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Cellular and molecular mechanisms of bioremediation by microbial mats

Sharifeh Mehrabi

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Microbial mats were developed in the Bioremediation Laboratory at CAU and have been used successfully to remediate heavy metals from surface water. The cellular and molecular mechanisms involved in the bioremediation processes are not well understood. The objective of this study was to provide basic understanding of the cellular and molecular basis of heavy metal remediation by mats. The central hypotheses are: (1) that there are metal resistant and/or reducing bacteria in the mats; and (2) that mats produce specific biomolecules that bind to and sequester heavy metals from solution. The specific aims of this investigation were: (1) to isolate, identify, and characterize metal resistant and/or reducing bacteria from the mats, and (2) to isolate, purify, and characterize specific metal-binding biomolecules (bioflocculants) secreted by microbial mats. In this study a facultative, photoorganotrophic, purple, non-sulfur bacterium was isolated from the mixed-species microbial mats that were dominated by cyanobacteria and contained heterotrophic and purple autorrophic bacteria. The isolated bacterium was a motile, gram negative rod. Electron micrographs of thin sections of the bacterium showed a lamellar intracytoplasmic membrane (ICM) system. Based on its morphology, nutrient
requirements, absorption spectra, GC content, RAPD-PCR fingerprints, and partial sequences of 16 S rDNA, this isolate has been identified as a new strain of *Rhodopseudomonas (Rhodopseudomonas mehrabi UME-1)*. This bacterium was resistant to high concentrations of several heavy metals (50-1000 mg/l) including As, Cd, Cr(III), Co, Cs, Cu(I), Cu(II), Fe, Hg(I), Mn, Pb, Sr, and Zn. The isolate reduced high concentrations (100 mg/l) of Cr(VI) and selenite. An extracellular, acidic polymer with metal binding and flocculating properties was also isolated from the microbial mats. Production of this acidic polysaccharide was highest at the end of the exponential growth phase, and decreased with increased formation of biofilm. The exopolymer has a high molecular weight (500,000 Dalton) and an isoelectric point of 4.2. Its carbohydrate composition was determined by GC/MS and fluorophore-assisted electrophoresis (FACE), and contained Arabinose, rhamnose, fucose, xylose, mannose, glucose, galactose, glucuronic acid, galacturonic acid, N-acetylglucosamine, and N-acetylgalactosamine. Rheological studies showed that bioflocculants produced a highly viscous solution, 1000 fold greater than the viscosity of glycerol at equivalent concentrations. Interaction of bioflocculant with mono- and divalent metals were examined using equilibrium and continuous flow dialysis. Bioflocculant was bound to 200-480 mg of metal per mg bioflocculant. Its strong metal binding and flocculating properties make this exopolysaccharide a good flocculating agent and may find useful commercial applications.
CELLULAR AND MOLECULAR MECHANISMS OF
BIOREMEDIATION BY MICROBIAL MATS

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY
SHARIFEH MEHRABI

DEPARTMENT OF BIOLOGICAL SCIENCES
ATLANTA GEORGIA
MAY 2000
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<td>FACE</td>
<td>Fluorophore-Assisted Carbohydrate Electrophoresis</td>
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CHAPTER 1

INTRODUCTION

Environmental pollution management involves both reduction in production and release of toxic chemicals into the environment, as well as decontamination of previously contaminated sites. In recent years biological treatment technologies have become popular environmental management tools, because biological treatment is generally inexpensive and ecologically clean.

Microbial mats, one of the oldest microbial communities on the earth, are stratified microbial communities that develop in shallow water, and at the interface of the water and solid substrates. They are composed primarily of photosynthetic microorganisms in which oxygenic cyanobacteria are dominant. A mixed microbial mat has been developed for bioremediation of mixed hazardous wastes in the environmental laboratory at Clark Atlanta University (CAU) (Bender, Vatcharapijarn et al. 1989).

Previous reports showed that microbial mats have been used successfully to remove heavy metals (Ag, As, Cd, Cu, Zn, Co, Cr, Fe, Pb, U) from water (Bender, Archibold et al. 1989, Bender, Rodriguez-Eaton et al. 1994, Bender, Vatcharapijarn et al. 1994) to reduce selenite to elemental selenium (Bender, et al. 1991), and to degrade several aromatic organic pollutants such as trichloroethylene and naphthalene (Phillips et al. 1994), and chlordane (Bender, Murray et al. 1994).
The microbial mats, which developed on the top of oil released into the sea during the Persian Gulf War had an active role in degradation of the oil (Sorkhoh et al. 1992). Although the mats have been successful in several bioremediation applications, little is known about the diversity of their microbial communities, their structural organizations, and the biochemical and molecular mechanisms of their bioremediation activities. As pointed out in a recent review by Bull et al. microbial biodiversity is potentially an important tool for bioremediation (Bull et al. 1992).

The use of the laboratory cultures of many types of mats in controlled experiments is an essential part of understanding various mechanisms of bioremediation occurring in mats. Isolation and identification of the member species of microbial mats and the elucidation of their roles in decontamination of hazardous compounds can provide important information to help us develop and improve the decontamination potential of the mat.

In this report a dominant member of the microbial mat community, a reddish purple, non-sulfur, facultative anoxygenic, photosynthetic bacterium has been identified as belonging to the genus *Rhodopseudomonas* (Imhoff and Trüper 1992, Pfennig and Trüper 1992). This strain was selected for isolation and characterization because it became dominant during the treatment process and gave the mat a reddish purple tint. The possible bioremediation potential and the functional role in the mat’s removal of heavy metals by the bacterium were also investigated.

Previous work in our laboratory suggested that some of the metal removal properties of microbial mats are related to the production and secretion of an acidic exopolysaccharide to the environment (Bender, Rodriguesz-Eaton et al. 1994). This exopolysaccharide has flocculation properties, which can flocculate suspended particles
and clarify water. Like other bacterial exopolysaccharides, it played major roles in adhesion of the bacteria to surfaces by interaction with the interfaces and eventually forming biofilms (Costerton and Irvin 1981, Christensen 1989, Christensen and Characklis 1990, Quintero and Weiner 1995, Becker 1996). Isolation and characterization of this exopolymer as a pure compound from our constructed mats will help us to relate some of the mat’s bioremediation functions to its chemical and physical characteristics.

1.1 **Objective**

This study attempted to:

a) Isolate and phenotypically as well as genotypically characterize a metal resistant bacteria from a microbial mat, and also demonstrate the metal removal properties of the strain thereby relating this to the metal sequestration property of the microbial mat.

b) Isolate and purify the exopolysaccharide from the mat and demonstrate its flocculating and bioremediation functions. In addition this study attempted: (1) to characterize the chemical and rheological properties, (2) to relate function to bioflocculant structure, and (3) to assess possible medical and industrial applications of isolated bioflocculant.

The goals of this study were designed to bring about some basic understanding of the molecular and cellular processes in the mats which are essential to their bioremediation applications, the specific goals of the project are to isolate and characterize the metal-binding bioflocculant, and to identify and characterize one of the important non-cyanobacterial members of the mat community.
CHAPTER 2
LITERATURE REVIEW

2.1 Microbial mat

Microbial mats develop in a wide variety of environments such as hot springs, hypersaline ponds, dry and hot deserts, alkaline lakes, coral reefs and coastal intertidal sediments. The majority of microbial mats are formed by oxygenic phototrophic cyanobacteria but mats of anoxygenic phototrophic organisms or eukaryotic algae are also known (Cohen and Rosenberg 1989).

Microbial mats are generally defined as laminated microbial systems with several cohesive, viscous layers that vary in thickness from a few millimeters to one meter. The biologically active layer is limited to the upper few millimeters or centimeters. The development of these communities causes steep environmental microgradients. The steep redox gradient is expressed at the oxygen/sulfide and often methane interface. In addition to redox gradient, steep gradients of other compounds and nutrients are developed that allow rapid internal cycling of carbon, nitrogen, sulfur, and phosphate within the mat and support the high rate of metabolism of microbial mats (Cohen and Rosenberg 1989).

Steep spectral light gradient and day-night fluctuation cause the microorganisms of microbial mats to either migrate diurnally along with the changing microgradient or
develop physiological flexibility which allow them to function under both oxidizing and reducing conditions (Cohen and Rosenberg 1989).

Microbial mats used in this study were established by enriching simulated laboratory ponds with ensiled grass clipping. After enrichment, mats develop spontaneously in the sediment and surface regions of the ponds by bacterial colonization of the silage, followed by cyanobacterial domination (Bender, Vatcharapijarn et al. 1989).

Previous reports (Bender, Archibold et al. 1989, Bender et al. 1991, Phillips et al. 1994, Bender, Murray et al. 1994, Bender, Vatcharapijarn et al. 1994) showed that the microbial mats have been used successfully to remove heavy metals (Ag, As, Cd, Cu, Zn, Co, Cr, Fe, Pb, U, Mn) from water, reduce selenite to elemental selenium, Cr(VI) to Cr(III) (unpublished), and to degrade several aromatic organic pollutants, TCE, TNT and chlordane. Previous work in our laboratory suggested that some of the metal removal properties of microbial mats are related to the production and secretion of an acidic exopolysaccharide into the environment (Bender, Rodriguez-Eaton et al. 1994).

Determination of bacterial species in microbial mats is a challenging and tedious task. Few microorganisms in microbial mats have enough distinct morphological and cultural characteristics to be recognized by conventional microbiology methods. The common selective enrichment methods to establish cultures of representative bacteria in mats and other microbial communities may often favor opportunistic species of the population. However, many if not most of the bacterial species in a microbial community may be unculturable. Identification of microbial components of mats and other microbial communities using new molecular techniques to characterize nucleotide
sequences of the conserved region of the 16 S subunit of ribosomal RNA, and in situ DNA/DNA and DNA/RNA hybridization, introduce a new concept of community microbiology. The sequences of ribosomal RNAs and the genes coding for rRNAs are widely used in phylogenetic and taxonomic studies (Stackebrandt 1992).

2.2 Purple non-sulfur bacteria

The purple non-sulfur bacteria are a group of phototrophic purple bacteria. Their diversity is reflected in morphological and physiological properties of the various species and also in the variation of chemotaxonomic characters such as cytochrome c structure, lipid and quinone composition, lipopolysaccharide structure and DNA-DNA hybridization data. Imhoff et al. (1984) carried out a rearrangement of the species of the phototrophic purple bacteria based on these characteristics.

According to 16S rDNA similarities, the majority of purple non-sulfur bacteria (the genera Rhodobacter, Rhodopila, Rhodomicrobium and Rhodopseudomonas) belong to the alpha group of Proteobacteria.

2.2.1 Morphology and physiological properties of Rhodopseudomonas

The species of Rhodopseudomonas which constitute the majority of photosynthetic purple non-sulfur bacteria, are gram negative rod, motile (polar or subpolar flagella) bacteria of 0.4-1.5 μm width, and 0.9-2.0 μm length. They form lamellar intracytoplasmic membranes, containing photosynthetic pigments under phototrophic conditions. They multiply by budding and the daughter cells originate at the opposite pole of the flagella-bearing end, giving a dumbbell shape to the bacterium before division (Pfennig and Trüper 1974).
Absorption spectra of cell extract or cell suspensions yield primary information on predominant bacteriochlorophyll a and b, with maximum absorption of 375, 590, 805, 830-890, and 400, 605, 835-850, 1020-1040 nm, respectively. The color of cell suspensions of many species grown under phototrophic conditions is indicative of major type of carotenoids (maximum absorption at 450-550 nm) present. Spirilloxanthin series (lycopene, rhodopin, and spirilloxanthin), as the major component, give a pink or red color, increasing amounts of rhodopsin turn the color to red-brown, okenone results in purple-red, and rhodopinal in purple-violet. Spheroidenone series give a green or brownish red and greenish brown under reducing conditions (Hiraishi and Yoko 1994).

The species of *Rhodopseudomonas* are anoxygenic phototrophic bacteria growing photosynthetically under aerobic conditions in light without producing oxygen. All species use organic substrates as an electron donor and carbon source. Most of the known species are facultative chemotrophs, in the presence of atmospheric oxygen. However some species are sensitive to oxygen. The growth of some species under anaerobic dark conditions is supported by respiratory electron transport (anaerobic respiration) in the presence of nitrate, nitrite, or nitrous oxide as electron acceptors. Denitrification is induced by nitrate either in dark or light but suppressed by oxygen. In the absence of external electron acceptors in anaerobic dark conditions, the growth is supported by fermentation of a number of substrates producing various organic acids as well as CO$_2$ and H$_2$ (Imhoff and Trüper 1992).

Most of the species are able to utilize a variety of different organic carbon sources. Organic carbon sources have different functions under phototrophic, respiratory and fermentative conditions. Under phototrophic growth they serve as a carbon source as
well as an electron donors. In the presence of inorganic electron donors, they are photoassimilated. One-carbon compounds such as CO, methanol and formate are used by strains of only a small number of species. When the reduced compounds serve as electron donors, they are first oxidized to CO₂, which in turn is assimilated. Some species also assimilate the aromatic organic compounds such as benzoate (Dutton and Evans 1967), and 3-chlorobenzoate (Krooneman et al. 1999).

In general some species of non-sulfur bacteria show a high level of resistance to toxic heavy-metal oxides and oxyanides, providing cells a sink for removing excess electrons and maintaining critical redox in vivo (Moore and Kaplan 1992, 1994). Autotrophic CO₂ fixation in all purple bacteria occurs via ribulose bisphosphate carboxylase and the Calvin cycle. Heterotrophic CO₂ fixation also occurs during assimilation of several reduced carbon sources by a number of carboxylating enzymes. The growth of most purple non-sulfur bacteria is inhibited by a low concentration of sulfide. Some species of Rhodopseudomonas are able to oxidize sulfide to sulfate without formation of elemental sulfur. None of the purple non-sulfur bacteria that oxidize sulfide deposit elemental sulfur inside the cell. Nitrogen fixation is a common property of all phototrophic purple bacteria. Most of the purple bacteria are able to photoproduce hydrogen. With dinitrogen or glutamate as a nitrogen source, a range of carbon sources may completely degrade to CO₂ and H₂, which in turn serve as a substrate for phototrophic growth.

*Rhodopseudomonas* species show a high degree of morphological and structural similarities, however they are phylogenetically quite diverse. Comparison of rRNA sequences, DNA-DNA, and DNA-RNA hybridization have demonstrated that these
bacteria belong to several different lines of descent within the alpha-2 subclass of
Proteobacteria (Hiraishi and Yoko 1994).

Imhoff et al. (1984) proposed a new arrangement for genus Rhodopseudomonas, based on morphological and physiological properties of species and also in variation of chemotaxonomic characters such as cytochrome c structure, lipid and quinone composition, lipopolysaccharide structure and DNA-DNA hybridization data. The proposed rearrangement was as follow: Rhodopseudomonas palustris (type species), Rhodopseudomonas viridis, Rhodopseudomonas sulfoviridis, Rhodopseudomonas acidophila, Rhodopseudomonas blastica, Rhodopseudomonas rutila and Rhodopseudomonas marina. The recent phylogenetic studies of Rhodopseudomonas species demonstrated that the type species Rhodopseudomonas palustris is more closely related to some chemotrophic taxa, such as Afipia felis, Bradyrhizobium japonicum, Blastobacter denitrificans and Nitrobacter winogradskyi, than to any of the other Rhodopseudomonas species for which rDNA sequences are available (Kawasaki et al. 1993, Hiraishi and Yoko 1994).

2.2.2 Habitats

Purple non-sulfur bacteria are widely distributed in nature and found in all kinds of stagnant water bodies in lakes, waste ponds, coastal lagoons, and paddy fields.

2.2.3 Application

Phototrophic non-sulfur bacteria have been used in sewage treatment processes (Kobayashi 1977, Irgens and Pfennig 1978) or bio-mass and vitamin production, as a valuable source for animal food (Sasaki et al. 1981), and production of molecular
hydrogen (Matsunaga et al. 1986). In bioenergetic research, they have been also used as cell-free system performing photosynthesis (Varga and Staehelin 1985, Svensson et al. 1996). Biodegradation of aromatic compounds such as benzoate (Dutton and Evans 1967), 3-chlorobenzoate (Krooneman 1999), and 4-hydrobenzoate (Gibson et al. 1997) by *Rhodopseudomonas* species have been reported. High heavy metal-resistance in some species of non-sulfur bacteria (Moore and Kaplan 1992) provide cells another capability to be used in bioremediation and bioreclamation of rare-earth metals.

2.3 Flocculation and flocculant

Flocculation is a common method used in many technologies dealing with the separation of a finely dispersed solid phase from a liquid suspension, by increasing the size of the particles. Flocculants are high molecular weight, water-soluble polymers, which promote coagulation of the particles in liquid, and facilitate gravity separation. Flocculants are used in food, coal, chemical, petrochemical metallurgy, textile, mining extraction, pulp and paper and other industries. The residue of the synthetic flocculant eventually enters the receiving water bodies after use. Depending on the median lethal concentration (LC$_{50}$) of the synthetic flocculant and complex pathological effects, it can be considered as a new micro pollutant class with high toxicity for aquatic organisms (Beim and Beim 1994).

Plant and fungal carbohydrates, such as plant gums, pectin, chitin, and chitosan, have been used in food and many other industries. Some of these carbohydrates have gradually been replaced in many larger-volume applications by the cellulosic derivatives, guar, and xanthan gum. The problems associated with the use of some of these carbohydrates are variability in quality, lack of adequate quality standards and variability

Many bacteria produce extracellular polymers, almost exclusively polysaccharide, as a hydrated capsule attached to the bacteria cell or as a viscous slime glycocalyx. Some bacterial carbohydrates have been used in various industrial processes, for example, xanthin in the food processing industry (Sanford and Baird 1983).

Native pectin, a plant carbohydrate, is highly methylated, and must be de-esterified to make low-methoxyl pectin to react strongly with metal ions and be used as a thickening agent (Norton et al. 1984). Plude et al. (1991) isolated a pectin-likes polysaccharide from the cyanobacterium Microcystis flos-aquae C3-40 which resembles pectin in sugar composition but not in sugar linkage, and is not detectably esterified and can accumulate metal ions without prior modification. Since the Microcystis flos-aquae capsule is an extracellular material, it can be readily washed from the surface of the cell and be used as an inexpensive alternative to pectin (Plud and Parker 1991).

Precipitation or flocculation of a dilute capsule was observed at various divalent cation concentrations. Fe" and Pb" increase the viscosity of the capsular carbohydrate at exceptionally low concentrations (Norton et al. 1984). A bioflocculant from the fungus, Aspergillus sp., was purified by Nam et al. (1996) which could flocculate all tested solids suspended in aqueous solution, including various microorganisms, organic acids and inorganic materials. This flocculation activity was not affected by pH from 3 to 8, but was stimulated by the addition of CaCl$_2$ (Moore and Kaplan 1994). Caccavo et al. (1996) showed the water turbidity in activated sludge decreased due to binding of the
cells to the floc in the presence of Fe\textsuperscript{+++}, but not Fe\textsuperscript{++}. After a lag of 6 hours (without aeration), the turbidity increased and Fe\textsuperscript{+++} reduction was observed. Deflocculation occurred due to the anaerobic reduction of Fe\textsuperscript{+++} to Fe\textsuperscript{++} by the microorganisms in the sludge (Caccavo et al. 1996).

There is a positive correlation with the concentration of the negatively charged polysaccharide with flocculant activity and the removal of the manganese from the water column beneath a microbial mat. Uronic acids with the charged carboxylic groups in bacterial exopolymers are the primary sites of metal interaction (Bitton and Freihofer 1978, Brown and Lester 1979, Houba and Remacle 1984, Cowen and Bruland 1985, Mitteelman and Geesey 1985, Stojkovski et al. 1986, Bender, Rodrigo-Eaton et al. 1994). The relationship between activated sludge-flocculation and polysaccharide concentration in gravity separation of activated sludge from a treated eluent was shown by Wahlberg et al. (1992). They suggested that a successful separation of suspended particles, which were incorporated into floc, was absolutely dependent on the cell-surface polysaccharide (Wahlberg and Keinath 1992). Takagi (1985) isolated and purified a strong flocculant agent from fungi (Paecilomyces sp). The polymer was composed almost purely of galactosamine, which was able to flocculate and sediment all kinds of microbial cells examined, and all suspended solids such as, red blood cells, charcoal powder, and cellulose powder, in aqueous solution (Takagi and Kadowaki 1985a, 1985b). A kaolin-flocculating extracellular glycoprotein was isolated from Byssochlamys nivea by Gomoiu et al., (1996). This polymer flocculated paper fibers, a constituent of white water effluent from paper manufacturing. The principal cross-linking moiety was a polysaccharide that binds to the kaolin fiber in the paper (Gomoiu and Catly 1996). Flocculation of the yeast
cells during the fermentation cycle was studied for its industrial interest. Suzzi et al. (1996) showed the involvement of glycoprotein surface factors in various patterns of flocculation, among different strains of *Kloeckera apiculata*.

Bar-Or (1987, 1988) screened flocculation properties of the many benthic cyanobacteria. They showed high levels of flocculant formation in cell extract and culture supernatants of *Phormidium sp.* strain J-1 and *Anabaenopsis circularis*. Analysis of the purified flocculant showed it to be a heteropolysaccharide consisting of sulfate, uronic acid, neutral sugar, protein and fatty acid. They also identified another exopolymer, from these bacteria, named emulcyan, which coated the cell surface and increase hydrophobicity of the cell, thus allowing detachment and dispersal of adherent cells.

### 2.3.1 Bacterial extracellular polymers

Most bacteria produce extracellular polymers in liquid or solid media in the form of capsules attached to the cell surface, or as a loose, soluble, viscous slime named glycocalyx. The chemical compositions of polymers are almost exclusively polysaccharide, and sometimes contain low proportions of other organic and inorganic compounds such as protein, lipid, pyruvate, sulfate, phosphate, etc. Because of their industrial applications (Sanford and Baird 1983, Beim and Beim 1994) and their involvement in a variety of specific biological interactions (Christensen 1989, Christensen and Characklis 1990), microbial exopolysaccharides have recently attracted some attention, but the chemical and physical properties of these valuable polymers are not well known.
2.3.2 Structure of bacterial extracellular polymers

The vast majority of bacterial exopolymers are polysaccharides (Christensen 1989). Based on the chemical composition, antigenic specificity, and the mode of biosynthesis the polymers are divided into two groups, namely specific and nonspecific polysaccharides (Kenne and Lindberg 1983).

2.3.3 Specific polysaccharides

Specific polysaccharides are specific to individual bacteria and contain common sugars such as glucose, galactose, mannose, rhamnose, N-Acetylglucosamine, glucuronic acid, and galacturonic acid. In addition to the common sugars, several unusual sugars have been found in specific polysaccharides, which may serve as a probe for polymer analysis (Cowen and Bruland 1985, Christensen 1989). Exclusive association of uronic acids with exopolysaccharide materials and its estimation as a qualitative measures of extracellular and cell wall polymers from environmental samples has been reviewed by Fazio et al. (1982). Detection and quantification of uronic acids from bacterial exopolymers has been reported extensively (Shabtai and Gutnick 1986, Quinterro 1989, Jikibara et al. 1992). Participation of negatively charged uronic acids, in metal binding property of bacterial exopolymers (Norton et al. 1984, Cowen and Bruland 1985, Stojkovski et al. 1986, Bender, Rodriguez-Eaton et al. 1994), account for its usage as a universal probe in bacterial carbohydrate studies.

The typical specific polysaccharide is composed of oligosaccharide repeating units, linear or branched, assembled into a polymer via glycosidic bonds. By traditional definition, oligosaccharides contain 2-10 sugar residues. However, since the naturally-occurring polysaccharide often contain more than 25-30 residues, it may be possible to
consider the range of the oligosaccharide between two to 20-25 sugar residues (Kennedy and Pagliuca 1994). The oligosaccharide repeating units may contain additional organic compounds such as pyruvate and acetate, as well as inorganic molecules such as sulfate, which seem to be less common in bacterial polysaccharide than in algal polysaccharide (Christensen 1989).

Despite the apparently regular structural pattern of polysaccharide consisting of oligosaccharide repeating units, structural variation and consequently variation in the solution properties are commonly observed. Both chain length and substituent pattern can vary, depending on bacterial strain, growth conditions, and chemical-physical processing of the samples (Sutherland 1977). Specific polysaccharides are sometimes called polysaccharide antigens (Jann and Jann 1977), because of their specific immunological properties which reside not only in individual monosaccharides, but also due to the position and configuration of glycoside linkages. Therefore, even a limited number of different monosaccharides give rise to a very large number of antigens (Christensen 1989). Serological techniques to detect antigenic differences have been used for very precise structural determination of polysaccharides (Jann and Jann 1977).

2.3.4 Examples of bacterial polysaccharides

A. Xanthan

Xanthan is a product of Xanthomonas campestris. It is one of the major commercial products obtained from bacteria and has a wide range of industrial and food applications. It contains five-sugar repeating units. The backbone is composed of a disaccharide (D-glucose) to which a trisaccharide side-chain (two mannose and one glucuronic acid) with an o-acetyl group, pyruvate ketal and acetate are attached
This polysaccharide has very useful solution property, including high viscosity over a wide range of temperatures and salt concentrations at low shear rates, but is considerably less viscous at high shear stress (shear thinning). This property is the basis for the successful commercialization of xanthan gum in many industrial processes (Sanford and Baird 1983).

B. Gellan series of polysaccharide

Eight chemically related deacylated commercialized polysaccharide products from bacterial strains, now considered to be *Sphingomonas paucimobilis* strains (Pollock 1993), possess linear structures in which there is at least an identical trisaccharide (D-glucose-D-glucuronic acid-D-glucose) sequence with the same configuration. The main structural differences are in the nature and the location of the monosaccharide or disaccharide side-chains. The polysaccharide forms a highly viscous aqueous solution and shows high thermal stability, but does not form a gel either before or after deacylation (Sutherland 1995).

C. Hyaluronan

Hyaluronan is a product of modern biotechnology methodology as well as an established product obtained from animal material. The bacterial product is a high value product, which can be synthesized by a number of *Streptococcus* spp., composed of repeating disaccharide subunits (Sutherland 1995).

D. Dextran

Dextran is the name given to a large class of extracellular bacterial polysaccharides composed of α-D-glucopyranosyl residues. Industrial dextran produced
by *Leconostoc mesenteroides* contains up to 95% of -D-(1v6) linkages, and a high molecular weight (in millions). The high proportion of (1v6) linkages gives it an unusual flexible backbone on which 3-hydroxyl groups in the consecutive position are available for binding to metal ions. Dextran is largely used in pharmaceutical and fine-chemical industries, and in gel precipitation for purifying, separating and concentrating metal for use as nuclear reactor fuel, etc (Sanford and Baird 1983).

2.3.5 **Non-specific polysaccharide**

The non-specific polysaccharide is found in a variety of bacterial strains and generally has simpler structure than the specific polysaccharide, containing only one monomer. For example, extracellular cellulose is produced by several *Acetobacter* species and is chemically similar to cellulose in higher plants. Bacterial dextran, bacterial glucan, bacterial fructan, and bacterial alginate are some other examples of bacterial nonspecific polysaccharides.
CHAPTER 3
MATERIALS AND METHODS

3.1 Isolation of bacteria

Microbial mats developed and used previously for heavy metal sequestering were used as the original cell sources. Mat cultures were exposed to a mixed heavy metals solution at 10 mg/l each (Cr(VI), Zn, Cd, Co, Hg). The red bloom bacterial growth appeared after a week of exposure to the metals solution. To isolate the red bacterium or bacteria, serial dilutions to $1 \times 10^{15}$ were prepared from the blended mats in 0.8 % NaCl solution under aseptic conditions. The diluted samples were dispersed over nutrient agar (Dickinson Microbiology Systems, Cokeysville, MD) plates, using the spread-plate technique.

Two sets of duplicate plates were prepared for aerobic/dark and anaerobic/light incubations. For the aerobic/dark condition, the plates were incubated at 30 °C. To provide the anoxygenic photosynthetic condition, one set of duplicate plates were incubated in an anaerobic chamber (Forma Scientific Inc. Marietta, Ohio) under 60 W incandescent bulbs at 50 cm distance. Colony formation was monitored daily over two weeks. No red colonies appeared on the plates incubated in aerobic/dark conditions. Several red colonies of different sizes and textures appeared after 5-7 days incubation under anaerobic/light conditions. The most dominant (above 80%) red colony with a
diameter of 2-3 mm was selected for isolation. Several representatives of these red colonies were individually transferred into screw-cap tubes filled with nutrient broth medium and incubated under the light at room temperature for enrichment. To ensure the purity of cell culture, streak dilution plates were prepared from each tube containing an originally isolated colony on nutrient agar medium, and checked for contamination. Two or three isolated colonies from each plate were individually transferred to filled nutrient broth tubes, and used as original stock culture. Streak dilution plates before each use confirmed the purity of each cell clone.

3.1.1 Metal resistance

The preliminary metal resistance properties of several isolated red colonies were determined by a rapid screening method described by Summers et al. (1977). A ditch of approximately 5 x 40 mm was cut in the center of the plate containing 20 ml of 15 g/l nutrient agar. Samples of bacterial culture at the log phase were streaked on the surface of the plates. After drying the streaks, the wells were filled with 1 ml of different concentrations (50-1000 mg/l) of metal solutions (As, Cd, Cr(III), Cr(VI), Co, Cs, Cu(I), Cu(II), Fe, Hg, Mn, Sr, Zn). The plates were incubated under an incandescent 60 W bulb at 50 cm distance in an anaerobic chamber (Forma Scientific, Inc.). After 7 days of incubation, the lack of any inhibition zone was considered as tolerance to the corresponding metal and related concentration.

The minimal inhibitory concentrations (MICs) were determined in triplicate by adding the isolate to several concentrations of metals in nutrient broth media (pH was adjusted to 6) in screw-capped flasks incubated in room temperature under light. Growth of the bacteria was monitored to the stationary phase by measuring the optical density at 620
nm against the blank containing medium and the same concentration of each metal, using a Beckman DU-650 UV/VIS scanning spectrophotometer (Beckman, Fullerton, CA) for two weeks. Growth factors were determined either directly from growth curves, or calculated using growth equations (Monod 1949).

3.1.2 Nutritional and physiological characteristics

The stock culture of isolated bacterium was maintained in nutrient broth medium under light. Experiments investigating the utilization of various carbon sources were carried out in triplicate in filter-sterilized AT medium containing: KH\(_2\)PO\(_4\), 1 g; MgCl\(_2\)•5 H\(_2\)O, 0.5 g; Ca Cl\(_2\)•2 H\(_2\)O, 0.1 g; NH\(_4\)Cl, 1 g; NaHCO\(_3\), 3.0 g; NaCl, 1.0 g; sodium acetate or other carbon source, 1.0 g and 1.0 ml of vitamin solution (Biotin, 10 mg; Niacin, 35 mg; Thiamine dichloride, 30 mg; p-Aminobenzoic acid, 20 mg; Pyridoxolium hydrochloride, 10 mg; Ca-panthothenate, 10 mg and 5 mg vitamin B\(_{12}\) per 100 ml distilled water) and 1.0 ml trace element solution (FeSO\(_4\)•7 H\(_2\)O, 1 g; H\(_3\)BO\(_3\), 300 mg; CoCl\(_2\)•6 H\(_2\)O, 190 mg; MnCl\(_2\) 4 H\(_2\)O, 50 mg; ZnCl\(_2\), 42 mg; NiCl\(_2\)•6 H\(_2\)O, 24 mg; Na\(_2\)MoO\(_4\)•2 H\(_2\)O, 18 mg, and 2 mg. CuCl\(_2\)•2 H\(_2\)O in one liter) as a base medium (Imhoff and Trüper 1992). Stock solutions of different carbon sources were filter-sterilized and added to the base medium to a final concentration of 1 g/l. The pH of the media was adjusted to 7-8, using 2N HCl or 2N NaOH.

Chemoorganotrophic cultures were grown in half-filled 250 ml flasks covered with sponges and incubated in a water bath shaker (30 °C, 100 rpm) in the dark. Photoorganotrophic cultures were grown in completely filled 150 ml screw-cap bottles and incubated under an incandescent 60 W bulbs at 50 cm. distance. Bacterial growth was monitored by measuring the optical density of cultures at time intervals at 620 nm, using a Beckman DU-650 UV/VIS scanning spectrophotometer (Beckman, Fullerton, CA). Viable
cell counts were estimated from a calibration curve relating the colony forming units (CFU) to OD$_{620}$. The growth rate constant (K), the number of generations (n) and the mean generation time (g) were calculated using the following standard mathematical growth equations (Monod 1949):

\[
k = \frac{n}{t} = \frac{\log N_t - \log N_0}{\log 2}\]

\[
g = \frac{1}{k}
\]

where $N_0$ is the initial population number; $N_t$ the population at time $t$, $n$ the number of generations in $t$, and $g$ the mean generation time.

3.1.3 Optimum temperature and pH

To determine optimum temperature, cells were cultured in nutrient broth medium and incubated in water baths adjusted to desired temperature under aerobic/dark, and anaerobic/light conditions. Optimum pH was measured in nutrient broth media adjusted to pH 5-9 at 30 °C. The cultures were prepared for aerobic/dark, and anaerobic/light conditions. The growth of the cultures was determined by monitoring the cell density at 620 nm.

3.1.4 Formation of intracellular sulfur globules

Detection of intracellular sulfur globules was performed according to method of Imhoff and Trüper, (1992). A drop of bacterial suspension was placed on the slide and a drop of neutralized sodium sulfide solution was drawn under the cover slip under light. The suspension was examined for the formation of intracellular sulfur globules after 20 minutes.
*Chromatium vinosum* (ATCC 17899), a sulfur oxidizing bacteria, was used as a positive control.

### 3.1.5 Absorption spectra

Absorption spectra of cell suspensions in sucrose (1.4 g/ml cell culture) were recorded against a blank containing 5 g of sucrose in 3.5 ml water, using Beckman DU-650 UV/VIS scanning spectrophotometer.

### 3.1.6 Electron microscopic analysis

Microscopic studies followed conventional methods of slide preparation and Gram staining. For thin-section electron micrographs, cells were fixed in 3% glutaraldehyde and placed in osmium tetroxide followed by several dehydration steps. Embedding was done by adding Epon and catalyst for twenty minutes. Electron micrographs were made using a Hitachi H-6000 Transmission Electron Microscope set at 0.3 nm point to point resolution, at 100,000 magnification.

### 3.1.7 DNA isolation and GC content

Six type species of *Rhodopseudomonas*, which share phenotypic characteristics with the isolated species were purchased from American Type Culture Collection (ATCC) (Table 1). These cells were cultured in media recommended by ATCC under phototrophic conditions. Cells were harvested at the exponential growth phase under phototrophic condition for DNA extraction. Genomic DNA was extracted with hexadecyltrimethylammonium bromide (CTAB) (Sigma Chemical Company, St. Louis, MO) using a method described by Doyle (1990). Bacterial cells were washed in washing
solution buffer (0.5 M NaCl, 0.05 % sodium sarkosyle, 0.05 M tris HCl and 0.02 EDTA, pH 7.5). Washed cells were incubated in CTAB buffer (4 % CTAB, 2M tris HCl, pH 8) at 65 °C for 60 minutes. The tubes were centrifuged at 15,000 x g for 10 minutes, and supernatants were collected. The genomic DNA was extracted from supernatant twice with phenol/chloroform/isoamyl (25:24:1 v/v), and once with phenol/chloroform. DNA was precipitated by absolute ethanol, and dissolved in TE buffer. DNA quantification was performed using spectrophotometric method. The optical density of the DNA sample at 260 nm was obtained using a Beckman DU-650 UV/VIS scanning spectrophotometer. An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA. The GC base content of the isolate was determined according to the method described by Meyer (1977).

3.1.8 Random amplified polymorphic DNA (RAPD-DNA) analysis

GeneAmp 2400 (Perkin Elmer, Cetus, Emeryville, CA) thermocycler was used in all the amplifications. All primers were obtained from Genosys Biotechnologies, Inc (The Woodlands, TX). The sequences of these primers are listed in Table 2. The GC content of these primers is 70%. RAPD-PCR typing was adapted from a method described by Pharmacia Biotech (1996) (Pharmacia Piscataway, NJ). PCR was carried out in a reaction pre-mix containing Ready-To-Go RAPD analysis beads (AmpliTaq and Stoffel fragment), dNTPs (0.4mM each dNTP), BSA (2.5µg), buffer, 3mM MgCl2, 30mM KCl and 10mM Tris, (pH 8.3); 5-10 ng of template, and 25pmol of primer in a 25µl reaction volume. The reaction mix was subjected to 1 cycle at 95° C for 5 minutes followed by 45 cycles of 95° C for 1 minute, 36° C for 1 minute and 72° C for 2 minutes. A negative control with no template was included. The RAPD-PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. A 100 bp and pGEM molecular
weight markers (GibcoBRL, Gaithersburg, MD) were used as reference. All the bands were scored in reference to the 100 bp ladder.

3.1.9 16S rDNA sequencing

16S ribosomal DNA (rDNA) fragments that correspond to positions 536 to 926 of Escherichia coli (Brosius et al. 1978) were amplified by PCR using colony PCR. Primers used were universal primers designated by Lane et al (1985) as A and B. Primer A (5'CAGCAGC-CGCGGTAATAC) hybridized at 519 - 536, and primer B (5'CCGTCAATTCCTTTGAGTTT) at 907-926 in E. coli 16S rRNA gene (1985). PCR conditions were as follow: 95°C for 4 minutes, 5 cycles of 95 °C/1 minute, 37°C/1 minute, 72°C/1 minute, then 25 cycles of 94°C/1 minute, 39°C/1 minute, 72°C/1 minute, followed by 72°C/7 minutes, and 4°C. Sequencing was done using ABI systems and Big Dye Termination kit by Perkin Elmer (Cetus, Emeryville, CA). The reactions were analyzed using DNA analysis program Lasergene, DNASTar (Madison, Wisconsin).

3.1.10 Phylogenetic analysis

Compilation of sequence data and calculation of sequence similarities were performed using BLAST search, Genbank, National Center for Biotechnology Information, National Institutes of Health. Phylogenetic analysis used was SEQUENCE_MATCH version 2.7, Michigan State University, Ribosomal Database Project II (HYPERLINK http://eme.msu.edu/RDP/egis.seqmatch.egi)

http://www.cme.msu.edu/RDP/egis.seqmatch.egi
3.2 Production of bioflocculant in microbial mat culture

3.2.1 Microbial mat culture

Previously developed microbial mats were blended using a kitchen blender and used as the original source for the microbial mat culture in this study. The blended mat was cultured in AA medium containing, per liter of deionized water: Fe/EDTA (Ferric-Sodium salt of Ethylenediaminetetraacetate), 0.3 g; NaHCO₃, 0.84 g; NaCl, 0.234 g; MgSO₄·7H₂O, 0.246 g; CaCl₂·2H₂O, 0.07 g; K₂HPO₄, 0.348 g; KH₂PO₄, 0.041 g, and 2.5 ml trace element solution containing, per liter: H₃BO₃, 2.86 g; MnCl₂·4H₂O, 1.181 g; Na₂MoO₄·5H₂O, 0.39g; CuSO₄·5H₂O, 0.08g; Co(NO₃)₉·6H₂O, 0.05g (2). Cultures were covered with plastic wrap and incubated under 60 W incandescent bulbs at 50 cm distance at room temperature for 8 weeks.

3.2.2 Bioflocculant production

Production of bioflocculant in the culture media was detected and monitored by performing Alcian Blue Binding test on cell free culture media as described by Fattom et al. (1984). Alcian Blue Binding Test is an indirect method for quantification of flocculant concentration in solution. A solution of 0.5 mg Alcian Blue /ml (Sigma St. Louis, MO) in 0.5 M acetic acid was prepared. Samples of culture media were centrifuged at 20,000 g for 20 minutes. A sample of 0.5 ml of culture supernatant was collected in each time interval and added to 4.25 ml of 0.5 M acetic acid and mixed. Then 0.25 ml of Alcian Blue solution was added to the mixture. After standing overnight, the solutions were mixed and centrifuged at 20,000 g for 20 minutes. Optical densities of supernatants were determined at 610 nm in a Beckman DU-650 spectrophotometer. A sample of AA medium was tested along with the culture samples.
as control. A solution of 0.5 M acetic acid was used as blank. The difference in absorbance between the control and the sample is proportional to the bioflocculant concentration. A linear relationship exists between flocculant concentration and the difference in absorbance in the range of 0-100 µg/ml (Bar-Or and Shilo 1986).

3.2.3 Biofilm formation and quantification

Biofilm formation was started by formation of a thin layer biofilm, underneath the mat, which grew gradually toward the bottom of the culture tube. Biofilm formation was quantified by cell density estimation and total carbohydrate measurement. To obtain biofilm, microbial mats were grown in vessels (25 x 10 x 5 cm), containing AA medium at the desired condition. Several acid washed glass slides (75 X 25 mm) were held vertically in the culture vessels. The slides were removed at time intervals from the vessels, rinsed with 0.8 % NaCl solution, and placed in 50 ml beakers. The biofilms were removed carefully off the slides and dispersed in 20 ml of 0.8 % NaCl solution by sonication for 5 minutes using an ultrasonic liquid sonicator (Fisher scientific) and used for quantification. The optical density of biofilm was estimated turbidimetrically against saline solution at 600 nm, using a Beckman DU-650 spectrophotometer (Beckman Instrument Inc.). Total carbohydrate concentration of biofilm was determined using phenol-sulfuric method (described below).

3.2.4 Effect of environmental factors on the bioflocculant production

A. Effect of pH and temperature

The microbial mats were grown in AA media which were adjusted to different pHs (4-13). The pH of the culture media, bioflocculant production, and biofilm
formation was monitored for 56 days as described above. Microbial mats were grown in AA medium at pH 7.5 and incubated at 4, 20, 35, 40, 45, and 50 °C. The bioflocculant production and biofilm formation were monitored during 56 days.

B. Effect of heavy metals concentrations

Microbial mats were grown in 100, 50, and 25 mg/l of each Zn, Se and Cr(VI) in AA media. The bioflocculant production and biofilm formation were monitored for 56 days.

3.2.5 Isolation of bioflocculant

Bioflocculant was isolated using a slightly modified method described by Doherty et al (1988). A four-week old mat culture (at the late log phase) was filtered with Whatman filter paper number 2, and centrifuged at 20,000 g for 20 minutes. The supernatant was precipitated by 0.1 volume of 0.1 % hexadecyltrimethylammonium bromide (CTAB) (Sigma Chemical Company, St Louis, MO.). The precipitate was dissolved in 10 % NaCl, and re-precipitated with two volumes of cold acetone, and dissolved in water. The isolated carbohydrate was dialyzed against deionized water for 5 days with several changes of deionized water, and lyophilized on Labconco Freezdry System Freezor (Labconco Corporation, Kansas City, MO.). Total carbohydrate content and bioflocculant activity were detected at each step of isolation.

3.2.6 Purification of bioflocculant

For further purification and fractionation of the isolated bioflocculant, 2 ml of bioflocculant (5 mg/ml) was applied to size exclusive chromatography using Sepharose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ) column (1,5 X 65 cm) and
eluted by 0.1 M NaCl in 2 mM tris buffer (pH 8.5). Fractions (3.0 ml) containing carbohydrate and showing bioflocculant activity were pooled, dialyzed against deionized water, and lyophilized using a Labconco Freezdry System.

3.2.7 Total carbohydrate content

Total carbohydrate was determined by phenol-sulfuric acid assay described by Kennedy (1994). A range of 5-60 nmol of methyl manno-lyranoside was used to prepare the standard curve. Several concentrations of purified bioflocculant were prepared and 200 μl of 5 % w/v phenol in water was added to 200 μl of samples, standards, and water as blank. One ml of concentrated sulfuric acid was added to each mixture and allowed to stand for 10 minutes. After shaking, the optical density of the mixtures was measured at 490 nm in Beckman DU-650 spectrometer (Beckman Instrument Inc, Fullerton, California). A standard plot was generated for OD versus concentration. The total carbohydrate of each sample was calculated from the standard curve.

3.2.8 Total protein determination

Total protein was measured using the Bio-Rad (Bio-Rad, Richmond, California) protein microassay. The procedure was performed according to the recommended protocol by Bio-Rad. The absorbency of the samples and standards (bovine serum albumin) were measured against the blank at 595 nm, using a Beckman DU-650 spectrophotometer. Total proteins of the samples were calculated from the standard curve.

3.2.9 Sulfate content determination

Sulfate content of the isolated bioflocculant was measured by sodium rhodizonate
method according to Terho (1971). Sodium rhodizonate solution was made by dissolving 5 mg sodium rhodizanate (Merck AG, Darmstadt) in 20 ml water, followed by adding 100 mg ascorbic acid (Merck). The volume was made up to 100 ml with ethanol. Sodium sulfate was used as the sulfate standard. Two ml ethanol, 1 ml of BaCl₂ buffer (10 ml of 2.0 M acetic acid, 2 ml of 0.005 M BaCl₂, 8 ml of 0.02 M NaHCO₃, in 100 ml dH₂O), and 1.5 ml of sodium rhodizonate solution were added to 0.5 ml of different concentrations of bioflocculant (5-500 μg/ml), standards (1-20 μg/ml sulfate), and water (as blank). The tubes were shaken well and allowed to stand 10 minutes in the dark at room temperature. The intensity of color was measured at 520 nm, using a Beckman DU-650 spectrophotometer. A standard plot was generated and sulfate content of samples were calculated using the standard curve.

3.2.10 SDS-PAGE electrophoresis

SDS-PAGE was used to verify the purity of the bioflocculant after fractionation by column chromatography. Electrophoresis was performed with some modification of the method of Gregory and Culp (1990) using a vertical slab gel apparatus with a discontinuous buffer system (model SE 400, Hoefer Scientific Instrument San Francisco, CA). The polyacrylamide stock solution was prepared by dissolving 29.2 g of acrylamide and 0.8 g of bisacrylamide in final volume of 100 ml water (30%). A 4-25% gradient gel was prepared as follows: 10 ml of 4% gel [6.05 ml water, 2.5 resolving gel buffer (1.5 M Tris-HCl pH 8.8), 0.1 ml 10% SDS, 1.33 ml acrylamide/bis stock solution, 50 μl 10% ammonium persulfate (APS) and 5 μl teramethylethylenediamine (TEMED)] and 10 ml of a 20% gel (1.5 ml resolving gel buffer, 0.1 ml 10% SDS, 8.33 ml acrylamide/bis stock solution, 50μl 10% APS, and 5μl TEMED) were poured into the
assembled plates in a casting stand using a gradient former (Model 385, Bio-Rad) apparatus. After polymerization of the resolving gel, the butanol layer was removed and washed by water, and a layer of 3 cm of a 4% stacking gel [6.05 ml water, 2.5 stacking gel buffer (0.5 M tris-HCl pH 6.8), 1.33 ml acrylamide/bis stock solution, 0.1 ml 10% APS, and 5 µl TEMED] was poured. The well forming comb was placed at the top of plates, and the gel allowed to polymerize. The gel was pre-run for 10 minutes at 30 mA immediately prior to loading samples. The sample was dissolved in sample buffer (4 ml of 0.6 M tris buffer pH 6.8, 5 mg bromophenol blue, 2 ml glycerol), and loaded on the gel. The gel was run at 30 mA until the samples reached the bottom of the stacking gel, then at 100 mA, until the dye front reached the bottom of plate.

3.2.11 Alcian-Blue silver stain

The gel was immediately immersed in Alcian Blue solution (0.005% w/v in a solution consisted of 40% ethanol, 5% acetic acid in water) for 30 minutes. The gel was rinsed in water for one minute and oxidized in sodium meta-periodate solution (0.7% in water) for 10 minutes. The oxidized gel was washed in dH₂O for 20 minutes and stained with 10% silver solution (Bio-Rad silver concentrate) for 10 minutes and was developed in Bio-Rad developer (3.0 g/100 ml dH₂O). The gel was washed and stored in dH₂O for photography.

3.3 Glycosyl composition analysis

3.3.1 Fluorophore electrophoresis

A fluorophore-Assisted Carbohydrate Electrophoresis (FACE) system was used to identify the monosaccharide composition of bioflocculant. Glyko FACE monosaccharide
composition kit (Glyko Inc., Novato, CA.) was used in this study. Purified bioflocculant was dialyzed against distilled water to remove buffers (buffers interfere with hydrolysis by changing the pH), and lyophilized by Labconco Freezedry System (Labconco Corporation).

A. Hydrolysis for neutral sugar content

Bioflocculant was suspended in dH2O (10-20 µg/50 µl dH2O) in 0.5 ml snap top tubes. An equal volume of 4N TFA was added to sample tubes and Mono Composition Control containing 1 nmoles of N-Acetyllactosamine. The Mono Composition Control was used to monitor the efficiency of hydrolysis reactions, re-N-acetylation reactions and fluorophore labeling reactions.

Hydrolysis reactions were incubated in a heat block with deep wells at 100 °C for 5 hours, then spun down briefly, cooled for 30 minutes to one hour at -20°C, and dried in centrifugal vacuum evaporator using SpeedVac Concentrator SVC100H (SAVANT Instrument Inc. Holbrook, NJ).

B. Hydrolysis for amine sugar content

Reaction mixtures containing 10-20 µg of bioflocculant in 50 µl dH2O and 50 µl of 8N HCl were incubated in a heat block at 100 °C for one hour. The reactions were cooled at -20 °C for 30 minutes and dried using a Speed Vac Concentrator. The dried pellets were re-N-acetylated by adding 10 µl of re-Nacetylation reagent (200 µl dry methanol, 20µl pyridine, and 20µl acetic anhydride) and incubated on ice for 30 minutes. The re-N-acetylated samples were dried in a centrifugal vacuum evaporator.
B. Fluorophore labeling

The dried monosaccharide pellets were resuspended in 2.5 μl of labeling diluent (Glyko), and labeled by 2.5 μl Monolabeling Dye, AMAC (2-aminoacridine) solution and incubated at 37°C overnight. The samples were dried to the viscose gel stage by a centrifugal vacuum evaporator.

C. FACE electrophoresis

Labeled samples and controls were resuspended in 15 μl of labeling solvent and mixed with the same volume of loading buffer. Four microliter of samples and control were loaded in a cassette, using FACE apparatus. The electrophoresis gel was run at a constant voltage of 400 at 4°C until the dye front reached the bottom of the gel.

3.4 GC/MS analysis

Preparation of trimethylsilyl derivatives: Several quantities of purified bioflocculant were prepared. Known quantities of myoinositol (20 μl of 1 μg/ml solution) were added to each sample as internal control. The samples and standards were dried under nitrogen gas. A mixture of 2 ml of dry methanol in 260 μl of acetylchloride was prepared in ice. After cooling the samples were removed from the ice, 500 μl of this mixture was added to each sample and standard, and incubated at 80°C for 16 hours in a chemical fume.

The samples were dried under air and heat (25-30°C), and re-acetylated by adding 200 μl of dry methanol, 20 μl of pyridine, and 20 μl of acetic anhydride, and incubating at 100°C for 20 minutes. The tubes were removed from the oven, cooled and air dried. The N-acetylated samples were sialylated using 200 μl of Tri-Sil (Tri-Sil no
48999, PIERCE for GC. 815- 964-0747) and run on a Hewlett Packard 5890-5970 GC-MS system (Hewlett Packard CA.) using a SP2330 column (Supelco, Bellefonte PA).

### 3.5 Molecular weight determination

Molecular weight of the flocculant was estimated by gel filtration chromatography using a Bio Gel A-1.5 m gel, 200-400 mesh (exclusion limit of 1.5 \( \times 10^5 \)) column (1.5 X 60 cm) (Bio-Rad Laboratory). The void volume of the column was measured by passing the excluded high-molecular weight material, dextran blue with molecular weight of 2 \( \times 10^6 \) through the column. Gel Filtration Standard, a mixture of protein markers ranging from 1,350-670,000 daltons (Bio-Rad) was used to calibrate the column. Bioflocculant was passed through the same column. The molecular weight of bioflocculant was estimated by extrapolation of parameter \( K \) versus \( \log M \) (molecular weight) of standard markers. The parameter \( K \) is defined as \( (V_e - V_o) / V_s \), in which \( V_e \) is the volume of the solvent required to elute the molecule of interest, \( V_o \) is the void volume, \( V_s \) is the volume of stationary phase that can be measured by \( V_t - V_o \), in which \( V_t \) is the total volume of the column (Freifelder 1976).

### 3.6 Determination of isoelectric point

The isoelectric point of bioflocculant was determined using a Bio-Rad Rotofor cell system model 120 (Bio-Rad laboratory). A multicharged structure (ampholyte) buffer was used to establish a pH gradient from 3 to 10 increasing in pH from anode to cathode. Cation Exchange membrane (equilibrated in 0.1M \( \text{H}_3\text{PO}_4 \)) was used to separate the negatively charged bioflocculant from electrolyte while allowing current flow. The chamber was pre run at 5 watts for 5 minutes before loading the sample. Two ml of
a 2.5 mg/ml bioflocculant was loaded to the sample cell. The chamber was connected to a recirculating coolant (Fisher Scientific) set at 4°C. The system was allowed to reach thermal equilibrium for 10 minutes before running. The power supply was set on 12 W constant and run for 90 minutes until the voltage stabilized at 1200 volts, and continued for 30 minutes at constant voltage. The fractions were collected inside the harvest box by applying vacuum. Total carbohydrate, flocculant activity and the pH of fractions were determined.

3.7 Viscosity and solution property

3.7.1 Viscosity determination

A 10 ml Cannon-Fenske capillary viscometer No 150, 46 ID (Fisher Scientific, Pittsburgh, PA) was used to measure viscosity. Kinematic viscosity is based on the value of the water adopted by the National Institute for Standards and Technology and the American Society for Testing Materials, July 1, 1953. The kinematic viscosity basis is 1.0038 mm²/s (Cst) for water at 20°C. The gravitational constant, g, is 980.10 cm/sec² at the Cannon Instrument Company (State College, PA 16804), and varies up to 1% in the United States. The correction of gravitational constant was ignored in this study.

The viscometer was suspended in precise vertical orientation in a circulatory chamber (Fisher Scientific). Bioflocculant was poured into the viscometer and allowed to reach to equilibrium temperature for 20 minutes. Bioflocculant was drawn to the upper chamber of the viscometer by applying vacuum. The time required for the meniscus of the solution to move from the upper to the lower marked level on the viscometer was measured by a stopwatch. The check run was made by repeating the above step at least 3 times. The kinematic viscosity in mm²/s (centistokes) was
calculated by multiplying the efflux time in seconds by the viscometer constant. The viscometer constant at 40 °C and 100 °C are 0.03216 and 0.03200 mm²/s² (cSt/s) respectively. The viscometer constant at other temperatures was interpolated from known values according to the company instructions. To obtain the viscosity in mPas (cP), the kinematic viscosity in mm²/s (cSt) was multiplied by the density in grams per milliliter. Due to low concentration of bioflocculant (less than 5 mg/ml), the density of bioflocculant was assumed equal to the density of the water.

The viscosity of the bioflocculant was monitored at several temperatures (0-80 °C), and concentrations (1-5.88 mg/ml). The viscosity data were analyzed according to standard equations (Chang and College 1996).

\[
K_v = (T_2 - T_1)K \\
\eta = K_v \eta_p \\
\eta_{sp} = \frac{\eta - \eta}{\eta} \\
\eta_{red} = \frac{\eta_{sp}}{c}
\]

where \(K_v\) is the kinematic viscosity in mm²/s (1 centistokes), \(T_2\) is the flow time of solution, \(T_1\) is the flow time of solvent in second, \(K\) is the viscometer constant, \(\eta\) is viscosity in mPa s (cP), \(P\) is density in g/ml, \(\eta_{sp}\) is the specific viscosity, \(\eta'\) is the solution viscosity, \(\eta\) is the viscosity of solvent, \(\eta_{red}\) is reduced viscosity, and \(c\) is unit polymer concentration.

### 3.7.2 Effect of concentration and temperature on viscosity

Viscosity of bioflocculant was measured at various concentrations (1-5.88 mg/ml) at 20 °C. Viscosity of bioflocculant at 5.88 mg/ml concentration was measured between 20 to 80 °C.
3.7.3 Effect of metals on viscosity

The effects of increasing concentration (0.005 - 100 mM) of Ca, CrVI, Zn, and Cu on viscosity of bioflocculant (5.88 mg/ml) at 20 °C were detected using a kinematic viscometer tubes no 150, 46 1D (Fisher Scientific, Pittsburgh, PA).

3.8 Bioflocculant-metal binding studies

Metal binding properties of bioflocculant were studied using equilibrium dialysis, flow dialysis and gel filtration chromatography.

3.8.1 Gel filtration

Biogel P6 (Bio-Rad Laboratory) column chromatography (1x25 cm) was used to separate metal and metal-bound bioflocculant. The column was equilibrated with deionized water. One ml of 5 mg/ml metal solution was added to one ml of 5 mg bioflocculant/ml and incubated in room temperature for 24 hours, the metal-bioflocculant complex (0.5 ml) was loaded on the column and was eluted by deionized water. Fractions of 0.5 ml were collected and tested for total carbohydrate content, bioflocculant activity, and metal concentration.

3.8.2 Equilibrium dialysis

Spectra/Por Macrodialyzer (Spectrum, Houston Texas) consisting of two 2-ml compartments (Fig 30) was used to assess metal-binding property of the carbohydrate (Dejong et al. 1976, Kenneth 1995). A semi-permeable Spectra/Por membrane (Spectrum Medical Industries, Inc., Houston TX) separated two compartments of the microdialyzer with MW cutoff of 12-14 KD. Two ml of 5.88 mg carbohydrate/ml was inserted into the one compartment and two ml of several concentrations of various metals...
(Zn and Cd) were inserted into the other compartment. The system was allowed to equilibrate at room temperature for 24 hours with continuous stirring. After equilibration a sample of each compartment was analyzed for metal concentration, using atomic absorption spectrometer SpectrAA.20 (Varian Instrument Group, Palo Alto, CA).

3.8.3 Flow rate dialysis

Flow rate dialysis was performed to achieve saturation of metal binding sites of the bioflocculant. Two ml of bioflocculant (5.88 mg/ml) was dialyzed against 750 ml of 47 and 132 mg Zn/l and 54 and 180 mg Cd/l solutions at 1.5 ml/minute flow rate and continuous stirring, using Spectra/Por Macrodialyzer. A sample of bioflocculant was analyzed for metal concentrations using SpectrAA 20 (Varian Instrument Group). To assess metal binding property of bioflocculant in seawater, bioflocculant (5.88mg/ml) was dialyzed against solutions of 55 mg/l Zn and 40 mg/l Cd in 0.15 and 0.3 M NaCl, and analyzed for metal concentration.

3.9 Flocculating property

Primary detection of flocculating activity by purified bioflocculant was performed using Alcian Blue Binding test as described earlier (Fattom 1984). A sample of 0.5 ml of several concentrations of purified bioflocculant was added to 4.25 ml of 0.5 M acetic acid and mixed. Then 0.25 ml of Alcian blue solution was added to the mixture. After standing overnight, the solutions were mixed and centrifuged at 20,000 g for 20 minutes. Optical densities of supernatants were determined at 610 nm using a Beckman DU-650 spectrophotometer (Beckman Instrument Inc., Fullerton, CA).
*E. coli* and *Rhodopseudomonas* cells were used as test microorganisms for quantification of flocculating activity. In this assay, described by Takagi and Kadowaki (1985a, 1985b) bacterial cells were washed three times and suspended in 0.8% NaCl solution to the OD value of 2 at 660 nm. Bioflocculant was added to the bacterial suspension to the final concentration 10 ng to 2.5 μg/ml. The turbidity of the dilutions was measured at different times on at 660 nm, using a Beckman DU-650 spectrophotometer. Equal dilutions of bacterial suspension were used as control.
CHAPTER 4

RESULTS

4.1 Cell and colony morphology

The bacteria formed dark red colonies with diameter of 2-3 mm on agar under phototrophic conditions. The strain appeared as facultative anoxygenic photoorganotrophs, motile, gram negative rod-shaped cells measuring 0.4-0.6 μm wide and 2.0-2.4 μm in length (Figure 1). The cells divided asymmetrically by budding at the ends of the mother cell. The dumbbell shaped dividing cells are shown in Figure 2. Electron micrographs of thin sections of the bacterium showed lamellar intracytoplasmic membranes (ICM) (Figure 3).

4.2 Phototropic properties

Cells grown under anaerobic, phototropic conditions formed photosynthetic pigments but cells grown under chemotrophic, aerobic conditions did not form photosynthetic pigments. Absorption spectra of heterotrophic cells suspended in sucrose, or isolated intracytoplasmic membrane in phosphate buffer, pH 7.0, had three major peaks at 590, 805 and 871 nm indicating the presence of bacteriochlorophyll a (375, 590, 805, 830-890 nm), and the absence of bacteriochlorophyll b (400, 605, 835-850, 1020-1040 nm) and bacteriochlorophyll c (745-755 nm), which are characteristic of purple non-sulfur bacteria (Figure 4). The optimal temperature for cell growth was about 30-32°C in both
Figure 1. Thin section electron micrograph of the isolate
(bar, 0.9 μm)
Figure 2. Scanning electron micrograph of the isolate
Circle shows the a budding cell; bar, 1 μm
Figure 3. Thin section electron micrograph of the isolate, showing ICM (bar, 0.3 µm)
Figure 4. Absorption spectrum of cell suspension of the isolate
heterotrophic and photoorganotropic conditions. The optimal growth occurred at pH 7-8.

4.3 Physiological and metabolic properties

Phototrophic and heterotrophic growth of isolated *Rhodopseudomonas* sp. was observed on several carbon and electron donor sources. Some growth parameters under phototrophic and heterotrophic conditions on various media are listed in Table 1. The growth of the bacteria on nutrient broth was significantly higher than on the medium with the single sole carbon sources. The isolate was able to utilize several carbon sources (Table 2). Morphological and physiological comparison analysis of the isolate and related species of *Rhodopseudomonas* genus is shown in Table 3.

<table>
<thead>
<tr>
<th>Media</th>
<th>Tₐ(h)</th>
<th>K (h⁻¹)</th>
<th>g (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient broth</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Photo</td>
<td>95</td>
<td>0.21</td>
<td>4.76</td>
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<tr>
<td>Heter</td>
<td>132</td>
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<tr>
<td>Acetate</td>
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<td></td>
<td>175</td>
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<tr>
<td>Succinate</td>
<td>168</td>
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<td>21.3</td>
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<td></td>
<td>258</td>
<td>0.041</td>
<td>24.7</td>
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<tr>
<td>Ethanol</td>
<td>234</td>
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<td>20.8</td>
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<tr>
<td>Glucose</td>
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<tr>
<td></td>
<td>258</td>
<td>0.040</td>
<td>25.0</td>
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<tr>
<td>Pyruvate</td>
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<tr>
<td></td>
<td>310</td>
<td>0.044</td>
<td>23.7</td>
</tr>
</tbody>
</table>

Value Tₐ is end of exponential phase in hours determined directly from growth curves; K (growth rate constant per hour) and g (mean generation time in hours) were calculated from Monod growth equations (Monod 1949); photo, phototrophic condition; heter, heterotrophic condition.
<table>
<thead>
<tr>
<th>Substrate, reaction or enzyme</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
</tr>
<tr>
<td>Malate</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Valerate</td>
<td>+</td>
</tr>
<tr>
<td>Benzoate</td>
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</tr>
<tr>
<td>Tween 80 hydrolysis</td>
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</tr>
<tr>
<td>Nitrate reduction</td>
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</tr>
<tr>
<td>Catalase</td>
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<td>3% NaCl</td>
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<td>Ornithine</td>
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<tr>
<td>Sodium citrate</td>
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<td>Sodium thiosulfate</td>
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<td>Urea</td>
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<td>Tryptophan</td>
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<td>Gelatinase</td>
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<td>Starch hydrolysis</td>
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<tr>
<td>Arabinose</td>
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</table>

+, positive growth or reaction; −, negative growth or reaction; ±, partially positive
Table 3. Comparison of the isolate and related species

<table>
<thead>
<tr>
<th>Property</th>
<th>Rp</th>
<th>Ra</th>
<th>Rr</th>
<th>Rro</th>
<th>Rv</th>
<th>Rm</th>
<th>Isolate</th>
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<td>Cell shape</td>
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<td>Rod</td>
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<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Cell width</td>
<td>0.6-0.9</td>
<td>1.0-1.3</td>
<td>0.4-1.0</td>
<td>0.8-1.0</td>
<td>0.6-0.9</td>
<td>0.7-0.9</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td>Cell length</td>
<td>1.2-2.0</td>
<td>2.0-6.0</td>
<td>1.5-3.0</td>
<td>2.0-3.0</td>
<td>1.2-2.0</td>
<td>1.2-2.3</td>
<td>2.0-2.4</td>
</tr>
<tr>
<td>Cell suspension color</td>
<td>Red to dark brown</td>
<td>Orange, brown, purple</td>
<td>Deep-red</td>
<td>Pink</td>
<td>Olive-green</td>
<td>Pink</td>
<td>Red</td>
</tr>
<tr>
<td>Relation to O₂</td>
<td>ac</td>
<td>ac</td>
<td>ac</td>
<td>m</td>
<td>m</td>
<td>m</td>
<td>ac</td>
</tr>
<tr>
<td>Type of Bchlo</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>GC content mol %</td>
<td>64.8-66.3</td>
<td>62.2-66.8</td>
<td>67.6-69.4</td>
<td>69.7</td>
<td>66.3-71.4</td>
<td>61.5-63.8</td>
<td>&lt;70</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzoate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>+</td>
<td>N</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
<td>N</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>+</td>
</tr>
</tbody>
</table>

R, *Rhodopseudomonas*; P, *palustris*; a, *acidophila*; r, *rutila*; ro, *rosea*; v, *viridis*; m, *marina*; ae, aerobic; m, micriaerophilic; Bchlo, bacteriochlorophyll; N, no data
4.4 RAPD-PCR profiles

RAPD-PCR showed different banding profiles under a range of precisely quantified DNA concentrations for all the seven bacterial species under study (Table 4).

Representative RAPD profiles obtained from amplifications using ten different primers (Table 5), are shown in Figure 5. Primer #70-03 produced a 350-bp fragment in samples 1 and 6. Sample 2 had a doublet band of 350-bp, while sample 3 had a unique 1900-bp. A 700-bp was shared by samples 1, 2, 4, 5 and 7. Sample 3 produced a distinctive high molecular weight band (Figure 5A). Primer #70-05 (Figure 5B) produced an intensive 600-bp band in sample 7 that is relatively weak in sample 6. Primer #70-14 (Figure 5C) produced 5 bands in samples 1 and 2, but weak bands of 550 and 650-bp in sample 2. Sample 3 had weak multiple bands with distinguishable 150-bp band shared with sample 7. Sample 5 had one high molecular weight band. Sample 6 had an intensive 300-bp band also shared by sample 4.

Although more amplicons were shared by samples 1 and 2 with Primer #70-14, sample 2 had an intensive band of 2072-bp. Sample 3 had multiple weak bands with a 1900-bp band shared by samples 4 and 7. Sample 7 had a distinguishable band of 300-bp.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Species</th>
<th>ATCC #</th>
<th>Reference #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rps. palustris (RPS p)</td>
<td>17001</td>
<td>Skerman 1980</td>
</tr>
<tr>
<td>2</td>
<td>Rps. acidophila (RPS a)</td>
<td>25092</td>
<td>Skerman 1980</td>
</tr>
<tr>
<td>3</td>
<td>Rps. rutila (RPS r)</td>
<td>33872</td>
<td>Akiba 1983</td>
</tr>
<tr>
<td>4</td>
<td>Rps. rosea (PRS ro)</td>
<td>49724</td>
<td>Jansson 1991</td>
</tr>
<tr>
<td>5</td>
<td>Rps. viridis (PRS v)</td>
<td>19567</td>
<td>Skerman 1980</td>
</tr>
<tr>
<td>6</td>
<td>Rps. marina (RPS m)</td>
<td>35675</td>
<td>List # 14 1984</td>
</tr>
<tr>
<td>7</td>
<td>Isolate</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
Figure 5. RAPD-PCR profile of *Rhodopseudomonas* species
Lane 1-6, known species; lane 7, the isolate; M, 100 bp ladder
Table 5. Sequences of RAPD-PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>70-02</td>
<td>CAGGGTTCGAC</td>
</tr>
<tr>
<td>70-03</td>
<td>ACGGTTGCCCTG</td>
</tr>
<tr>
<td>70-05</td>
<td>GAGATCCGGCG</td>
</tr>
<tr>
<td>70-07</td>
<td>ATCTCCGGGGG</td>
</tr>
<tr>
<td>70-12</td>
<td>GGCCTACTCG</td>
</tr>
<tr>
<td>70-14</td>
<td>CGGGTTCGATC</td>
</tr>
<tr>
<td>70-21</td>
<td>TCCCTGTGCCC</td>
</tr>
<tr>
<td>70-22</td>
<td>GCTCTGGGTTG</td>
</tr>
<tr>
<td>70-09</td>
<td>TGCAAGCACCAG</td>
</tr>
<tr>
<td>70-01</td>
<td>CATCCCGAAG</td>
</tr>
</tbody>
</table>

4.5 Partial sequencing of 16S rDNA

The nucleotide sequences of the 16S rRNA gene ranging from 536 to 926 corresponding to the *E. coli* numbering system (Brosius et al. 1978) are as follows:

```
CCGTCAATTC CTGGTAAAGC CATTCATCGT CCCACGCTTT CCACGTGGGT GAGTTGAGCT CTTTACGCAC TGCAGCACCG CATCCCGAAG
```

4.6 Phylogenetic relationships

The sequences determined were partial sequences (407 base pairs). The 16S rRNA sequences of these organisms were compared with the sequences found in the Genbank database. The sequence was 97% homologous to *Nitrobacter winogradskyi* strain W and several *Bradyrhizobium* and *Rhodopsuedomas palustris* strains. Using SEQUENCE_MATCH from the Ribosomal Database Project II, we determined that this
new isolate belongs to the purple bacteria, alpha subdivision. Sequence homology corresponded with the *Rhizobium-agrobacterium* group, *bradyrhizobium* sub-group.

4.7 Strain identification

The phenotypic characteristics of the bacterium are listed in Table 3. The bacterium has been identified based on the aggregate of these characteristics, as a new strain of mesothermophilic Gram negative rod shaped, facultative anoxygenic, photoorganotrophic, non-sulfur organism that share general characteristics with *Rhodopseudomonas* (*Rps*), a genus of Rhodospirillaceae family (Table 3).

Two commercial organizations ATCC (Rockville, MD) and Analytical Services, Inc (Essex Junction, VT) were sent the bacterium for their independent confirmation of the identification. Analytical services, Inc (Essex Junction, VT) using fatty acid profile was unable to obtain a match on its microbial identification system (MIS) database. They suggested that microorganisms, which yielded low similarity indices, could be isolated from a unique habitat or belong to strains, which are not represented in the database. ATCC using microscopic examination, cultural requirements, and metabolic properties identified the bacterium as an unknown species of *Rhodopseudomonas*.

4.8 Metal resistance

The bacterium is resistant to several heavy metals. The list of resistant metals and their minimum inhibitory concentration are presented in Table 6.
Table 6. MIC of metals for the isolate

<table>
<thead>
<tr>
<th>Salt</th>
<th>MIC Metal concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl</td>
<td>200 (125)</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>100 (60)</td>
</tr>
<tr>
<td>Cr(NO₃)₂</td>
<td>400 (200)</td>
</tr>
<tr>
<td>K₂Cr₂O₇</td>
<td>&gt;200ᵃ</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>100 (60)</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1000 (750)</td>
</tr>
<tr>
<td>AuCl</td>
<td>500 (250)</td>
</tr>
<tr>
<td>AsH₃O₄</td>
<td>500 (250)</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>750 (500)</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>&gt;1000ᵃ</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>&gt;100ᵃ</td>
</tr>
</tbody>
</table>

The numbers in parentheses indicate the next lower concentration of metal at which growth was observed after one week incubation; ⁿ, higher concentrations were not tested.

Some growth parameters of the isolate at sub-inhibitory concentrations of resistant metals are presented in Table 7.
Table 7: Effect of metals at sub-inhibitory concentrations on growth parameters

<table>
<thead>
<tr>
<th>Metal (mg/l)</th>
<th>Tₐ (h)</th>
<th>Tₓ (h)</th>
<th>K (h⁻¹)</th>
<th>g (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>120</td>
<td>0.182</td>
<td>5.48</td>
</tr>
<tr>
<td>Cr III (200)</td>
<td>72</td>
<td>264</td>
<td>0.075</td>
<td>13.33</td>
</tr>
<tr>
<td>Cr VI (200)</td>
<td>81.6</td>
<td>216</td>
<td>0.090</td>
<td>11.11</td>
</tr>
<tr>
<td>Mn (500)</td>
<td>85.2</td>
<td>288</td>
<td>0.062</td>
<td>16.12</td>
</tr>
<tr>
<td>Zn (750)</td>
<td>84.6</td>
<td>288</td>
<td>0.060</td>
<td>16.66</td>
</tr>
</tbody>
</table>

Values of Tₐ (lag time) and Tₓ (end of exponential phase) were determined directly from the growth curves (Monod 1949); K (growth rate constant) and g (mean generation time) were calculated from the growth equation explained in text.

This bacterium was able to reduce high levels of Cr(VI) in the culture media. The rates of Cr(VI) reduction at varying concentrations of Cr(VI) are shown in Figure 6. The isolate was also able to degrade 2,4,6-trichlorophenol through a dehalogenation process under photosynthetic conditions. Dehalogenation occurred at carbon 6, 2, and 4 respectively (Figure 7).

4.9 Bioflocculant production

The screening for bioflocculant production was performed on a cell free water column of microbial mats cultured for 8 weeks. No significant bioflocculant production was detected in the first two weeks. The bioflocculant concentration reached the highest level during the fifth week, followed by a rapid reduction through the sixth week, and another increase during week 7-8 (Figure 8).

A visible biofilm layer in the microbial mat was formed by the beginning of the fourth week and continued to grow rapidly during weeks 4-6. Total carbohydrate and optical density of sonicated biofilms showed a moderate increase between 25-32 days and
Figure 6. Cr(VI) reduction by the isolate
Figure 7. Degradation of 2,4,6-trichlorophenol by the isolate
a rapid increase during 35-45 days which corresponded to decreasing bioflocculant concentration (Figure 8). Biofilms grew thicker and greener after 6 weeks, due to the attachment and growth of filamentous cyanobacteria.

4.9.1 Effect of temperature and pH on bioflocculant production

To determine the optimum temperature for bioflocculant production, the microbial mats were grown at different temperatures (4, 15, 25, 30, 35, and 46 °C). The cell free water column of the microbial mat cultures was tested for bioflocculant concentration daily for 8 weeks. The results presented in Figure 9, showed that the lag phase during bioflocculant production at 35 °C was less than that of the culture incubated at 25 °C.

A thin layer of biofilm formed slowly in the cultures grown at sensitive temperatures compared to cultures incubated at room temperature. Quantitative measurement of biofilm revealed a moderate increase during the 3-6 week period. Optical density and total carbohydrate of biofilm showed rapid increase between 35-45 days. No significant bioflocculant production or biofilm formation were observed in cultures incubated at 4 °C or lower and 46 °C or higher (Figure 9).

To determine the effect of pH on bioflocculant production and biofilm formation, the microbial mats were grown on AA media at pH values of 4, 5, 6, 7, 7.5, 8, 9, 10, 11, 12, and 13. The bioflocculant concentrations of the cultures at pH values of 10-12 increased continuously. There was no evidence of biofilm formation. The cultures at pH values of 7-8 showed the normal pattern of bioflocculant and biofilm production.
Figure 8. Bioflocculant and biofilm production in microbial mat cultures
Figure 9. Effect of temperature on bioflocculant production
The highest levels of biofilm formation were observed at pH 7-8. The bioflocculant production at pH values below 6 were low and relatively slow, and a thin layer of biofilm with low density were detected by the end of the 7th week (Figure 10).

### 4.9.2 Effect of metals on bioflocculant production

Microbial mats were grown on AA media containing three different concentrations (100, 50, and 25 mg/l) of each Chromium(VI), Selenium, and Zinc metals. The bioflocculant concentration and biofilm formation of each sample were monitored for 8 weeks. Zinc at 50 mg/l increased the concentration of bioflocculant concentration in solution over twice the amount in the control partly due to uncoupling of biofilm formation. Bioflocculant production decreased when the Zn concentration in the media was increased to 100 mg/l. (Figure 11). Increasing the selenium concentrations from 25 to 100 mg/l in culture media caused an increase in bioflocculant concentrations. At a selenium concentration of 100 mg/l, the bioflocculant concentration increased to twice the amount in the control (Figure 12).

Addition of Cr(VI) at 25 mg/l decreased the growth rate, and caused slight disintegration in mat formation in the culture. The growth of microbial mat was affected by chromium concentration above 25 mg/l. The flocculating activity and total carbohydrate assays were dramatically affected by the presence of Cr(VI) in the cultures. However microbial mat cultures could grow at Cr(VI) concentrations below 25 mg/l. Biofilm formation did not occur in all experimental samples containing heavy metals.
Figure 10. Bioflocculant production as a function of pH
Figure 11. Effect of Zn on bioflocculant production by the microbial mats
Figure 12. Effect of Se on bioflocculant production by the microbial mats.
4.10 Isolation and purification of bioflocculant

To isolate and purify the bioflocculant from culture media, the cell free water column of a 5-week-old culture was collected. The bioflocculant was isolated using two (cetrimide and acetone) precipitation steps. The isolated carbohydrate was dissolved in water and dialyzed against deionized water. The ratio of collected carbohydrate to culture medium was approximately 200 mg/l (w/v). The solubility of lyophilized isolated carbohydrate in water was about 5.88 mg/ml. The isolated bioflocculant contained no detectable protein or sulfate.

For further purification one ml of bioflocculant (2.85 mg/ml) was applied to a Sepharose CL-4B column (1.5 x65 cm) and was eluted by 50 mM Tris-buffered saline. Three ml fractions were collected and assayed for total carbohydrate content and flocculant activity. Two distinct carbohydrate peaks A and B were eluted. The fraction A eluted at the void volume (Figure 13), and represented over 91% of total carbohydrate applied to the column. Fraction B represented less than 10% of total applied carbohydrate with no flocculant activity (Table 8).

<table>
<thead>
<tr>
<th>PEAK</th>
<th>CARBOHYDRATE (%)</th>
<th>FLOCCULANT ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>91.5</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>8.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 13. Size exclusive chromatography of bioflocculant using Sepharose column
4.11 Alcian Blue-silver stain SDS-PAGE

The purity of the purified bioflocculant was confirmed on SDS-PAGE. Alcian Blue-silver stained SDS-PAGE gel of crude bioflocculant and fraction A are presented in Figure 14. Lane 1 and 2 represented different concentration of crude bioflocculant and lane 3 represented purified bioflocculant (Figure 14). Special problem associated with SDS-PAGE of the bioflocculant was its high viscosity. Only very low concentrations of bioflocculant could be used to run the gel, because high concentrations of bioflocculant did not enter the gel.

4.12 Monosaccharide composition analysis

4.12.1 Fluorophore-assisted carbohydrate electrophoresis (FACE)

The sugar composition of the bioflocculants was determined using Fluorophore Electrophoresis (Figure 15). The following monosaccharides were detected based on the available standards: N-acetylgalactosamine (GalNAc), mannose, glucose, galactose, and N-acetylglucosamine (GlcNAc). There were several bands on the gel that could not be identified because of lack of standard monosaccharides in the kit. Our laboratory designed standard mixture which was run with the company standard marker that revealed identification of glucuronic acid and galacturonic acid.
Figure 14. SDS-PAGE gel of bioflocculant
Lane 1 and 2, crude bioflocculant; lane 3, purified bioflocculant
Figure 15. FACE gel of bioflocculant
Lane 1, 2 and 6, Mono Ladder Standard (top to bottom, GalNAc, Man, Fuc, Glu, Gal, GlcNAc); lane 3, neutral hydrolysis reaction product; lane 4, amine hydrolysis reaction product; lane 5, monosaccharide control (glucose)
4.12.2 GC/MS

GC/MS analysis of N-acetylated and sialylated bioflocculant was performed using column SP2330 (Supelco, CA) revealed the presence of 11 monosaccharides. The GC/MS spectra of N-acetylglucosamine, glucuronic acid and galacturonic acid are presented in figures 16-18. The monosaccharide composition and the relative ratios of bioflocculant are presented on Table 9. The weight ratios of monosaccharides were extrapolated from the peak surface areas of a known internal control (inositol).

<table>
<thead>
<tr>
<th>MONOSACCHARIDE</th>
<th>WEIGHT RATIO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>2.9</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>10.3</td>
</tr>
<tr>
<td>Fucose</td>
<td>12.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>7.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>16.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>33.1</td>
</tr>
<tr>
<td>Galacturonic Acid</td>
<td>0.6</td>
</tr>
<tr>
<td>Glucuronic Acid</td>
<td>5.2</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>9.5</td>
</tr>
<tr>
<td>N-acetylgalactosamine</td>
<td>0.6</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 16. GC/MS spectra of galacturonic acid from bioflocculant
GLUCURONIC ACID

Figure 17. GC/MS spectra of glucuronic acid from bioflocculant
Figure 18. GC/MS spectra of N-acetylg glucosamine from bioflocculant.
4.13 Physical properties

4.13.1 Molecular weight determination

The molecular weight of the bioflocculant was estimated by gel filtration chromatography. To determine the approximate molecular weight of the bioflocculant, 0.25 ml of Bio-Rad Gel Filtration Standard Mixture and 0.5 ml of the bioflocculant (1mg/ml) were applied to Bio-Gel A-1.5 m column (1 x 65 cm), and eluted by 50 mM Tris-buffered saline pH 7.6, at a flow rate of 0.25 ml/minute. Fractions (0.5 ml) were collected and analyzed for total protein and bioflocculant activity.

The range of molecular weight standards is presented in Table 10. The bioflocculant eluted close to the void volume (Figure 19). The plot of K values versus log of MW of the standards yielded a straight line (Figure 20) (Freifelder 1976). The approximate molecular weight of the bioflocculant (500,000 D) was extrapolated from the standard curve.

Table 10. Bio-Rad Gel Filtration Standard components

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>MOLECULAR WEIGHT (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin</td>
<td>670,000</td>
</tr>
<tr>
<td>Gama globulin</td>
<td>158,000</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>44,000</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,000</td>
</tr>
<tr>
<td>Vitamin B-12</td>
<td>1,350</td>
</tr>
</tbody>
</table>
Figure 19. Gel filtration chromatography profiles of molecular weight standards and the bioflocculant
Figure 20. Plot of Log MW verses K value
4.13.2 Viscosity

The bioflocculants produced highly viscous solutions in water at low concentrations. The effects of the bioflocculant concentrations on viscosity were studied at 20 °C, and the results are presented in Figure 21. Viscosity of bioflocculant was compared to that of glycerol. The concentration of bioflocculant was 1000-fold less than glycerol at the same viscosities (Figure 22).

A. Effect of temperature on viscosity

The effect of temperature on the viscosity of the bioflocculant is presented in (Figure 23). The viscosity decreased as the temperature increased. However the more significant decrease occurred between 20 and 40 °C.

B. Effect of metals on viscosity

The changes in viscosity as a result of metal binding were conducted by direct viscosity measurement following addition of the desired salt. Several concentrations of Ca and Co (0.001-1 mM) and Zinc (0.01-1000 mM) were added to the biflocculant solution (5 mg/ml) at 20 °C. The results presented in Figures 24 and 25 showed that each increased metal caused the viscosity to decrease.

4.14 Isoelectric point

The bioflocculant is a polyanionic compound. The glycosyl composition analysis showed the presence of amino and carboxyl functional groups. The net charge of the bioflocculant was acidic with an isoelectric point of 4.2 (Figure 26).
Figure 21. Bioflocculant viscosity as a function of concentration.
Figure 22. Comparison of viscosity of the bioflocculant against glycerol
Figure 23. Effect of temperature on viscosity of the bioflocculant
Figure 24. Effect of Zn on bioflocculant viscosity
Figure 25. Effect of Co and Ca on bioflocculant viscosity
Figure 26. Preparative isoelectric focusing profile of the bioflocculant
4.15.1 Gel filtration

Gel filtration gave information about the ability of carbohydrates to bind to metals strongly enough to elute together through the size exclusive column. Excess Zn, Cr(VI), and Cu(II) salts were added to bioflocculant and left overnight at room temperature. A sample of 0.5 ml of the metal-bioflocculant solution was loaded to a Bio-Gel P6 (Bio-Rad Laboratory) column (1 X 25 cm) and was eluted with distilled water in 0.5 ml fractions.

The fractions were assayed for total carbohydrate, bioflocculant activity, and metal concentration. As controls, samples of metals were individually applied to the same column and eluted with distilled H₂O and tested for related metals concentrations under the same condition. The metal concentrations were determined by ICP.

Approximately 65 % of zinc co-eluted with the bioflocculant, and the rest eluted as the free unbound metal (Figure 27). Most of the Cr(VI) co-eluted with the bioflocculant (Figure 28). Cu(II) did not co-elute with the bioflocculant, indicating no strong bond between Cu(II) and the bioflocculant (Figure 29).

4.15.2 Equilibrium dialysis

Equilibrium dialysis technique has been used to show the specific binding between a macromolecule and a small-molecule or ligands. The semi-permeable dialysis membrane allows water and other small molecules to pass through freely but prevents passage of macromolecules (Figure 30). Therefore equilibrium can be reached for all species except the macromolecule. At equilibrium if the macromolecule binds one of the small molecules, concentration of this molecule will be higher in that cell compartment.
Figure 27. Elution profile of Zn-bioflocculant solution
Figure 28. Elution profile of Cr(VI)-bioflocculant solution
Figure 29. Elution profile of Cu(II) and the bioflocculant
Figure 30. Schematic diagram of equilibrium dialysis cell
When there is no binding between macromolecule and ligand, the concentration of ligand remains the same in both compartments (Kenneth 1995).

Two ml of metal (Zn and Cd) solutions at various concentrations were inserted in compartment 1 and the bioflocculant (5.88 mg/ml) in compartment 2 of the microdialysis cell. The system was allowed to reach equilibrium overnight. The metal concentrations in both compartments were measured using atomic absorption spectrometer (SpectrAA, Varian).

The results of Zn binding to bioflocculant using equilibrium dialysis are presented in Table 11. The concentration of Zn after equilibrium in compartment 2 was increased by 94-97%.

Table 11. Analysis of Zn binding to the bioflocculant using equilibrium dialysis

<table>
<thead>
<tr>
<th>Initial Zn concentration at T₀ (mg/l)</th>
<th>Zn concentration at equilibrium at T₂₄ (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compartment 1</td>
<td>Compartment 2</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>0</td>
</tr>
</tbody>
</table>

The results of Cd binding to the bioflocculant are summarized in Table 12. After 24 hours compartment 2 contained 70 -80% of initial Cd concentration.
Table 12. Analysis of Cd binding to the bioflocculant using equilibrium dialysis

<table>
<thead>
<tr>
<th>Initial Cd concentration (mg/l)</th>
<th>Cd concentration at equilibrium state (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compartment 1</td>
<td>Compartment 2</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>0</td>
</tr>
</tbody>
</table>

4.15.3 Continuous flow dialysis

Metal binding was studied under continuous flow conditions, using the flow dialysis method, to determine the saturation of metal binding capacity of the bioflocculant. The concentration of metal bound to the bioflocculant was increased by continuously increasing the metal concentration of the dialysis solution (Table 13). Zn binding to the bioflocculant was significantly decreased by the presence of NaCl in the metal solution. At equilibrium no binding occurred in the presence of 0.15 and 0.30 M NaCl (Table 13).

Table 13. Analysis of Zn binding to the bioflocculant using continuous flow dialysis

<table>
<thead>
<tr>
<th>Zn concentration of dialysis solution (mg/l)</th>
<th>Zn bound to bioflocculant (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>304</td>
</tr>
<tr>
<td>132</td>
<td>387</td>
</tr>
<tr>
<td>55 in 0.15 M NaCl</td>
<td>38</td>
</tr>
<tr>
<td>55 in 0.30 M NaCl</td>
<td>60</td>
</tr>
</tbody>
</table>

By increasing Cd concentration of dialysis solution, from 47 to 132 mg/l, the concentration of metal bound to the bioflocculant increased from 304 to 387 mg/l (Table 14). Cd binding capacity of bioflocculant was also affected by the presence of NaCl in
the metal solution. At equilibrium point, concentrations of Cd were almost equal in both compartments (Table 14).

<table>
<thead>
<tr>
<th>Cd concentration of dialysis solution (mg/l)</th>
<th>Cd bound to bioflocculant (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>268</td>
</tr>
<tr>
<td>180</td>
<td>482</td>
</tr>
<tr>
<td>40 in 0.15 M NaCl</td>
<td>36</td>
</tr>
<tr>
<td>40 in 0.31 M NaCl</td>
<td>46</td>
</tr>
</tbody>
</table>

4.15.4 The flocculation of bacterial cells

The flocculating properties of bioflocculant were detected by flocculating charged particles (Alcian Blue dye) and bacterial cells. Bacterial suspension at OD of 2 was cleared in 20 minutes in presence of as low as 0.02 mg/ml concentration of the bioflocculant (Figure 31).
Figure 3. Flocculation of bacterial (E. coli) suspension by the biofloculant
CHAPTER 5
DISCUSSION

There is a dearth of information on the mechanisms of bioremediation by the microbial mats. In this study two mechanisms were investigated. One was to establish the presence of some specific non-cyanobacterial species in the mats and the second was to monitor the production of metal-binding expolysaccharide by mats.

A metal-resistant bacterium was isolated from the microbial mats. The results, taken together, characterized the isolated strain as a Gram negative, motile, rod shaped bacterium that shares the general characteristics with *Rhodopseudomonas* (*Rps*), a genus of non-sulfur purple bacteria, *Rhodospirillaceae* family. This bacterium had a budding type of cell division and was a facultative anoxygenic phototroph, which, formed a lamellar type of interacytoplasmic membrane system (ICM). Electron micrographs of *Rhodopseudomonas* sp. described in this work revealed these characteristics (Figure 3).

The photosynthetic pigments in the isolate consisted of bacteriochlorophyll a and carotenoids which are characteristic of most species of non-sulfur, purple bacteria. The isolate was unable to utilize sodium thiosulfate and accumulate elemental sulfur inside the cells. These features distinguished the isolate from the purple, sulfur bacteria.
Based on these morphological and physiological characteristics, the isolate was identified tentatively as a new species of *Rhodopseudomonas* genus. Cellular and morphological similarities of the isolate to the previously described species in the genus *Rhodopseudomonas* are presented in Table 5 (Janssen and Harfoot 1991, Imhoff and Trüper 1992).

Neither BIOLOG (Rockville, MD) nor FAME has photosynthetics in their microbial identification database; therefore, these methods could not be used to identify this bacterium. Analytical Services, Inc (Essex Junction, VT) was unable to obtain a match on its microbial identification system (MIS) database. However, ATCC identified the bacterium as an unknown species of *Rhodopseudomonas* genus.

The identification of the bacterium as a new strain of nonsulfur purple bacteria was based on its cellular, morphological, and physiological characteristics and DNA analyses. The closest species of this genus to the isolate was *Rps. palustris* based on shared phenotypic characteristics such as red pigmentation, GC content, inability to utilize benzoate, and ability to hydrolyze tween 80 (Pfennig and Trüper 1974, Imhoff and Trüper 1992). However, the cells of this isolate were 0.4-0.6 µm wide and 2.0-2.4 µm long, while the cells of *Rps. palustris* are reported to be 0.4-1.0 µm wide and 1.5-3.0 µm long (Pfennig and Trüper 1974). Unlike *Rps. palustris*, this new strain can utilize acetate, valerate and glucose, and reduce nitrate to nitrite and does not grow on citrate.

RAPD-PCR was used to identify genetic differences between the isolate and other *Rhodopseudomonas* species. The RADP-PCR results are in agreement with results obtained from biochemical and morphological studies (Figure 5). The RAPD-PCR is valuable in
differentiating and confirming that the isolate is indeed a new species of purple bacteria.

Our results showed no evidence of significant change in the banding profiles under a range of DNA dilutions already described in the methods. It was previously shown that variations in the RAPD-PCR fingerprinting method could be attributed to primer length or concentration, dNTP concentrations, commercial origin of Taq polymerase, and purity of DNA (Williams et al. 1994). Some weak and fuzzy amplified fragments obtained with primer #70-14 may be attributed to the above mentioned problems. Many additional fragments that are not visible on ethidium bromide stained gels might be visualized by radioactive labeling or silver staining of polyacrylamide gels (Williams et al. 1990, Welsh and McCelland 1990, McCelland and Welsh 1994). While these techniques might be used for individual typing for specific purposes, they are expensive and time consuming processes, and are not suitable for confirmation purposes.

This study utilized commercially premixed reactions, where only primer concentrations and DNA quality were crucial for reproducibility of RAPD-PCR patterns. We were able to demonstrate with ten RAPD-PCR primers that distinct differences existed between the seven bacterial strains. Our RAPD-PCR typing system appears to be as effective in distinguishing between species as systems based on the use of microbiological methods.

Partial 16S rRNA sequences (407 base pairs) of this organism were compared with the sequences found in the Genbank database. The sequence was 97% homologous to *Nitrobacter winogradskyi* strain W and several *Bradyrhizobium* species, photosynthetic *rhizobium sp Afipia* species and *Rhodopseudomas palustris* strains. Using
SEQUENCE_MATCH from the Ribosomal Database Project II we determined that this new isolate belong to the purple bacteria, alpha subdivision.

*Rhodopseudomonas* genus is phylogenetically a heterogeneous genus. Our results and several others (Young et al. 1991, Hiraishi and Yoko 1994, Vinuesa et al. 1998) showed a high degree of homology of 16S rRNA sequences between type species *Rps palustris* and some chemotrophic bacteria such as *Nitrobacter winogradskyi*, several *Bradyrhizobium* species, *Afipia* species, and *Photorhizobium thompsonium* BTAi.

The isolate is differentiable from the above chemotrophic species based on distinct phenotypic differences and especially the phototrophic property of the isolate. Additionally, *Photorhizobium sp*, the phototroph with which the isolate has high homology, is an oxygenic photosynthetic bacteria (Janssen and Harfoot 1991).

In spite of high homology with, the isolate differs clearly from *Rps palustris*, based on the results from RAPD profiles, GC contents, and a number of phenotypical characteristics such as size of the cells, color of the cell suspension, etc. Unlike the isolate, *Rps palustris* is able to utilize benzoate and thiosulfate, or hydrolyze tween 80. The isolate is able to reduce nitrate to nitrite under anaerobic/dark conditions, however, Hiraish (1994) could not find denitrifying growth in any test strains of *Rps palustris*. According to phenotypic and genotypic data, the new isolate tentatively can be classified as new species of *Rhodopseudomonas* genus.

The isolate has shown tolerance to a wide array of heavy metals (Table 6). Many members of the *Rhodospirillaceae* family have been reported to show a high level of resistance to toxic heavy metal oxides and oxyanions and to have remarkable enzyme
systems to detoxify potentially lethal environmental contaminants (Moore and Kaplan 1992, 1994). Mats containing these strains have been used successfully to remove heavy metals from contaminated waters (Bender, Archibold et al. 1989, Bender et al. 1991, Bender, Vatcharapijarn et al. 1994). Although the mechanism of metal removal from aqueous matrix by the mats maybe complex, including metal sequestration by extracellular flocculents (Bender, Rodriguez-Eaton et al. 1994), this report suggests that the *Rhodopseudomonas* sp plays an important role in mat removal of toxic heavy metals and degradation of organic chemicals from aqueous media.

This strain may act as a resistant member, providing and maintaining the integrity of the microbial mat consortium, when exposed to metal contaminated fields. The great diversity of biochemical and physiological capabilities of the *Rhodopseudomonas* group of bacteria as agents of bioremediation and bioreclamation should be studied in depth as suggested by Moore and Kaplan (1994).

The bioflocculation of heavy metals from the solution is the second mechanism of bioremediation applicable to the mats. The bioflocculants production by the microbial mats in a base salt media was monitored. We used the microbial consortia as the source for bioflocculant production because our attempts to screen bioflocculant production from cyanobacteria isolated from the mats were unsuccessful. Also we were not able to successfully grow cyanobacterial species obtained from ATCC to monitor their flocculant production. The justification for using mat rather than a single cyanobacterial strain, in addition to the technical difficulties discussed above, may be advanced by ecological relevance of mats bioremediation applications. For example, Costerton (1987), found
that many important population growths in adherent biofilms and structured consortia are not seen in pure culture (Costerton and Irvin 1981, Costerton 1987).

Bioflocculant production in microbial mat cultures started after two weeks, and was sharply decreased by the sixth week. Biofilm formation started on the fourth week and grew rapidly during week 4 to 6, which was synchronized with a sharp decrease in bioflocculant concentration in culture. This pattern was seen in all of the mats cultures that grew in optimum conditions (pH 7-8, temperature 25 °C), suggesting that the exopolymers may provide an organic precursor for biofilm formation, as has been reported by others (Costerton 1987, Quintero 1989, Becker 1996, Langelli and Weiner 1998). Our study suggested that an inverse cyclic relationship may exist between bioflocculant and biofilm formation (Figure 8). Biofilms are basically embedded cells in exopolymer matrices. The function of bacterial extrapolymer in primary adhesion of the bacteria to the surfaces has also been well documented (Costerton 1987, Cohen and Rosenberg 1989, Quintero 1989, Quintero and Weiner 1995, Becker 1996, Langelli and Weiner 1998).

The bioflocculant production and biofilm formation were significantly lower at non-optimal conditions, such as temperature, pH or presence of stressors. The highest levels of the bioflocculant concentration and biofilms thickness were seen at 25-32 °C (Figure 9).

The bioflocculant productions and biofilm formations were uncoupled at pH > 9. The highest amount of biofilm was formed at pH 7-8, and decreased gradually on both sides of this pH scale (4-6 and 9-13). Taking together, these results reveal that the
optimum conditions for a typical layered-structural mat culture in a minimum salt media would be at pH value of 7-8 and at temperatures of 25-30 °C. However in field conditions, mats have been shown to grow in acid mine drainage (pH < 4).

The addition of Zn and/or Se increased the level of the bioflocculant concentrations in the culture over time. However the absence of a typical biofilm structure in culture might account for elevation of bioflocculant concentration. Under this condition, the metals ions might shield the negative charges of bioflocculant, and inhibit the initial adhesion and as a result, prevent biofilm from forming.

Chemical analysis showed that bioflocculant was a polysaccharide with an average molecular weight of approximately 500,000 D. Since polydispersity of polysaccharides represent a continuous variation in molecular size, experimentally determined values for molecular weight are obtained as averages (Aspinall 1992).

The purified bioflocculant contained exclusively carbohydrate and did not have detectable protein or sulfate. The bioflocculant was an anionic polymer with an isoelectric point (pI) of 4.2. The compositional analysis of the bioflocculant and the proportion of sugar constituents are listed in Table 9. Most, if not all, polysaccharides are polydisperse molecules, so that information on both molecular size and composition can be obtained only as average value. Since polysaccharides contain repetitive features, compositional analysis give information on relative proportions of sugar constituents (Aspinall 1992).

The bioflocculant is composed of eleven monosaccharides which is not common among bacterial exopolysacharides. Seven aldose (two pentose and five hexose) sugars,
2 amino sugars and two acidic sugars were detected as constituents of the bioflocculant. Arabinose and xylose are two pentose sugars that were detected in bioflocculant. Xylose is one of the most abundant sugars in plant polysaccharides and is a rare component of bacterial polysaccharides (Lindberg 1990). However, because Cyanobacteria is photosynthetic, it may explain the presence of xylose in the bioflocculant. Five other aldose found in the bioflocculant are hexose sugars such as glucose, galactose, mannose, fucose (6-deoxygalactose) and rhamnose (6-deoxymannose) which are common sugars of bacterial polysaccharides. N-acetylglicosamine (GlcNAc) and N-acetylgalactoseamine (GalNAc), glucuronic acid, and galacturonic acid are charged monosaccharides that made up over 15 % by weight of the bioflocculant and contributed to its anionic characteristics.

Based on the sugar composition and proportion, and molecular weight of the bioflocculant, the bioflocculant has been estimated to consist of roughly 30 repeating units per molecule. The mass of each repeating unit \( (m_0) \) has been estimated to be about 16,500 Dalton.

Rheological studies showed that the bioflocculant at low concentrations produced highly viscous solutions in water. There was a linear relationship between concentrations and viscosity at low concentrations of the bioflocculant. The viscosity of the bioflocculant was 1000-fold greater than the viscosity of glycerol at equivalent concentrations.

The bioflocculant viscosity decreased as temperature increased, due to increased free energy to the molecules, leading to rupture of temporary cross-linkages. The transient non-covalent bonds cross-linked the simple linear polymer into a network.
These temporary, physical cross-linkages relate to the temperature, pH and cation species (Ross-Murphy and Shatwell 1993). The melting point of the polymer (5.88 mg/ml) was in the 20-50 °C range. However this polymer at 5.88 mg/ml concentration and 60 °C temperature has viscosity approximately equal to the viscosity of glycerol at 83% saturation (1.04 g/ml). The highly viscous nature of the solutions at low concentrations and relatively high temperatures, make this polymer potentially useful for a number of commercial applications.

The viscosity of the bioflocculant decreased in the presence of various concentrations of different divalent metals tested. This decline in viscosity correlated with bioflocculant-metal interaction and formation of the loose flocs (Brown and Lester 1979, Caccavo et al. 1996, Jumel et al. 1997, Norton et al. 1984, Ross-Murphy and Shatwell 1993).

The bioflocculants interact with a variety of monovalent and divalent metals, such as, Se, Cr(VI), Zn, Ca, Cd and Co in cultures, and in vitro as pure elements. The charged carboxylic acid residues of uronic acids in the bioflocculant might be the primary sites of metal ion interaction (Quintero 1989, Stojkovski et al. 1986). Elution of bioflocculant-Zn and bioflocculant-Cr VI from size exclusive columns suggested relatively strong bonds between the bioflocculant and the metal ligands. Cu(II) did not co-elute with bioflocculant, indicating a weak or no interaction between these molecules.

Flow dialysis data further confirmed specific interaction between the bioflocculant and metals ions. In equilibrium dialysis, the bioflocculant was bound to two divalent metals Zn and Cd, at the ratio of 94-97 % and 70-80 % respectively, suggesting some
degree of specificity in metal interaction properties. The saturation of metal-binding sites of the bioflocculant was not achieved by increasing metal concentrations in the equilibrium and continuous flow dialysis experiments. The ratio of bound-metals increased significantly by increasing the metal concentrations in the flow-through dialysis. It seemed that no saturation was reached after 24 hours dialysis. The metal binding properties of the bioflocculant explicitly account for one of the metal removing mechanisms in microbial mats.

The interaction between the bioflocculants and both Zn and Cd were affected by the presence of excess concentrations of NaCl (0.15 and 0.3 M) in the metal solutions during flow through dialysis experiments (Table 13 and 14). The metal binding sites of bioflocculant were preferentially occupied by Na ion because of the high concentration of this ion (20-40 times) in dialysis solution. This effect of NaCl on bioflocculant binding may not reflect the in situ situation with the microbial mats. It, however, suggested that purified bioflocculant may not be used to remove heavy metals in physiological fluids or seawater with high NaCl concentrations.

The biflocculant can flocculate several charged particles and dyes at very low concentration. In nature, microbial mats flocculate and sediment suspended particles, thus allowing light to reach to the benthic interface in turbid waters, which is indispensable for several photosynthetic members in mats communities. Secretion of these polysaccharides into the environment by the members of the microbial mats communities are one of the survival strategies of the mats that are attached to various submerged surfaces in aquatic habitats as opposed to free living planktonic species.
CHAPTER 6
CONCLUSION

The identification of a new bacterium was based on phenotypic and genotypic characteristics. The isolate showed a high level of resistance to toxic heavy metals. This bacterium was able to reduce Cr(VI) and selenite and degraded a number of organic chemicals. The remarkable ability of the isolate to detoxify environmental pollutants, suggest an important role by this organism and the mechanisms of bioremediations by microbial mats. The bioflocculant is a high molecular weight heteropolysaccharide with a rare sugar composition. Its metal binding capacity explains another mechanism of bioremediation by the mats. The bioflocculants produce highly viscous solution in water at very low concentrations, and in the presence of cations, initiate aggregation and flocculation. Based on rheology, metal binding, and flocculating properties of bioflocculant, we concluded that this polymer is not a good thickening and gelling compound like some other bacterial polysaccharides such as gellan and xanthan rather, it is a strong flocculating agent. Flocculating and metal binding properties of the bioflocculant, make this polymer useful for a number of commercial applications.
REFERENCES


Gibson J, Dispensa M, Harwood CS. 1997. 4-hydroxybenzoate (dehydroxylating) is Required for Anaerobic Degradation of 4-hydroxybenzoate
by *Rhodopseudomonas palustris* and Shares Features with Molybdenum-

Gomoiu I, Catley B J. 1996. Properties of a kaolin-Flocculating Polymer Elaborated by

Proteins on Sodium Dodecyl Sulfate-polyacrylamide Gels: Enhancement by

Bacteria Including *Rhodopseudomonas rosea* as *Rhodoplanes roseus* Comb. Nov.

Holm H W, Vennes J W. 1971. Occurrence of Purple Sulfur Bacteria in a Sewage


Transfer in Biology. EMBO J. 8 (8):2125-2147.

Imhoff J F, Trüper H G, Pfennig N. 1984. Rearrangement of Species and Genera of

Imhoff J F, Trüper H G. 1992. The Genus of *Rhodospirillum* and Related Genera". In:

Irgens R L, Pfennig N. 1978. Phototrophic Purple and Green Bacteria in Sewage

Jann K, Jann B. 1977. Bacterial Polysaccharide Antigen. In; Sutherland W. Surface

Glycoprotein of *Fusarium sp.* M7-1:III. The Primary Structure of the Acidic


Jumel K, Geider K, E Harding S. 1997. The Solution Molecular Weight and Shape of
the Bacterial Exopolysaccharides Amylovoran and Stewartan. Int. J. Biological
Macromolecules 20:251-258.


