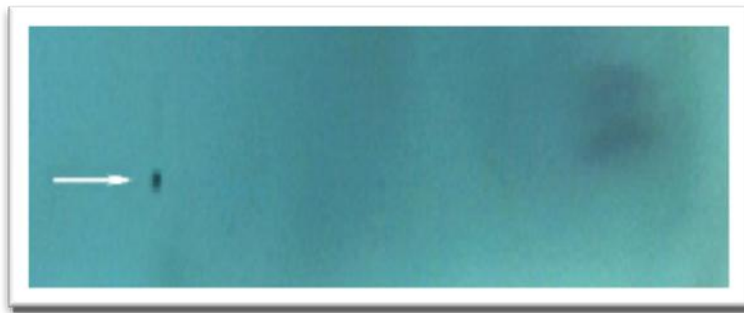


Fig 4.4 Cellulose acetate paper electrophoretogram of purified bioflocculant produced by *Klebsiella terrigena*

4.5 Optimization of factors effecting flocculant ativity

4.5.1 Effect of bioflocculants dosage

The typical flocculation curve of the purified bioflocculant as shown in fig. 4.5 with its varing concentration was obtained. The flocculating activities of kaolin suspension were estimated for the flocculant concentrations in the range of 1-10 mgL^{-1}. As shown in fig. 4.5, the corresponding maximum flocculating activity was achieved at bioflocculant dose of 2 mgL^{-1} (93.6±3.9%). However, bioflocculant dosage exceeding that of 6 mgL^{-1} decreased its flocculating activity.

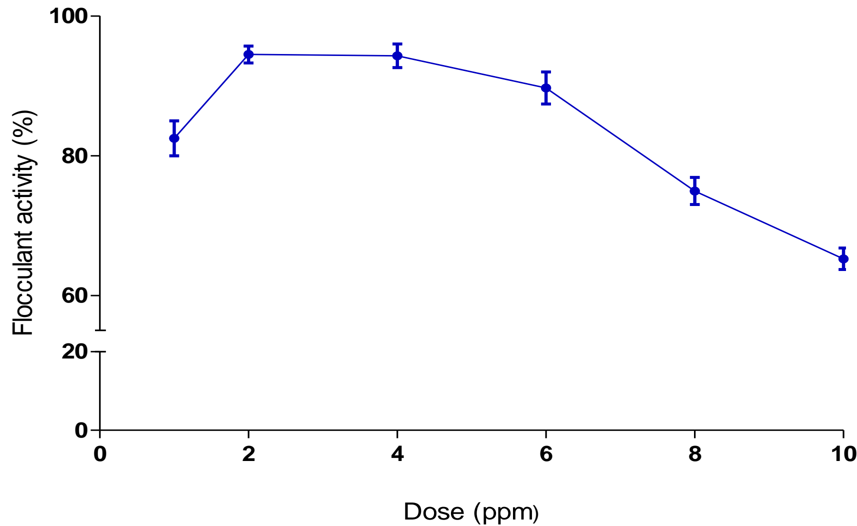


Fig. 4.5 Optimization of bioflocculant dose

4.5.2 Optimization of Cations

The influence of cations and their concentration on flocculating activities was studied in the kaolin suspension containing 2 ppm of the bioflocculant.The flocculating activity of KBF markedly increased by the addition of divalent cations as compare to other monovalent or trivalent cations used. The maximum flocculating activity of 93.6±3.2 % was obtained with Ca^{2+}

at 5 mM followed by Fe^{2+} at the same concentration. Flocculating activity decreased when the cation were used above this optimum concentrations (Fig. 4.6).

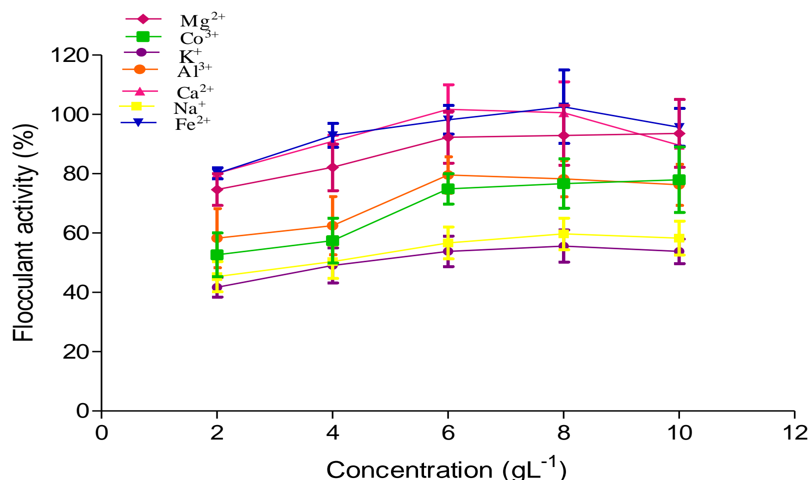


Fig 4.6 Effect of cations on flocculating activity of the bioflocculant *Klebsiella terrigena* RD4

4.5.3 Effect of medium pH and bioflocculant stablity in solutions

The effect of pH on flocculating activity of the KBF was evaluated at pH values ranging from 4.0 to 9.0 while keeping other conditions constant. Flocculating activity of *K. terrigena* flocculant varied with pH. The highest flocculating activity (93.6±3.2%) occurred at pH 7.0 (Fig. 4.7). KBF was also found to be stable at this pH for least 96 hrs. The pH stablity experiments illustrated that the purified bioflocculant was relatively stable over a wide pH range 6-8 compared to that of the control at pH 7 (Fig.4.8).

4.5.4 Incubation temperature

The effect of temperature on flocculating activity illustrated in fig. 4.9, the activity was above 80% in a temperature range of 35–85°C. A gradual decrease in the activity was observed thereafter. The bioflocculant was thermally stable at 100°C for 10 min.

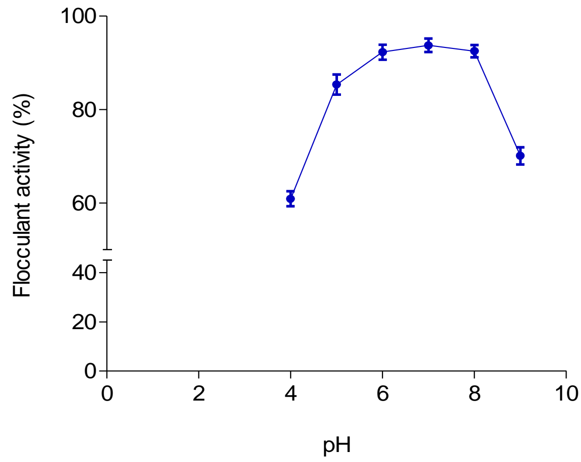


Fig. 4.7 Optimization of medium pH for maximum flocculating activity

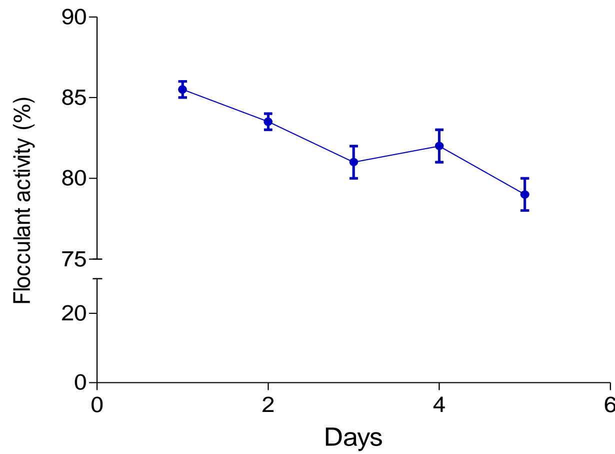


Fig. 4.8 Stability profile of bioflocculant in solution

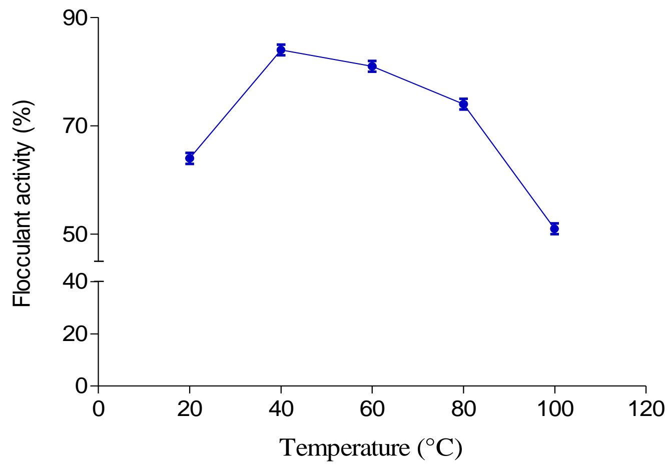


Fig. 4.9 Optimization of temperature conditions for maximum flocculant activity

4.6 Evaluation of bioflocculant efficiency

4.6.1 Comparative study with some natural and synthetic flocculants

The activity of the purified biopolymeric flocculant was compared of its efficacy with other natural and synthetic flocculants under their most optimal conditions of dose, pH, temperature and cations. KBF showed better flocculating activity than other synthetic and natural flocculants and activity was approximately similar to that of polyacrylamide (92.6±4.2%) (Table 4.6). The flocculating activites of other polymers like xanthan, chitosan and polyglutamic acid were (87.6±4.4 %), (77.9±2.6%) and (73.5±3.4%) repectively.

Table 4.6 Comparision of flocculating efficiency of bioflocculant with other flocculants

Flocculants	Dosage	Cations	Temp	pH	Activity (%)
KBF	2mg/ml	Ca^{2+}	30°C	7.0±0.02	93.4±3.4
Xanthan	1mg/ml	Ca^{2+}	40°C	7.0±0.02	87.6±4.4
Γ-PGA	8 mg/l	Ca^{2+}	30°C	7.5±0.02	73.5±3.4
Chitosan	1mg/l	Not required	40°C	7.5±0.02	77.9±2.6
Polyacrylamide	30 mg/ml	Not required	35°C	8.0±0.02	92.6±4.2
CHEMFLOC985W	8mg/ml	Fe^{3+}	RT	8.0±0.02	93.8±3.9

4.6.2 Flocculation of various suspended solids

The efficiency of the bioflocculants to agglomerate different sized colloidal particles ranging from (0.5 µ - 100 µ) was assessed. Although, the bioflocculant could aggregate a wide range of colloidal particles, large variations in the degree of flocculation were observed. The effective bioflocculant concentration in removing supensions that are composed of colloids of known size was also optimized. The varied size of colloidal particles used [~0.5-0.8µm (bacterial cell suspension), ~4 µ -7 µ (Kaolin, yeast cell suspension, cellulose), ~15 µ-~20 µ (silica), ~100 µ (Active carbon)] were broadly differentiated into very low, low, medium and large colloidal

sizes respectively. A mixture of all particles in equal amount was also used for better insights of the performance under realtime condition.

The bioflocculant concentration ranged from 2-10 ppm. As illustrated in the fig. 4.10 that most effective flocculation was achieved in suspensions comprising very low and low sized particles at even low concentration of the bioflocculant. Whereas the efficacy of flocculation of suspension of medium sized particles ranged from 30-40% that too at relatively higher concentration of 8-10 ppm of the bioflocculant. The suspensions of large sized particles showed 50-65% flocculant activity at the same concentration range that flocculated in the range of 70-90% in case of low and very low sized particle suspension.This might be due to non uniform distribution of pore size on the bioflocculant surface also the number of sites available for binding low and very low sized particles might be more than that of binding medium and large sized particles.

In order to have a practical understanding of bioflocculants efficiency, an evaluation was carried out in terms of turbidity. The four different category of particle size were used to obtain turbid solution ranging from 50-2000 NTU. Fig. 4.11 revealed at dose of 6 ppm, the bioflocculant was found to be most effective for a turbidity range from 500-1000 NTU. The degree of flocculant activity achieved with various size of particle suspension was found to be in agreement with the results of the above study. As compared to super turbid solution i.e., above 1000 NTU the flocculant activity was lower in suspension with very low turbidity (< 80 NTU), especially in the suspension comprising very low and low sized particles.

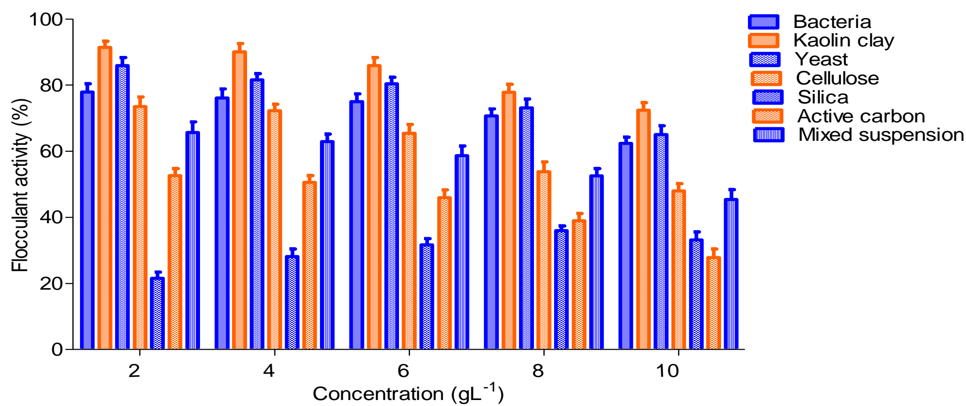


Fig. 4.10 Efficiency of removal of varied size colloidal partricles in solution applied with different doses of bioflocculant ranging from 2ppm -10ppm. The corresponding size of the particles are following [~0.5-0.8µm (bacterial cell suspension), ~4-7µ (Kaolin, yeast cell suspension, cellulose), ~15-20µ (silica), ~100µ (Active carbon)]

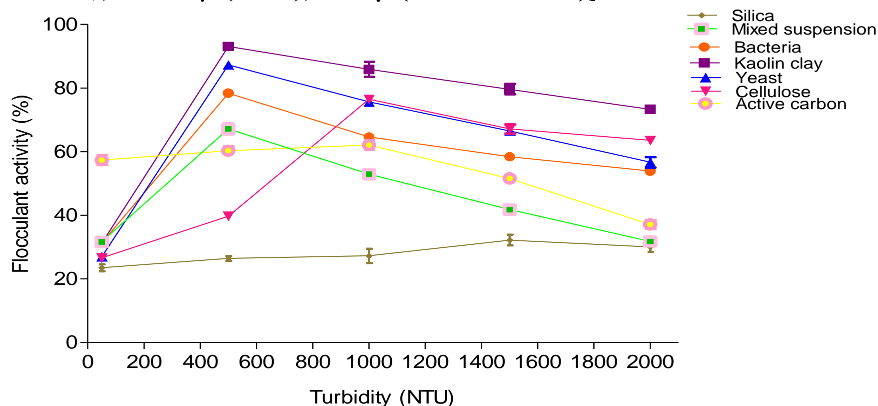


Fig. 4.11 Evaluation of bioflocculant efficiency in turbid solutions (low, high and super turbid) (50-2000 NTU). Different turbid solutions comprised of varied sized particles ranging from (0.5-100µ) also a mixture of them was used

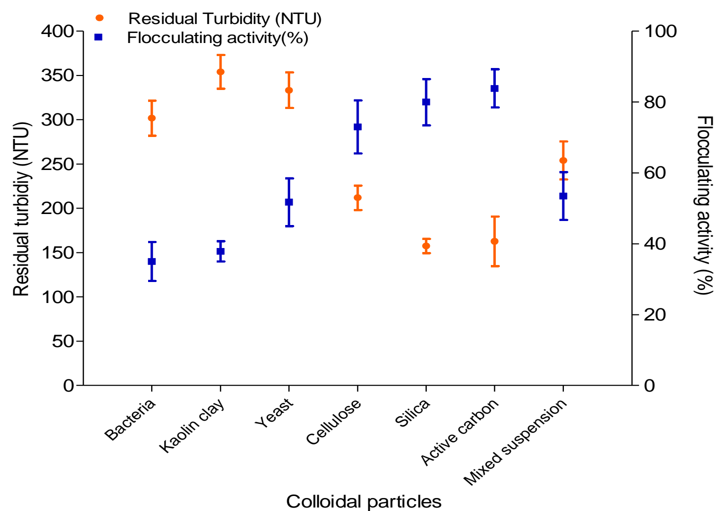


Fig. 4.12 Maximum efficiency of bioflocculant at most optimal turbidity (500 NTU) and correlation between the flocculating activity and residual turbidity

This might be due to insufficient binding with the particles on account of very low number of collisions required to initiate flocculation by the biopolymeric flocculant. However, flocculation was observed to be effective even at lower tubidity (< 80 NTU) of suspension samples with large sized particles.

The removal rate of all these suspended particle samples were also compared with aluminium sulphate at 10 ppm the efficiency of turbidity removal was better with the bioflocculant for all particles sized suspended samples (Fig. 4.12). The removal by the bioflocculant for lower particle size showed drammatically high as compared to its synthetic counterpart. Also the relationship between the spectrums of flocculating activity corresponding to the turbidity removal could be successfully established. The measurements of the residual levels of turbidity referred to approximately equal rate of flocculating activity.

The use of EDTA as a chelating agent reduced the flocculating activity of KBF from 93.6±3.2% to 42±2.8%. EDTA combined with Ca^{2+} to form more stable complex Ca-EDTA and therefore eliminated the effect of Ca^{2+}. The results show that it could be Ca^{2+} mediated bridges between kaolin particles and the flocculant molecules.

4.7 Partial Characterization of the purified polymeric flocculant (KBF) of *Klebsiella terrigena* RD4 strain

4.7.1 Surface properties of *K. terrigena* bioflocculant

The scanning electron micrograph of the purified KBF revealed the polymeric surface to be highly porous structure with randomly distributed small pores, interconnected with channels (Fig. 4.13).

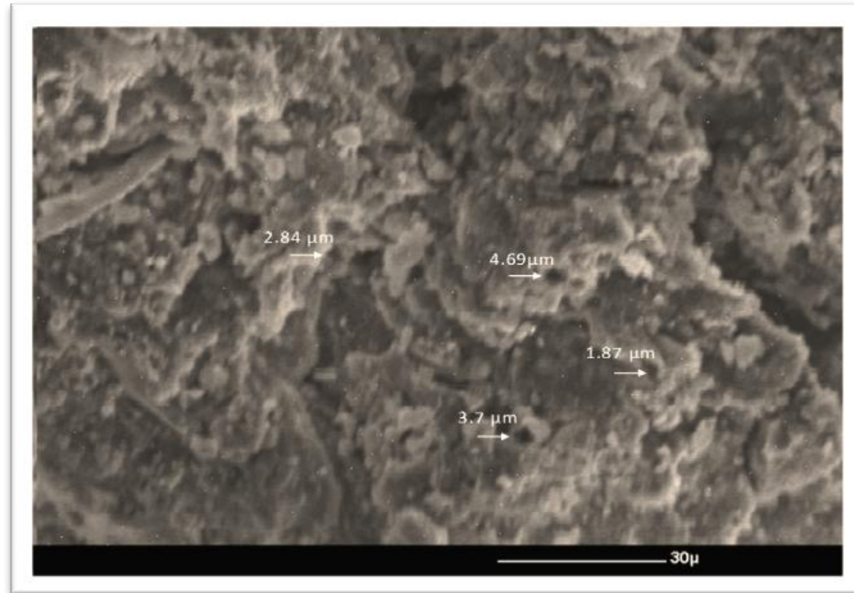


Fig. 4.13 Scanning electron microphotograph (SEM) of the *Klebsiella terrigena* flocculant from *strain* RD4 Magnification 500X, bar size 30 µ

4.7.2 Biochemical composition and physical properties of *K. terrigena* bioflocculant

The Molecular weight of KBF was determined using gel permeation chromatography using blue dextran as standard. The chromatogram (Fig. 4.14) revealed the presence of two carbohydrate-containing peaks. The first of these peaks, eluting at approximately 50 min, was detected using a refractive index detector as well as by UV absorbance. Its size was estimated to be 260,000 Da by comparison to the elution times of blue dextran standards of known size. A second peak eluting at approximately 76 min was also detected by refractive index. This peak, however, had no UV absorbance. The size of the material in the second peak was estimated to be approximately 1200 Da. The presence of a second smaller peak led to the inclusion of a membrane filtration step using a 100 KDa MWCO membrane in the bioflocculant extraction procedure in order to focus on the 260 KDa polymer of interest. A molecular mass greater than 260 Da was observed. An important reason for high flocculating activity of KBF produced by *K. terrigena* is its high molecular weight.

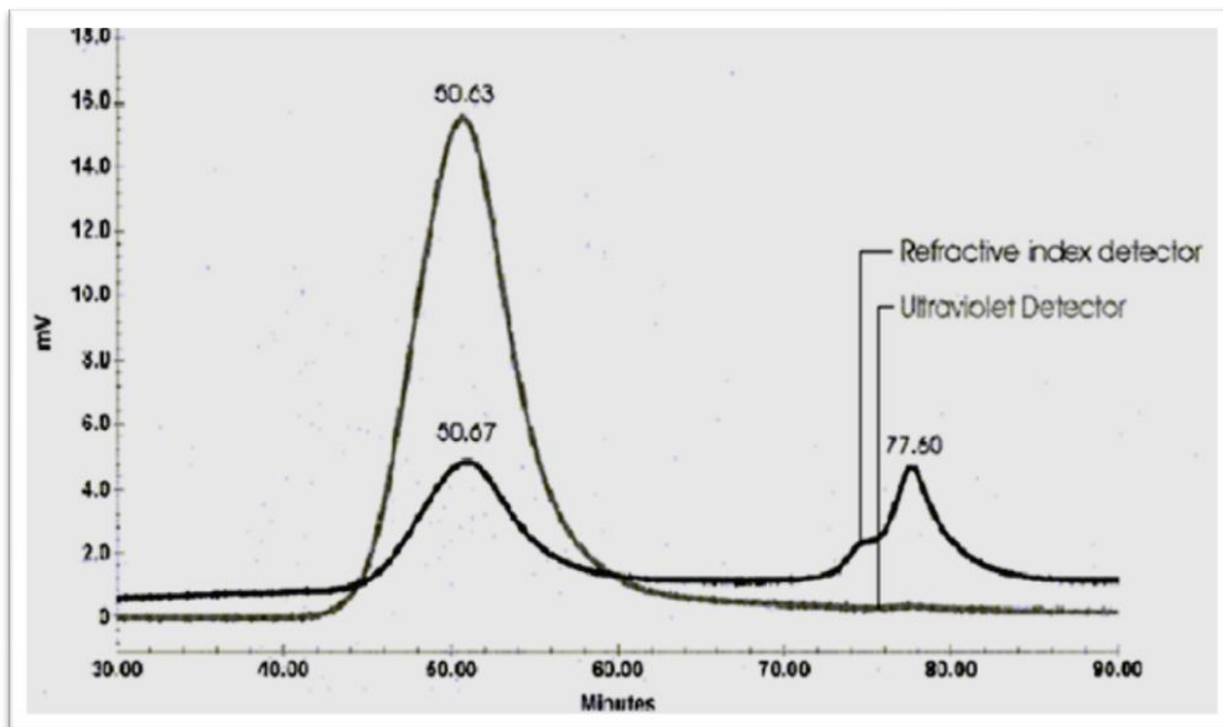


Fig. 4.14 Gel permeation chromatogram of the polymeric flocculant from *Klebsiella terrigena* RD4. The large peak at 50.67 min was estimated to be approximately 260 KDa in size. The smallest peak at 77.60 min is approximately 1200 Da

4.7.2.1 Functional group analysis

Functional groups present in the biopolymer are important determinants for the flocculating activity (Kumar et al., 2004). The FTIR spectrum recorded for purified KBF is given in fig. 4.15 which displayed more complex pattern of peaks from 2350 to 1300 cm^{-1}. The IR spectra revealed characteristic functional groups such as a broad-stretching hydroxyl group at 3403 cm^{-1} and, a weak C-H stretching peak of methyl group at 3029 cm^{-1} (Xia et al., 2010); the spectrum also displayed an asymmetrical stretching band at 1626 cm^{-1}. The absorption peak at 1056 cm^{-1} is known to be characteristic for all sugar derivatives. A weak symmetrical stretching band near 1415 cm^{-1}, showed the presence of uronate. Biopolymer such as xanthan components are recognized by peaks at wave numbers of 1040 cm^{-1} (C–O bond from the alcohol group), 2940 cm^{-1} (C–H stretch) and 3400 cm^{-1} (–OH stretch) (Cetin and Erdincler, 2004).

Polysaccharides contain a significant number of hydroxyl groups, which exhibit a broad rounded absorption band above wave number 3000 cm^{-1}. The absorption in that region has the rounded trait typical of hydroxyl groups (Howe et al., 2002), which suggests that the substance is polysaccharide. A band stretch of C–O–C, C–O at 1021–1200 cm^{-1} corresponds to the presence of carbohydrates (Bremer and Geesey, 1991), so in the fingerprint region (region below 1500 cm^{-1} where bands characterize the molecule as a whole), the absorption band at 1056 cm^{-1} is attributed to that substance is polysaccharide (Nataraj et al., 2008). Strong absorption at 1626 cm^{-1} which corresponds to amide > C= O stretch and C–N bonding of protein and peptide amines, and a peak at 1398 cm^{-1} could be assigned to >C= O stretch of the COO− and C–O bond from COO− (Haxaire et al., 2003; Helm & Naumann, 1995). The results clearly indicate the KBF to be predominantly composed of polysaccharides.

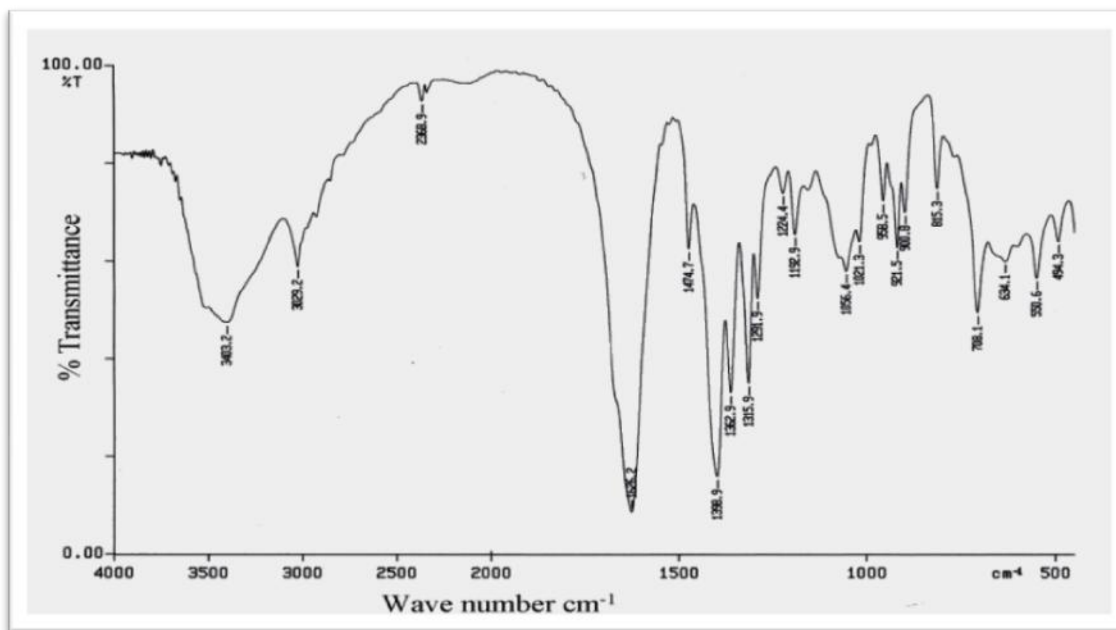


Fig. 4.15 Fourier transform infrared spectra (FT-IR) transmittance spectrum from 4000-500cm^{-1} of the purified *Klebsiella terrigena* flocculant

4.7.2.2 DNase, RNase, Protease activity on *K. terrigena* bioflocculant

Treatment of biopolymers with nucleases, proteases and lipases may be used for a preliminarily confirmation of compostion. Digestion by nuclease and enzymes DNase, RNase

and proteases marginally affected the flocculating activity. Thus the associated protein or nucleic acids of biopolymeric flocculant appear not to be related for flocculating activity.

4.7.2.3 Compositional characteristics

The bioflocculant was mainly composed of 69.8% (w/w) carbohydrate (Table 4.7). Thus, KBF could be an acidic heteropolysaccharide. KBF binding to the alcian blue indicated it to be polyanionic. The C-H-N mass analysis results are also shown in table 4.7.

Table 4.7 Compositional analysis of the bioflocculant KBF

S.No.	Components	Composition (%)
1	Total sugar	69.8
2	Amino sugar	6.87
3	Uronic acid	14.73
4	Pyruvic acid	0.6
5	Protein	1.12
Elemental analysis		
6	Carbon	14.73
7	Hydrogen	1.23
8	Nitrogen	0.64

The monomeric units of KBF were analyzed by TLC and HPLC analysis. Following TLC analysis of TFA hydrolyzed samples, four spots with R_f values of 0.559, 0.572, 0.598 and 0.639 corresponding to D-Glucose, D-Mannose, D-Galactose and D-Glucuronic acid respectively (Fig. 4.16). This was further confirmed by the HPLC analysis of the acid hydrolysate identified D-Glc, D-Man, D-Gal and D-GlcA as its constituent monosaccharides (Fig. 4.17). Thus the bacterial polymer KBF of RD4 has been observed to be rich in hexoses like glucose mannose and galactose. Apart from polysaccharides, KBF also contain fair amounts of proteins 1.12% (w/w). However, the protein moiety present was not responsible for flocculating activity of KBF,

since treatment of KBF with protease did not lead to a loss of flocculating activity. Other observed non-sugar moieties like uronic acid (14.73%), pyruvates (0.6%), hexosamines (6.87%).

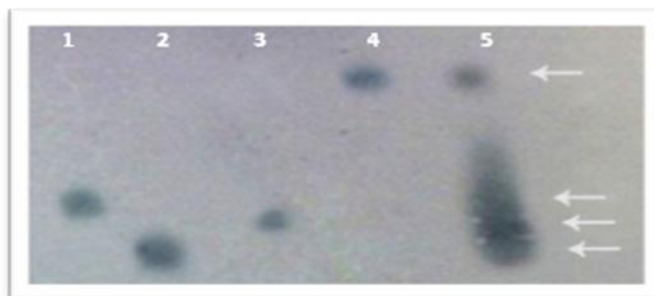


Fig. 4.16 Thin Layer Chromatograph of TFA hydrolyzate of biopolymeric flocculant polysaccharide (Lane 5) resolved in its monomeric sugars. Lanes (1-4) indicate standards of Galactose(R_f 0.599), D-Glucose(R_f 0.572), D-Mannose(R_f 0.598), D-Glucuronic acid (R_f 0.639) respectively

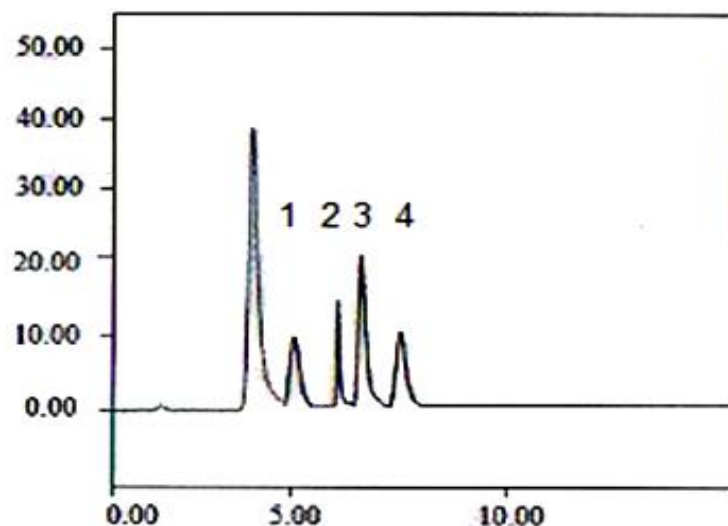


Fig. 4.17 High performance liquid chromatographic (HPLC) separation of acid hydrolysates of KBF 1) Mannose 2) Glucuronic acid 3) Glucose 4) Galactose

4.7.3 Thermal and rheological property of KBF

Thermal stability studies revealed that the purified bioflocculant KBF could be classified a thermal-stable bioflocculant since its flocculating activity did not decrease in the temperature range of 60–100°C. The pyrolysis characterization of KBF was investigated using thermogravimetric analysis (TGA), carried out dynamically (weight loss vs. temperature). The

experimental results are illustrated in fig. 4.18. A degradation temperature (Td) of around 250°C was estimated from the TGA curve for KBF. The polymer showed an initial weight loss due to moisture content. The initial moisture content in the sample may be attributed to the increased level of carboxyl groups in the polysaccharide, which had the greater affinity for interaction with water molecules. The decline in weight above 130°C might be due to the degradation of the sample. The onset of decomposition occurred at about 220°C and the recorded mass loss was 22%. The polymer dramatically lost weight around 270°C and gradually decreased further and retained about 15% of the weight after exposure at 750°C.

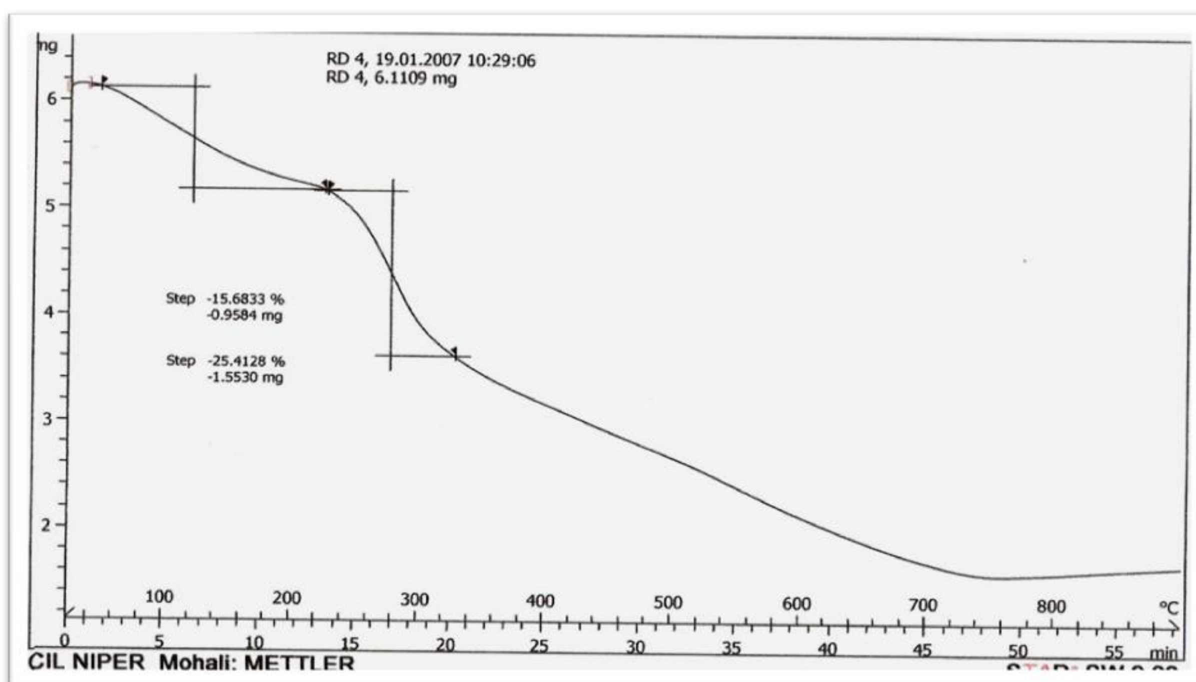


Fig. 4.18 Thermal gravimetric analysis of the purified biopolymer from *Klebsiella terrigena* RD4

4.7.4 Rheological studies of the bioflocculant

The rheological characterization of KBF was carried out with respect to its viscosity and elasticity. The experimental results are illustrated in fig 4.19 (a-f). KBF, when undergo a shear rate and shear stress at constant frequency and at a temperature range of 25°C to 85°C it

exhibited constant effect or no effect upto 65°C. Further, with increasing temperature from 65°C to 85°C, its storage modulas i.e elasticity increased from 2000 Pa to 70000 Pa (Fig. 4.19 a).

Thebioflocculant material did not show any viscosity effect upto 62.8^{o}C. Its viscosity increased upto 10,000 Pascal/sec at 81.7^{o}C. A illustrated in figure 4.19 b, temperature increased the viscosity of the polymer increased upto that point and then starts decreasing. The viscoelastic behavior of the flocculant biopolymer solution from dynamic oscillatory shear measurements is illustrated in fig. 4.19 c. The viscoelasticity showed the temperature increase from 25°C to 85°C led to decrease in the tan delta values. Tan delta decreased from 0.3 to 0.15. This means the elasticity is less and viscosity of KBF is more. To monitor possible depolymerisation of KBF at higher temperature, control measurements were performed by cooling the sample solutions from 85°C to 25°C. During cooling of KBF from 85°C to 25°C it exhibited reverse effect of temperature on storage modulas. The study showed that the storage modulas of the polymer decreased from 130000 Pa to 60000 Pa which is due to the lower temperature that causes more shear effect at constant frequency (Fig. 4.19 d). As temperature decreased from 85°C to 25°C, the viscosity of KBF again decreased upto 56.4^{o}C then start increasing at 26.8^{o}C (Fig. 4.19 e). Viscoelasticity study showed that as temperature decreased from 85°C to 25°C the tan delta exhibited an increasing trend (Fig. 4.19 f). Tan delta increased from 0.08 to 0.13. This showed that the viscosity is less and elasticity of the polymer is more.

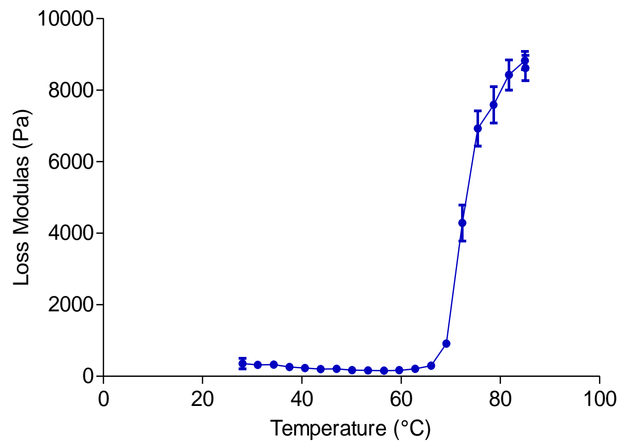


Fig. 4.19a Effect of temperature (heating) on loss modulas of bioflocculant

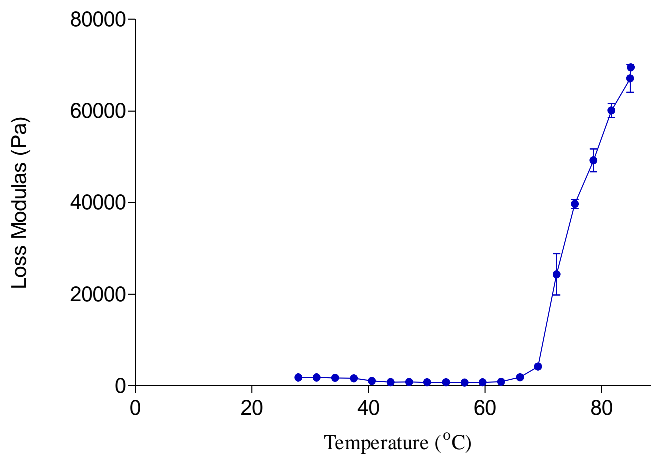


Fig. 4.19b Effect of temperature (heating) on storage modulas of bioflocculant

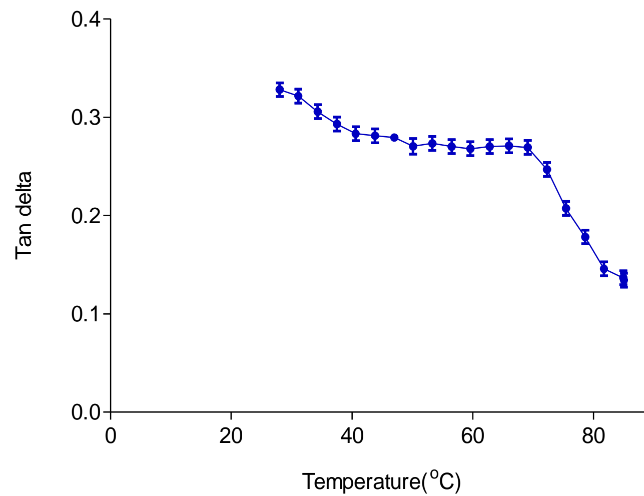


Fig. 4.19c Effect of temperature (heating) on tan delta of bioflocculant

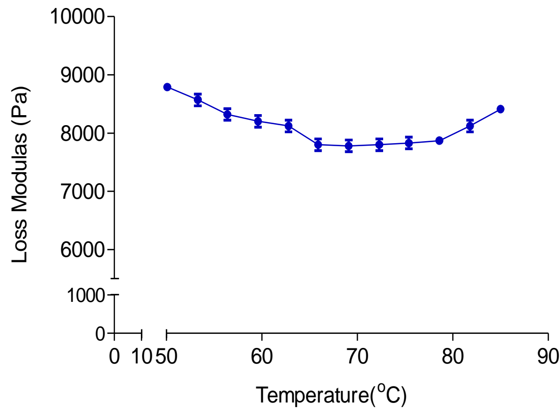


Fig. 4.19d Effect of temperature (cooling) on loss modulas of bioflocculant

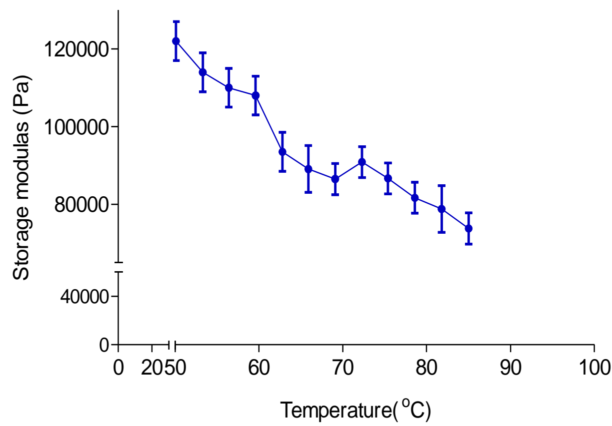


Fig. 4.19e Effect of temperature (cooling) on storage modulas of bioflocculant

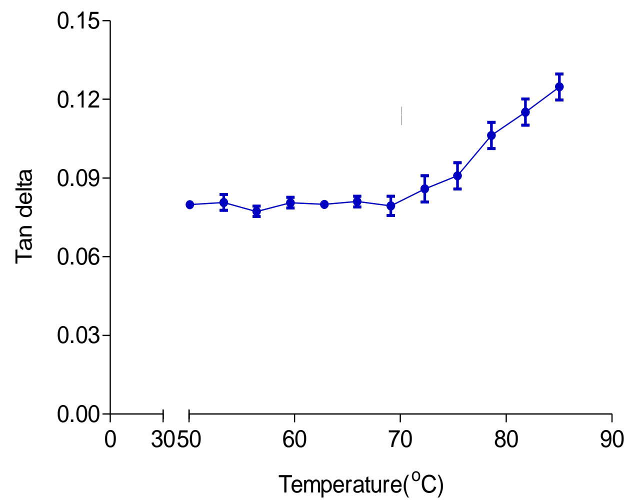


Fig. 4.19f Effect of temperature (cooling) on tan delta of bioflocculant

4.8 Effect of nutritional factors and culture conditions of *Klebsiella terrigena* RD4 on bioflocculant production, growth and bioflocculant activity

The effect of nutrients and their concentations (Carbon and Nitrogen) and culture conditions (the initial pH of the production medium, temperature of cultivation, agitation and inoculum size) on bioflocculant production and its flocculant efficiencies of *Klebsiella terrigena* strain RD4 were investigated.

4.8.1 Carbon source(s)

Based on preliminary carbon source utilization profile of this test strain (data not shown), five carbon sources galactose, rhamnose, sucrose, glucose and maltose were selected for examining production of the bioflocculant of *K. terrigena* (KBF). An upper limit of 2.5% was found to be sufficient since higher concentrations of carbon did not result in an increase in highest achievable cell numbers. One noteworthy result was that the bacterium grew and produced bioflocculant with all the carbon sources assayed. Growth remained unimpeded when glucose was increased to above 1% (w/v) in the culture but without any significant increase in synthesis (Fig. 4.20 a-f) indicated different types of carbon source showed relatively little influence on biomass and biopolymer productivity. The influences were not significantly different ($P<0.05$). However, the flocculating activity varied significantly not only with the carbon sources but also with their concentration. The cell growth observed at higher concentrations of carbon sources could not be correlated with flocculating activity. The latter activity was highest with glucose as carbon source; when sucrose or rhamnose was used individually as sole source of carbon in the growth media, the polymer produced showed a poor flocculating activity.

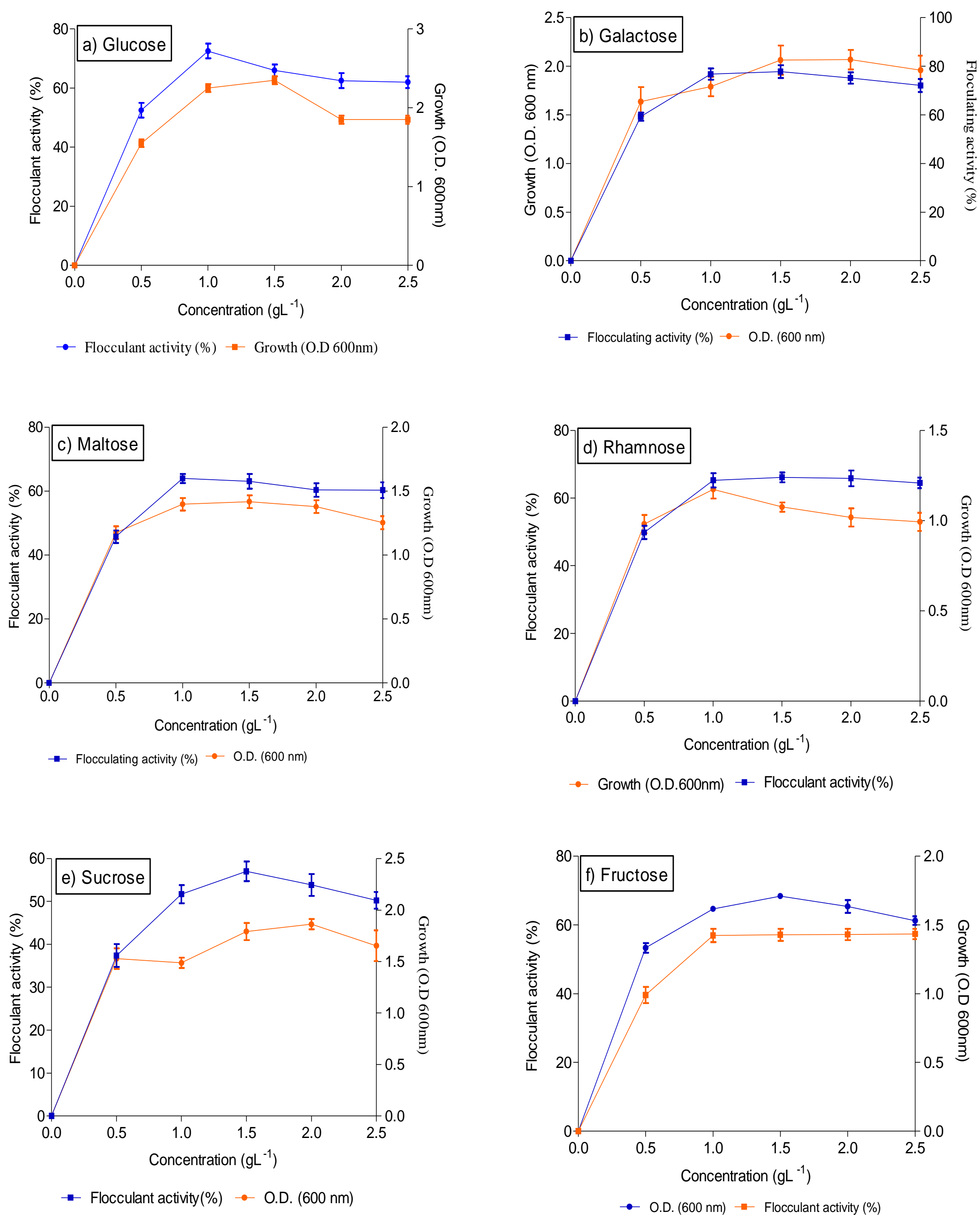


Fig. 4.20 (a-f) Effect of different carbon source(s) on growth and flocculant activity of *Klebsiella terrigena* RD4

The best carbon source for biopolymer production was glucose, followed by galactose. The data incorporated in table Table 4.8 indicated the variations of production with this series of five carbon sources were from 0.112 to 0.221 corresponding to 50.6%. Interestingly these two carbon sources also supported the best growth. Variations in biomass were limited, ranging from 5.48 to 8.48, corresponding to 54%. Yields were little influenced, with a minimum of 0.059 and a maximum of 0.078 gg^{-1} cells, corresponding to a variation of 7.5%. Glucose proved a good carbon source for KBF production, other sugars galactose, rhamnose, maltose sucrose and fructose had stimulatory effect on biopolymer production when added at 2 gL^{-1} to the medium. They have led to the production of 0.14, 0.43, 0.38, 0.34 and 0.24 times less biopolymer respectively than was achieved with glucose. Also, their relative reduction in the ability to flocculate restricted them to be the choice of carbon source. Glucose was used as the carbon source in the production optimization studies because the highest production of KBF (221±9.1 mgL^{-1}) was obtained from glucose at a concentration of 1 gL^{-1} .

Table 4.8 Effect of different carbon sources on flocculant activity and yield of bioflocculant produced by *Klebsiella terrigena*

Carbon source	Concentration (gL^{-1})	Doubling time (min)	Growth rate	Yield (gg^{-1} cells)	Yield (gL^{-1})	Flocculant Activity (%)
Glucose	**0.5**	**37.3**	**0.0186**	**0.068±.007**	**0.167±.018**	**62.3±3.2**
	1	30.5	0.0227	0.078±.003	0.221±.021	81.3±2.9
	1.5	33.1	0.021	0.076±.008	0.218±.014	78.7±3.8
	2	43.2	0.0161	0.075±.011	0.217±.012	75.6±4.2
	2.5	41.8	0.0166	0.074±.002	0.215±.017	74.1±3.7
Galactose	0.5	34.2	0.0202	0.059±.003	0.151±.010	74.1±4.6
	1	38.5	0.018	0.064±.006	0.182±.017	74.4±2.8
	1.5	34.2	0.0203	0.066±.002	0.186±.018	75.2±4.9
	2	37.3	0.0186	0.067±.007	0.191±.013	72.9±3.7
	2.5	42.2	0.0164	0.067±.004	0.190±.017	69.4±4.1
	0.5	43.7	0.0159	0.056±.008	0.112±.012	43.8±2.9
Maltose	1	47.5	0.0146	0.060±.006	0.134±.019	62.6±4.6
	1.5	41.3	0.0168	0.064±.007	0.136±.016	60.8±4.3
	2	44.4	0.0156	0.065±.003	0.139±.018	58.3±3.7
	2.5	44.4	0.0156	0.065±.010	0.138±.013	57.9±3.6
	0.5	**42.9**	0.0161	0.052±.008	0.116±.019	47.9±4.0
Rhamnose	1	41.5	0.0167	0.054±.007	0.121±.012	63.2±2.7
	1.5	42.9	0.0161	0.057±.006	0.124±.011	64.7±4.8
	2	49.1	0.0141	0.062±.005	0.128±.013	63.5±4.3
	2.5	46.5	0.0149	0.063±.004	0.127±.015	62.9±3.6
	0.5	38.1	0.0182	0.054±.008	0.122±.019	34.7±3.9
Sucrose	1	38.5	0.018	0.058±.006	0.141±.014	49.6±4.5
	1.5	34.8	0.0199	0.060±.007	0.142±.018	54.7±3.6
	2	39	0.0178	0.063±.004	0.144±.013	51.3±3.9
	2.5	40.8	0.017	0.063±.006	0.142±.017	48.3±3.0
	0.5	37.5	0.0185	0.059±.009	0.146±.013	37.3±3.8
Fructose	1	39.1	0.0177	0.062±.002	0.167±.019	58.9±4.7
	1.5	38.9	0.0178	0.065±.005	0.158±.011	54.7±3.8
	2	51.5	0.0135	0.065±.009	0.156±.017	53.2±3.4
	2.5	52.7	0.0131	0.066±.007	0.152±.012	52.8±2.6

± SD of triplicate data

4.8.2 Nitrogen source(s)

Both organic and inorganic nitrogen sources are utilized by *Klebsiella* sp, therefore an attempt was made to understand the effect of nitrogen sources on flocculating activity also an optimum organic and inorganic nitrogen source in the medium for the production of KBF was examined. Beef extract, yeast extract, peptone and tryptone were tested as organic nitrogen source and ammonium sulphate, ammonium chloride and ammonium phosphate were tested as inorganic nitrogen source.

The quality of nitrogen sources played an important role on biopolymer production which varied from 0.132-0.216 gL^{-1}. The best nitrogen source for production was peptone to give a yield of 0. 216 gL^{-1} at a concentration of 5 gL^{-1} (Fig. 4.21a), followed by beef extract, tryptone and yeast extract at concentrations 5 gL^{-1} and 7.5 gL^{-1} and 7.5 gL^{-1} respectively and maximum growth recorded was 1.5 1.8 and 1.3 OD units whereas the yield was 0.177, 0.172 and 0.152 gL^{-1} respectively. Biomass seemed to be less influenced by the types of nitrogen, but variation in the flocculating activity was observed; especially highest flocculating activity was observed when peptone and yeast extract both were present together as components in the media although peptone alone was the best source for higher exobiopolymer yields but yeast extract was required to achieve maximal flocculation activity. It also should be noted that the strain showed good growth on ammonium nitrogen sources but with low yields. The high flocculating activity was observed as shown in fig.4.21.b when ammonium sulfate was used as inorganic source of nitrogen in the media. Thus ammonium sulphate was found to be a favourable inorganic nitrogen source for high yield of bioflocculants.

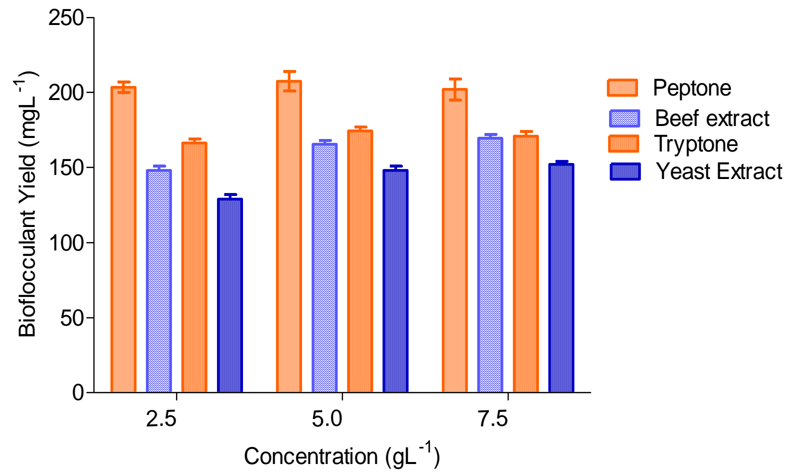


Fig. 4.21a Effect of organic nitrogen source on yield and activity of bioflocculant (peptone, tryptone, beef extract, yeast extract) on bioflocculant production by *Klebsiella terrigena* RD4 strain

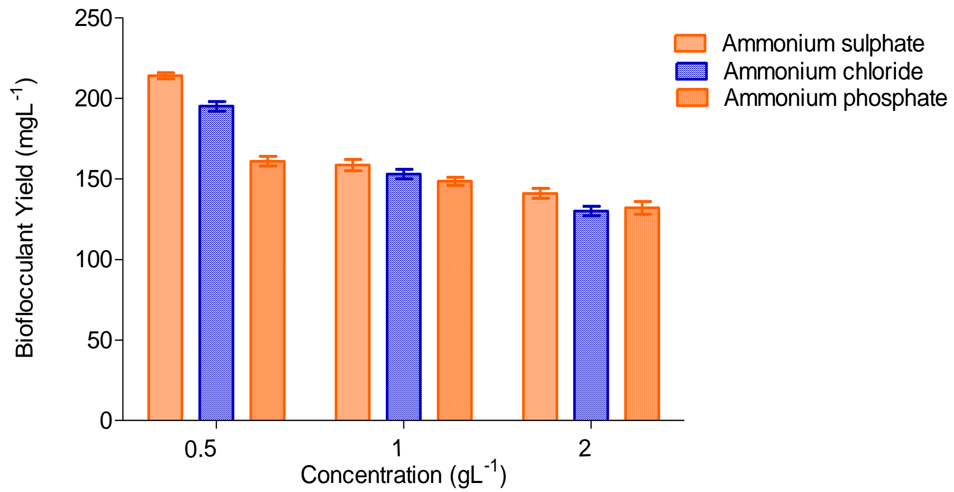


Fig. 4.21b Effect of inorganic nitrogen source on yield and activity of bioflocculant (ammonium sulphate, ammonium chloride, ammonium phosphate) on bioflocculant production by *Klebsiella terrigena* RD4 strain

4.8.3 Carbon/Nitrogen (C/N) ratio

The extracellular biopolymer's synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium. An optimum C:N ratio in the medium for the production of bioflocculant by *K. terrigena* RD4 strain was examined The highest KBF production was achieved at a ratio of 10:1 (221±9.1 mgL^{-1}). Further increase of C/N ratio did not yield a higher amount of bioflocculant (Fig. 4.22). It is apparent from these results that low level feeding of ammonium at relatively higher C/N ratio was more effective for the production of the bioflocculant by *Klebsiella terrigena*.

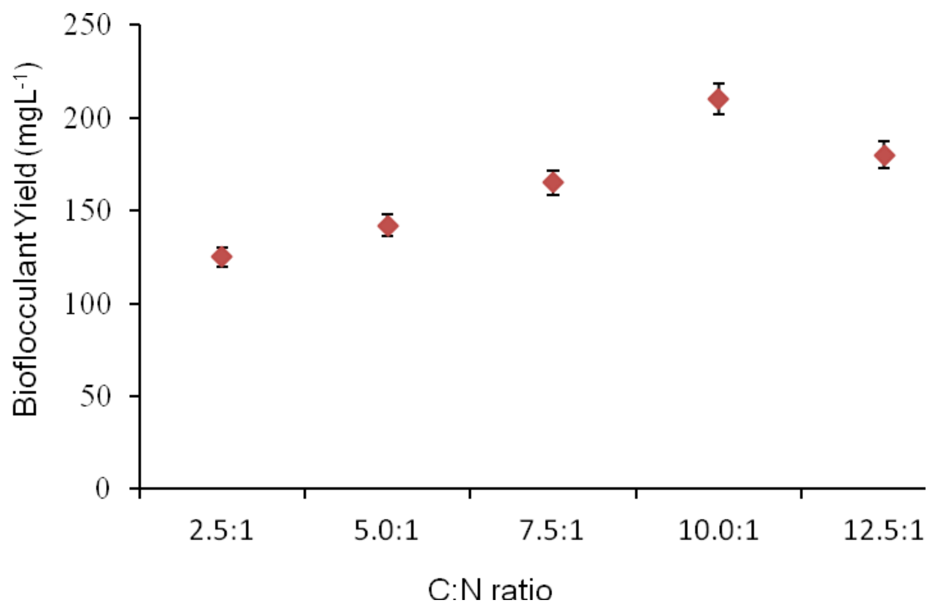


Fig. 4.22 Optimization of C:N ratio for bioflocculant production. Each bar represents mean ± SD of triplicate data

4.8.4 pH of culture medium and cultivation temperature

The effect of initial pH of the production medium on the flocculating activity and biopolymer synthesis in *K. terrigena* RD4 strain was investigated. The optimal initial pH for *K. terrigena* growth and flocculating activity was found to be 7.0±0.2 (Fig. 4.23).

The temperature also plays a dominant role in growth and associated metabolic activities of microorganisms (Ratkowsky et al., 1982; Cavari et al., 1981). To elucidate the influence of temperature optimal for bioflocculant production *K. terrigena* was incubated at a temperature range of 28°C to 42°C (Fig. 4.24). The optimal temperature for *K. terrigena* growth and flocculating activity was recorded at 30°C which was subsequently used in the present study. The bioflocculant production in *K. terrigena* is linked with its growth; therefore optimal growth at 30°C plays an important role biofloculant synthesis 30°C (221±9.1 mgL^{-1}).

4.8.5 Agitation speed and inoculum size

It was found that the shaking speed of 120 rpm/min was the most preferred shaking condition (Fig. 4.25). Either higher or lower shaking speed than 120 rpm/min caused a decrease in the flocculating activity,

When the inoculum size of the strain was 1% (4 log CFU/mL), the flocculating activity was up to 81.3% ± 3.9 (Fig. 4.26). A small inoculum prolong the stagnant time, whereas a large inoculum make niche of *K. terrigena* overlap excessively and restrain the bioflocculant production and hence low flocculating activity.

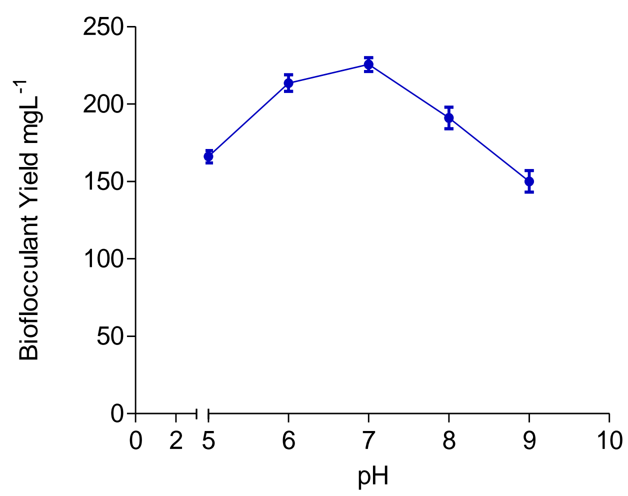


Fig. 4.23 Effect of culture pH on *Klebsiella terrigena* bioflocculant production

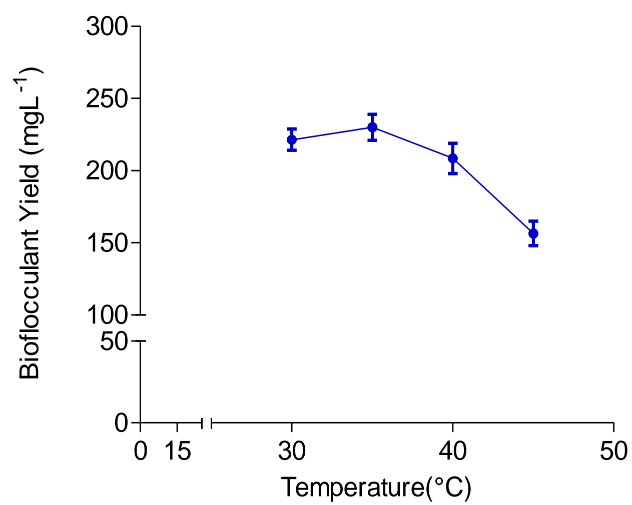


Fig. 4.24 Effect of incubation temperature on *Klebsiella terrigena* bioflocculant production

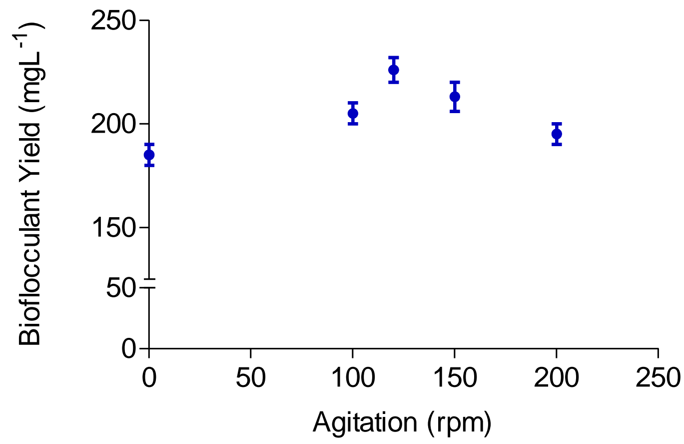


Fig. 4.25 Effect of agitation speed on *Klebsiella terrigena* bioflocculant production

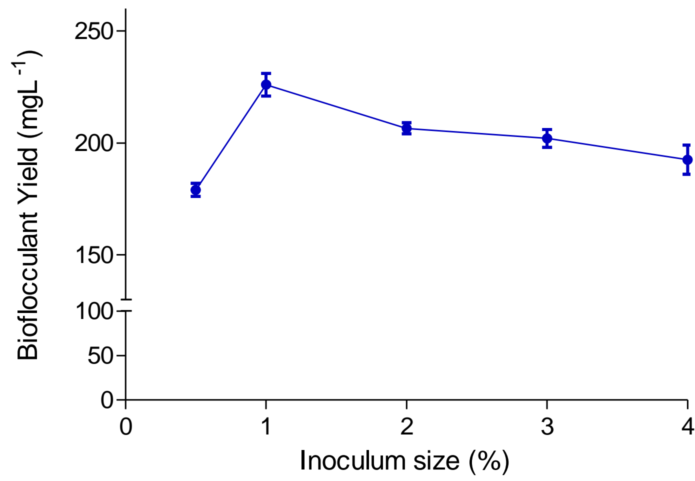


Fig. 4.26 Effect of inoculum size on *Klebsiella terrigena* bioflocculant production

4.8.6 Effect of metal ion(s)

The effect of various salts on bioflocculant production was examined (Fig. 4.27). The divalent ions like Cu^{2+}, Fe^{2+} and Mg^{2+} were found to be more favourable for *K. terrigena* bioflocculant production. Mg^{2+} showed the highest bioflocculant production among the tested salts. Conclusively the media composition, their concentation and other physical parameters was optimised for maximum bioflocculant production consists of: peptone 5 gL^{-1}, ammonium sulphate 0.5 gL^{-1}, yeast extract 1 gL^{-1}, $CaCl_2.2H_20$ 0.7 gL^{-1}, NaCl 0.1 gL^{-1}, $MgSO_4.7H_2O$ 0.3 gL^{-1}, K_2HPO_4 1 gL^{-1}, glucose 1 gL^{-1} and agar 3 gL^{-1}. KBF production was maximum when the strain was incubated at 30°C, 120 rpm at neutral pH 7.0. Under optimum conditions, the production of KBF was 476±3.5 mgL^{-1} with a flocculating rate of 93.4±2.7%.

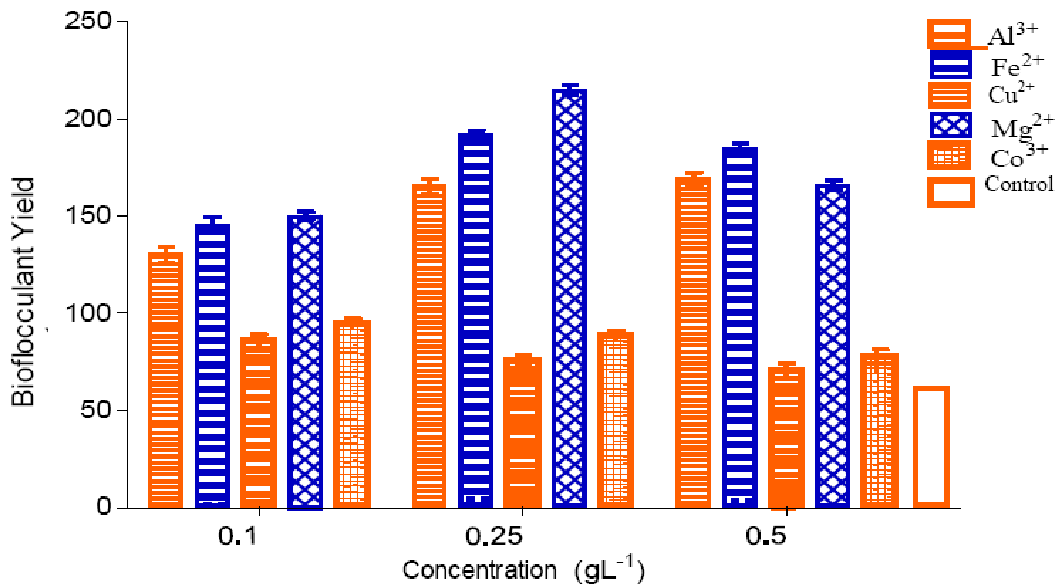


Fig 4.27 Effect of cations (Cu^{2+}, Fe^{2+}, Mg^{2+}, Al^{2+}, Co^{2+}) on *K. terrigena* bioflocculant production

4.9 Cellular response of *K. terrigena* RD4 to environmental modulators and its impact on flocculant exopolysaccharides

To have a better insight into the role of sugars in *Klebsiella terrigena* RD4 strain on its flocculant activity, a chemical mutagenesis using NTG, followed by ampicillin enrichment of *K. terrigena* was carried out to raise auxotrophic mutants (Huang et al., 2005). This approach allowed generating variant ($Glu^{-}Mal^{+}$) of *K. terrigena* RD4 strain, that had lost its glucose utilization ability. More than 1200 non mucoid putative mutants were screened for EPS biosynthesis using the lipophillic dye Sudan Black B. Amongst all non EPS producers, only one putative variant could be found that stained black with the used dye, which was subsequently selected from medium containing malate as carbon source. (Fig. 4.28). The mutant (designated as KTM1) could produce reduced amounts of EPS only after three days of incubation. Whereas the wild type of the test strain result in mucoid colonies with copious amounts of EPS with overnight incubation. The biopolymer of the mutant completely lacked flocculating activity (3.2±2.7%). It was therefore essential to characterise the polymer produced by the mutant to understand the cellular mechanism of biopolymers that possess flocculating ability.

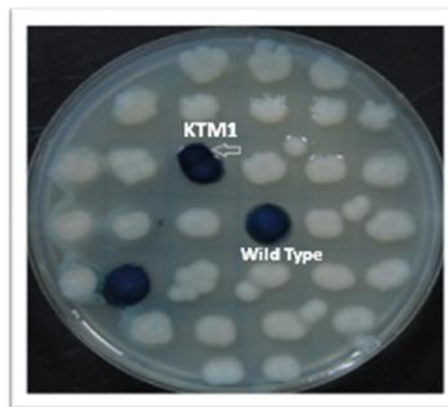


Fig. 4.28 Screening of glucose auxotrophic mutants. one variant KTM1 (indicated by an arrow) was Sudan Black positive amongst the negative that remained unstained

The results of compositional analysis as shown in (Table 4.9) and HPLC chromatogram (Fig. 4.29) revealed that the polymer produced by the mutant KTM1 that could not utilize glucose is probably different from that of the polymer produced by wild type strain. The HPLC

analysis of the acid hydrolysate of the bioflocculant of mutant type identified its constituent monosaccharides as D-Glc, D-Man, and D-GlcA. Exopolysaccharide of the variant lacked D-Gal in its polymeric chain.

Table 4.9 Compositional analysis of auxotrophic mutant KTM1

S.No.	Components	Composition (%)
1	Total sugar	67.8
2	Amino sugar	7.63
3	Uronic acid	10.28
4	Pyruvic acid	0.79
5	Protein	2.12
Elemental analysis		
6	Carbon	12.73
7	Hydrogen	1.01
8	Nitrogen	0.44

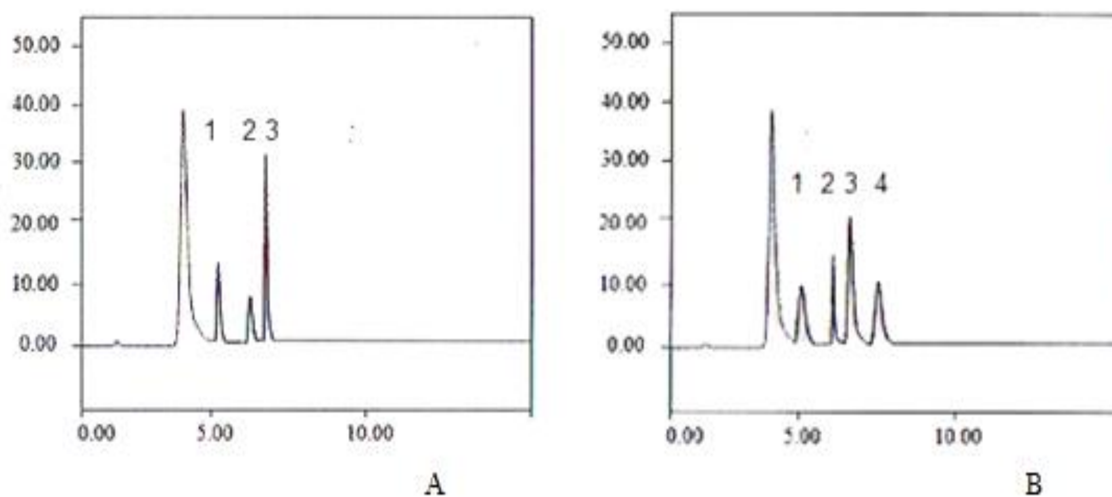


Fig. 4.29 High performance liquid chromatographic (HPLC) separation of acid hydrolysates of extracellular polysaccharides constituent monosaccharides. (A) *Klebsiella terrigena* mutant KTM1 1) Mannose 2) Glucuronic acid 3) Glucose. (B) Wild type 1) Mannose 2) Glucuronic acid 3) Glucose 4) Galactose

Both low water activity and oxidative stress are likely to be encountered by bacterial cells in the environment. Therefore the role of these two stressors in production of the biopolymeric flocculant by *Klebsiella terrigena* RD4 was examined. Initially experiments were conducted to ascertain viability of the cells to the stressors. Then a tolerable range of each stressor was chosen and both bioflocculant production and activity by the strain were determined. The experimental results indicate that hydrogen peroxide completely inhibited *K. terrigena* RD4 at 40 mM and 30 mM concentration; with 10 and 20 mM hydrogen peroxide concentration, *K. terrigena* RD 4 cells exhibited a prominent lag phase, the specific growth rates were not notably different (Fig. 4.30). Both flocculant production as well as floculant activity was not significantly different from control, where cells were grown in FIB without H_2O_2.

In medium adjusted to water activity values of 0.6 and 0.7, *Klebsiella terrigena* RD4 was unable to grow; the cells barely survived in medium adjusted to water activity value of 0.8. A higher water activity value of 0.95 supported growth of *Klebsiella terrigena* RD4 and the bioflocculant production as well bioflocculant activity did not differ significantly than the control (Fig. 4.31). It has been shown earlier that microorganisms have a limiting water activity level below which they will not grow. For *K. terrigena*, the limiting water activity value seems to be 0.95.

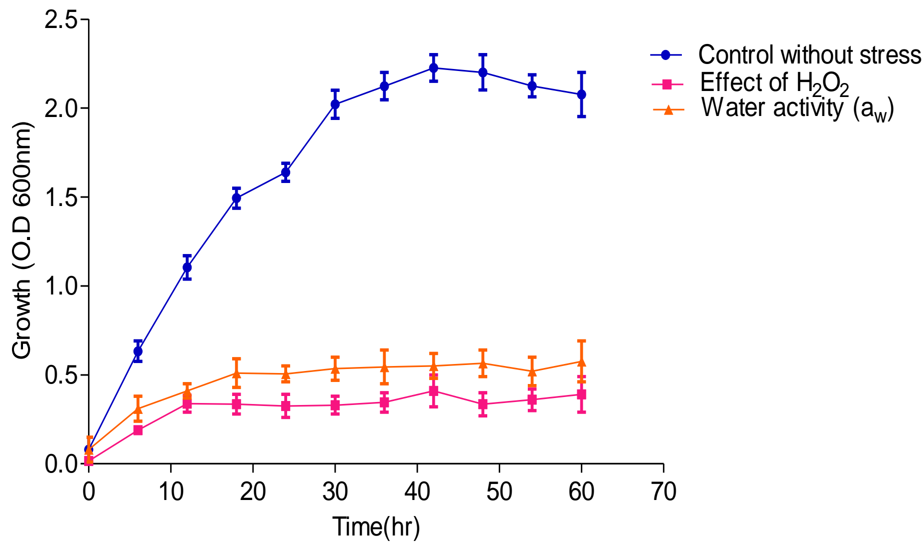


Fig. 4.30 Effect of H_2O_2 and water activity on growth kinetics of *Klebsiella terrigena*

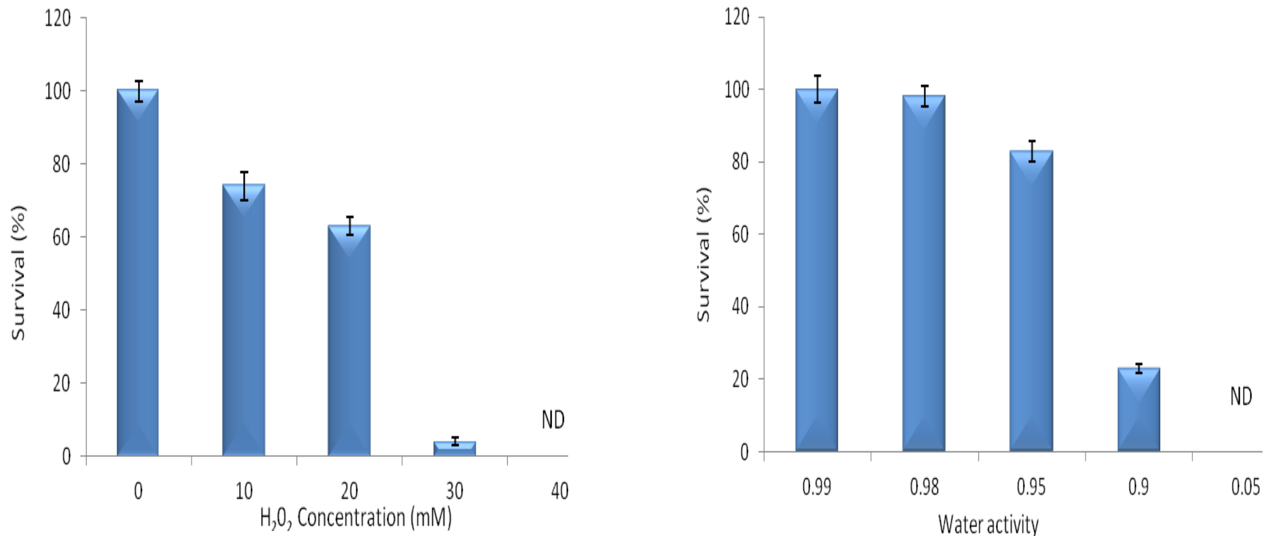


Fig. 4.31 Stress sensitivity of *Klebsiella terrigena* to H_2O_2 and water activity. The percentage of resistance of H_2O_2 and water activity data was calculated by comparison to the number of viable cells in FIB alone. Data represent mean values from three independent determinations. ND = Not detected

4.10 Simulated and real time evaluation of pathogen removal efficacy by bioflocculant

The high flocculating activity obtained with Kaolin by the KBF indicated size range 2–5μm, falls within the size range of bacteria, (Ghosh et al., 2009). It was therefore anticipated that this biopolymeric flocculant may be useful for removal of bacterial cells as well from waterbodies. The initial counts of spiked enteropathogens (*S. aureus* ATCC 9144, *E. faecalis* ATCC 35550) and *E. coli* DH5α in tap water samples are presented in table 4.10.

Table 4.10 Selective removal of *S. typhimurium* ATCC 23564 from mixed cultures in tap water by purified *Klebsiella terrigena* biopolymer

Microorganisms	Initial counts (log cfu/mL)	Final counts (log cfu/mL)
S. typhimurium ATCC 23564	3.0	A
S. aureus ATCC 9144	3.0	2.91
E. coli DH5α	3.0	2.93
E. faecalis ATCC 35550	3.0	2.98

Results are average of three independent trials. Each trial comprised of three replicates
A: none detected; Biopolymer for removal studies was dosed at 2 mgL^{-1}

Following addition of biopolymer and subsequent to the treatment process, aliquots of tap water samples were diluted in 0.1% MRD and suitable dilutions plated onto respective selective media as well as on TAL (Thin Agar Layer plates). The Thin agar layer method has been successfully used to recover injured pathogens from food and environmental samples (Wu et al, 2001).The presence of both rich medium as well as the respective selective medium helps in resuscitating the injured pathogen thus enabling accurate enumeration. Thus application of this method for assessing biopolymer treated water samples along with both selective and rich medium for pathogens provided a realistic estimation of residual pathogens.

Significant reduction ($P<0.5$) in numbers of *Salmonella* (3 log reduction) were observed in both selective and TAL plates within a period of 30 mins and an optimum dose of $2mgL^{-1}$ (Table 4.11). Counts of *S. aureus*, *E. faecalis* and *E. coli* DH5α remained unaltered.

Table 4.11 Optimization of dose of purified *Klebsiella terrigena* biopolymer for flocculating *S. typhimurium* ATCC 23564, spiked in tap water

Biopolymer dose (mgL^{-1})	Initial Counts[a] (log cfu/mL)	Final counts (log cfu/mL)
0. 1	3.0	2.65
0.8	3.0	2.41
1	3.0	2.0
2	3.0	B
4	3.0	2.98
6	3.0	2.97
10	3.0	3.0

Results are average of three independent trials. Each trial comprised of three replicates
a: optimization was carried out with 3 log cfu/ml *Salmonella typhimurium;*B: None detected

4.10.1 Selective removal of *Salmonella sp.* from the mixed cultures by KBF demonstrated by Fluorescent *In Situ* Hybridization (FISH)

After the mixed cultures in tap water was treated with the biopolymer, visualization and simultaneous detection of the biopolymer matrix was carried out directly, using FISH in combination with CLSM. Hybridization with probe EUB338, which is complementary to a portion of the 16S rRNA gene conserved in the domain bacteria, was used to visualize the entire bacterial population in the specimens (Amann et al., 1990). Also all detected cells stained with the DNA intercalating dye DAPI demonstrated that they were permeable to probes and therefore good candidates for *in situ* hybridization. Also by using the control probe non EUB338 and DAPI staining, non-specific binding was excluded between the probe and constituents of the cellular matrix, which gave confidence to the results achieved; in addition, autofluorescence was

excluded by viewing the sections prior to the FISH procedure. Repeated microscopic evaluation confirmed the unambiguity of the interpretation of the images obtained.

Hybridizations with probe EUB338 and the -proteobacterial probe GAM42a resulted in strongly positive fluorescence hybridization signals for *E. faecalis* and *S. typhimurium* (Fig 4.32 A,B,C). The more specific probe *Sal*3, which binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (excepting only subspecies IIIa) (Fang et al., 2003), was then used to verify the presence of *Salmonella* in the biopolymeric matrix. Sensitivity testing showed that the probes were able to detect as low as 10^3 cfu/mL. Fig. 4.32 demonstrate that with the universal eubacterial probe EUB 338-Cy5, bacteria could be detected by FISH, the observed bacteria were located within the bioflocculant matrix; FISH positive signals obtained using simultaneous EUB338-Cy5 and *Sal*3-Cy3 oligonucleotide probe signals revealed distinct morphotype of small bacterial rods, detected as *Salmonella*. Phase contrast microscopic analysis allowed 3-dimensional reconstruction of the biopolymer matrix and confirmed that the cells observed were bacteria; the spatial distribution of the bacteria was also evident. The organisms were located enmeshed on the surface and were also visible within layers of the biopolymer. FISH of *E. faecalis* ATCC 35550 was carried out by using ENF191–Fluos probe with EUB338-(Cy5). On hybridization with probe EUB338, no hybridization signals of the active bacterial population were observed in the fixed biopolymer specimen. Before fixation, the sample was subjected to flocculate *E. faecalis* (10^7 cfu/mL) in tap water. Although hybridization with probe ENF191 was successful in the tap water sample without biopolymer addition as shown in fig. 4.32. FISH positive signals for the test organism were visualized as light blue in the unflocculated control water sample. This clearly indicated that the biopolymer possessed specific

binding affinity for the cells of *Salmonella* spp., although taxonomically both the organisms belong to same family and subclass.

The results of *Salmonella* removal through direct plating on selective media were confirmed by the TAL method, which can resuscitate injured microorganisms exposed to varied environmental matrices, by simultaneously enriching them as well as providing selectivity, thus allowing adequate enumeration of cells. Absence of *Salmonella* in both cases confirmed complete removal of the cells. To further elucidate the observations, FISH and CLSM of fixed biopolymer samples were carried out. The bright signal intensities of the bacteria indicated a high amount of rRNA, which is an evidence for physiological activity of the cells at the time of sampling (Wallner et al., 1996; Montgomery and Kirchman, 1994). CLSM, which was carried out in conjunction with FISH allowed optical sectioning and 3-dimensional reconstruction for localization and observation of the spatial distribution of bacteria in different layers of the bioflocculant material.

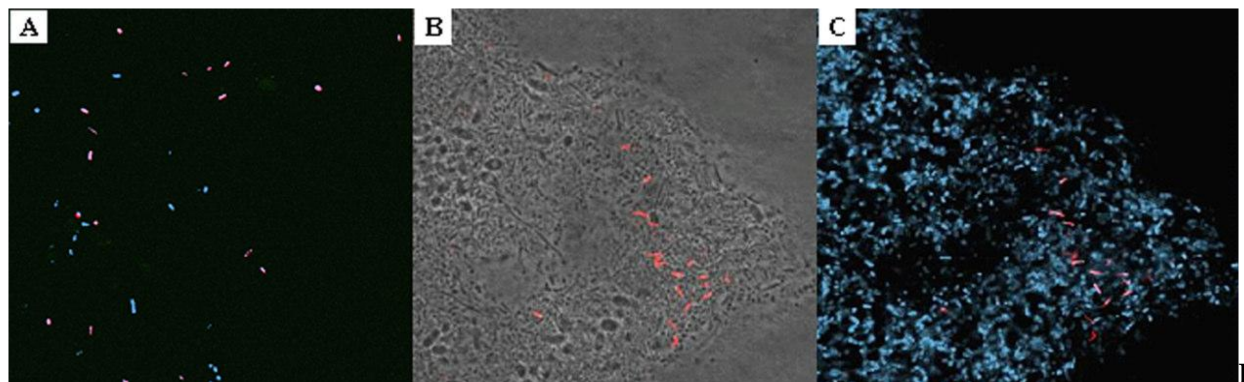


Fig. 4.32 Confocal laser scanning micrograph of FISH of the mixed cultures.. Hybridization with oligonucleotide probes ENF191-FLUOS, *Sal*3-Cy3 and Eub338-Cy5. Excitation wavelengths: 488 nm (FLUOS), 543 nm (Cy3), and 633 nm (Cy5) for green, red and infrared fluorescence, respectively. Signals displayed as an rgb image. *E. faecalis* appear in light blue, *S. typhimurium* in pink; (B) & (C). Phase contrast and Confocal laser scanning micrographs, respectively of FISH of the sample showing selective binding of *Salmonella* to biopolymer. Phase contrast and CSLM analysis of agglomerated biopolymer matrix after FISH with *S. typhimurium* have aggregated on the biopolymer matrix. Hybridization with oligonucleotide probes Eub338-Cy5 and *Sal*3-Cy3 excitation wavelengths were 543 nm (Cy3), and 633 nm (Cy5), red and infrared fluorescence, respectively. Signals displayed as an rgb image by overlay of infrared fluorescence in both channels, blue and red. The signals of the *S. typhimurium* appear pink. Measure bars-5 µm

4.10.2 Removal of *Salmonella* sp. from poultry wastewater

The removal efficiency of KBF on *Salmonella* from poultry wastewater was evaluated. The total aerobic counts of the water samples from wastewater from an unorganized poultry processing unit were 7.3 log cfu/mL whereas counts of *Salmonella* were 3.0 log cfu/mL. The optimum dosage of the *K. terrigena* bioflocculant was 2 mgL^{-1} for achieving a removal of 80.3±2.8% from the initial number of 3.0 log cfu/mL *Salmonella* spp. in poultry water. However, the removal of *Salmonella* spp. by the bioflocculant at a dosage exceeding that of 2 mgL^{-1} remained unaltered (Fig. 4.33). The high removal of *Salmonella* spp. was noted within a period of 30 min (Fig. 4.34). On the other hand, a mean removal efficiency of 47.5% (±2.44) was achieved by alum (10 mgL^{-1}), used for comparison in this study (Table 4.12). Thus, *Salmonella* removal was not only significantly low but a higher dose (10 mgL^{-1}) of the synthetic flocculant was required.

Table 4.12 Comparison of flocculation efficiency between bioflocculant and alum for removal of *Salmonella* from poultry wastewater

Flocculant used	Initial *Salmonella* counts	Mean removal efficiency*
K. terrigena bioflocculant[1]	2.1×10^3	80.3 % (±2.21)
Alum ($Al_2(SO_4)_3 \cdot 18H_2O$)[2]	2.1×10^3	47.5 % (±2.44)

[1]Dosed at 2 mgL^{-1}, [2]Dosed at 10 mgL^{-1}, *Results represent the average of three independent trials ± represent standard deviation from triplicate data

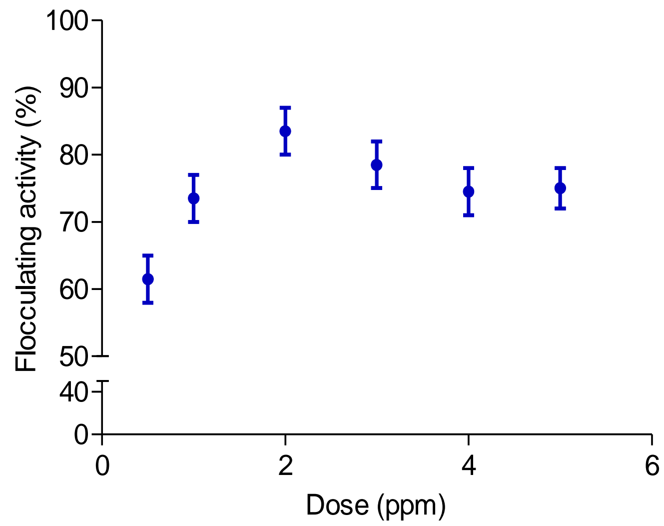


Fig. 4.33 Optimization of dose of purified *Klebsiella terrigena* biopolymer for flocculating *Salmonella sp.* from poultry wastewater

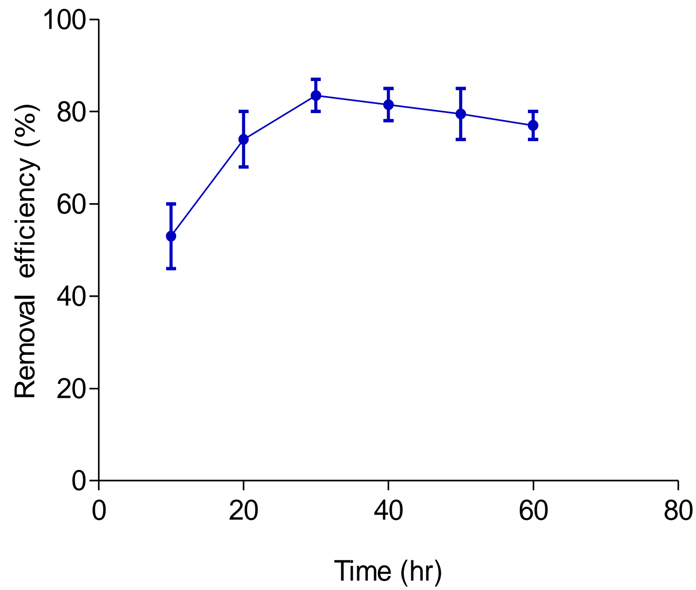


Fig. 4.34 Optimization of time for maximal removal of *Salmonella sp.* by *K. terrigena* flocculant (KBF)

4.10.3 FISH for the visualization of bacteria agglomerated by the bioflocculant, detection and removal of *Salmonella* from poultry wastewater

After the poultry wastewater was treated with the bioflocculant, visualization and simultaneous detection of *Salmonella* spp. in the bioflocculant matrix was carried out directly, using FISH in combination with CLSM. Fig. 4.35 demonstrate that, with the universal eubacterial probe Eub 338-FLUOS (green), co-aggregated bacteria located within the bioflocculant matrix could be detected by FISH; bacteria of different morphologies could also be detected with DAPI staining in the treated bioflocculant sample. In addition, a distinct morphotype of small bacterial rods were detected as *Salmonella* with the *Sal3*-Cy3 oligonucleotide probe in a manner similar to as decribed in the previous study on selective remval of *Salmonella* fro mixed culture.

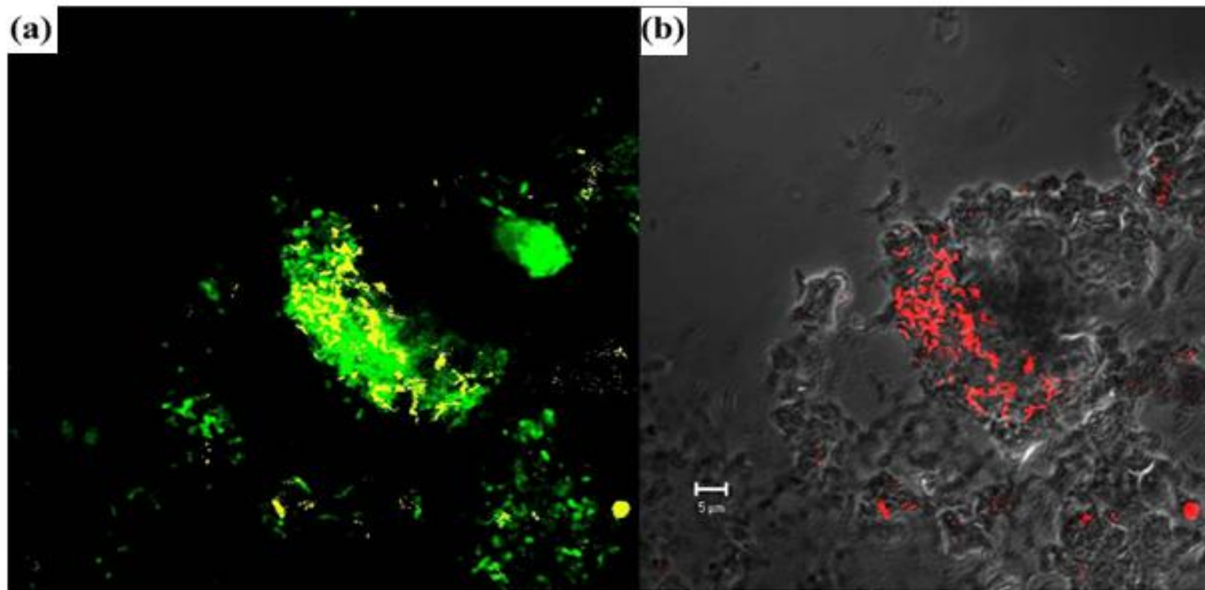


Fig. 4.35 Confocal laser scanning micrograph of FISH of the sample showing distribution of cells in poultry-processing wastewater within biopolymeric flocculant. (a) The image shows a flocculant-treated poultry-processing wastewater sample hybridized with the probe Eub338 labelled with FLUOS (green, targeted for the domain bacteria) and with *Sal3* labelled with Cy3 (red, targeted for the *Salmonella*). The dual combination of the red (Cy3) and the green (FLUOS) colours results in yellow. (b) The image shows specific detection of *Salmonella* hybridized with *Sal3*-Cy3 oligonucleotide probe in the biopolymeric matrix. Measure bars in (a) represents 10 µm and (b) represents 5 µm

4.11 Removal of *Cryptosporidium* oocysts

The removal of *Cryptosporidium* in the spiked samples of water was done. Since no high molecular weight flocculant of natural origin have been reported to remove *Cryptosporidium* oocysts, the removal efficiency was compared with aluminium sulphate. The dose of KBF was 2 mgL^{-1} for achieving removal of 62.3% (S.D. ±2.8) for 1 X 10^6 seeded oocysts, by pH adjustment to 5.4 to 5.8 (Table 4.13). Increase in KBF concentration increased the removal of *Cryptosporidium* oocysts till a concentration of 2 mgL^{-1} (Fig. 4.36). Removal efficiency of oocysts by the bioflocculant at a dose of 2 mgL^{-1} remained unaffected at pH values of 6, 7.2 and 8 respectively (Table 4.14).

Table 4.13 Comparison of removal of *Cryptosporidium parvum* oocysts

Flocculant	Number of seeded oocysts[a]	Mean recovery*
K. terrigena bioflocculant	1 X 10^6	62.3% (±2.28)
$Al_2 (S0_4)_3$	1 X 10^6	62.5% (±2.44)

* Five independent trials, [a] Oocysts were counted by Nebuarer cell counter. Seeding was done in 1 L of tap water in each case

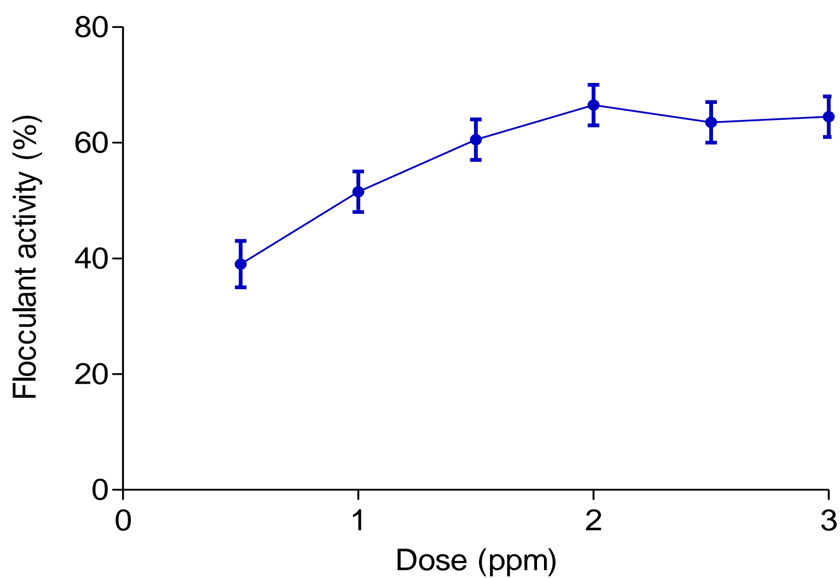


Fig. 4.36 Dose optimization for removal of *Cryptosporidium* oocysts

Table 4.14 Optimization of medium pH for the removal of *Cryptosporidium parvum* oocysts by bioflocculant

pH	Number of seeded oocysts[a]	Mean removal efficiency*
6	1 X 10^6	61.9% (±2.21)
7.2	1 X 10^6	62% (±2.16)
8	1 X 10^6	61.6% (±2.44)

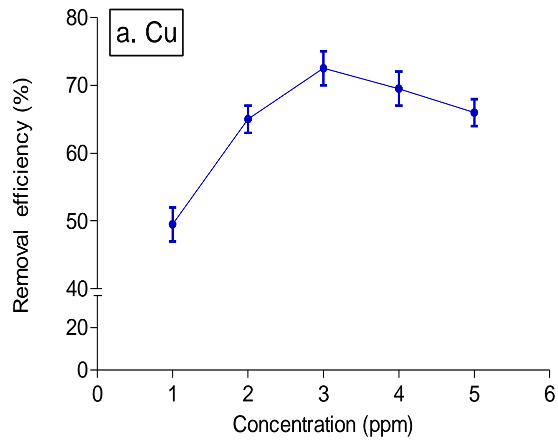
* Five independent trials.
[a] Oocysts were counted by Nebauer cell counter. Seeding was done in 1 L of tap water in each case

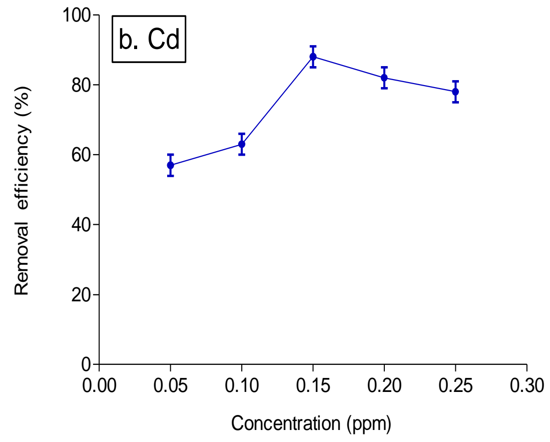
4.12 Flocculation of some heavy metals

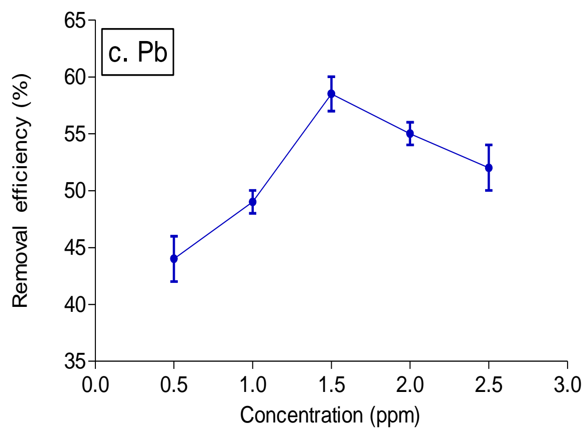
The potential of KBF in the removal of some heavy metals (cadmium, copper, lead and zinc) in water samples was evaluated. The highest flocculating activity of Cu at metal concentration of 3.0 ppm (71%±3.6), Cd at a concentration of 0.15 ppm (84%±3.1), Pb at a concentration of 0.01 ppm (64%±2.3) and Zn at a concentration of 1.5 ppm (57%±4.2) was observed (Fig. 4.37 a-d). Metal ion concentration plays an important role in determining the metal removal by KBF. It was observed that an increase in the initial metal concentration resulted in an increase in the metal removal, which culminated in a plateau at high metal concentration.

Dose optimization results revealed that about 2.5 mg/L of KBF were sufficient to remove these heavy metals from water samples. As observed during the study on the effect of varying KBF concentrations, with initial low concentration, the rate of adsorption was low (Fig. 4.38).

Removal efficacies of the biopolymers were not altered within a temperature range of 28-45°C and a pH range of 5-8 (Fig. 4.39; Fig. 4.40). At higher pH, metal ions are precipitated which decreases the amount of adsorbed metals. Increase of incubation time after 6 hr did not affect metal flocculation significantly ($p>0.0.5$) (Fig. 4.41). 65-70% of heavy metals were adsorbed from the solution within the first 2 hr of the treatment. Subsequent 2 hr of contact resulted in a further adsorption of 10-15%. There was a further increase in metal adsorption by 10-15% in the next 2 hr.







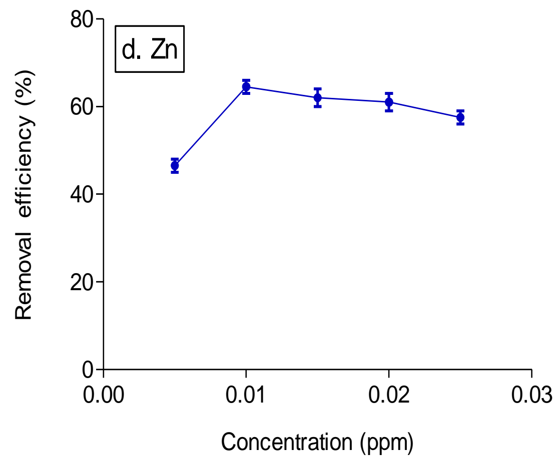


Fig. 4.37 (a-d) Heavy metals (Cu, Cd, Pb, Zn) removal by bioflocculant

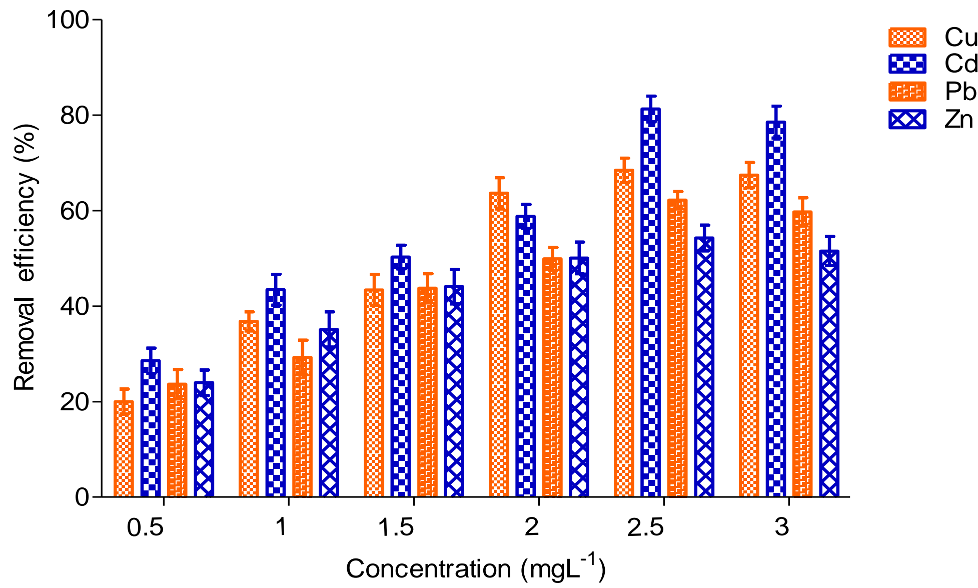


Fig. 4.38 Optimization of dose of bioflocculant for removal of heavy metals (Copper, Cadmium, Lead, Zinc) from aqueous solutions

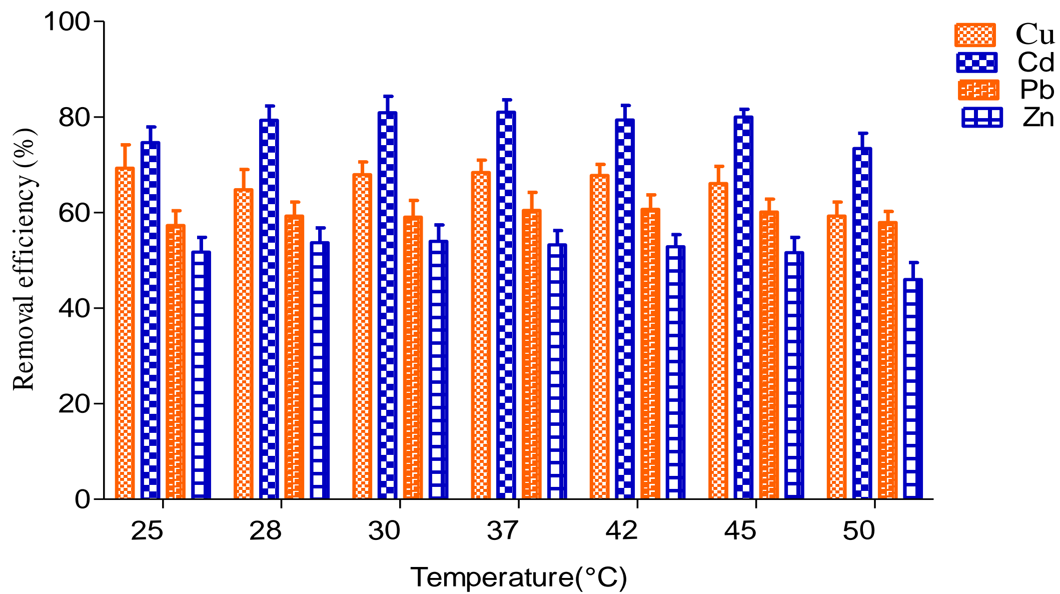


Fig. 4.39 Effect of incubation temperature on removal of heavy metals (Copper, Cadmium, Lead, Zinc) from solution by *Klebsiella terrigena* bioflocculant

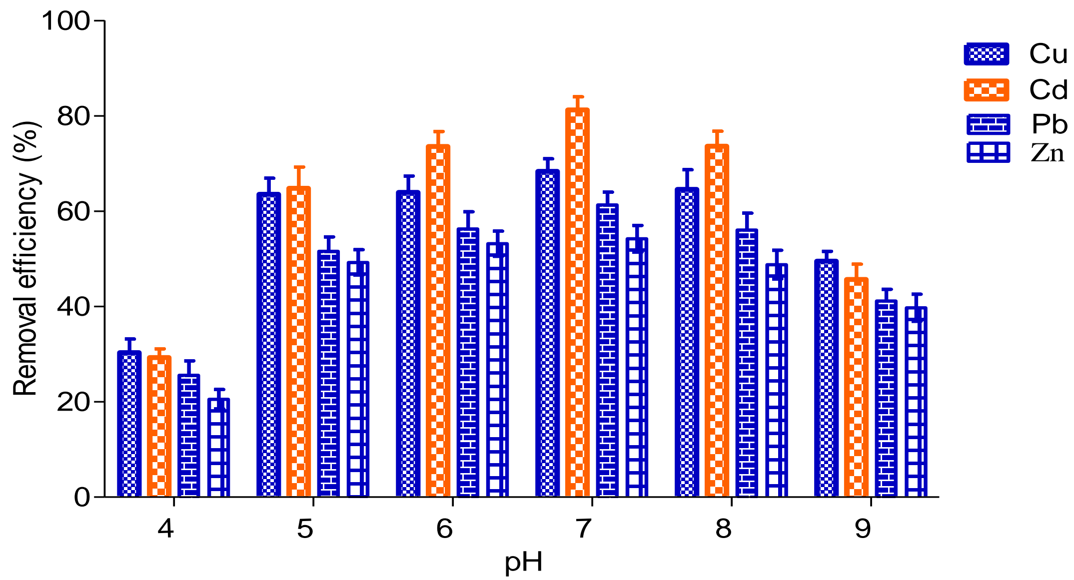


Fig. 4.40 Effect of medium pH on removal of heavy metals (Copper, Cadmium, Lead, Zinc) from solution by *Klebsiella terrigena* bioflocculant

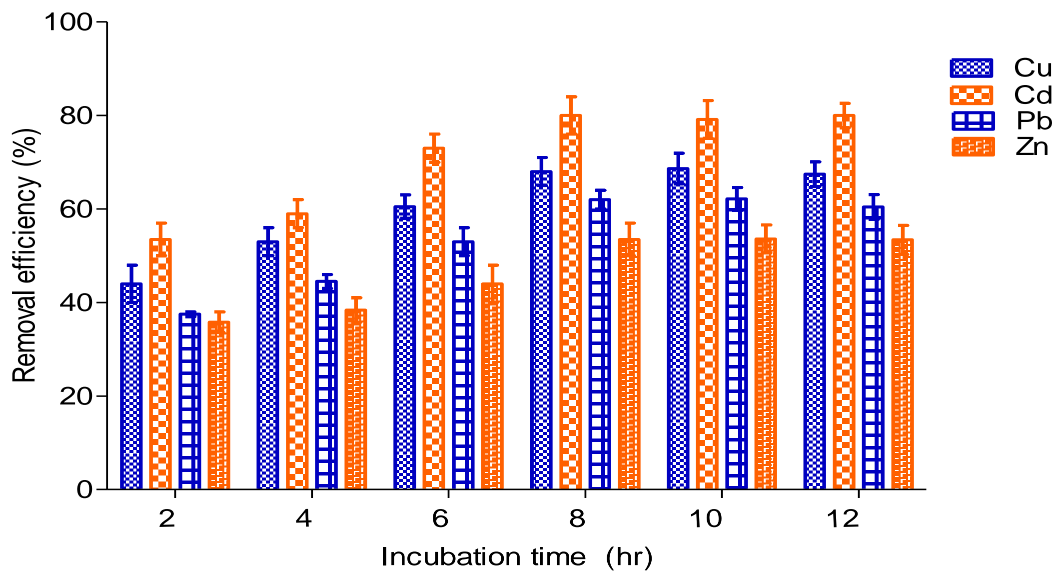


Fig. 4.41 Effect of incubation time on removal of heavy metals (Copper, Cadmium, Lead, Zinc) from solution by *Klebsiella terrigena* bioflocculant

Chapter 5

Discussion

Discussion

In view of the crucial need for efficient and robust microbial flocculant, bacterial strains were screened from diverse environmental locations which included activated sludge samples from industries. Natural processes of indigenous agglomeration and autoflocculation are predominant in activated sludge (Tenney and Verhoff, 1973) therefore they are considered as ideal sources for isolating and screening exopolymer producing bacteria.The 632 bacterial strains initially isolated from sludge samples were based on their mucoidy nature exhibited on solid nutrient plates. Generally, the extent of mucosity observed in colonies are correlated to their ability to produce exopolysaccharides, while this may be true in several instances, the flocculating ablity for 43 bacterial isolates wasw notable but could not correlated to the degree of mucosity. The surface of gram-negative bacterial cells is covered with a variety of carbohydrates. These carbohydrates, including most prominently the extracellular polysaccharide (EPS) may either be in the form of a capsule or a free slime or both and have been mostly correlated to mucoidy apprearance of bacterial colonies on solid nutrient media. Exopolysaccharides produced in higher quantities especially in broth cultures are usually dissociated from the the producer cells. It is therefore appropriate to analyze the cell free supernatants of broth cultures for desired exopolysaccharides.

The cell free culture supernatants of the bacterial isolates were therefore presumed to contain the exoploymeric flocculant and subsequent investigations to locate the flocculating principle indicated it to be primarily extracellular and occur in the culture supernatants. This finding was similar to previous studies which reported flocculants to be primarily extracelluar (Salehizadeh and Shojaosadati, 2001) in case of microorganisms. An important prerequisite for applicability of microbial flocculants is their ability to retain functionality under environmental

upheavals of temperature and pH. Culture supernatants of the bacterial isolates subjected to different temperatures and pH exhibited variations in the flocculating rates. The variation in flocculation rate may imply a variation in chemical properties or chemical composition (Deng et al., 2003) of the flocculants produced by different strains. The preceding studies was used to shortlist 11 isolates with high flocculating rates and were further examined for the ability to remove pathogenic bacteria, parasitic protozoans and heavy metals. Finally the strain selected for subsequent studies possessed the desired set of attributes namely high flocculating activity, and ability of flocculant to remove pathogens and selected heavy metals. Both biochemical and molecular methods subsequently characterized the strain as *Klebsiella terrigena.*The *Klebsiella* sp has documented evidence for exopolysaccharide production upon growth on a variety of sugars ; production and composition of exopolysaccharides in members of *Klebsiella* strains studied has been reported to be a function of strain, carbon source and growth stage(Bryan etal, 1986). The high rate of flocculation, pathogen and heavy metal binding ability observed in the strain of *K.terrigena* was unique amongst flocculant producing strains of *Klebsiella* studied thus far.

Growth kinetic studies in batch culture offer a simple and convineint method for studying the relationship of culture age and extracellular metabolites produced by microorganisms. A batch kinetic study carried out with *K.terrigena* RD4 using the screening medium FIB (Flocculant Isolation Broth) provided information on both flocculating activity as well as bioflocculant synthesis. As opposed to an increase in flocculating rate from mid log phase onwards a decline in flocculating rate was observed during later phase.It is possible that the excess biopolymer production led to a poor dispersiblity that could not expose all its binding sites (Roussey, et al., 2004). Thus the nonavailablity of such sites to colloidal particles reduced

the flocculating rate. According to Li et al. (2007) concentrated solution of high molecular weight biopolymeric flocculant showed low flocculating activity because of the presence of charged groups of the biopolymer in close proximity. These charged groups create either an intra molecular attractive or repulsive forces which act upon already bound colloidal paricles. Finally the latter get dislodged from their binding sites. Interestingly even a decrease in the amount of polymeric flocculant was observed after the culture was incubated beyond 60 hours. The possible reason for decrease in the polymer amount could be as cells had reached the decline phase where the dead biomass might have undergone autoflocculation with its own extracellular polymeric flocculant, resulted in the loss the biopolymer (***Tenney*** and Verhoff, ***1973***). Hence it could not be recovered in the same or in higher amount from the cell free supernatant after 60 hours.

Flocculating activity is regarded as a function of the quantity of flocculant or dosage. However dosage exceeding that of 6 mgL^{-1} decreased the flocculating activity in case of *K.terrigena* flocculant. This could be due to over-saturation of several binding site of kaolin surface particles which were reduced leading to a decrease in flocculating activity (Kwon et al., 1996). The depression in flocculating activity at high concentration could also be due to incomplete dispersion of excess bioflocculant (Suh et al., 1997). Thus, either the deficiency or excess amount of bioflocculant and kaolin clay decreased the flocculating activity (Lee et al., 1995) which indicated KBF to be an effective flocculating agent with low dosage requirement. Both dosage and stability of KBF could be compared favorably with flocculant produced especially by *Bacillus* sp DYU1 and thus seemed worthy for further studies especially in terms of functional modulators.

Cations are the most frequented functional modulators of flocculants in suspensions. Evidence of positive effects of cations on flocculation with microbial flocculants has been

documented in earlier studies (Kumar et al., 2004). Cations stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups due to bridging formed between particles. However, the stimulating effect of the added cations on flocculation is highly dependent on both the concentration and valency of the ions (Wu and Ye, 2007; Yokoi et al. 1995). Modulation of bioflocculant activity by medium pH has been noted by several researchers (Gao et al., 2006; Oh et al., 2001; Li et al., 2007) thus stablity studies at different medium pH is important for predicting desired flocculating activity of flocculants. A decrease in the flocculant activity in acidic solution (below pH 6) might be either due to the glycosidic bonds in the polysaccharide chain being hydrolyzed; it is equally probable that at low pH, both bioflocculant and kaolin particles adsorb hydrogen ions (H^+), thus weakening the formation of complexes between KBF molecules and kaolin particles mediated by Ca^{2+}. Similarly, in basic solutions either hydroxide ions (OH^-) interfere with the combination of the flocculant molecules and kaolin particles at high pH, resulting in lower flocculating activity. In basic solutions beyond a pH value of 9 the flocculant activity decreases due to alkaline degradation of the polysaccharide causing several changes like molecular rearrangement of its residue or fragmentation of the polysachharide chain (Aspinall, 1982). Moreover, the mediating effect of Ca^{2+} is the strongest at neutral pH values (Li et al., 2008) and may account for the observed stability of the KBF. An extrapolation of these results suggests bridging to be important in flocculating kaolin particles by KBF and account as well for the stability of KBF in solutions.

Temperature is an intrinsic factor used in seperation processes and therefore one of the essential modulators of flocculating activity. The structural changes occurring upon increasing temperatures may be deemed to affect the overall structural configuration of polymeric flocculants thereby resulting in differential activity. Few studies have in fact, attempted to

elucidate the mechanism of thermal degradation in biopolymeric flocculants.However based on existing interpretations of biopolymer stability under varied thermal conditions, the ability to form helical aggregates may be assumed probable; such a condition supposedly protects the repeating acetal linkages of the polymer backbone(Glass etal, 1983). The observed thermal stability of KBF maybe explained on the foregoing discussions.The thermal stability of other bioflocculant by virtue of the polysaccharide backbone have been reported recently(He et al., 2009).

Colloidal particles are fundamental to the activity of bioflocculants. Colloidal particles [~0.5-0.8µm (bacterial cell suspension), ~4 µ -7 µ (Kaolin, yeast cell suspension, cellulose), ~15 µ-~20 µ (silica), ~100 µ (Active carbon)] were broadly differentiated into very low, low, medium and large colloidal sizes respectively in this study. Besides, all particles were mixed in equal amount for better insights of the performance under realtime condition. The most effective flocculation occurred in suspensions comprising very low and low sized particles at even low concentration of the bioflocculant. With suspensions comprising of medium sized particles flocculating activity ranged from 30-40% that too at relatively higher concentration of 8-10 ppm of the bioflocculant. The suspensions of large sized particles showed 50-65% flocculant activity at the same concentration range that flocculated in the range of 70-90% in case of low and very low sized particle suspension.This might be due to non uniform distribution of pore size on the bioflocculant surface. It is also possible that the number of sites available for binding low and very low sized particles might be more than that of binding medium and large sized particles.

Surprisingly, as compared to super turbid solution i.e., above 1000 NTU the flocculant activity was lower in suspension with very low turbidity (< 80 NTU), especially in the suspension comprising very low and low sized particles. An insufficient binding with the

particles due to very low number of collisions required to initiate flocculation by the biopolymeric flocculant may be attributed to this effect. However, flocculation was observed to be effective even at lower tubidity (< 80 NTU) of suspension samples with large sized particles.On the other hand, removal by the bioflocculant for lower particle size was drammatically high as compared to its synthetic counterpart. Also the relationship between the spectrum of flocculating activity corresponding to the turbidity removal was successfully established. The measurements of the residual levels of turbidity referred to approximately equal rate of flocculating activity.These observations also indicate that flocculation is probably responsible for destabilization of negatively charged colloids by polyanions, and conditions for optimum flocculation occur when a certain fraction of the available adsorption sites on the particle are occupied by polymer segments (Gregory and Sheiham, 1974).

Studies' involving structural elucidation of the exocellular polymeric flocculants has revealed crucial information regarding the activity of flocculants, however in most cases a complete structural determination, requires time as well as sufficient quantities of purified biofloccculant. The low amount of KBF hindered a complete elucidation of structure in this study.The surface morphology is an valuable structural feature; besides, data obtained from surface topology is important in predicting the behavior of the biopolymers in interfaces and within a natural environment to a certain extent (Levy et al., 2004). Scanning electron micrographs of purified KBF enabled acquisition of vital data especially small pore structure considered important for the compactness of the polymer and the maintenance of the textural properties during storage. An extremely small pore size distribution may enable the web structure to have much higher capillary forces to retain water in the gel (Kumar et al., 2004). The varied distribution of pores as observed in KBF may be responsible for flocculating wide range

of colloids as discussed previously.The importance of scanning electron microscopy in Ultra structural analyses of purified biopolymers in clearly understanding of the surface morphology of purified biopolymers have been suggested in recent studies(Xiong et al.2010).

It has been observed that high molecular weight flocculants form stronger flocs compared to lower molecular weight alternatives. An explanation to this effect lies in the molecular weight of a flocculant, which in turn is related to the chain length of the polymer and is an important factor in the flocculating reaction. A large molecular weight flocculant usually has long chain length and has a sufficient number of free functional groups, which can act as bridge to bring many suspended particles together and hence cause a larger floc size in the flocculating reaction (Shih et al., 2001). The observed high flocculating activity of KBF may be correlated to its high molecular weight (>260Da); the enhanced flocculation by higher molecular weight biofloculants have been demonstrated by several investigators (Salehizadeh and Shojaosadati, 2002; Li et al., 2009; Suh et al., 1997; Xiong et al., 2010).

Key to the activity of the flocculant lies in its functional groups, which intercat elctrostatically with colloidal particles (Kumar et al., 2004). Fourier transform infrared spectroscopy (FTIR) has been an useful tool in identifying the functional groups and monitoring structural changes in (Yu et al., 2005) the biopolymer structure(Cetin and Erdincler, 2004).The presence of carboxyl groups visualized in the FT-IR spectra of KBF may serve as binding sites for divalent cations (Bramhachari et al., 2007). Further, the spectrum showed the presence of carboxyl, hydroxyl, and amine groups, are the preferred groups for the flocculation process similar to that observed in polyelectrolyte (Zajic & Knetting, 1970). The hydroxyl group present within the polysaccharide had the possibility of hydrogen bonding with water molecules. These

observations explain the polymers ability to imbibe water, swell, and even dissolve partially or completely with uniform dispersibility.

Extracellular polymeric substances produced by microorganisms generally have ***heteropolymeric*** composition (Kumar et al., 2007).Thus the biopolymer may be composed of nucleic acids, proteins or lipids, in addition to polysaccharides. It is essential therefore to know the chemical component important in imparting the flocculant activity. For ascertaining the latter KBF was subjected to treatment with proteases, lipases ad nucleases and treated samples examined for flocculant activity.However, elimination of associated protein, lipids or nucleic acids did not alter the flocculating activity suggesting their non involvement in flocculation mechanism by KBF.The polysaccharide chain itself may be unbranched or branched with side chains of other compounds attached to the polymeric chain (Sutherland, 1977). Generally, the polysaccharides are made of monosaccharides – with hexoses and pentoses forming the bulk of biopolymer. Compositionally KBF is rich in hexoses like glucose, mannose and galactose. Such variations in monomer composition can alter the properties of polymer. The presence of sugars like galactose in the chain, aid in cell aggregation in bacteria (MaCleod et al., 1995). However, the contribution of different monomers to the total polysaccharide varies with the source and such variations in the polysaccharide chain composition can alter its physicochemical properties (Wang et al., 2007).

This observation correlates with the recent findings that the most effective bioflocculants were polysaccharide-like substances (Deng et al., 2003; Taniguchi et al., 2005). Although the nonsugar components are present in relatively smaller quantities, they are generally attached to the sugar residues and are important in imparting unique characteristics to the biopolymer. The KBF was polyanionic due to the presence of either uronic acids (either D-glucuronic acid or D-

galacturonic may be present). Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status (Sutherland, 1990). The uronic acid may constitute up to 20–50% of the polysaccharide fraction. The uronic acid and other moieties, including pyruvates and acyl groups gives an overall negative charge to the polymer, thereby imparting binding and adsorptive properties to the polymer (Smidsrod, 1974). Furthermore, the presence of pyruvates and acyl groups prevents the epimerization of uronic acid, thereby ensuring higher content of uronic acid in the polymer (Decho, 1990).

The composition and structure of the polysaccharides determines their primary conformation. In a polysaccharide structure, monomeric units are linked together either in branched or non branched form, it is important to understand the linkage between the monomeric units in order to decipher the complete structure.A simple method to achieve this utilizes the binding ability of the fluorescent dye Calcofluor white. Also referred to as a 'fluorescent probe', calcofluor white binds specifically by hydrogen bonding to ß (1-4) and ß (1-3) glycosidic bonds in polysaccharides(Bar-Or and Shilo, 1987), the inability of calcofluor white to bind to KBF indicated that the composition in the polymeric chain may be similar to that of galactoglucans also reported in many other Gram negative bacteria (Ruberg et al., 1991; Zevenhuizen and Ebbink, 1974). Generally, polymers, with the backbone composition of sequences of 1, 4-β or 1,3-β linkages confer considerable flexiblity (Meisen et al., 2008) and has been ascribed as better flocculants (Vanhooren et al., 1998).

The behavior of polymers in solution is probably one important characteristic wehich cannot be overlooked since separation is a primary objective of bioflocculants. The viscosity of a solution is the measure of its resistance to flow when a shearing force is applied. It reflects the frictional forces of all the molecules in the solution. Measurement of solution viscosity allows

determination of the intrinsic ability of the polymer to increase the viscosity of a particular solvent at a given temperature. Dilute solution viscometry is one of the simplest and quickest methods for characterizing polymers in solution (Kulicke and Clasen, 2004; Lovell, 1989).

Vangani and Rakshit, (1996) described two effects that an increase in temperature can have on a polymer system: firstly, increase in temperature can enhance the solubility of the polymer resulting in increased salvation and higher intrinsic viscosity. Secondly, increase in temperature may enhance the flexibility (degree of rotation) of the bonds making up the polymer structure by lowering the rotational barrier. This means the elasticity is less and viscosity of KBF is more. To monitor possible depolymerisation of KBF at higher temperature, control measurements were performed by cooling the sample solutions exhibited reverse effect of temperature on storage modulas. The study showed that the storage modulas of the polymer decreased which is due to the lower temperature that causes more shear effect at constant frequency. Viscoelasticity study showed that as temperature decreased the tan delta exhibited an increasing trend. These observations indicate that the viscosity of KBF is less and elasticity of the polymer is more.

Bacteria can utilize the nutrients in the culture medium to synthesize high molecular weight complex polymers internally within the cell under the action of specific enzymes, and these polymers can be excreted and exist in the medium. One of the very unique properties exhibited by such polymers is their ability to flocculate colloidal suspensions (Tripathy and De, 2006). The complexity in both structures and chemical compositions of biopolymers render their chemical synthesis both inefficient and expensive. Nevertheless, the establishment of a process to produce bioplymer economically has been pressing (Hamer, 1985, Rehm, 2010, Mooibroek et al., 2007), therefore, the development of biotechnological processes is an inevitable route towards the

economic production of biopolymers. As understanding of the biosynthesis of biopolymers and fermentation process development has advanced, it has become now possible to produce an increasing number of biopolymers (Khanafari and Sepahei, 2007; ***Shibazaki et al*., *1993***; ***Watanabe et al*., *1998***). Culture parameters are important in adjusting growth of microorganisms and their metabolic products (Button, 1998). In view of these, the effect of nutrients and their concentations (Carbon and Nitrogen) and culture conditions (the initial pH of the production medium, temperature of cultivation, agitation and inoculum size) on bioflocculant production and its flocculant efficiencies of *Klebsiella terrigena* strain RD4 were investigated.

Both the nature and concentration of carbon source in media is recognized as a key determinant for growth metabolic activity and production of exopolymers (Rehm, 2010). The different types of carbon sources used had relatively little influence on biopolymer production, biomass and productivityin case of *K.terrigena* RD4. However flocculating activity varied significantly not only with the carbon sources but also with the concentration, interestingly the cell growth observed at higher concentrations of carbon sources could not be correlated with flocculating activity. The latter activity was highest with glucose as carbon source; this may be attributed to the preferential utilization of glucose by *K. terrigena* over the other carbon sources, which not only enables a high final cell mass but probably serves as the desired precursors for the biopolymeric flocculant, leading to a very high flocculating activity (Bajaj and Singhal, 2009). When sucrose or rhamnose individually was used as sole source of carbon in the growth media the polymer produced, showed a poor flocculating activity. According to Ji and Ciobanu,(2003) the biopolymers can be classified according to the monomers that constitute them, therefore it was speculated that the monomeric composition of the referred biopolymer might be altered than that of the biopolymer produced with glucose, galactose or sucrose when

used individually as carbon source in the growth media. Both levels of the individual monomers and the order of their monomeric sequence in the polymer chain may affect the flocculating activity (Zajic and Leduy, 1973). However, further studies are required to establish the same.

In general extracellular polysaccharide production increased under conditions where growth was extended by the glucose content in the culture medium (Fleming and Wingender, 2001). These observations suggest that production of the flocculant is probably delinked from the cell growth after its initiation.

Bacteria generally use either ammonium salts or amino acids as their source of nitrogen (Gandhi et al., 1997; Looijesteijn et al., 1999; Czaczyk et al., 2003). Organic nitrogen sources are a rich source of amino acids and may be an important adjunct to normal nitrogen requirement by the cell particularly during production of exopolymers (Unz and Farrah, 1976). Both organic and inorganic nitrogen sources are utilized by *Klebsiella* sp, therefore an attempt was made to understand the effect of nitrogen sources on flocculating activity. The high flocculating activity observed when ammonium sulfate was used as inorganic source of nitrogen in the media, may be possibly due to the fact that an ionic environment in the cell is maintained for the exopolymer synthesizing enzymes in addition to satisfying cells own nitrogen requirement (Sheng et al., 2005). Although low nitrogen content in the growth environment are reported to influence the extensive microbial synthesis of extracellular biopolymers (Sleytr, 1997) but this was not in agreement with that of KBF production. The above deliberations suggested that biopolymer synthesis in *K.terrigena* follows a typical C/N ratio as evident in previously reported biopolymer producing bacteria.True to this assumption, a low level feeding of ammonium at relatively higher C/N ratio was found to be more effective for the production of KBF by this strain.Feasibility of bioflocculant production lies in the economics of the overall production process. Carbon source

contributes significantly to the overall production cost of biopolymer, and using a cheaper carbon source can lower such costs, particularly in industrial scale fermentations. The cost of a complex nitrogen source can also contribute significantly to the overall production cost. Yeast extract, peptone and casamino acids are good complex nitrogen sources, but are rather expensive. The use of corn steep liquor, and molasses as cheap media components did not show any notable improvement in the yield of KBF. This may be due to the lack of a favourable C/N ratio. Thus the present optimization studies may need further investigation before exploiting the feasibility of KBF for commercial production.

The initial pH of the production medium determines the electric charge of the cells and the oxidation-reduction potential, which can affect absorption of nutrients, nutrition utilization rate, enzymatic reaction, and cell structure (Salehizadeh and Shojaosadati, 2001; Nakata and Kurane, 1999). These factors in turn, are important in modulating the enzymatic activities leading for synthesis of the production of biofloculant .The initial pH of the fermentation medium affected bioflocculant synthesis (Nakata and Kurane, 1999). The paeking in bioflocculant production at pH7 and decline therafter at increased pH values may have happened due to the high electric charge on the cells or the nutrient absorption potentials which affected the enzymatic reactions (Xia et al., 2008). As noted in previous studies, the optimum pH for bioflocculant accumulation varied with different strains. In the case of *Corynebacterium xerosis*, the flocculant was produced at relatively low pH (Esser and Kues, 1983), whereas alkaline medium was much more favorable for bioflocculant production by *Aspergillus sojae* (Nakamura et al., 1976). The extreme pH profiles of the medium (pH 2.0-3.0 or pH $\geq$10) inhibited not only the process of microbial growth but also the biosynthesis of extracellular polymers (Stredansky and Conti, 1999).Likewise, incubation temperature plays a dominant role in growth and

associated metabolic activities of microorganisms (Ratkowsky et al., 1982; Cavari et al., 1981). Thus its role in production of extracellular metabolites or products by microorganisms require due consideration. It was observed that bioflocculant production in *K. terrigena* is linked with its growth; therefore optimal growth at 30°C plays an important role, it is obvious that under optimal growth conditions synthetic enzymes and as well as precursors would be adequate for maxiam flocculant production.This justification is supported by Nakata and Kurane (1999) who suggested that maximum enzymatic activation for bioflocculant production can be obtained at an optimal temperature of growth of the producer bacterial strains.

Oxygen is central to metabolic activity of aerobic microorganisms, thus its availability may affect the production of metabolites and extracellular products. The shaking speed/agitation determines the concentration of the dissolved oxygen as well as uniformly distributes the nutrients (Xia et al., 2008). An adequacy of nutrient absorption as well as oxygen concentrations by the bacterial cell is expected to lead to an optimal synthesis of bioflocculant. Agitated cultivations in scale up processes for biopolymer production have been carried out and compared with static cultivation condition. The productivity of KBF was relatively improved in agitated batch culture, the biopolymer produced and activity observed suggested the suitablity for agitated cultivation (Jang et al., 2001; Patil et al., 2010). The productivity achieved was thus comparable with that obtained in a stirred-tank fermentor.However a high agitation led to decline in flocculant activity, this might have caused shearing in the polymer chain therefore due to the loss in the biopolymer.An inoculum size may play an important role in enhancing flocculating activity was reported in previous studies.Thus the flocculating activity of KBF was determined using different initial inoculum sizes. Highest activity (81.3% ± 3.9) was observed with an inoculum size of 1%.; these results were in agreement with those described by Salehizadeh and

Shojaosadati (2001). It has been suggested that a small inoculum may prolong the stagnant time, whereas a large inoculum make niche of producer cultures overlap excessively and restrain the bioflocculant production and hence lower the flocculating activity. This proposition explains the observations in case of *K.terrigena* RD4.

In studies on polysaccharide biosynthesis, frequent use has been made of mutants unable to synthesize the exopolysaccharide characteristic of the parent organism (Norval and Sutherland, 1969). The results of compositional analysis revealed that the polymer produced by the mutant KTM1 that could not utilize glucose lacked D-Gal in its polymeric chain, is probably different from that of the polymer produced by wild type strain. The formation of exopolysaccharides actually is a complex process involving a series of reactions that begin with the formation of intermediates such as UDPglucose. The spatial configurations of the enzymes accept UDP-glucose only, modify it and lead to polymer synthesis. UDP-glucose passes through the cell membrane to reach the membrane-bound dehydrogenase and epimerase enzymes. After modification by these enzymes the sugar nucleotides so formed are utilized by the transferases to form precursors for polymerization and finally, leads to polymer extrusion into the extracellular environment. Thus, the biosynthesis of glycans is primarily determined by glycosyl transferases that assemble monosaccharide moieties into linear and branched glycan chains. As might be expected from the complex array of glycan structures found in nature, the glycosyl transferases constitute a very large family of enzymes. However, they have in common the ability to catalyze a group-transfer reaction in which the monosaccharide moiety of a simple nucleotide sugar donor substrate is transferred to the acceptor substrate. The specificity of glycosyl transferases, with respect to nucleotide sugar donor and glycan acceptor, led early on to the concept that each glycosidic linkage is the product of a single enzyme. This so-called "one enzyme–one linkage"

hypothesis was advanced by Saul Roseman and coworkers. Thus, those mutants unable to transfer glucose or mannose or galactose did not result in EPS. The changes in the genetic regulatory elements might have resulted in delayed onset of biopolymer production and in decrease in the polymer yield observed in the mutant KTM1. In natural systems where nutrients levels in close proximity to the bacterial cell may vary considerably, shifts in the physiological state of the cell result in variable EPS compositions (Geesey, 1982). It is anticipated that the nutrient stress of glucose in the mutant was translated to a type of EPS which possessed no flocculating activity. EPS in the mutant phenotype was structurally altered in some way, possibly by changes in the type of aglycone substitution. It is presumed that the due to the absence of galactosyltransferase activity in the mutant the enzyme might be unable to transfer galactose and thus have resulted in the failure to incorporate galactose into the polymeric chain. Nevertheless the mechanism of polymerization of the repeating unit and its subsequent export from the cell is still unclear in *Klebsiella terrigena* RD4 and its mutant. Therefore, this proposition further needs more extensive study. An important conclusion that could be drawn from the study is that galactose probably serves as an essential monomeric repeating unit of the polymer that renders the bioflocculant, the flocculating ability. Thus, the loss of flocculating activity may be ascribed to absence of galactose moieties in the polymeric chain of exopolymeric polysaccharide of *K. terrigena* mutant.

While investigating the role of sugars in the synthesis of the bioflocculant, it was deemed appropriate to consider stress as a probable eliciting mechanism for the flocculant. A vast body of evidences has indicated environmental stressors to have an important role in the physiology and metabolism of bacteria. In order to overcome the stressors, several protective mechanisms are elaborated by the cells including exopolysaccharides. Both low water activity and oxidative

stress are likely to be encountered by bacterial cells in the environment. Therefore the role of these two stressors in production of the biopolymeric flocculant by *Klebsiella terrigena* RD4 was examined. Initially experiments were conducted to ascertain viability of the cells to the stressors. Then a tolerable range of each stressor was chosen and both bioflocculant production and activity by the strain were determined. Both flocculant production as well as floculant activity was not significantly different from control, where cells were grown in FIB without H_2O_2. It is possible that the cells were protected by intracellular mechanisms for instance reduced glutathione (GSH; L-glutamyl-L-cysteinyl glycine) (Alcaraz et al., 2004; Ziegler and Poulsen, 1977) and enzymes such as catalases and superoxide dismutases (Cabiscol et al., 2000) reported in many mirocorganisms (Lynch and Kuramitsu, 1999). In *Pseudomonas aeruginosa, E.coli* K12, and *Bacillus subtilis* oxidative stress elevated SOD activityas well as new SOD isozymes was observed during oxidative stress.

Besides, the above mentioned mechanisms, the role of glutathione transferases (GTP; also known as glutathione-S-transferases) may also be implicated. Gsts are are major phase II detoxification enzymes found mainly in the cytosol (Tang et al., 2008) of many gram negative bacteria. They catalyze the conjugation of electrophilic substrates to glutathione (GSH), and posess peroxidase and isomerase activities (Sheehan et al., 2001), thereby inhibiting the Junn terminal kinase (thus protecting cells against H_2O_2 induced cell death). The role of GSTs in bacterial growth was reported in many studies (Piccolomini et al., 1989; Vuilleumier and Pagni, 2002; Feil et al., 1996; Tang et al., 2008). The up regulation of GST's in *Ochrobactrurm anthropi, E.coli* and *Proteus mirabilis* during exposure to H_2O_2 has been demonstrated (Tamburro etal, 2004). The combined role of both SOD and GST in conferring cellular protection to *Klebsiella oxytoca* during oxidative stress encountered by succinonitrile exposure

(Tang et al., 2008). More recent studies have demonstrated the association of *oxyR* regulon for cellular protection against oxidative stress. (Hennequin and Forresteir, 2009). It is possible that oxidative stress is encountered in *K. terrigena* RD4 by the aforementioned mechanisms; a clear understanding however requires detailed stduies beyond the scope of the the current work. Ba It was aply clear from the above discussions that oxidative stress had no role of bioflocculant production in conferring resistance to cellular protection.

The presence of solutes capable of binding to water is a prevailing phenomenon in the environment, this becomes important for a microbial cell since adequate mechanisms need to be trigerred for encountering the lowered amounts of available water. The water activity which describes the degree to which the water is "bound" to a solute, describes its availability to act as a solvent and participate in chemical/biochemical reactions and growth of microorganisms and hence its availability to act as a solvent and participate in chemical/biochemical reactions and growth of microorganisms. It is established that microorganisms have a limiting water activity level below which they will not grow. For *K. terrigena*, the limiting water activity value was observed to be 0.95. Several non halophilic microorganisms (*S.typhimurium*, *E.coli*) are protected in media with high salt concentration (low water activity) by accumulation of chemical compounds like glycine betaine, gamma amino butyric acid and proline within the cells has been shown to confer ability to sustain salt or osmotic stress caused by low water activity. Compatible solutes like betaine were reported to be predominant in countering high salt concentration (low water activity) in *K.pnuemoniae* (Rudulier and Bouilard, 1983). At limiting water activity level, the production of bioflocculant as well as its activity of *K. terrigena* RD4 did not differ from control indicating that stress exerted by low water activity do not influence the production of bioflocculant. These observations were similar to results obtained by Looijesteijn and

Hugenholtz, (1999), who showed that exopolysaccharide production in *L.lactis* subsp *cremoris* NIZOB40 was not affected by low water activity. Low water activity stress however resulted in increased biosynthesis of alginate by *Pseudomonas putida*, *Pseudomonas syringae* as well as *Pseudomonas aeruginosa* PAO1 (Chang et al., 2007). In the fungi *Ganoderma lucidum*, low water activity was found to be correlated to exopolysaccharide biosynthesis and lowering of water activity acted as enhancer of exopolysaccharide synthesis (Looijesteijn et al., 2001). Whereas, exopolysaccharide biosynthesis is believed to be a protective mechanism for microbial cells during water activity mediated stress in the above cases, the flocculant production of *K. terrigena* RD4 do not appear to be specifically related to protection of the cells during low water activity. It ispossible that either a single or multiple other mechanisms confer protection during low water activity to *K.terrigena*.

Previous observations with KBF and colloidal particles of varied sizes provided a crucial 'cue' for designing further studies related to applicability. In fact high flocculating activity observed with Kaolin by the KBF indicated size range 2–5µm; this was within the size range of bacteria, (Ghosh et al., 2009). It was therefore anticipated that this biopolymeric flocculant may be useful for removal of bacterial cells as well from waterbodies. Enteric *Salmonella* infection is a global problem both in humans and animals, and is regarded to be the most important bacterial etiology for enteric infections worldwide (McCormick et al., 1993). For treatment of such wastewater, flocculation is an easy and effective method where flocculants are used to remove the suspended solids, colloids, cell debris as well as the waterborne/enteropathogens pathogens including *Salmonella* spp. (Sproul, 1976; Payment and Armon, 1989; Sobsey, 2002). Significant reduction ($P<0.5$) in numbers of *Salmonella* (3 log reduction) were observed. Counts of *S. aureus*, *E. faecalis* and *E. coli* DH5α remained unaltered, suggesting selectivity to Salmonella.

….To ascertain whether Salmonella cells have were actually removed further detailed studies using CLSM, was carried out in conjunction with FISH; this method allowed optical sectioning and 3-dimensional reconstruction for localization and observation of the spatial distribution of bacteria in different layers of the bioflocculant material. FISH technique also provided potential information in elucidating the specificity and thus the mechanism of binding of biopolymeric material to *Salmonella.* The bright signal intensities of the bacteria indicated a high amount of rRNA, which is an evidence for physiological activity of the cells at the time of sampling (Wallner et al., 1996; Montgomery and Kirchman, 1994). It is difficult to explain why hybridization signals of the active bacterial population of *E. faecalis* were not observed in the fixed biopolymer matrix, when the sample was allowed to flocculate *E. faecalis* (10^7 cfu/mL) in tap water before fixation. Nevertheless, explanation of the observed selective binding cannot be made considering purely electrostatic interactions as key players. There is also increasing evidence that many of the surface appendages, especially of pathogens, carry specific adhesins involved in specific interactions with receptors on the host cell (Montgomery and Kirchman, 1994). It is possible that attachment of *Salmonella* to the biopolymer occurred through a specific binding mechanism. Absence of such mechanisms in *S. aureus*, *E. faecalis* or *E. coli* might be the possible explanation for this observation (Otto et al., 1999; Yuda et al., 2001). However, these propositions require detailed information of the biopolymer structure.

A validation of the above observed results were attempted in untreated poultry wasate water containing Salmonella. Removal of *Salmonella* was found to be remarkably low with the standard dose of alum (Sobsey, 2002; Sobsey et al., 2002) which was used for comparative purposes. Similar observations on the low flocculation efficiency of alum in abattoir wastewaters have also been reported (Amuda and Alade, 2006). Moreover, the effective dose was nearly five

times higher than that of the used bioflocculant. Besides the inability of alum to aggregate microorganisms present in low counts, the difference in effectiveness between alum and the bioflocculant for flocculating *Salmonella* may be attributed to hydrogen bonding between the polysaccharide flocculant and cell surface polymers (Harris and Mitchell, 1973) in addition to electrostatic interaction; also the high concentration of divalent cations (e.g. Ca^{2+}) present in the poultry wastewater (Murthy et al., 1996) may have reduced the steric repulsion between surface charges of the *Salmonella* cells. Another tempting speculation for the observed flocculation of *Salmonella* to the biopolymer could be a specific interaction of cell surface components (specific adhesins, receptors or inducible proteins) with the biopolymer itself (Montgomery and Kirchman, 1994). The presence of specific sites on the biopolymer structure may have been responsible for facilitating this binding (Kobayashi et al., 2002). However, this proposition requires detailed information of the biopolymer structure (Otto et al., 1999).

Subsequent to bioflocculant treatment of the poultry wastewater, visualization and simultaneous detection of *Salmonella* spp. in the bioflocculant matrix was carried out directly, using FISH in combination with CLSM. The bright signal intensities of the bacteria indicated a high amount of rRNA, which is evidence for physiological activity of the cells at the time of sampling (Kemp et al., 1993; Wallner et al., 1993). Also by using the control probe non EUB338 and DAPI staining, non-specific binding could be excluded between the probe and constituents of the cellular matrix, which gave confidence to the results achieved; in addition, autofluorescence was excluded by viewing the sections prior to the FISH procedure. Repeated microscopic evaluation confirmed the unambiguity of the interpretation of the images obtained.

Additionally with the universal eubacterial probe Eub 338-FLUOS (green), co-aggregated bacteria located within the bioflocculant matrix could be detected by FISH; bacteria

of different morphologies could also be detected with DAPI staining in the treated bioflocculant sample. In addition, a distinct morphotype of small bacterial rods were detected as *Salmonella* with the *Sal3*-Cy3 oligonucleotide probe in a manner similar to as discussed in the previous study on selective removal of *Salmonella* from mixed culture.

Flocculation by polymers may be viewed upon as a process where bacteria adhere to each other through the adsorbed flocculant on their surfaces. The attachment or aggregation is controlled by a complex interplay of interaction forces between the bacterium in a bulk phase and a substratum and other bacterium. The most relevant interactions are *van der Waals* forces (VDW), electrostatic interactions, hydrophobic interactions and polymer interactions (Strand et al., 2002). Since microbes as well as naturally occurring surfaces usually carry negative charge, the electrostatic interactions between them are repulsive and the bacterial adhesion must occur as a result of attraction due to other interactions, such as VDW, hydrophobic or polymer interactions. The above results demonstrated the remarkable ability of KBF for the removal and binding of *Salmonella* from poultry wastewater. Further insights on the mechanism of interaction of *Salmonella* with this biopolymer might help in the possible engineering of the functional groups so as to generate promising new and/or modified polysaccharide variants (Ha et al., 1991).Another persistent source of threat to human health is Cryptosporidiasis on account of the ubiquitous distribution of *Cryptosporidium* spp. in humans, animals, and water. Chemical flocculants such as aluminum sulfate or polyaluminum chloride have been used for concentration and removal of *Cryptosporidium* oocysts from water via aggregation, settling, and filtration. In addition to health concerns, the failure of these flocculants to remove *Cryptosporidium* oocysts because of floc stabilization and resulting floc breakage during filtration (Salehizadeh and Shojaosadati, 2001; Bolto and Gregory, 2007), has been documented. Application of the

bioflocculant in *Cryptosporidium* spiked samples of water indicated a comparable removal efficacy as achieved with alum.

Earlier, removal studies attempted by Vesey et al. (1993) reported 59% flocculation of seeded *C. parvum* oocysts (10^3 in 1 L tap water, pH adjusted to 9.0) using alum as the flocculant. Wheras, Kimura et al. (2000) reported recovery rates of 67.8 and 58.1% for 1 X 10^6 and 2.5 X 10^5 oocysts respectively at pH 6. The results of *C. parvum* oocysts removal in this study, resembled with those obtained by Karanis and Kimura, (2002).However, the difference in rates noted may be attributed to several reasons such as source and age of *Cryptosporidium* oocysts and surface charge, which probably differentiates different *C. parvum* isolates (Klonicki et al., 1997). The oocyst surface is believed to contain proteins that extend out into solution, causing both electrostatic and steric forces to be involved in oocyst–surface association. A possible explanation for the observed binding of oocysts to bioflocculant could be the reduction in steric repulsion mediated by dissolved cations, for instance, Ca^{2+} to the negatively charged (because of the presence of uronic acid) bioflocculant (Wu and Ye, 2007). Binding of cations (Ca^{2+}) to oocyst surface proteins, leading to a neutralization and collapse of such proteins have been reported (Kuznar and Elimelech, 2004). It is plausible that the presence of calcium-facilitated bioflocculant binding to the oocysts, possibly through entrapment. Thus the application of bioflocculant prior to filtration may allow an effective filtration. However, exact mechanism through which this exopolysaccharide bioflocculant interacts with *Cryptosporidium* oocyst requires specifically designed studies.

Removal of heavy metals from the environment using microbial biosorbents has been studied extensively as a major challenge in bioremediation. Extracellular polymeric flocculants produced by many microorganisms are of particular relevance to the bioremediation process

because of their involvement in flocculation and binding of metal ions from solutions (Salehizadeh and Shojaosadati, 2003), also they are known to have greater binding capacity for metals than any other known mineral sorbent (Quigley et al., 2002) and form multiple complexes with metal ions where the functional groups (e.g. hydroxyl, carboxyl) associated with polymer exhibit a high affinity towards certain metal ions. (Mittelman and Glesey, 1985).

Moreover, the use of isolated biopolymers in biosorption phenomenon seems to be more economical, effective and safe alternative to chemical methods (such as precipitation, coagulation, ion exchange, electrochemical etc). Biopolymeric flocculants exhibit excellent metal binding properties with varying degrees of specificity and affinity (Pal and Paul, 2007). They contain ionizable functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups, which enable biopolymer to sequester heavy metals, complexation with functional groups of negatively charged followed by adsorption and precipitation are the mechanisms involved in metal biosorption. The flocculant biopolymer are assumed to have multiple metal binding sites with different binding strengths (Rudd et al., 1984) as it possess multiple functional groups to which metals sequentially bind, first occupying high binding energy site configurations and subsequently weaker site configurations as the metal concentration in solution is increased. However, the ability of biopolymeric flocculants to bind metals may be anticipated to vary considerably in both capacities of binding as well as the type of metals.

Heavy metals (e.g. lead, copper, cadmium, zinc, etc.) are toxic even at low concentrations. Since they are non-biodegradable, their threat is multiplied by their accumulation in the environment through the food chain. The potential of KBF in the removal of some heavy metals (cadmium, copper, lead and zinc) in water samples was evaluated. The trend observed was an increase in the initial metal concentration culiminating in a plateau at high metal

concentration. At lower concentrations, all metal ions present in the solution interact with the binding sites and thus facilitate maximum removal. At higher concentrations, more metal ions are left in the solution due to the saturation of binding sites.

Upon dispersion of the purified flocculant in the heavy metal solution, an increase on the availability of possible cation binding groups may be expected. Dose optimizations revealed that about 2.5 mg/L of KBF were sufficient to remove these heavy metals from water samples. As observed during the study on the effect of varying KBF concentrations, with initial low concentration, the rate of adsorption was low. However, as the amount of biosorbent increased adsorption also increased. It has been reported for metal adsorption studies that an increase in biosorbent concentration results in a corresponding increase in the total metal adsorption accompanied by decrease in specific uptake (Sameer and Zdravko, 1996). This is due to the fact that total adsorption is dependent upon the number of available binding sites, whereas specific uptake is calculated as the amount of metal adsorbed per weight of the biosorbent. Removal efficacies of the biopolymers were not altered within a temperature range of 28-45°C and a pH range of 5-8; the thermal and pH stability of KBF may be accounted for its adsorption efficacy at a variable temperature and pH range. Since the functional groups responsible for metal binding are protonated at low pH, increasing the pH increases the amount of adsorbed metals because binding sites on the cell wall surface become more available due to deprotonation of the functional groups responsible for binding. At higher pH metal ions are precipitated which decreases the amount of adsorbed metals. Say et al. (2001) have also reported more than 60% adsorption of Cd, Pb and Cu following an exposure for 2 h using *Phanerochaete chrysosporium*.

The differential metal removal property of KBF may be attributed to the ordered conformation and consequent intermolecular interactions with different metal ions. These groups

act as polyanions under natural conditions by formation of salt bridges with carboxyl groups of acidic polymers (polysaccharides containing uronic acids) or by forming weak electrostatic bonds with hydroxyl groups on polymers containing neutral carbohydrates. Electrostatic interactions between negatively charged functional groups (i.e. uronic acids) of KBF and the cationic heavy metals could promote heavy metal removal effectively from solution. Presence of carboxyl or hydroxyl functional groups in KBF may also be primarily involved in metal binding by forming coordination bonds that facilitate the stability of ion polymer complex. The factors able to affect metal biosorption are the ones having an influence on the environment of the binding sites or on their chemical nature (Artola and Rigola, 1992). Metal content of wastewater (Mehrotra and Tandon, 1991), pH (Nelson et al., 1981; Baudu et al., 2000), ionic strength and temperature of the solution, surface properties (Sag and Kutsal, 1995), EPS composition, metals speciation (Rosin et al., 1982) have been suggested important for affecting metal biosorption.

Conclusions

In summary, the work aimed at examining the following objectives:

- Isolation and characterization of flocculant producing microorganisms and their exopolymeric flocculant
- Optimization for maximal bioflocculant production and elucidation of cellular mechanisms
- Evaluation of the exopolymeric flocculants for removal of pathogens and metals.

The salient findings in the study are as follows:

A. Isolation and characterization of flocculant producing microorganisms and their exopolymeric flocculant

1. Sludge samples examined from 23 industrial units for exopolysaccharide producing bacteria resulted in 957 isolates which were distinguished preliminarily on their mucoidy appearance. As many as 632 isolates exhibited copious exopolysaccharide production as visualized on solid media. One prospective strain, finally selected from amongst these isolated bacteria satisfied a stringent set of criterion which included: high flocculation rate (>80%), pathogen and metal removal.
2. Both conventional biochemical and molecular methods were applied to completely characterize the strain, which was designated as *Klebsiella terrigena* RD4. Kinetic analysis of growth characteristics of *K.terrigena* RD4 revealed highest flocculant yield (81.3%±4.9) and flocculation activity in 48 hours (stationary phase); the bacterial growth and flocculant production thereafter, could not be correlated.

3. The purified bioflocculant was anionic and electrophoretically homogenous with high molecular weight ~$2.6X10^5$ Da; low dosage of purified bioflocculant had highest flocculating activity, the latter being stimulated by divalent cations, Ca^{2+} and Fe^{2+} at 5mM concentration.
4. Bioflocculant activity remained unaltered over a wide range of pH (6-8) and temperature (35-85^{0}C); although high flocculation was observed with a wide range of colloidal particles, the activity declined at low concentrations of colloidal particles.
5. Chemically the bioflocculant was primarily a heteropolysaccharide comprising of D Glucose, D Mannose, D Galactose and DGlucuronic acid units linked with either 1,4 ß or 1,3 ß linkages. The bioflocculant was thermostable with excellent viscoelastic properties-notable at higher temperature; surface structure of the bioflocculant was compact with abundant, randomly distributed small pores interconnected with channels.

B. Optimization for maximal bioflocculant production and elucidation of cellular mechanisms

1. For *K.terrigena* RD4, glucose, peptone and yeast extract at a C/N ratio of 10:1 were found to be preferred carbon and nitrogen sources for optimal yield of the bioflocculant; Mg^{2+} appeared to be important nutritionally since its presence led to an enhancement of flocculant production. Further optimization studies of culture variables indicated that highest flocculant production occur at a temperature of 30°C, pH 7, inoculum size of 1% and agitation speed of 120rpm.
2. The flocculant was predominantly a polysaccharide comprised of monomeric sugar units, a clearer understanding for preferential sugar monomer was deemed important to understand flocculant functionality during either absence /limiting concentration of

preferred sugars in media. Thus, a variant ($Glu^{-}Mal^{+}$) incapacitated for glucose utilization was selected and investigated from amongst several mutants generated by chemical mutagensis. The mutant exhibited delayed flocculant production with virtually no flocculating activity.

3. Chemical structure analysis of the flocculant produced by this mutant strain revealed a complete absence of DGal units from the polymeric backbone-signifying the importance of glucose limited cultures and concomitantly highlighting the functionality of DGal monomeric units in flocculation.
4. Two prominent environmental stressors such as water activity and oxidative stress were evaluated for exopolysacharide production by *K. terrigena* RD4. However, in *K. terrigena* RD 4, these stressors could not related to the flocculant production.

C. Evaluation of the exopolymeric flocculants for removal of pathogens and metals.

1. The *K.terrigena* RD4 flocculant possessed both selectivity and efficacy demonstrable by significantly high removal (80.3% $\pm$ 3.4) of *Salmonella* from mixed bacterial cultures in simulated experiments. The above results could be successfully validated in actual poultry waste water contaminated with *Salmonella typhimurium*. Likewise, trials carried out in potable water spiked with the persistent parasitic oocysts of *Cryptosporidium,* indicated notable removal (62.3%) at a low bioflocculant dose ($2mgL^{-1}$) the presence of Ca^{2+} being highly effective.
2. Fluorescent in situ hybridization with genus specific *Sal3* probe hybridized with the Salmonella present in the agglomerated matrix of the biofloccuant.Confocal laser scanning micrographs showed the removed *Salmonella* to be bound and embedded within the flocculant matrix.

3. Specificity testing of the *Sal 3* oligonucleotide probe confirmed the high specificity of the probe towards the target pathogen.

4. The contribution of *K.terrigena* RD4 flocculant in removal of heavy metals: Cu, Cd, Zn and Pb were examined. The removal efficacy was variable from moderate to high depending on the metals as well as the concentration. For instance, with 3.0 ppm Cu , 0.15 ppm Cd and 0.01 Pb, the removal were, 71%(±3.6), 84%(±3.1), and 64%(±2.3) respectively whereas removal was comparatively low (57%±4.2) in case of Zn, even at a concentration of 1.5 ppm. A consistent observation in both the removal studies was the low dose requirement in comparison to high dosages required by chemical flocculants.

In conclusion, the present study carried out could primarily:

(a) Identify a prospective bacterial strain capable of producing an exocellular polymeric flocculant. In comparison to reported biofloculants, this newly characterized bioflocculant exhibited superiority in terms of robustness and performance. The flocculant was characterized chemically and physically and for its characteristics.

(b) The culture parameters were optimized for maximal production of the bioflocculant, these conditions were also appropriate for high activity of the bioflocculant. The role of preferred sugar in the biopolymeric activity as well as two important environmental stressors was elucidated.

(c) The efficacy of the purified exopolymeric flocculant was examined for removal of selected heavy metals and pathogens under laboratory conditions- the results were validated in actual field samples.

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Appendix I

MEDIUM COMPOSITION

*1. Luria-Bertani (LB) medium**

Composition	*Quantity (gL^{-1})*
NaCl	10.0
Beef extract	5.0
Tryptone	10.0
Agar	10.0

*2. Flocculant Isolation Broth (FIB) medium**

Composition	*Quantity (gL^{-1})*
Peptone	5.0
Ammonium sulphate	0.5
Yeast extract	1.0
$CaCl_2.2H_2O$	0.7
NaCl	0.1
$MgSO_4. 7H_2O$	0.3
K_2HPO_4	1.0
Glucose	1.0
Agar	3 .0

*3. Medium composition for NTG mutagenesis**

Composition	**Quantity (gL^{-1})**
Peptone	5.0
Ammonium sulphate	0.5
Yeast extract	1.0
$CaCl_2.2H_2O$	0.7
NaCl	0.1
$MgSO_4. 7H_2O$	0.3
K_2HPO_4	1.0
Malate	1.0
Agar	3 .0

*Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

BUFFERS AND SOLUTIONS

1. TBE buffer (10X)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

2. Citrate buffer (0.1M), pH 5.5*

Citric acid (0.2 M)	116.25 mL
Sodium citrate(0.2 M)	383.75 mL

3. 0.1M Phosphate buffer

Monobasic sodium phosphate, monohydrate (1 M)	61.5 mL
Dibasic sodium phosphate, monohydrate (1 M)	38.5 mL
Dilute to 1 L with distilled water	

4. Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

5. ***Ethidium Bromide*** 0.5µg mL^{-1}

REAGENTS

1. Phenol Sulfuric acid method

Phenol	5%
Sulfuric acid (reagent grade)	96%
Sugar standards (reagent grade)	1 mg mL^{-1}

2. Folin Lowry method

Reagent A (alkaline solution) (for 50 mL)	
NaOH	0.1 M
Na_2CO_3	2.0g
Reagent B (for 50 mL)	
$CuSO_4.5H_2O$	0.25g
Na_2Tartarate.$2H_2O$	0.5g
Lowry solution	(fresh; 0.7mL/sample)
Reagent A+ Reagent B	

Folin and Ciocalteu's Phenol Reagent	1:1

3. *Elson Morgan method*

Reagent A (100 mL)

di-Potassium tetraborate tetrahydrate	6.1g

Reagent B

4-N,N-dimethyl-p-aminobenzaldehyde	1 g
Glacial acetic acid	50 mL
Hydrochloric acid (10 N)	1.5 mL
Standard solution (D-glucosamine)	1 mg mL^{-1}

4. *Carbazole assay*

Reagent A

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

Reagent B

Carbazole	100 mg
Absolute ethanol	100 mL
Standard solution (D-glucouronic)	0-1 mg mL^{-1}

5. *Friedman method*

Perchloric acid	50%
DNP reagent	
2,4-dinitro phenylhydrazine	500 µmoles
Hydrochloric acid (2N)	100 mL
Sodium hyroxide	2.2N
Standard solution (pyruvic acid)	0-3 $\mu g mL^{-1}$

*Diluted to 1 L with distilled water and store 2°C-8°C

STAINING SOLUTIONS**

Sudan Black B	0.02%
Alcian Blue	
3% glacial acetic acid	100.0 mL
Alcian Blue 8 GX	1.0gm

**Mix, adjust pH to 2.5 using acetic acid

Appendix II

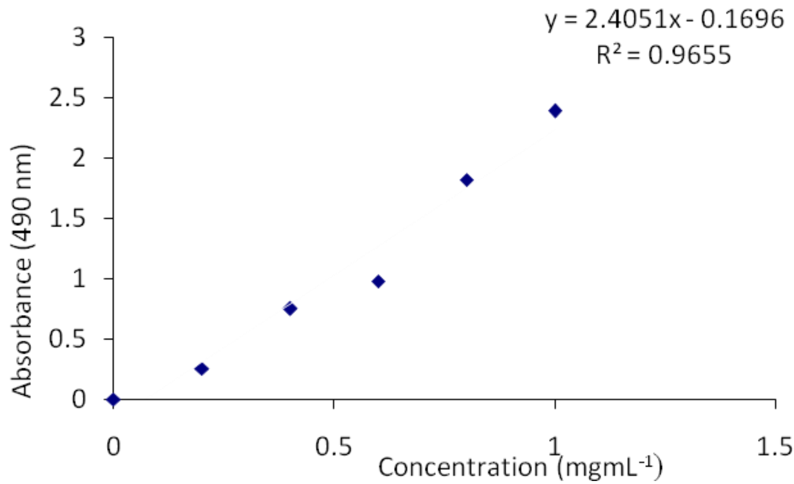

Fig. 3.1. Standard curve of carbohydrate assay. Relationship between carbohydrates (as mg glucose) and absorbance using phenol-sulfuric acid assay. R^2 = 0.9655

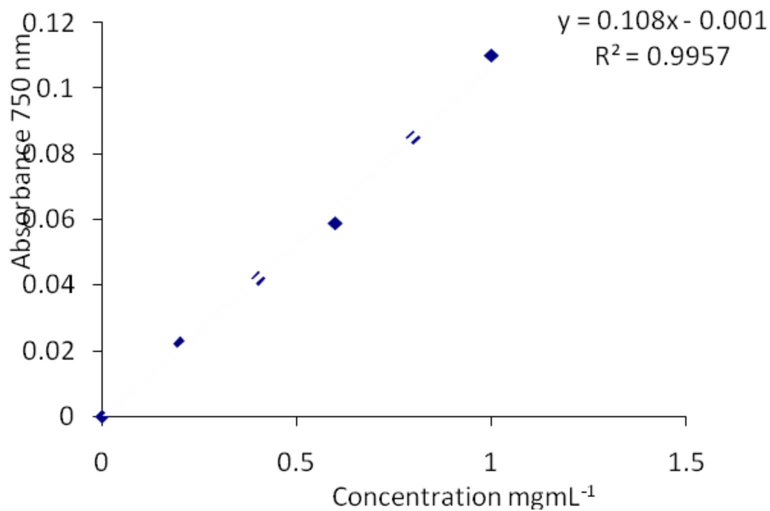

Fig. 3.2. Standard curve of protein assay. Relationship between protein (as mg bovine serum albumin) and absorbance using the Folin Lowry assay. R^2 = 0.9957

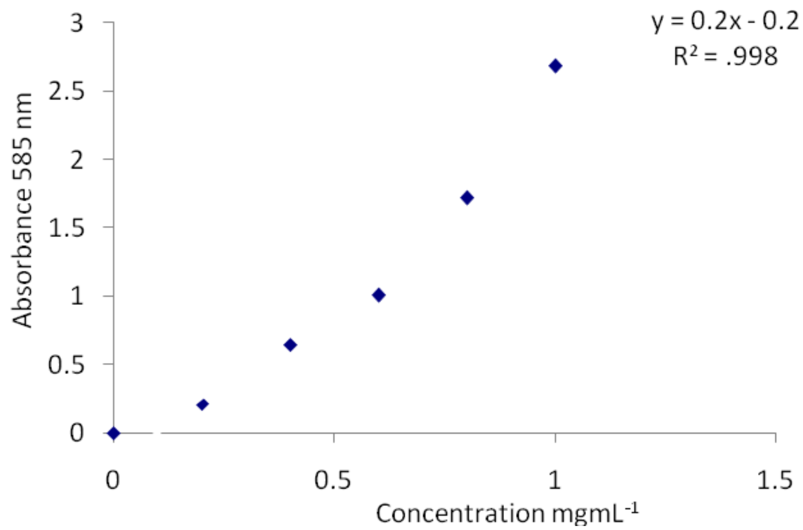

Fig. 3.3. Standard curve of hexosamine assay standard curve. Relationship between hexosamine (as mg glucosamine) and absorbance using the Elson Morgan method. R^2 = 0.998

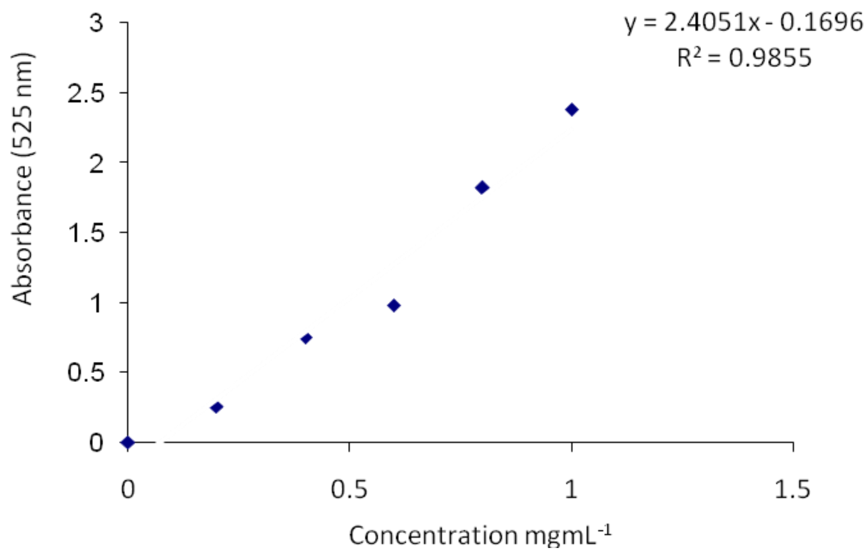

Fig. 3.4. Standard curve of uronic acid assay. Relationship between uronic acid (as mg glucuronic acid) and absorbance using uronic acid assay. R^2 = 0.9855

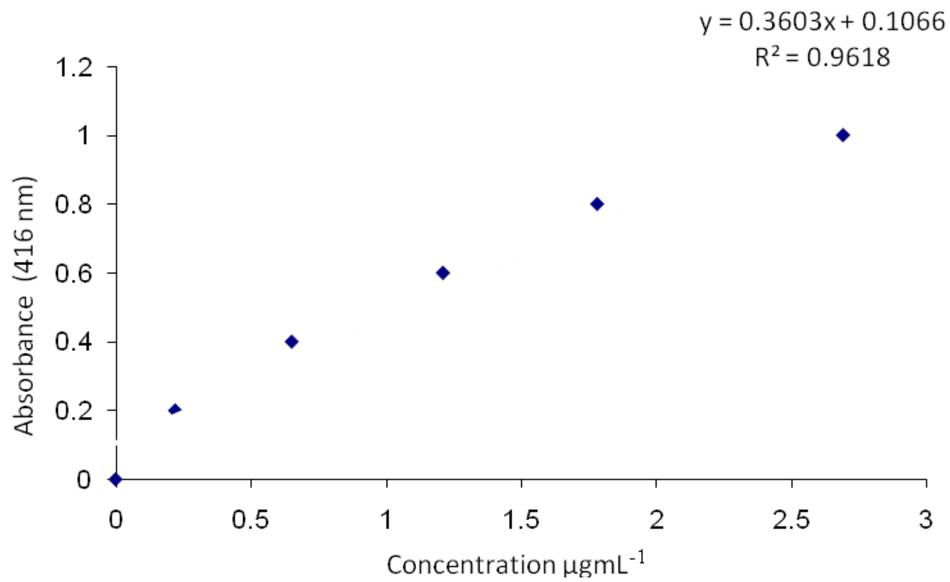

Fig. 3.5 Standard curve of pyruvic acid assay. Relationship between pyruvic acid (as µg) and absorbance using the Friedman method. R^2 = 0.9618