THE REMOVAL OF TOXIC HEAVY METALS FROM AQUEOUS SOLUTIONS BY ALGAL EXTRACELLULAR POLYSACCHARIDES

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by

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This thesis is dedicated to

my family

ABSTRACT

This study investigated the possible use of algal extracellular polysaccharide as a biosorbent for removal of heavy metals (copper and lead) from aqueous solutions as a means of bioremediation for metal containing effluents. This biopolymer has good biosorbent properties and a potential to provide a cost effective, selective and efficient purification system.

A variety of environmental conditions induce the production of extracellular polysaccharides in algae. The production of exopolysaccharides by *Dunaliella* cultures was induced by nitrogen deficient conditions. A high ratio of carbon to nitrogen source considerably enhanced the polysaccharide release. Purified extracellular polysaccharide samples exhibited a monosaccharide composition consisting of the following sugars: xylose, arabinose, 2-O-methyl mannose, mannose, glucose and galactose. The relative abundance (%) of these sugars were calculated relative to xylose. The major sugar constituent was 2-O-methyl mannose, which was present at approximately 160 % relative to xylose. The percentage relative abundance of other sugars was as follows: 18.8; 86.8; 85.3 and 22.3 % for arabinose; mannose; glucose and galactose respectively. The identity of the various constituents were confirmed by mass spectrometry.

The ability of *Dunaliella* exopolysaccharides to accumulate metals was investigated. The

following parameters were studied because they affect metal uptake: solution pH, biomass concentration, temperature, time and metal concentration. The uptake of both copper and lead were pH dependent. However, metal uptake was not significantly affected by temperature. Kinetic studies showed that *Dunaliella* extracellular polysaccharides exhibit good bioremediation properties. Metal uptake was rapid. In addition, the exopolysaccharide has good metal binding capacity with an uptake capacity for lead of 80 mg/g from a solution containing initial lead concentration of approximately 40 mg/*l*.

Competition studies revealed that the presence of a second metal in solution inhibits uptake of the other metal compared to uptake in single metal solution of that particular metal. The presence of lead inhibited the uptake of copper from approximately 65 % in single metal solution to 10 % in binary metal solution. The presence of copper also inhibited lead uptake, though not to the same extent. Higher concentrations of lead could not completely prevent removal of copper from solution and visa versa. The same was true for lead which could not be displaced by a four-fold concentration of copper. Instead, a certain percentage of copper was always removed showing that lead did not compete with copper for these binding sites. In conclusion it appears that, copper and lead bind to different sites on *Dunaliella* exopolysaccharides and that they exhibit selective or preferential removal of lead.

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ABBREVIATIONS

AAS	-	Atomic Absorption Spectrometer
EPS	-	Extracellular polysaccharides
GC-MS	-	Gas Chromatography-Mass Spectrometer
IR	-	Infrared Spectrophotometer
MAC	-	Maximum Admissible Concentrations
TFA	-	Trifluoroacetic acid

CHAPTER 1

LITERATURE REVIEW

1.1 Potable water supply in South Africa

It is generally agreed that the quality of water to be used for human consumption or in food preparation is of paramount importance. The legislations of a number of countries establish mandatory, health-related standards for water quality including those for human consumption and for the fabrication of foods and beverages (Muzzarelli *et al.*, 1989) . Maximum admissible concentrations (MAC) are specified for a wide range of compounds, although different countries differ often over the precise value they put upon a particular MAC. A sample of some MAC of ions in Italy, South Africa and the USA is given in table 1.1.

Indeed there may be quite wide discrepancies as to the level at which it is considered that a particular ion or substance presents a possible health hazard and becomes undesirable (Marr and Cressser, 1983). Sometimes this stems from genuine inadequate knowledge of the short term and long term physiological effects of the pollutants at different levels. Sometimes they arise from economic considerations, since it is futile to produce legislation which, in its enforcement, would make prohibitively expensive demands upon national resources, and compromise levels may thus have to be adopted (Marr and Cresser, 1983).

Table 1.1. Maximum admissible concentrations (μg/m*l*)of ions in waters to be used for food production and in drinking water for Italy, South Africa and USA

Metal	Definition	Concentration (μg/m*l*)
Lead	Toxic	100
Chromium	Toxic	200
Cadmium	Toxic	20
Nickel	Toxic	500
Mercury	Toxic	10
Arsenic	Toxic	300
Iron	Undesirable	1000
Manganese	Undesirable	1000
Copper	Undesirable	1000
Zinc	Undesirable	5000
Silver	Undesirable	50
Barium	Undesirable	4000
Fluoride	Undesirable	2400
Nitrate	Undesirable	23 000

(Pieterse, 1989; Muzzarelli *et al.*, 1989)

Because of these restrictions and costs, the use of impoundments to retain wastes has increased rapidly in the United States, but this solution has brought a new concern regarding air pollution and ground water contamination by impounded wastes. As a result, new laws requiring covers and lining of all impoundments known or suspected of containing toxic wastes were passed (Oswald, 1988).

Growth of the population, urbanisation and industrialisation serve to increase the demand for water. Water supply is related to a basic increase in quality of human life, and one of

the aims of the reconstruction and development programme in South Africa is to supply everyone in the country with 25 *l* of potable water per day within a radius of 200 m of their residence (Stephenson, 1995). The cost of supplying 12 million people in South Africa who at present do not have clean drinking water is estimated to be between R1 billion and R10 billion (Stephenson, 1995).

The United Nations designated the 1980s as "The International Drinking Water Supply and Sanitation Decade"(Dean and Lund, 1981). The objective was to make good water available for everyone by 1990. However, this objective has not been met, especially in developing countries. The availability of an adequate supply of water of suitable purity is undoubtedly essential to any community. This simple chemical plays a vital role in domestic, municipal and industrial use as well as in all ecological systems. It is without question, man's most precious resource. It is important therefore that as far as possible deliberate or accidental actions of man which adversely affect the best use of water for whatever purpose should be avoided (Marr and Cresser, 1983).

In the 1980s more than 1.6 billion people gained access to water of reasonable quality. Yet data from the World Health Organisation suggest that at least 170 million people in urban and 855 million in rural areas still lack a source of potable water (World Bank, 1994). To provide a continuous safe water supply calls for ample and reliable water sources and adequate capacity for treatment. Wealthier households, like industrial firms, can install private facilities such as reservoirs, recycling equipment, and private wells, though at additional cost to the economy. These options are beyond the poorer

consumers, who suffer disproportionately when water supply is unreliable (World Bank, 1994).

1.2 Water reuse

South Africa is a semi-arid country with an average annual rainfall (500 mm) well below the world annual rainfall (860 mm) (Gordon *et al.*, 1996). The distribution of subsurface waters varies profoundly while droughts are not uncommon. This scarcity of water has resulted in the management of water resources being entrenched in the new constitution. It has been estimated that even if all the potentially viable water sources in South Africa are fully developed, the supply will not be sufficient to meet the demands of the year 2025 (Gordon *et at.*, 1996). All these factors underline the fact that South Africa is a water deficient country.

This problem is further exacerbated by the depletion and degradation of water resources by pollutants from industrial processes. Accordingly, it has become imperative to recycle water, and the future will see more and more of this out of necessity rather than choice (Gordon *et al.*, 1996). In many parts of the world, increasing demands on available water resources, due to the growth of the world's population and the expansion of industrial needs, make the unintentional recycling of domestic water quite a common practice (Dean and Lund, 1981).

In 1985 for instance, 72 % of water consumption in Saudi Arabia was for agriculture with wastewater treatment contributing only 5 % of the Saudi water balance. It has been

projected that by the year 2000, wastewater could account for more than 20 % of the water supply (Al-Mutaz, 1989). The projection was based on a rapid growth of the cities and a growing demand of water for agriculture which has seen an increase in annual wheat production from 141 732 tons in 1980 to 2 048 000 tons in 1985 (Al-mutaz, 1989).

Although the reuse of wastewater for agricultural purposes is an attractive alternative, its use often brings about risks to the environment caused by accumulation of heavy metals (Labrecque *et al.*, 1995). In other words, the use of industrial wastewater containing low levels of metals will result in accumulation of trace levels of metals, which may not be sufficiently toxic to affect the simple plant physiology, but can be magnified by passage through the food chain, thereby posing a grave threat to higher organisms. In addition, even if they are present in dilute, undetectable quantities, their recalcitrance and consequent persistence in the environment imply that through natural processes such as biomagnification, concentrations may begin to exhibit toxic characteristics (Atkinson *et al.*, 1998). Thus, it should be emphasized that proper disinfection or treatment is an absolute requirement for the reuse of wastewater for potable or agricultural purposes (Dean and Lund, 1981).

1.3 Metal pollution of the environment

Almost all industries discharge at least one trace metal into the environment i.e. soil or water (table 1.2). This is the case even in south Africa where most of these industries are available. The mining of valuable metals and the deposition of waste products above ground gave rise to continuous leaching of minerals from mine dumps into streams, lakes

and rivers. The effluents thereof contribute significantly towards the acidification, mineralisation and metal contamination of the affected water bodies. Until recently, the removal of dissolved minerals from wastewater has been given relatively little attention, because minerals have been considered to be less of a pollution hazard than other constituents, such as organic matter and suspended solids (Dillman, 1979). Particles, turbidity, colour, even odour and taste, that make water aesthetically unacceptable, are seldom of themselves very hazardous. Industrial waters are also subject to regulations and their treatment for metal abatement is compulsory in many countries (Muzzarelli, 1989).

In developing countries, many industries are operated at small or medium scale, or even as a family business within the residential premises of the owner (Chiu *et al.*, 1987; Quek *et al.*, 1998). For example, according to the census and statistic department of the Hong Kong Government there were approximately 847 electroplating establishments in Hong Kong at the end of June 1984 (including buffing and polishing establishments) and about 98 % of them were predominantly small and medium-scale operations employing less than 50 employees (Chiu *et al.*, 1987). These smaller units without any facilities for waste water treatment can generate a considerable pollution load which, in many cases, is discharged directly into the environment (Quek *et al.*, 1998).

In Taiwan, the largest concentration of tanners is in Tainan, and other centres include Taoyuan, Taichung and Kaohsiung. A survey of these tanneries indicated that on a nationwide basis, an estimated 85 % had no effluent treatment facility (Hayward, 1990).

Table 1.2. Production of trace metals by various industries

	Al	Ag	As	Cd	Cr	Cu	Fe	Hg	Mn	Pb	Ni	Sb	Sn	Zn
Pulp, paper industries					*	*		*		*	*			*
Organic chemicals, petrochemicals	*		*	*	*		*	*		*			*	*
Alkalis, chlorine, inorganics	*		*	*	*		*	*		*			*	*
Fertilizers	*		*	*	*	*	*	*	*	*	*			*
Petroleum refining	*		*	*	*	*	*			*	*			*
Steel works, foundries			*	*	*	*	*	*		*	*	*	*	*
Non-ferrous metal works, foundries	*	*	*	*	*	*		*		*		*		*
Vehicle, aircraft plating, finishing	*	*		*	*	*		*			*			
Glass, cement, asbestos products					*									
Textile mill products					*									
Leather tanning, finishing					*									
Steam power plants					*									*

(Volesky, 1990)

This is because the capital investments, turnover and profit for these countries are also small. In Malaysia, a similar situation exists, and the discharge of waste water containing chemicals and metallic ions into nearby watercourses is well documented (Sohaili, 1990, cited in Quek *et al.*, 1998).

The potential hazardous materials that may occur in drinking water are not obvious. As more is learnt about the causes and effects of pollution, the importance of reducing the quantity of certain types of inorganic matter, which industry is permitted to discharge, becomes apparent (Dillman, 1979). Pollution from industrial sources can easily create conditions of elevated metal presence which could lead to disastrous effects on animals and humans (Volesky, 1990), since heavy metals are not biodegradable and will tend to accumulate in the environment or through the food chain. The injurious effects of heavy metals to humans are well established, for example, excessive amounts lead in the human body cause hypertension and brain damage (Ho *et al.*, 1996). Well-known cases of metal poisoning have been recorded in Japan where the local inhabitants around the Minimata Bay suffered neurologic illness after consuming seafish and shellfish contaminated with methyl mercury (Ting *et al.*, 1989).

Man's exploitation of the world's minerals resources and his technological activities tend to unearth, dislodge, and disperse chemical elements. The mobility of these metallic elements have recently been brought into the environment in unprecedented quantities and concentrations and at extreme rates (Volesky, 1990). Since the advent of industrial revolution there has been a trend of processing an increasing tonnage of metals for

manufacture of products. Consequently, there are numerous opportunities for metal release into the environment during the sequential mining, refining, and final processing of metals. This problem is compounded by the habits of the modern "throw-away society" which encourages built-in obsolescence in manufactured articles, yet with limited infrastructure for recycling these materials (Duncan *et al.*, 1994).

Metals are expensive to locate, mine, and refine (Duncan *et al.*, 1994). If metals could be reclaimed from waste or if losses during each step in metal processing could be reduced, then the opportunity is presented to produce cheaper goods with high profit margin. It should be borne in mind that metals are considered to be a non-renewable source and should be managed with care (Duncan *et al.*, 1994). Hence this area of research is of singular importance to metal ore mining and refining countries such as South Africa.

While industry in South Africa only consumes 9 % of the total water usage annually, it does produce significant quantities of effluent, which in turn pollute the rivers and oceans around the country (Henry *et al.*, 1998). Until recently, wastewater treatment has been seen as a centralised process, whereby all effluent streams were mixed together and treated as one effluent (end-of-the-pipe treatment), sent to off-site municipal treatment facilities or discharged in a pipeline. This method of wastewater treatment is expensive, impractical and unsustainable because different effluents require different treatments (Henry *et al.*, 1998). Over the last few years, the focus by industry has changed from this "end-of-the-pipe" treatment, to a cleaner and more integrated approach , whereby the production of wastes are minimised, yielding less effluent and requiring less raw

materials and water in order to produce the same product (Henry *et al.*, 1998).

These methods are often ineffective and/or very expensive when used for the removal of heavy metals to very low concentrations (Wilde and Benemann, 1993). As a result these methods unavoidably leave trace amounts of pollutants in wastewaters. This necessitated research into developing alternative, efficient and cost-effective wastewater purification methods.

1.4 Conventional wastewater purification methods

The major processes currently used to treat metal containing solutions include the addition of chemicals (lime or sulfide) for precipitation of metals and the use of ion exchange resins to bind metals to a substrate or biomass (Bhattacharyya and Cheng, 1987). Several other methods are also available, although less frequently employed by industry.

1.4.1 Chemical precipitation

1.4.1.1Hydroxide precipitation

Acidic effluents are produced from many industrial operations, including mining and metallurgical operations, the fertiliser, paper and chemical manufacturing processes as well as off-gas treatment by power stations. The neutralisation of acid water is usually required before discharge to municipal sewers or the environment to prevent aggressive attack of or corrosion to pipelines and treatment reactors, and to eliminate/reduce the

accumulation of acid and toxic heavy metals in the environment (van Tonder and Maree, 1998).

Alkaline precipitation is the most generally applied method, particularly where complex chemical compounds are not involved and economic recovery is not a consideration. It is relatively simple and the cost of the precipitant is low. The removal of metals such as copper, zinc, iron, manganese, nickel, and cobalt requires almost complete precipitation with no special modifications. Although lime (calcium oxide or hydroxide) is generally used for neutralisation of acid water, limestone is a promising lower cost alternative. Limestone neutralisation has had limited application as a result of low neutralisation rates compared to other alkalies and the phenomenon of surface sealing, which inhibits the reaction rate (van Tonder and Maree, 1998).

The major disadvantage of this method is the generation of large volumes of wet sludge, which is very difficult to dry. The costs of hazardous landfills is increasing rapidly, therefore, the generation of larger amounts of sludge is a problem. In addition, the low solubility of lime, which requires a carefully designed dosing system is another disadvantage of this method (Binnie and partners, 1987).

1.4.1.2 Sulfide precipitation

Sulphide precipitation has several attractive features compared with hydroxide precipitation and has been demonstrated to be an effective alternative (Binnie and Partners, 1987). Sulphides have a high reactivity with heavy metal ions and form highly

insoluble precipitates over a wide range of pH (Binnie and Partners, 1987; Pollution Research group, 1984). This method can also achieve low metal solubilities in the presence of certain complexing and chelating agents. It is accomplished with either the 'soluble' or 'slightly soluble' process. In the soluble process, sulphide is added in the form of a water soluble sulphide, such as sodium sulphide or sodium hydrogen sulphide. A sulphide ion-selective electrode is employed to control excessive addition of sulphide and the resultant odour of hydrogen sulphide gas.

The slightly soluble process uses a freshly prepared solution of ferrous sulphide as the source of sulphide ions needed to precipitate metals from wastewater (Binnie and Partners, 1987). In alkaline solutions, the excess ferrous ions are precipitated as ferrous hydroxide, whilst the heavy metals precipitate out as sulphides. The major operational problems associated with this method is poor settleability of the sludge and the need for control of addition of reaction chemicals (Chiu *et al.*, 1987).

1.4.2 Ion exchange

A rapid increase in the price of precious metals in the last few years, has made the recovery of even minute amounts of these elements of great interest to the metal finishers (Gold, 1979; Waitz, 1982). Physical treatment techniques, such as evaporation, reverse osmosis (ultrafiltration), reduction and precipitation or electrodialysis are known to be fairly effective in recovery of precious metals from aqueous solutions. However, these methods cannot be used to concentrate the dilute metal effluent streams to give a concentrate which can be returned to the plating bath (Spearot and Peck, 1984). Ion

exchange resins on the other hand are well known for their high degree of efficiency for the recovery of uranium from uranium ores (Dean *et al.*, 1972) and other metals from dilute solutions (Dillman, 1979; Waitz, 1982).

It has been reported that ion exchange will continue to find increased usage as industry seeks ways to extract metals from effluents on order to meet more stringent pollution control laws (Dillman, 1979). Although these wastes can be treated by methods other than ion exchange (chemical precipitation, for example), ion exchange appears to be the only practical technique currently available that offers the metal processing industry three advantages, namely: metal recovery of metal of value, rapid volume reduction of waste with minimum space requirement, and recovery of water for reuse.

All of these advantages are not always available in a particular situation. However, even one or two justify the employment of ion exchange in most instances (Dillman, 1979). Notwithstanding that, ion exchange resins are rarely used for effluent treatment because of the high capital and operating costs compared with the value of the recovered materials (Pearson, 1983).

1.4.3 Solvent extraction

Solvent or liquid-liquid extraction is based on the principle that a solute can distribute itself in a certain ratio between two immiscible solvents, one of which is usually water and the other an organic solvent such as benzene carbon tetrachloride or kerosene (De *et al.*, 1970). In certain cases the solute can be more or less completely transferred into the

organic phase. The solvent reacts preferentially with the heavy metal ion of interest and converts it to a form which is soluble in appropriate organic solvents. The solution is mixed with water, and the two phases separated. The metal is subsequently released in a concentrated water-soluble form from the organic fraction by acid treatment. This method was successfully adopted by the uranium and copper industries and has the possibility of removal and recovery of metals from industrial wastes (Dean *et al.*, 1972; Kim, 1984). However, solvents cannot be reused once they have been stripped with acid because of loss of extraction capacities (Clevenger and Novak, 1983). Furthermore, the cost of solvents (chelating agents) needed to recover a metal was found to be higher than the value of the metal, which is a severe limitation of this method of recovery (Clevenger and Novak, 1983).

1.4.4 Activated carbon

Activated carbon adsorption has shown to be effective for the removal of organic and inorganic pollutants from wastewater (Bhattacharyya and Cheng, 1987). Although poor adsorption may be exhibited for some heavy metals, in the presence of organic complexing agents, adsorption can be improved substantially. When complexing agents (EDTA, Citrates, etc) are present in the wastewater, heavy metal hydroxide precipitation is inhibited, thus causing adverse effects on removal efficiencies (Bhattacharyya and Cheng, 1987). Therefore, in the case of removal of heavy metal chelates or by deliberate addition of chelates to heavy metals, adsorption by activated carbon provides an alternative separation technique.

Acidic powdered activated carbons have been shown to have high sorption capacities for the inorganic ions of mercury, lead and copper (Huang and Blankenship, 1984), whereas basic granular activated carbon has exhibited high sorption capacities for chromium (Bowers and Huang, 1981).

The use of activated carbon is similar to the resin-in-pulp technique, however, it employs activated carbon instead of synthetic resins. It is used successfully in the commercial operation for extraction of gold from cyanide solution (Dean *et al.*, 1972). Activated carbon is relatively coarse (10 mesh) and contained in stainless steel cylindrical screens which are submerged in the pulp, and the flow of suspended carbon from one screen to the next is counter current to pulp flow. The loaded carbon is subsequently eluted with hot cyanide solution or sodium sulphide which dissolves the gold for later precipitation with zinc, and the carbon is recycled. The carbon is periodically reactivated by heating in a small rotary kiln (Dean *et al.*, 1972).

Desorption of various metal ions from activated carbon has not been investigated adequately despite the importance of such studies, which provide information on the strength of sorptive bonds and the extent of sorption reversibility. The use of activated carbon for removal of metals has considerable promise in industrial waste treatment as a polishing step since it can remove metals in the range of 1-2 ppm.

1.4.5 Reverse osmosis

Reverse osmosis (RO) or ultrafiltration consists of semipermeable membranes which act

basically as 'molecular sieves'. A high pressure system is used to force pure water through these membranes, leaving behind a concentrated chemical solution (Bhattacharyya *et al.*, 1976; Crampton, 1982). The membranes are synthetic organic materials and are frequently laminated (Dean *et al.*, 1972). The RO system is generally skid-mounted and can include a prefilter with micron elements, high pressure pumps, a system for chemical additions, a pH controller, and instrumentation devices for measuring flow, conductivity, temperature, pressure and any additional desired data (Crampton, 1982).

The metal finishing industry employ RO systems in the following distinct applications:

1) Purification of water supply for process needs
2) Recovery of plating drag-out from segregated rinses and reuse of chemical concentrate and purified rinse water
3) Purification of mixed wastewater to allow recycle of the purified water and achieve reduction of the waste volume requiring treatment.

One of the most significant operating problems common to all of the membranes is gradual reduction in performance because of plugging by suspended solids (Binnie and Partners, 1987). However, the problem is normally overcome by the proper pretreatment of the system. Another potential problem is associated with the precipitation of dissolved solids in the feed solution as it is concentrated in the RO system. In such cases, pretreatment involves either precipitation of the solids before the prefilters or addition of a solubilizing compound to the feed solution to prevent precipitation during

concentration. The operating cost of a RO system is one of the most attractive features of the technology i.e. reduced cost for plating chemicals and water; reduced costs for wastewater treatment; and reduced volumes of solid waste for disposal.

1.5 Remediation of wastewater using biomass of biological origin

The treatment of low charge effluents, with economic and technical constraints, is impossible with traditional physico-chemical processes. For example non-selective precipitation generates metallic sludges containing various metals difficult to valorize, while ion exchange techniques require large volumes of resins for treatment of high delivery of low metallic concentration flows and generate a high concentration of salt residue (Guibal *et al.*, 1992).

These methods are capable of reducing the concentration of metal pollutants to any desired degree, but the cost increases rapidly as the desired concentration gets smaller (Dean and Lund, 1981; Volesky, 1990; Wilde and Benemann, 1993). As a result, these methods unavoidably leave trace amounts of pollutants in wastewaters. This has necessitated research in developing alternative, efficient and cost effective wastewater purification systems based on metal-sequestering properties of certain natural materials of biological origin (Volesky, 1987; 1990). Indeed, there are indications that clean-up processes based on microbial technology can be more economical than existing treatments and some are in commercial operation (Gadd, 1990a).

The degree of treatment may range from a main process stream for seriously polluted

industrial waste to a polishing process to remove the trace concentrations which can remain after the main treatment (Forster and Wase, 1997). Microbial removal of metals from solutions is divided into three categories: The first is metabolism independent binding to surfaces of biomass termed adsorption. However, the term biosorption is now often used to describe the non-directed binding that occurs between metals and cellular components. The second involves metabolism-dependent intracellular uptake termed bioaccumulation. The third involves the biological conversion of the metals species by microbes such as sulphate reducing bacteria and fungi.

1.5.1 Biological transformation and reduction

The biological transformation of certain metals is an important process that can occur in many habitats and be carried out by a wide variety of microorganisms, chiefly bacteria and fungi (Gadd and Griffiths, 1978). Microorganisms can transform metal and metalloid species by oxidation, reduction, methylation and dealkylation. The process of biological transformation has been associated with microbial detoxification of metals. Metals cannot be converted into other products but may, as a result of biological action, undergo changes in valence and/or conversion into, organometallic compounds, which are often less toxic. For example, some yeast strains can also methylate mercury. In addition, microbial hydrogen sulfide production has significant effects on metal toxicity since most heavy metals form insoluble sulfides with H_2S. Consequently, H_2S-producing organisms often exhibit tolerance to heavy metals.

Both processes can be considered to be detoxification mechanisms since volatilization

and removal of the metal may result. Several types of bacteria and yeast have been shown to effect the reduction of Hg^{2+} to Hg^{0} (Gadd and Griffiths, 1978). This usually results in the mercury being volatilized from the medium.

1.5.2 Metabolism-dependent intracellular accumulation

Metabolism-dependent uptake of metal ions is usually a slower process than biosorption although greater amounts of metal may be accumulated by some microorganisms, e.g. yeast (Gadd, 1990a). This process occurs mainly in living cells and may be accompanied by toxic symptoms. In some cases intracellular uptake is due to increased membrane permeability arising from toxic interactions. It should be noted that many metals are essential for microbial growth at low concentrations e.g. Cu, Zn, Mn, Co, whereas many others have no essential biological role e.g. Cd, Pb, Hg, As, Au.

Once inside cells, metal ions may be preferentially located within specific organelles and bound to proteins such as a metallothionein (Wood and Wang, 1983; Gadd, 1990a). A copper-tolerant strain of *Scenedesmus* has been reported to produce a metallothionein type protein, while *Chlorella pyrenoidosa* and *Dunaliella* produced metallothionein type proteins when exposed to high concentration of cadmium (Wood and Wang, 1983; Gadd, 1990b). Since the uptake of excessive amounts of heavy metal will result in death of the microorganism, microorganisms therefore accumulate metals to a limited extent.

1.5.3 Metabolism-independent biosorption

Biosorption is the property of biomass of biological origin to retain and concentrate metallic elements from relatively dilute solutions (Tsezos and Volesky, 1982). This process has recently been receiving a great deal of attention for both its specific novelty and application potential. The biosorption of heavy metals by certain types of microbial biomass and algae is a well established phenomenon (Volesky, 1987; Volesky and Prasetyo, 1994; Ramelow *et al.*, 1992).

Metabolism-independent binding of heavy metals to cell walls, extracellular polysaccharides, or other materials occur in living and dead cells and is generally rapid (Gadd, 1990a; Wilhelmi and Duncan, 1996). However, in using this system, nonviable biomass or a derived product is preferable for metal removal and recovery since nonviable biomass offer several advantages over viable biomass. Dead biomass is not subject to metal toxicity or adverse operating conditions such as elevated temperature or nutrient limitation. It can be stored or used for extended periods of time at room temperature without the onset of putrification (Brady *et al.*, 1994a; 1994b).

Some methods of killing cells may actually improve biosorption properties of the biomass. Rapid and efficient metal uptake occurs and the biomass also behaves as an ion exchanger (Sağ and Kutsal, 1996a). For example, killing methods such as immersion in formaldehyde, would cross-link the cells thereby simultaneously immobilizing them (Brady *et al.*, 1994b). In addition, the recovery of metals may be by relatively simple nondestructive treatments (de Rome and Gadd, 1991). Comparatively, nonviable biomass

accumulates more metals than viable biomass (Volesky, 1987). This observation is attributed to the fact that nonviable biomass lacks the resistance mechanisms that prevent metal uptake in viable biomass. Moreover, the membrane integrity is also lost.

The ability of some microorganisms to bind metallic elements is well documented (Volesky, 1987). Biomass of brown marine alga *Ascophylum nodusum* has also been shown to possess a remarkable biosorption property to bind and remove dissolved cadmium from aqueous solutions (Volesky and Prasetyo, 1994). The high performance of the biosorption process featuring short contact time and high adsorptive capacity of the biosorbent was also indicated in the same study, where the biomass effectively removed approximately 100 % of cadmium ions from dilute (10mg/*l*) aqueous solutions (Volesky and Prasetyo, 1994).

The microbial biomass can chemically attract and sequester metals from solutions. The biosorption process depends not only on the chemical composition of the cell or its composition such as the cell wall but also on external physico-chemical factors and solution chemistry of the metal (Volesky, 1987). Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids offer abundant metal-binding functional groups such as carboxylate, hydroxyl, sulphate and amino groups (Kuyucak and Volesky, 1988).

Chlorella vulgaris has the ability to bind a variety of metal cations, and offer an advantage for selective desorption of metals from the biomass (Darnall *et al.*, 1986).

Most metals, such as Cu^{2+}, were eluted by reducing the pH to 2, while others, such as Au^{2+}, Ag^{+} and Hg^{2+} remained bound. These bound metals could then be selectively eluted by addition of mercaptoethanol, which presumably dissociates the metals which bind more firmly to thiol groups on the biomass. This added selectivity may make the biomass more viable than synthetic ion exchange resin which usually possess a single ligand type (Brady *et al.*, 1994a).

1.6 Algal exopolysaccharide - A potential metal biosorbent

There are indications that microbially-synthesized polymers extending from the outer membrane of a cell are in some cases responsible for the binding of metal ions. For example, a number of heavy metal ions were found to be chelated by chitosan-glucan from mycelia of filamentous fungi in higher amounts than those observed with animal chitosan (Kuyucak and Volesky, 1989). Consequently, the ability of microbial biomass to bind metals in solution has been attributed to the presence of biopolymers associated with the cell wall. Extracted membrane polymers have higher metal binding capacities than whole microbial cells (Macaskie and Dean, 1989), though quantitative polymer extraction is difficult to achieve (Sterritt and Lester, 1986).

Both ionic and covalent binding are involved in biosorption with proteins and polysaccharides playing important roles. A comparison of cation binding by polymers extracted from the yeast cell wall showed that mannans are generally the most significant accumulators of heavy metal cations compared to the two other carbohydrates of the cell wall, glucan and chitin (Duncan *et al.*, 1994). The postulated mechanism of binding is

that metal cations are complexed by negatively-charged sugar units of a polysaccharide chain. For instance, polysaccharides rich in phosphate groups complex ions by chelation through negatively charged oxygen atoms (Kuyucak and Volesky, 1989). These phosphate groups were also found to be the responsible functional groups for binding of uranium.

The precipitation of alginate by calcium was ascribed to crosslinkage through chelate formation involving carboxyl and hydroxyl groups. Some attention has also been given to the ion-exchange properties of sulfated polysaccharides from the brown seaweeds (Kuyucak and volesky, 1989). An ascorbic acid derivative of chitosan was found to have increased metal adsorption capacity which was orders of magnitudes higher than with other biopolymers (Muzzarelli, 1985). Bacterial exopolysaccharides are also vital for the process of flocculation, and indeed, the adsorption of ions from wastewater. The process of flocculation is generally affected by adsorption of metal ions which alter the water binding properties of the extracellular polymers (Dugan and Pickrum, 1972).

Conventional industrial applications of polysaccharides did not include their potential use as metal biosorbents. The application of polysaccharides as potential adsorbents for hazardous metals or strategically important metals has been a topic of intense research since this potential was realised (Jang *et al.*, 1991). Since natural biopolymers such as exopolysaccharides are becoming increasingly attractive due to their potential application in the metal removal process, the identification of new sources of these materials is extremely important (Vincenzini *et al.*, 1990a).

1.7 Research objectives

Effluent treatment processes are designed to ensure that when wastewaters are discharged into natural water courses, any adverse effects are reduced or prevented. The extent of any such effect will be a function of the volume and composition of the influent wastewater and the dilution capacity of the receiving water. Traditional wastewater treatment procedures become prohibitively expensive to operate when the metal concentration is below 100 mg/*l*. This has necessitated research into developing additional, alternative and cost-effective methods of removing heavy metals from dilute solutions.

The objective of this study was three-fold: Initial studies focussed on parameters that promote extracellular polysaccharide production as well as the kinetics of extracellular polysaccharide release. This was followed by isolation, purification and characterization of the polymer. Lastly, the efficiency of extracellular polysaccharides for removal of metals from aqueous solutions and the effect of parameters affecting metal removal from solutions was studied.

CHAPTER 2

ALGAL GROWTH AND PRODUCTION OF EXOPOLYSACCHARIDES

2.1 Introduction

Recently, interest in mass cultivation of microorganisms from marine and hypersaline environments has grown considerably, because this represents an innovative approach to the biotechnological use of under-exploited resources (Sudo *et al.*, 1995). Microalgae represent a new source of unique polysaccharide products, better known as biopolymers (Anderson and Eakin, 1985). A variety of studies on both unicellular and filamentous cyanobacteria of freshwater or marine origin, have examined either the chemical composition of the polysaccharides, and the influence of different growth conditions on exopolysaccharide production (Panoff *et al.*, 1988; Phlips *et al.*, 1989; Vincenzini *et al.*, 1990a; 1990b; De Philippis *et al.*, 1991), or rheological properties of both whole cyanobacterial cultures (Lapasin *et al.*, 1992) and aqueous solutions of purified polysaccharides (Navarini *et al.*, 1990).

Although polysaccharides of plant origin (such as starch, alginate, or agar) have been used in industry as stabilizers, thickeners, and emulsifiers for many years, microbial polysaccharides have become widely used over the past several decades (Crueger and

Crueger, 1990). Around twenty different microbial polysaccharides with market potential have been described, but the largest part of the market is held by xanthan, with production of about 10 000 tons per year. However, microbial polysaccharides are really only a small part of the polysaccharide market because xanthan represents only 4 % of the market (Crueger and Crueger, 1990). Studies have been, however, limited to a few cyanobacterial strains and additional work is necessary to evaluate the potential of cyanobacteria as a new source of these biopolymers (De Philippis *et al.*, 1993).

2.1.1 Algal growth

Many arid and desert areas offer important advantages for cultivation of algae, under conditions which usually impose severe limitations on conventional agriculture (Richmond and Preiss, 1980). Arid zones lack sufficient water and such water resources as may be available are often of a quality unsuitable either for human consumption or for use in agriculture, since they contain high concentrations of salt or other contaminants (Richmond and Preiss, 1980). A considerable number of unicellular algal species are considered euryhaline, since they can adapt to varying external salinity (Hellebust, 1985). In cultures grown outdoors in open ponds under arid and semiarid conditions, daily evaporation of 1-2 cm occurs leading to a progressive increase in the salt concentration (Vonshak, 1988).

The cyanobacteria constitute a large group of oxygen evolving photosynthetic prokaryotic microorganisms which include unicellular as well as filamentous species. Some genera possess the capacity to fix gaseous nitrogen so that they are able to grow exclusively at

the expense of simple inorganic molecules such as H_2O, CO_2 and N_2. Photosynthetic apparatus provides the cyanobacterial cells with needed ATP and reducing power (Castenholz and Waterbury, 1989, cited in Vincenzini *et al.*, 1990b). Cyanobacteria are found in all types of ecosystems, some species being able to survive even under extreme environmental conditions. Their presence has been demonstrated in both fresh, salted and alkaline waters, where they proliferate as free living cells in suspension and in non-aqueous habitats (soil, rocks, plant roots or leaves), where they grow as colonizing populations attached to solid surfaces (Vincenzini *et al.*, 1990b).

It is generally believed that several metabolic strategies may have been developed by these microorganisms to cope with the selective pressures of these varied natural environments. Many of these metabolic strategies remain unclear, but it is perceived that the synthesis of exocellular polysaccharides plays a key role in allowing the survival and growth of cyanobacteria under such wide ranging conditions (Vincenzini *et al.*, 1990b). Some of the most frequently mentioned functions attributed to polysaccharide production are:

1) protection against dessication, predation and antibacterial agents (Balkwill and Stevens, 1980; Arad, 1988; Flaibani *et al.*, 1989),
2) chelation of cations essential to cell life (Balkwill and Stevens, 1980) and
3) adhesion to solid surfaces (Balkwill and Stevens, 1980; Robins *et al.*, 1986).

Indeed, photosynthetic microorganisms are exposed in nature to a continuous fluctuation in growth conditions such as temperature, nutrients, light intensity, and an increase in

osmotic pressure due to evaporation (Lang and Brown, 1981; Vonshak *et al.*, 1996). Light is considered to be one of the most important limiting factors in outdoor dense cultures (Torzillo and Vonshak, 1994). When the nutritional requirements of mass cultured algae are satisfied and the environmental conditions are not growth-limiting then mixing, designed to create a turbulent flow, constitutes the most requisite condition for consistently high yields of algal mass. The three major effects of mixing have been established:

1) It prevents cells from sinking to the bottom of the pond. When this is allowed to happen, it leads to the accumulation of organic material and consequently to anaerobic decomposition and the release of unwanted substances and metabolites.
2) It prevents the formation of nutritional and gaseous gradients. Apart from lowering of oxygen supersaturation, it also reduces the boundary layer around the cells.
3) Mixing also helps move the cells through an optically dense gradient, with variations in the quantity and quality of light energy (Grobbelaar, 1994).

However, some natural populations of cyanobacteria have been reported to carry out vertical migration in fresh water bodies to compensate for lack of mixing in their natural environment (van Rijn and Shilo, 1985). The buoyancy regulation underlying this vertical migration is influenced by several environmental factors, of which light and nutrient availability are the most important. A change in these environmental factors causes the cyanobacterial cells to float up or sink down, a phenomenon that can be explained by two proposed mechanisms:

1) Gas vacuole synthesis and collapse (Walsby, 1969) and

2) Fluctuations in the amount of high density cell components (van Rijn and Shilo, 1985).

Indeed, it has been amply demonstrated to date that mixing represents one of the most important factors which govern overall culture productivity (Richmond and Grobbelaar, 1986; Terry, 1986). *Spirulina* culture is decisively affected by the extent of turbulence, which is due to a more favourable light/dark cycle. This was ascribed to the fact that the time period of each interval becomes smaller with increasing turbulence (Richmond and Vonshak, 1978). Contrary to these findings it was found, using ^{14}C-uptake rates, that on the average lower productivities were measured in a rotating system compared to those obtained in static bottle incubations over similar depth profiles and light fields (Grobelaar, 1989; 1991). The observations were accounted for by the fact that turbulence does not only move cells through an optically dense medium at varying rates, but it also influences the boundary layer. The effect would be an increase in the rates of exchange of metabolites and nutrients between algae and the environment, consequently the algae will be able to utilize light more efficiently (Grobbelaar, 1991).

There is no doubt that the genus *Dunaliella* is unique. It is distributed all over the world, wherever salty ponds are to be found. It has no rigid cell wall, thus it is one of the only naked cells in the plant and bacterial kingdoms (Ginzburg, 1978). Interestingly, this alga can adapt to a very wide range of salt concentrations. It is assumed that *Dunaliella* adjust to the external salinity by accumulating glycerol as an osmolyte and compatible solute

(Bental *et al.*, 1990). At constant salinity, the turnover rate of the glycerol pool is relatively slow. However, when subjected to a hypoosmotic or hyperosmotic shock, the cells react within seconds, like osmometers, swelling or shrinking, respectively, due to very rapid water fluxes. This is followed by a metabolic phase, which lasts for approximately two hours, during which the cell carries out massive glycerol elimination or synthesis in order to regain its original volume (Bental *et al.*, 1990).

Algae of the genus *Dunaliella*, especially *D. salina* and *D. tertiolecta*, are among the microalgae most studied for mass culture. They are grown as a food source in aquaculture and *D. salina* is the richest algal source of β-carotene and glycerol (Borowitzka and Borowitzka, 1988). *Dunaliella salina* is the first microalga to be used commercially to produce fine chemicals, because its extreme salinity tolerance simplifies maintenance of a uni-algal culture, relatively free of competitors, pathogens and predators (Borowitzka and Borowitzka, 1988).

2.1.2 Production of exopolysaccharides

The production of extracellular polysaccharide will be referred to as liberation, release, or excretion without attributing different meanings to these terms. In addition, polysaccharides released into the surrounding media will be referred to as extracellular polysaccharides or exopolysaccharides again without attributing any different meanings to these terms.

The photosynthetic processes whereby phytoplankton synthesise cellular components do not show 100 % efficiency (Myklestad, 1995). A fraction of these organic molecules, small as well as large, are released from algal cells into the ambient water (Myklestad, 1995). This has been consistently observed in the laboratory for more than 60 years. Serious doubt has been expressed as to the evidence of this process in healthy cells, but it was later suggested that excretion is a normal process in healthy phytoplankton (Fogg, 1977).

Moreover, Myklestad *et al.*, (1989) showed that the rate of release of carbohydrates and amino acids per cell in the exponential phase of growth was as high or higher than that in the stationery phase. These researchers subsequently concluded that exudation takes place during all phases of growth, and that it is not per se a consequence or symptom of cell death or environmental stress. A study by Vincenzini *et al.*, (1990a) using *Cyanospira capsulata,* a helically coiled heterocystous cyanobacterium, also demonstrated this phenomenon. *Cyanospira capsulata* is characterised by a thick mucilaginous capsule and during photoautotrophic growth, it releases large amounts of mucilaginous material into the surrounding medium. As a direct consequence of this massive release, old cultures of this organism become highly viscous.

However, some microalgae including cyanobacteria, have been shown to modulate the production of exopolysaccharides in response to several environmental factors including among other things, salt stress, nutrient depletion and light intensity (Hellebust, 1965; Tischer and Davis, 1971; Kroen and Rayburn, 1984; Thepenier *et al.*, 1985; Adda *et al.*,

1986; Arad *et al.*, 1992; De philippis *et al.*, 1993; De Zoysa and Ragusa, 1995). The observation that some microorganisms produce exopolysaccharides only in response to environmental conditions while others produce exopolysaccharides regardless of the influence of environmental conditions strongly suggests that the excretion of polysaccharides may be species specific.

Capsular polysaccharides are intimately associated with the cell surface, are structurally coherent, so as to exclude particles (e.g. indian ink) and appear as a thick, regular, cloud-like zone with sharp outlines (Whitfield, 1988). They are detectable only under a light microscope after positive or negative staining (Balkwill and Stephens, 1980). The polysaccharides can also be associated with the cell as slimes (Whitfield, 1988; Vincenzini *et al.*, 1990a; 1990b). Contrary to capsular polysaccharides, slimes may be detached from the cell surface, do not completely exclude particles and appear as a broad layer less dense towards the outer margins and unrelated in form to the shape of the cells (Whitfield, 1988). In both cases, if large quantities are produced in to the culture medium, the cultures become more viscous (Vincenzini *et al.*, 1990a; 1990b).

Porphyridium, Rhodella, and Rhodosorns are unicellular red microalgae encapsulated in sulfated polysaccharide, the external part of which dissolves in medium (Arad *et al.*, 1992). Accordingly, it has been found that the polysaccharides contiguous with the cell membrane are qualitatively identical with the polysaccharides released into the medium (Jones, 1962; Ramus, 1972). It was found (Vincenzini *et al.*, 1990a) using Cyanospira capsulata that polysaccharide released into culture media, monitored by quantifying

soluble carbohydrates, occurred continuously throughout batch growth, constituting a significant proportion of the total synthesized carbohydrates. However, some algae have a propensity to produce polysaccharides mainly in the stationary phase of growth when free cells are cultured (Thepenier *et al.*, 1985).

2.1.3 Factors affecting algal polysaccharide production

Elemental nitrogen is one of the most important nutrients required for plant growth (Layzell, 1995). Acquisition and assimilation of nitrogen is second in importance only to photosynthetic carbon assimilation for plant growth and development (Vance and Griffith, 1995). It is a key constituent of proteins, nucleic acids and other cellular components and its availability in the environment frequently limits the growth and yield of photosynthetic organisms (Layzell, 1995).

There are conflicting reports about the exact conditions that stimulate the production of polysaccharides. There are reports that the release of polysaccharide occurs only during the stationary phase. Exponentially growing cultures of *Thalassiosira fluviatilis* release only about 5 % of the photoassimilated carbon, while the proportion increased to over 20 % when the cells enter the stationary phase (Hellebust, 1974). Kroen and Rayburn (1984) argued that phosphate-starved cells show similar response (produce polysaccharide) to nitrogen-starved cells, indicating that the beginning of a stationary phase and not nitrogen depletion causes the stimulation of polysaccharide synthesis. They also illustrated that the addition of nitrogen to stationary phase cultures causes renewed growth and a temporary lag in polysaccharide synthesis until growth again ceases. Adda

et al., (1986) also noted that when cultures are grown at high nitrogen regimes, an accumulation in the polysaccharide concentration preceded the depletion of nitrogen.

On the other hand, it is reported that this process is mediated by nutrient depletion, particularly nitrogen. Nevertheless, it cannot be unequivocally concluded that there is a casual relationship between nitrogen depletion and polysaccharide production, since the level of other nutrients, not being analysed, may also have dropped below the threshold during the growth period (Adda *et al.*, 1986). The depletion of other nutrients such as phosphate, sulphate and magnesium have been shown to stimulate the synthesis of polysaccharides. At fairly low nutrient concentrations the stationary phase coincides with nutrient depletion, in particular, nitrogen and phosphorus. When these nutrients are depleted, the synthesis of proteins and cell division stops (Arad *et al.*, 1988; 1992). This is followed by accumulation of extracellular polysaccharides (Arad *et al.*, 1988).

In other words, whereas protein is accumulated during the log phase, the extracellular polysaccharide is produced mainly in the stationary phase when no nitrate and phosphate is left in the medium. This is biochemically possible because no nitrogen or phosphorus is incorporated into polysaccharide and the cells use their intact machinery to channel the carbon source into the synthesis of polysaccharide. It was thus suggested that polysaccharide production takes place at the expense of protein synthesis (Spoer and Milner, 1949; De Philippis *et al.*, 1991).

Adda *et al.*, (1986) reported that polysaccharide yield generally depends on both algal

growth and polysaccharide synthesis. In addition, they indicated that the optimal conditions for both processes are not necessarily identical. They demonstrated the requirement for high nitrogen levels for growth, whereas nitrogen depletion and arrest of cell growth were found to be appropriate for polysaccharide synthesis. Interestingly, the beginning of the stationary phase of growth seemed to coincide with the depletion of nitrogen from medium in cultures initially containing nitrate concentrations below 10 mM. However, at higher nitrate concentrations, the growth reached a plateau before nitrate was depleted from the medium. This finding was confirmed by Arad *et al.*, (1988) who found that daily additions of nitrate to the medium do not prevent the onset of the stationary phase. It became evident that a factor other than nitrate could be growth limiting. It was reported that such a factor was likely to be light limitation due to high cell density, in other words, mutual cell shielding. It should be noted that the pattern of excretion of polysaccharide is species-specific and that the effects of nutritional limitation are often very different (Myklestad, 1995).

One of the most important findings in pilot plant production studies (Anderson and Eakin, 1985) was the influence of temperature control. To achieve maximum polysaccharide production, the culture temperature should be maintained at optimum levels. For example, these authors recorded a decrease from 23 g/m^2 day to 14.5 g/m^2 day when the temperatures was allowed to decrease below 25 °C using *Porphyridium* cruentum.

The objective of this section of the study was to establish the conditions that induce

exopolysaccharide production by *Dunaliella* as well as to study the kinetics of polysaccharide release.

2.2 Material and Methods

2.2.1 Materials

Chemicals

Chemicals of high (analytical) grade were supplied by the following companies:

BDH Chemicals Ltd., Poole, England - Zinc chloride, Sodium potassium tartrate.

Merck, Darmstadt, Germany - Potassium nitrate, Calcium chloride, Ferric sulphate, Manganese chloride, Sulphuric acid, Hydrochloric acid, Magnesium sulphate, Sodium carbonate.

Saarchem (Pty) Ltd., Johannesburg, S. Africa - Sodium hydrogen carbonate, Sodium chloride, Potassium dihydrogen orthophosphate, EDTA, Boric acid, Cobaltous chloride, Ammonium chloride, Sodium hydroxide.

Fluka Chemie AG., Buchs, Switzerland - Folin ciocalteau reagent.

Equipment

The following equipment was used in carrying out experimental work:

- UV/VIS Spectrophotometer (UV-160A) - Shimadzu Corporation
- Freeze Dryer (Modulyo) - Edwards Corporation
- Centrifuge (J2-21) - Beckman
- Nitrate Selective Electrode - Eutech Cybernatics

2.2.2 Methods

2.2.2.1 Algal culture

Dunaliella bardawil was routinely grown in Baam medium at 28°C under continuous illumination provided by white fluorescent lamps of 85 watts, with constant stirring. The pH of the culture medium was adjusted to 8.82. Fresh medium was added every 4-5 days depending on the rate of growth.

2.2.2.2 Assessment of cell growth

Cell growth was determined by reading the optical density of the culture at 560 nm (Maxwell *et al.*, 1994; Pirt and Pirt, 1977). In another set of experiments, cell growth was measured by following increases in total protein concentration (Vonshak *et al.*, 1982). This was achieved by using the Lowry method for protein determination (Lowry *et al.*, 1951). Briefly, 1 m*l* aliquots were sampled from experimental cultures, placed in eppendorf tubes and centrifuged at 1400 g. The pellet was resuspended in 0.5 M NaOH (1 m*l*) and boiled for 15 min. The supernatant was collected after 10 min centrifuging (1400 g). The procedure was repeated and the supernatants were combined.

Aliquots (0.5 m*l*) of the supernatant were put in clean dry test tubes followed by the addition of 2.5 m*l* reagent C (see appendix B for preparation of Folin Lowry reagents). The tubes were mixed and allowed to stand at room temperature for 10 - 15 min. Thereafter, 0.25 m*l* reagent E was added and the tubes vortexed immediately after addition. The tubes were allowed to stand for 30 min at room temperature and absorbance

read at 540 nm. All samples were prepared in triplicate.

2.2.2.3 Assay for residual nitrate concentration in culture media

Determinations were done using the Merck Nitrate Spectroquant Kit by following instructions provided by the supplier. Nitrate (0-90 mg/*l*) dissolved in Baam medium was used as a standard. Culture aliquots (2.5 m*l*) were centrifuged in eppendorf tubes and the pellet discarded. Aliquots (1.5 m*l*) of the supernatant were put in clean dry test tubes followed by the addition of one microspoon of NO3-1A reagent. Five milliliters of NO3-2A reagent were then added and the tubes were vortexed immediately to effect dissolution. The tubes were allowed to stand for 10 min and absorbance measured at 515 nm. The nitrate concentration of the individual samples were determined by extrapolation from the standard curve. All the samples were done in triplicate assay. The remaining supernatant was used for determination of extracellular polysaccharide concentration. A nitrate specific electrode was also used for assay of residual nitrate concentration.

2.2.2.4 Induction of exopolysaccharide production

For experimental purposes, cultures were exposed to various concentrations of KNO_3 (half and one fold strengths relative to normal Baam medium), $NaHCO_3$ (one and two fold strengths relative to normal Baam medium) and high light intensity. Various combinations of carbon to nitrogen ratio were investigated for their effects on exopolysaccharide production. For KNO_3/$NaHCO_3$ experiments, the cultures were

allowed to run for 22 days without replenishing nutrients. In another set of experiments, the cells were exposed to high light intensity at light/dark cycles of 16h/8h. Samples were taken every other day for determination of exopolysaccharides in medium. All samples were prepared in triplicate.

2.2.2.5 Quantitative determination of exopolysaccharides

Polysaccharide concentration was estimated from culture supernatants by the phenol-sulphuric acid method (Dubois *et al.*, 1956) and an experimental factor of 2 was utilized to convert the amount of soluble carbohydrates into actual exopolysaccharides (De Philippis *et al.*, 1991). Determinations were done every other day using glucose (dissolved in water) as a standard. One ml of culture supernatant was put in a test tube followed by the addition of 1 m*l* of 5 % phenol (w/v) solution. Concentrated sulphuric acid (5ml) was then quickly added to the surface of the suspension while mixing in the fume cupboard. The suspension was allowed to cool to room temperature. Any sample that was too high in polysaccharides was diluted with sulphuric acid. Absorbance was read at 488 nm on a UV/VIS spectrophotometer (Shimadzu). The remaining culture supernatant was used for the determination of residual nitrate concentration.

2.2.2.6 Isolation and purification of EPS

For isolation of EPS, the cultures were centrifuged (JA 20 centrifuge, Beckman instruments) at 8 000 g for 10 min at 10 °C. Polysaccharide precipitation was effected by addition of two volumes of 2-propanol to the supernatant (Smith and Pace, 1982;

Vincenzini *et al.*,1990a). The precipitate was isolated and dialysed overnight in 2 x 5 *l* deionised water, followed by freeze drying.

2.3 Results

2.3.1 Growth of *Dunaliella* cultures under different nutritional conditions

The effects of nutritional depletion and growth phase on algal growth were investigated, as nutrient status appears to be one of the most important factors controlling the growth of algae in the natural environment. Growth of *Dunaliella* was assessed by reading the optical density of the culture at 560 nm (figure 2.1). The growth profile in all conditions was found to increase in a time dependent manner reaching the stationary phase of growth after eighteen days. However, growth in experimental cultures was higher than in control cultures with the highest growth recorded in cells grown in medium containing a high (twice as in control cultures) concentration of the carbon source.

Growth in cells cultured in medium containing half the concentration of the nitrogen source and normal levels of the carbon source (figure 2.1) was slightly higher than in control cells. Low levels of the nitrogen source did not adversely affect growth of cells. These observations suggest that the utilization or consumption of the nitrogen source in these cells is relatively slow, since at no stage throughout the experiment was the growth of experimental cultures lower than that of control cells. On the other hand, an increase in the concentration of the carbon source was found to stimulate growth beyond that of control cells. The cultures remained viable throughout the experiment.

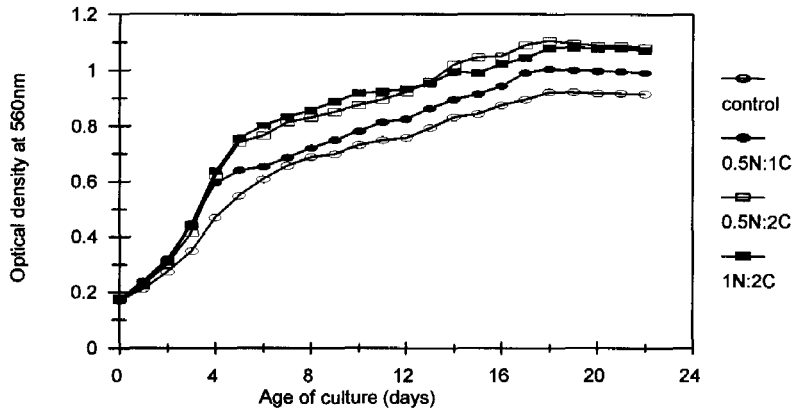


Figure 2.1: The effect of different nutritional conditions on growth of *Dunaliella* cultures growing in Baam medium. Growth was assessed by reading optical density of the culture at 560 nm. Various conditions indicate the ratio of N to C source relative to that of control (○), where (●) indicates half [N] and normal [C]; (❑), half [N] and twice [C]; (■), normal [N] and twice [C] relative to control. Results represent the mean of triplicate assays.

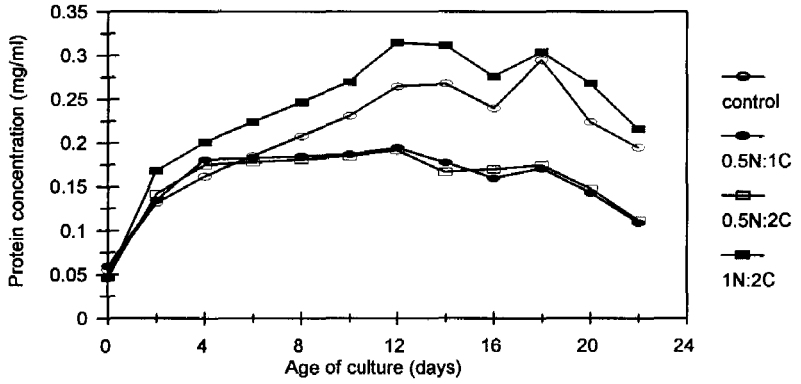


Figure 2.2: The effect of nutritional conditions on growth of *Dunaliella* cultures growing in Baam medium. Growth was assessed by following increases in protein content. Symbols as described in figure 2.1. Results represent the mean of triplicate assays.

An accumulation of particulate matter was observed after day eight, which could give false negative results because particulate matter also absorbs light. Consequently, the determination of cell growth was done by following increases in protein concentration (figure 2.2). The final cell density and length of time required to reach the stationary phase are directly related to the original amount of nitrogen present (Kroen and Rayburn, 1984; Lupi *et al.*, 1994). The present author also established that growth in cultures that were subjected to lower concentrations of the nitrogen source was inhibited within the first six days of the experiment whereas growth of cultures growing in normal concentrations of the nitrogen source was only inhibited after twelve days. This suggests that growth was inhibited due to depletion of the nitrogen source which is important for both protein and nucleic acid synthesis. A high concentration of the carbon source did not cause any significant difference in cell growth when the concentration of the nitrogen source was low.

In the presence of normal concentrations of the nitrogen source, a high concentration of the carbon source promoted growth above that of control cells. This finding was in agreement with observations (Borowitzka and Borowitzka, 1988) that addition of inorganic carbon as CO_2 in air or as $NaHCO_3$ stimulates the growth of *Dunaliella* as long as there is no precipitation of carbonates. It is not clear whether the arrest of cell growth after day 12 occurred as a result of nitrogen depletion or light limiting conditions that are obtained at high cell density. Vonshak (1988) found that KNO_3 concentration below 2 mM limits growth of *Porphyridium*, indicating the existence of threshold levels of the nitrogen source. Therefore, it was necessary to follow the utilization of nitrate in culture

media.

2.3.2. Quantitative determination of residual nitrate concentration

Samples for determination of residual nitrate concentration were done every other day. This would explain whether the stationary phase of growth was reached after nitrate depletion or not. Furthermore, it would also provide evidence regarding whether the production of exopolysaccharide occurs as a result of nitrate depletion or due to the influence of other parameters such as light. A gradual decrease in nitrate concentration was observed until day 12, followed by an increase (figure 2.3).

When the increase in nitrate concentration was observed, the level of nitrate in media was still relatively high. The same profile was followed for conditions with normal nitrate concentrations as well as those with lower concentrations. This trend was not expected because nitrate is a nutrient and should continue to decrease from culture media to indicate its utilization. On analysis it was found that simple sugars, in particular glucose, form colour when subjected to methods for nitrate determination. This problem made it difficult to establish if there is any coincidence between the depletion of the nitrogen source and the beginning of the stationary phase and/or exopolysaccharide production.

2.3.3. Induction of extracellular polysaccharide release

Figure 2.4 shows the induction of exopolysaccharide production by subjecting *Dunaliella* cultures to different nutritional conditions. The cells were cultured aerobically for 22

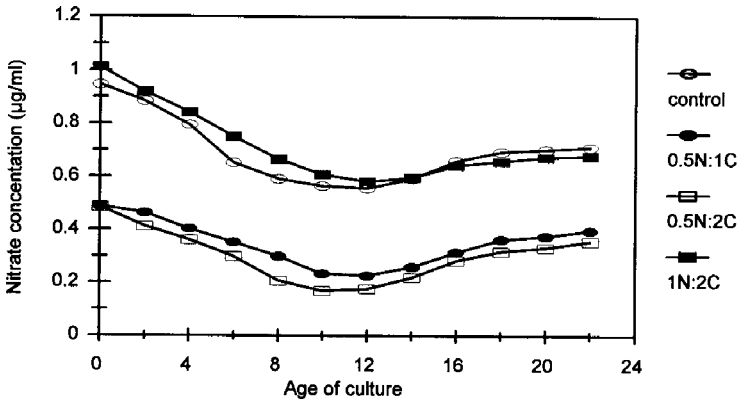


Figure 2.3: Determination of nitrate concentration remaining in culture medium. Symbols as described in figure 2.1. Results represent the mean of triplicate assays.

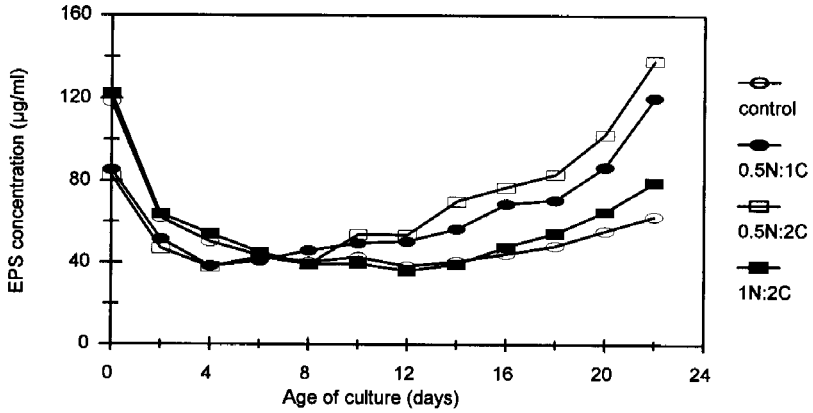


Figure 2.4: The effect of nutritional status on extracellular polysaccharide release by *Dunaliella* cultures. Symbols as in figure 2.1. Results represent the mean of triplicate assays.

days in Baam medium. It was to be expected that EPS concentration in culture media will be almost zero at the beginning of the experiment, subsequently increasing when conditions are suitable for EPS production. Contrary to expected profiles an initial decrease in EPS content was recorded for controls as well as experimental culture. This discrepancy was found to occur as a result of nitrate interference with the method for polysaccharide quantification.

A slight increase in EPS production was observed after twelve days in cultures growing in normal concentration of the nitrogen source. A low concentration of the nitrogen source resulted in an increase in EPS release, with an even higher EPS production being obtained when the concentration of the carbon source was doubled. The increase in profiles obtained after 12 days clearly indicated the accumulation of EPS in culture media, and not nitrate interference because nitrate should be depleted as a nutrient. However, the exact time during which EPS production starts could not be established, due to the problem mentioned above. These observations seem to suggest that the supply of nitrogen influences exopolysaccharide release. Owing to nitrate interference and consequent inability to accurately quantify exopolysaccharides, an alternative nitrogen source (ammonium chloride) was used. However, this was not successful because the cultures died within 10 days (results not shown).

2.3.4. The effect of high light intensity on growth of *Dunaliella* cultures

Cultures were exposed to light/dark cycles of 16h/8h to allow them to recover from the high light intensity stress. As shown in figure 2.5, an increase in growth occurred in a

time dependent fashion up to day 8, followed by cell death due to stress. Growth of experimental cultures was lower than that of control cultures. It is not clear why control cultures were protected from the adverse effects of high light intensity. However, it is assumed that the high concentration of the nitrogen source in controls compared to experimental cultures allowed the control cells to grow faster, which would cause mutual cell shielding due to high cell density, thus ameliorating the toxic effects of high light intensity. The death of cultures was confirmed by viewing the cells under the microscope.

2.3.5. The liberation of EPS under high light intensity by *Dunaliella* cultures

The assay of EPS released into media by *Dunaliella* under high light intensity (figure 2.6) shows a gradual increase in EPS concentration. The high ratio of carbon to nitrogen source seemed to enhance EPS production beyond that of the control under similar conditions. A steady accumulation of EPS in control cultures was also observed. Interestingly, the initial decrease in EPS concentration that was observed in figures 2.4 was not observed under these conditions. However, it was noted that on day 0 the concentration of EPS was not zero. This further confirmed the nitrate interference since nitrate is present in the initial medium. It is not clear why the expected initial decrease was not observed, but it was presumably due to an early release of EPS at very high concentration. This is very likely to occur under high light intensity stress, and if it happens, the high EPS concentration will mask the nitrate interference. The cultures lost viability by day 12 and cell death was confirmed by viewing cultures under the microscope.

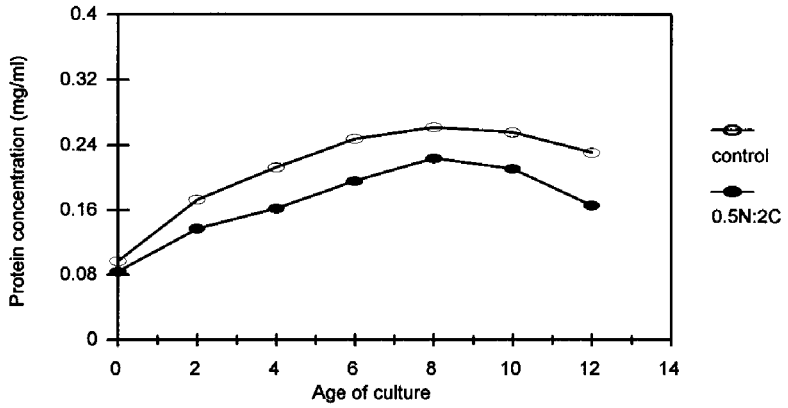


Figure 2.5: The effect of high light intensity on growth of *Dunaliella* cultures. Cultures were exposed to high light intensity/ darkness for 16h/8h respectively. Control (○), normal Baam medium; (●), half [C] and twice [N] sources. Results represent the mean of triplicate assays.

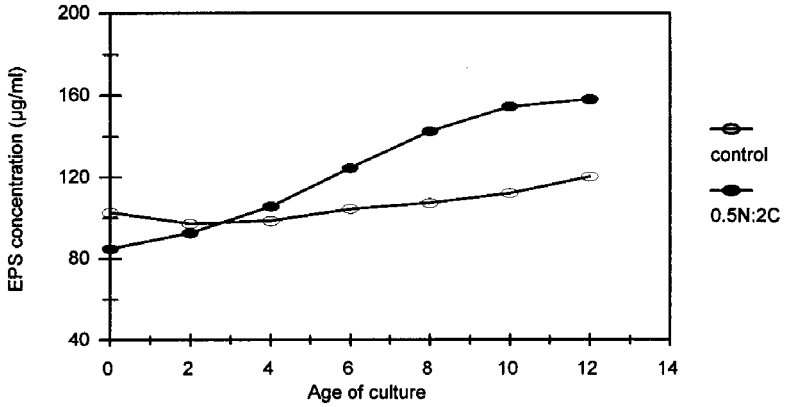


Figure 2.6: The production of extracellular polysaccharide by *Dunaliella* cultures under high light intensity. Cultures were exposed to high light intensity/ darkness for 16h/8h respectively. Control (○), normal Baam medium; (●), half [C] and twice [N] sources. Results represent the mean of triplicate assays.

2.3.6 The use of artificial brine medium for production of EPS by *Dunaliella* cultures

Due to cross interference between nitrate and carbohydrates, it became impossible to normalise for the contribution of colour by nitrate in the determination of polysaccharide concentration (see sections 2.6.2 and 2.6.3). It was decided that artificial brine medium, which has a lower nitrate concentration, would be used. The use of this medium allowed the quantification of EPS without assaying for nitrate.

However, the slight decrease in EPS concentration at the beginning of the experiment was still observed, although less pronounced than when a medium containing a high nitrate source was employed (figure 2.7). This was followed by steady accumulation of EPS in the surrounding media. The highest EPS concentration was obtained in cultures exposed to high concentrations of the carbon source (twice the level in control cultures), followed by cultures growing in medium containing normal levels of the carbon source and half the concentration of nitrogen source. These findings confirm the influence of nitrogen supply on EPS production. In addition, the ratio of carbon to nitrogen source was found to have profound effects on EPS production.

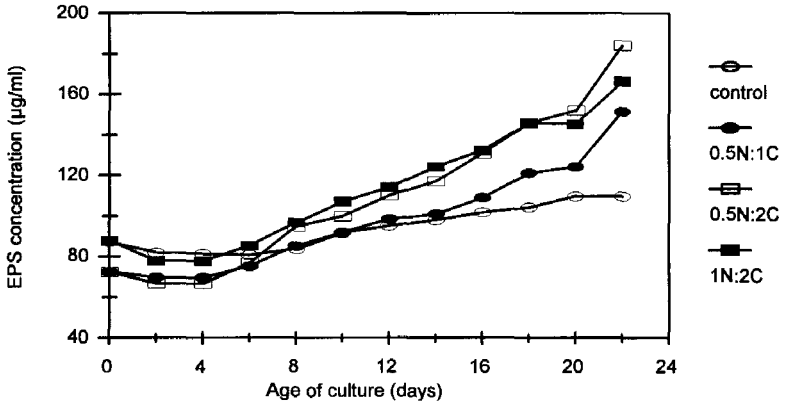


Figure 2.7: The effect of different nutritional conditions on extracellular polysaccharide production by *Dunaliella* cultures growing in artificial brine medium. Symbols as described in figure 2.1. Results represent the mean of triplicate assays.

2.4 Discussion

An algal species considered for outdoor cultivation should be able to survive the expected fluctuations in nutritional conditions, light intensity and temperature. It should be possible to grow the algae at high cell concentrations and to obtain a high productivity. A major requirement is for the algal culture to exhibit steady continuous growth and undergo several harvesting cycles without becoming contaminated with other microorganisms and without deterioration caused by predators or by self excreted growth inhibitors. It was therefore relevant to study the effects of various parameters on growth of *Dunaliella.*

Growth was affected by low levels of the nitrogen source with the stationary phase being reached within eight days, presumably due to nitrate depleting conditions. This

observation supports the finding by Adda et al., (1986) who found that at relatively low nitrogen concentrations (below 10 mM KNO_3), growth assessed by following protein content, increased until nitrate was depleted from the medium. A higher cell growth was obtained in the presence of high concentration of the nitrogen source.

Increasing (doubling) the carbon source enhanced growth even more. Under these conditions cell growth was only arrested after twelve days. This finding confirmed a suggestion (Adda et al., 1986) that the length of the logarithmic phase as well as cell number is positively correlated with the initial nitrogen concentration. However, at this stage the cell density was very high. Thus, it is likely that mutual cell shielding could have also played a role in the arrest of cell growth. For example, when *Spirulina* cultures are grown at optimum temperature and nutrient conditions, light is considered to be the only limiting growth factor (Vonshak *et al.*, 1996). Furthermore, Arad *et al.* (1988) have demonstrated that daily additions of the nutrients do not prevent the onset of the stationary phase of growth from occurring. This led them to suggest the involvement of light as a contributing factor.

The influence of temperature on growth and EPS production by *Dunaliella* was not feasible in this study because of lack of enough constant environment (CE)- rooms set at different temperatures. The adverse effects of not stirring cultures have been discussed in section 2.2. This rules out the possibility of employing water-baths for the regulation of temperature. Another drawback of using water-baths is that when cultures are immersed in water they do not get sufficient light. Incubators could not be used either

because they do not have a light source.

The obvious effects of temperature on growth of algae cannot, however, be ignored. The algal species which have been considered for commercial production are warm-water species, thus the effect of temperature on the performance of outdoor cultures is of prime importance (Richmond and Grobbelaar, 1978; Vonshak *et al.*, 1982). Both studies reported a sharp decline in *Spirulina* growth rates during winter. The importance of temperature is underlined by the occurrence of algal blooms in summer.

Research has shown that when algae are exposed to nutrient deficient conditions, growth is inhibited (Thepenier *et al.*, 1985). In most algae this is followed by an accumulation of polysaccharide material in the surrounding media (Sutherland, 1982; Myklestad, 1995). In particular, this phenomenon becomes more pronounced if there is a high ratio of utilizable carbon to limiting nitrogen (Sutherland, 1990). These reports were confirmed by our studies. Due to nitrate interference with carbohydrate analysis, it was not possible to express polysaccharide production on a per cell basis. However, the results obtained were satisfactory and it was found that more polysaccharides were produced by cultures grown in the presence of low concentrations on the nitrogen source. This was particularly interesting to note since most algae that excrete exopolysaccharide into surrounding media are encapsulated. The mechanism of release is not clearly understood and needs further investigation.

The interference of nitrate in carbohydrate quantification and the inability to follow its utilization prompted the use of an alternative nitrogen source. An attempt to use ammonium as an alternative source of nitrogen was not successful because the cells died within 10 days (results not shown). This unexpected phenomenon could not be explained. However, it could be attributed to the fact that ammonium salts such as ammonium acetate, ammonium nitrate, ammonium citrate, and ammonium chloride are generally less effective nitrogen sources (Borowitzka and Borowitzka, 1988). It is, therefore, possible that *Dunaliella* cultures could not utilise this form of nitrogen source.

An artificial brine medium was used as an alternative to normal Baam medium which has a high level of nitrates. It was also an alternative to the use of other sources of nitrogen, which were not very useful. The significance of using this alternative medium was to avoid interference due to high levels of nitrate in Baam medium. Because of its low nitrate concentration, artificial brine medium would allow determinations of polysaccharides with some measure of accuracy after a few days of culturing, i.e. when the levels of nitrate are depleted. However, the beginning of polysaccharide production normally starts before the depletion of nitrate in culture media. Consequently, the exact time when polysaccharide production begins and nitrate is depleted remains unclear due to interference. It is, however, assumed that by day 12, nitrate will be depleted.

Studies on the effect of light intensity have revealed a higher polysaccharide exudation in full light compared to cultures that were exposed to light for shorter periods (Hellebust, 1965). This finding was supported by Phlips *et al.* (1989) who recorded a 25 to 30 %

increase in carbohydrate released by cultures continuously exposed to light as opposed to cultures grown under light/dark regimes. High light intensity at light/dark cycles of 16h/8h was toxic to *Dunaliella* cultures. The cells lost viability after ten days. It is not clear why control cultures grew better than experimental ones. It is, however, assumed that the level of nitrate in culture media played a crucial role in ameliorating the toxic effects of exposure to high light intensity. The EPS accumulating in media of cultures exposed to high light intensity was probably released from ruptured cells as well.

2.5 Conclusion

This study provide evidence for the production of polysaccharides by *Dunaliella* under nutrient depleting conditions. Preliminary results show that the highest EPS produced by *Dunaliella* occurred when the cells were grown at low initial nitrogen concentration supplemented with high concentration of carbon source. Due to high salt concentration in media and interference by carbohydrate material accumulating in culture media, the rate of nitrate consumption could not be determined. Optimization of high light intensity conditions as well as investigation of the effects of temperature on EPS production could prove useful in optimising the production of EPS by *Dunaliella*.

It was not possible to measure/calculate the efficiency of EPS production, that is, express EPS production on a per cell basis. This would give insight into the understanding of EPS production and optimization of the system. Nitrate interference also made it very difficult to accurately measure the kinetics of EPS release. It will be noted that even with the use of artificial brine medium, it remains inconclusive as to when exactly polysaccharides

begin to be produced. It is suggested, however, that after 12 days of culture in artificial media, measurements reflect only polysaccharides and not nitrate.

CHAPTER 3

CHARACTERIZATION OF *Dunaliella* EXOPOLYSACCHARIDES

3.1 Introduction

Studies on the characterisation of algal exopolysaccharides has indicated that the most common constituents of exopolysaccharides and heteropolysaccharides are: glucose, galactose, mannose, rhamnose, fucose, arabinose, xylose, and uronic acids (Jones, 1962; Hellebust, 1974; Augusto *et al.*, 1986). In addition, the exopolysaccharides of algae are also sulfated, and contain small amounts of protein and sometimes pyruvate (Nunn *et al.*, 1973; Painter, 1983; Lewis *et al.*, 1988; Arad, 1988; Sudo *et al.*, 1995).

Most studies have dealt mainly with the monosaccharidic composition of the exopolysaccharides, and only a few investigated the kinetics of polysaccharide release and its productivity despite the fact that the structure and composition of microbial exopolysaccharides depend on a number of different factors, such as microbial species, nature of substrate and other fermentation conditions (Margaritis and Pace, 1985).

A complete characterisation of a polysaccharide includes specification of which monomers are present and the sequence of monomers. It also requires that the glycosidic

bond be specified. This can be achieved by complementary analyses, the most important of which are graded hydrolysis, by acids or enzymes, followed by isolation and identification of the oligosaccharides formed (Björndal *et al.*, 1970a). This information is of vital importance in the classification of polysaccharides as alginate, xanthan, etc. However, sequence analysis and bond specification were beyond the scope of this study, while the determination of functional groups found in exopolysaccharide was the aim of this study.

Infrared spectrometry, which involves examination of the twisting, bending, rotating and vibrational motions of atoms in a molecule, is crucial for achieving this objective. Upon interaction of molecules with infrared radiation, portions of the incident radiation are absorbed at specific wavelengths. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum that is uniquely characteristic of the functional groups that make up the molecule as well as the overall configuration of the molecule (Willard *et al.*, 1988).

For qualitative analysis, one of the best features of an infrared spectrum is that the absorption or lack of absorption in specific frequency regions can be correlated with specific stretching and bending motions and, in some cases, with the relationship of these groups to the rest of the molecule. Thus, when interpreting the spectrum, it is possible to state that certain groups are present in the material and certain others are absent. With this data, the possibilities for the unknown sometimes can be narrowed so sharply that comparison with a library of spectra of pure compounds permits identification (Willard

et al., 1988).

Also crucial in the functional group analysis is mass spectrometry, which involves the separation and measurement of ions according to their mass-to-charge (m/z) ratio. The production of fragment ions leads to useful information concerning the structure of the parent molecule (Brown *et al.*, 1988). It is often observed that a certain feature of molecular structure will give rise to a characteristic peak in the mass spectrum such that the presence of an ion at that (m/z) value may be taken as good evidence for the feature (Rose and Johnstone, 1982). For example, an ion at m/z 43 is often evidence for the presence of the grouping CH_3CO in a molecule. It is of course better to determine the elemental composition of the ion at m/z 43 by accurate mass measurement to support the diagnosis.

It should be borne in mind that in mass spectrometry, rearrangement reactions accompanying fragmentation are common, i.e. the original molecule is considerably disturbed on ionization (Rose and Johnstone, 1982). For instance, although an ion of composition C_2H_3O at m/z 43 suggests the CH_3CO grouping in a molecule, it is found that many oxygen-containing compounds without this grouping also give abundant ions at this m/z value (Rose and Johnstone, 1982).

A systematic investigation of mass spectra of partially methylated alditol acetate sugar derivatives has led to the following generalizations regarding fragmentation:

1) Derivatives with the same substitution pattern (e.g. 2,3,4,6-O-methyl derivatives of hexitol acetate) give very similar mass spectra, typical of that substitution.

2) The base peak of the spectrum is generally, but not always, of mass fragment m/z 43, and this ion is accompanied by diacetyl (m/z 103) and triacetyl (m/z 145) oxonium ions.

3) Primary fragments are formed by fission or cleavage between carbon atoms in the chain. There is a marked tendency for cleavage to occur between two adjacent carbon atoms bearing methoxyl groups, with less tendency to cleave between carbon atoms respectively bearing acetoxyl and a methoxyl group, and considerable resistance to cleavage between adjacent carbon atoms bearing acetoxyl groups. The lack of any functional groups to direct the point of cleavage of the backbone results in a condition where, in general, it is equally possible for cleavage to occur at any carbon-carbon bond on the backbone.

4) Secondary fragments are formed from the primary fragments by single or consecutive loss of acetic acid (m/z 60), ketene (m/z 42), methanol (m/z 32), or formaldehyde (m/z 30) (Björndal *et al.*, 1970a; Seymour *et al.*, 1979).

The purpose of this section of the study was to investigate the monomeric composition of *Dunaliella* exopolysaccharides as well as to establish the various functional groups found in them. The importance of functional groups in metal removal from solution will be discussed in the next chapter.

3.2 Materials and Methods

3.2.1 Materials

Chemicals

Chemicals of high (analytical) grade were supplied by the following companies:

BDH Chemicals Ltd., Poole, England - Hydroxylamine, Acetic anhydride, Sodium sulphate, Silver nitrate, Sodium borohydrate, Zinc chloride, Sodium potassium tartrate .

Merck, Darmstadt, Germany - Chloroform, Methanol, 2-Propanol, Ethanol, Potassium nitrate, Calcium chloride, Ferric sulphate, Manganese chloride, Sulphuric acid, Hydrochloric acid, Magnesium sulphate, Sodium carbonate.

Sigma Chemical Co., St Louis, Mo, USA Rhamnose, Fucose, Arabinose, Ribose, Xylose, Mannose, Glucose, Galactose, Cation exchange resin IR-120 (H^+), Sodium rhodizonate.

Saarchem (Pty) Ltd., Johannesburg, S. Africa - Copper sulphate, Sodium hydrogen carbonate, Sodium chloride, Potassium dihydrogen orthophosphate, EDTA, Boric acid, Cobaltous chloride.

Fluka Chemie AG., Buchs, Switzerland - N-Methylimidazole, Trifluoroacetic acid, Folin ciocalteau reagent.

Rhodes University (School of Pharmacy), S. Africa - *Klebsiella* K69 monosaccharides (generously donated by Dr. L.A.S. Parolis)

Equipment

The following equipment was used in carrying out experimental work:

- Rotary Evapotator - Bücchi
- Gas Chromatography (6890 model) - Hewlett Packard
- Reactitherm Heating Block (18780 model) - Pierce Chemical Company
- FTIR Spectrometer (Spectrum 2000) - Perkin Elmer
- Mass Spectrometer (MAT GCQ-MS) - Finningan

3.2.2 Methods

3.2.2.1 Qualitative determination of sulphate groups in exopolysaccharides

Five milligrams of the polysaccharide (purified as described in section 2.2.2.6) was carefully weighed out and hydrolysed for 16 h at 100 °C in 2 M trifluoroacetic acid (TFA). The acid was removed by evaporation under vacuum at 40 °C, washed in water and evaporated as described above. The washing was repeated three times. The monosaccharides were then resuspended in 50 m*l* of water and 0.5 m*l* pipetted into a clean dry test tube for determination of the sulphate content using sodium sulphate as a standard (Terho and Hartiala, 1971).

Two millilitres of ethanol (spectrograde) were added to the sample and standards. Barium chloride buffer (1 m*l*) was added followed by 0.5 m*l* rhodizonate solution. The solution was well mixed and allowed to stand at room temperature for 10 min in the dark. Absorbance was read at 520 nm (the colour remains stable for 30 min). Different

dilutions (triplicate assays) of the sample were used.

3.2.2.2 Assay for protein content

The Lowry method was employed to assay for protein concentration in exopolysaccharide solution. See section 2.2.2.2 for details of the Lowry method for protein determination.

3.2.2.3 Constituent analysis

a) Direct hydrolysis - The polysaccharide (3-5 mg) was hydrolysed in 4 M trifluoroacetic acid (TFA) for 1 h at 125 °C in a heating block. The hydrolysate was then transferred into a 50 m*l* round bottom flask and the acid removed by rotary evaporation. The residue was washed with deionised water and evaporated to dryness. The procedure was repeated three times. The monosaccharides were then redissolved in 0.2 m*l* deioinised water followed by the addition of 0.4 m*l* of hydroxylamine prepared in N-methyl imidazole. The solution was heated in an oil bath at 80 °C for 10 min and cooled in ice. One millilitre of acetic anhydride was added dropwise with gentle swirling in ice, yielding peracetylated aldononitrile (PAAN) derivatives (McGinnis, 1982). The solution was allowed to stand at room temperature for 5 min and the monosaccharides extracted in chloroform and water using a separation funnel. The chloroform layer was washed with 10 % sulphuric acid, deionised water, saturated sodium hydrogen carbonate, and deionised water respectively in that order. The solution was dried by addition of anhydrous sodium sulphate. The solution was filtered, concentrated by rotary evaporation

and used for gas chromatography (GC) analysis.

Analytical GC was performed with a Hewlett Packard 6890 gas chromatograph, fitted with flame ionisation detectors and a recording integrator, with helium as carrier gas. The monosaccharides were separated isothermally at 220 °C on a DB-225 column (J & W Scientific). The relative proportion of the peak areas was calculated to estimate the monomer composition (De Vuyst *et al.* 1998). For the purpose of comparison, the following standards were run separately under identical conditions: glucose, fucose, arabinose, rhamnose, galactose, mannose, and xylose. In addition, laboratory isolated *Klebsiella* K69 monosaccharides were also used as standards (Hackland *et al.*, 1988). Unless otherwise stipulated, all GC analyses were run under identical conditions.

b) Reduction and hydrolysis (alditol acetate derivatives) - The polysaccharide (3-5 mg) was hydrolysed in 4 M TFA for 1 h at 125 °C in a heating block. The hydrolysate was then transferred to a 50 m*l* round bottom flask and the acid removed by rotary evaporation. The acid was washed with deionised water and evaporated to dryness. The procedure was repeated three times and followed by reduction with sodium borohydride in water at room temperature for 1 h. The hydrolysate was passed through an IR-120(H^+) column (10 cm x 0.5 cm) prepared in methanol. This cation exchange resin coverts sodium borohydride into borate ions. The borate ions were washed in methanol (borate ions dissolve in methanol to form methyl borate which is volatile) and dried by rotary evaporation. The procedure was repeated three times.

The monosaccharides were then acetylated with a 1:1 (v/v) solution of acetic

anhydride/pyridine for 1 h at 100 °C and allowed to stand at room temperature for 5 min. The monosaccharides were then extracted in chloroform and water using a separation funnel. The chloroform solution was washed in 10 % sulphuric acid, deionised water, saturated sodium hydrogen carbonate, and deionised water. The solution was dried by addition of anhydrous sodium sulphate. The solution was filtered, concentrated by rotary evaporation and used for GC analysis. The BD-225 column was employed for the separation of these derivatives. Appropriate standards were run separately. Column conditions were as described previously.

3.2.2.4 Mass Spectra of the various monosaccharides

For identification of monosaccharides, each peak in the GC trace was analysed by electron impact mass spectrometer using the same columns as for the quantitative analysis, at an ionisation energy of 70 eV. The profile of each spectrum was compared with that of authentic standards for confirmation of identity.

3.2.2.5 Functional group analysis (Infrared Spectrum)

For determination of the various functional groups found in the polymer, FTIR spectrum was done by dissolving approximately 5 mg of freeze dried polysaccharide in a mulling agent (nujol or hexachlorobutadiene). The sample was applied on NaCl discs and scanned at MidIR (4000- 400 cm^{-1}) wavelength. Reference charts were used to identify the absorption bands of various functional groups.

3.3 Results

3.3.1 Sulphate group analysis

The sample was resuspended in 10 ml of distilled water and mixed thoroughly (extract). From this solution, two dilutions were made, namely 5 and 10 fold dilutions prepared in deionised H_2O. For plotting the standard curve, the absorbance values of standards and test samples were subtracted from that of blank. The presence of sulphate groups was demonstrated in table 3.1. A 5x and 10x dilution of the sample resulted in a concentration dependent decrease in absorbance, indicating the dilution of sulphate containing functional groups occurring concomitantly with dilution of the polysaccharide.

Table 3.1 The determination of sulphate groups in *Dunaliella* exopolysaccharides

Sulphate conc (μg/ml)	Absorbance 520 nm (1)	Absorbance 520 nm (2)	Mean Abs 520 nm	Standard Deviation
0	0.000	0.000	0.000	± 0.000
1	0.054	0.066	0.060	± 0.008
2	0.098	0.093	0.096	± 0.004
3	0.154	0.156	0.155	± 0.001
4	0.208	0.203	0.206	± 0.004
5	0.255	0.255	0.255	± 0.000
6	0.304	0.318	0.311	± 0.010
Extract	0.396	0.400	0.398	± 0.002
5x dilution	0.084	0.090	0.087	± 0.004
10x dilution	0.028	0.031	0.030	± 0.002

The purpose of the experiment was to demonstrate qualitatively, the presence of sulphate groups in the polysaccharide.

3.3.2 Determination of protein content

In order to establish whether *Dunaliella* exopolysaccharide has protein associated with it, an EPS solution was subjected to the Lowry method. An increase in colour formation relative to blank was observed indicating the presence of protein in *Dunaliella* exopolysaccharide (De Philippis *et al.*, 1991; 1993). This was a qualitative test rather than quantitative.

3.3.3 Constituent analysis

The separation of constituent monosaccharides was achieved by GC analysis on DB-225 column (high polarity). Appropriate standards were run separately for the purposes of comparison. The monosaccharides used as standards are those reported to occur very frequently in algal exopolysaccharides. The chromatogram for PAAN derivatives of monosaccharide standards is shown in figure 3.1.

The identity of various sugar components of *Dunaliella* exopolysaccharide was achieved by comparing the retention times with those of standards (see tables 3.2 and 3.3). Gas chromatogram of the hydrolysate of *Dunaliella* EPS (figure 3.2) revealed the presence of arabinose, xylose, mannose, glucose, galactose and an unusual sugar (designated sugar Y). The retention time of sugar Y did not compare with any of the standards used. The

relative peak area of the various monosaccharides is given in table 3.3. The percentage peak area was calculated relative to the xylose peak. Several other small peaks were also observed, the retention times of which cannot be correlated with any of the standards.

The alditol acetate derivatives of the monosaccharide standards and hydrolysate of *Dunaliella* exopolysaccharides were also run and the retention times compared (figures 3.3, 3.4 and 3.5). It should be mentioned that it is not possible to identify an unknown molecule by mass spectrometry alone, since stereoisomeric substances give similar profiles. The identity of a molecule can only be achieved by using mass spectra in conjunction with gas chromatography for comparison of retention times with those of standards. Because of the presence of an unknown monomer in *Dunaliella* polysaccharide, whose retention time did not coincide with any of the commercial monosaccharide standards, it became necessary to obtain other standards, preferably of microbial origin, for the purposes of comparison.

The only available source was *Klebsiella* K69 whose monosaccharides had previously been isolated (Rhodes University, School of Pharmacy). Therefore, in addition to commercial monosaccharide standards, known monosaccharide (alditol acetate derivatives) components isolated from *Klebsiella* K69 were used as standards (figure 3.3), and their retention times compared with that of *Dunaliella* monosaccharides (figure 3.4).

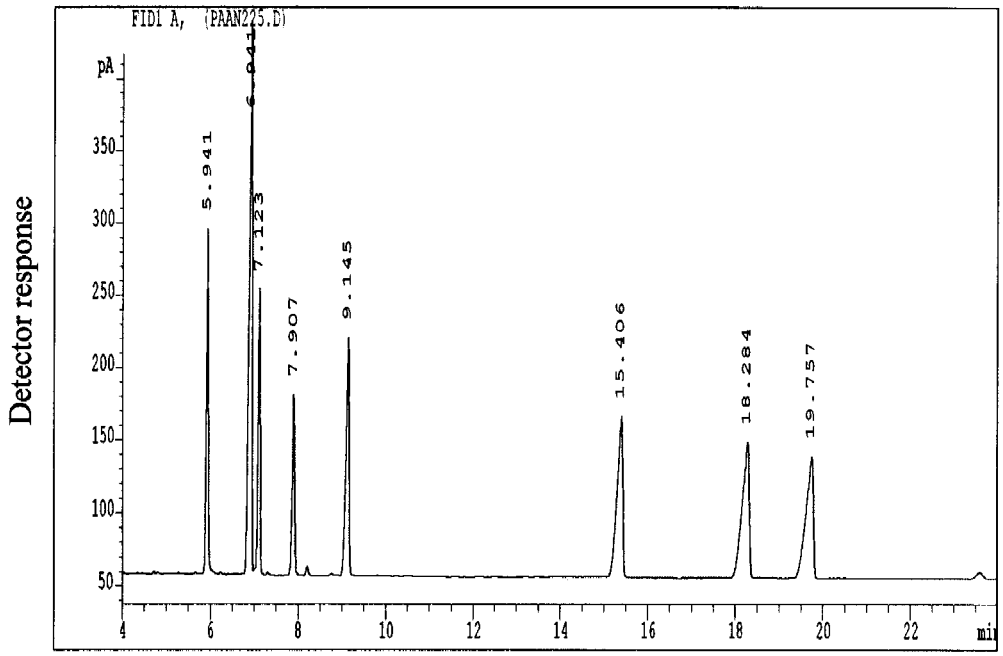


Figure 3.1: Gas chromatogram of commercial monosaccharide standards (PAAN derivatives) run on a DB-225 column.

Table 3.2 Retention times for commercial monosaccharide standards (PAAN derivatives) run on a DB-225 column

Name of sugar standard	Retention time (min)
Rhamnose	5.941
Ribose	6.941
Fucose	7.123
Arabinose	7.907
Xylose	9.145
Mannose	15.406
Glucose	18.284
Galactose	19.757

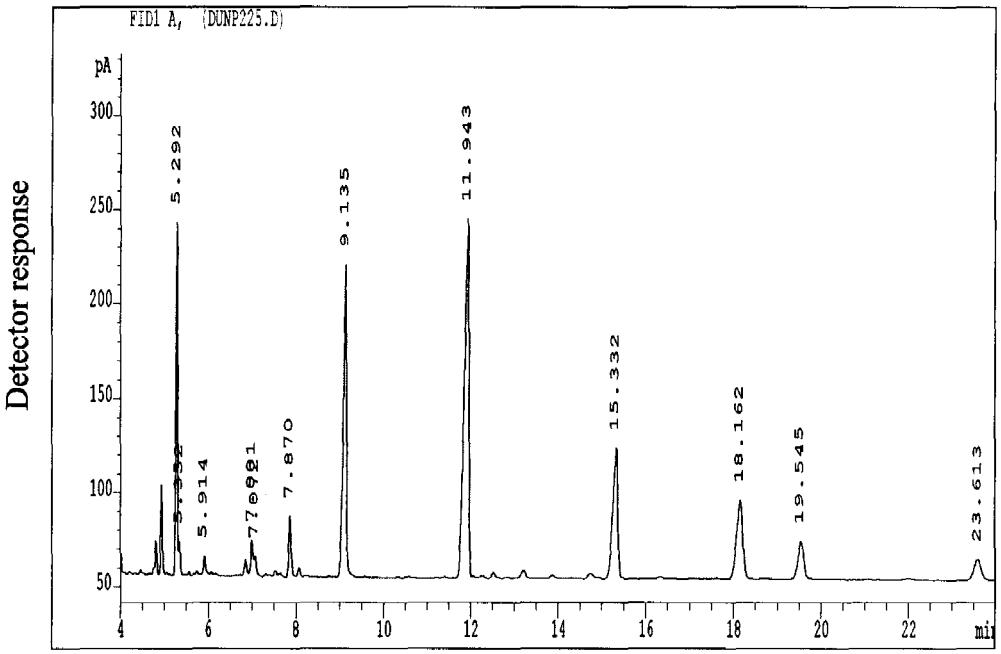


Figure 3.2: Constituent analysis of *Dunaliella* extracellular polysaccharides (PAAN derivatives) by gas chromatography on a DB-225 column.

Table 3.3 Identification of *Dunaliella* monosaccharides by comparison of retention times with commercial standards. PAAN derivatives were produced after methanolysis reduction and hydrolysis.

Retention time (min)	Identity of the monosaccharide	Relative area of peak (%)
7.870	Arabinose	15.1
9.135	Xylose	100
11.943	Y	166
15.332	Mannose	64.4
18.162	Glucose	48.7
19.545	Galactose	14.7

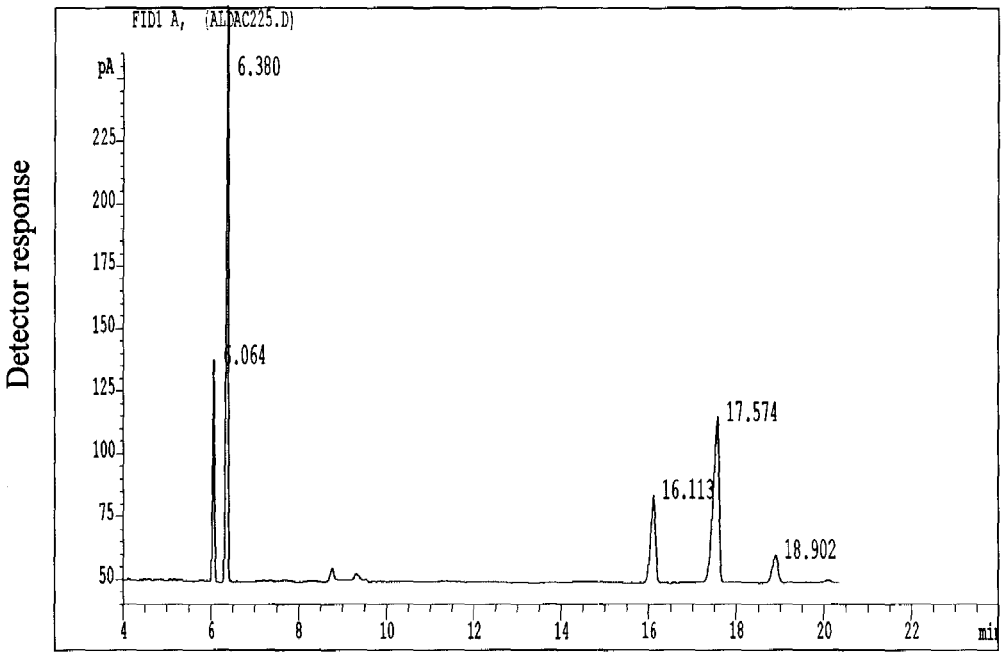


Figure 3.3: Gas chromatogram of commercial monosaccharide standards (alditol acetate derivatives) run on a DB-225 column.

Table 3.4 Retention times for commercial monosaccharide standards (alditol acetate derivatives) run on DB-225 column.

Name of sugar standard	Retention time (min)
Rhamnose	6.064
Ribose	6.380
Arabinose	7.692[a]
Xylose	9.280[a]
Mannose	16.113
Galactose	17.574
Glucose	18.902

[a] see chromatogram in appendix D

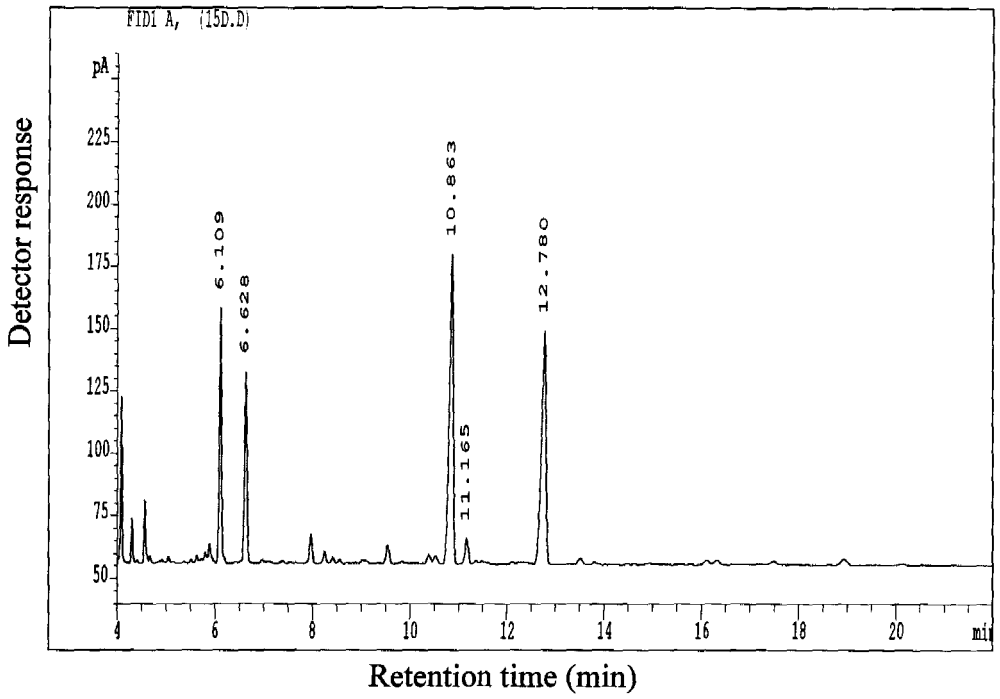


Figure 3.4: Alditol acetate derivatives of *Klebsiella* K69 monosaccharides run on a DB-225 column.

Table 3.5 Monosaccharide standards (alditol acetate derivatives) produced by hydrolysis from *Klebsiella* K69 polysaccharide and run on a DB-225 column

Name of sugar standard	Retention time (min)
1,2,3,5,6 penta-O-methyl mannose	6.109
2,3,4,6 tetra-O-methyl glucose	6.628
2,3 di-O-methyl galactose	10.863
2-O-methyl mannose	12.780

(Hackland *et al.*, 1988)

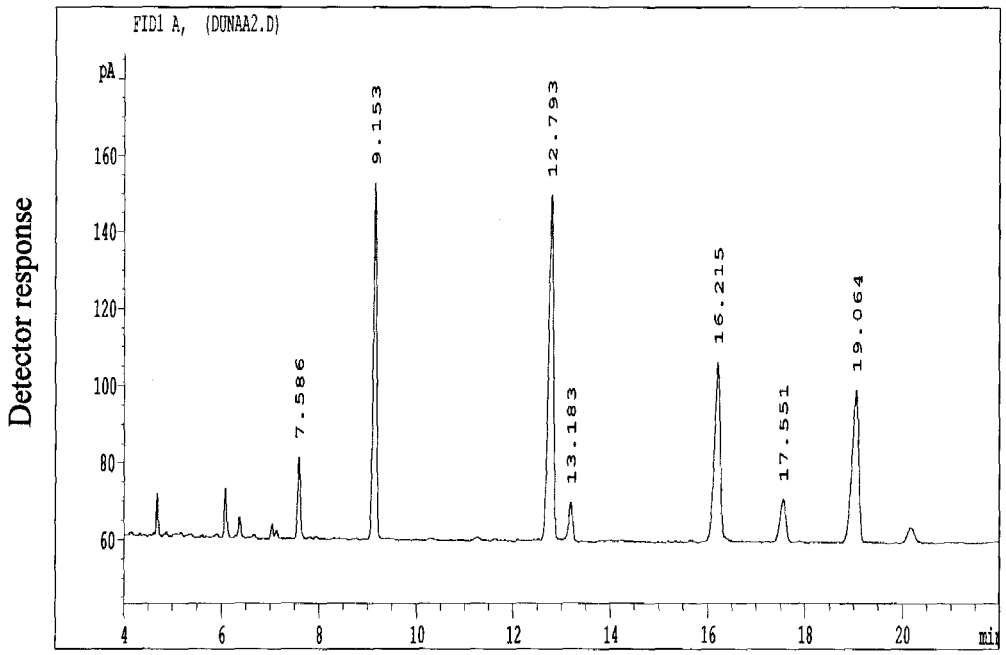


Figure 3.5: Constituent analysis of *Dunaliella* extracellular polysaccharides (alditol acetate derivatives) by gas chromatography on a DB-225 column.

Table 3.6 Identification of sugar Y (alditol acetate derivatives) by comparison of retention time with monosaccharide standards isolated from *Klebsiella* K69.

Retention time (min)	Identity of the monosaccharide	Relative area of peak (%)
7.586	Arabinose	18.8
9.153	Xylose	100
12.793	2-O-methyl Mannose	141.3
16.215	Mannose	86.8
17.551	Galactose	22.3
19.064	Glucose	85.3

The retention time of sugar Y was found to compare well with that of 2-O-methyl mannose (tables 3.5 and 3.6). The alditol acetate derivatives of glucose and galactose were found to swop their order of appearance compared to the PAAN derivatives. The reason for this observation is not clear because both sugars have the same number and type of functional groups. This finding was also reported by McGinnis (1982).

3.3.4 Mass spectra of *Dunaliella* exopolysaccharide

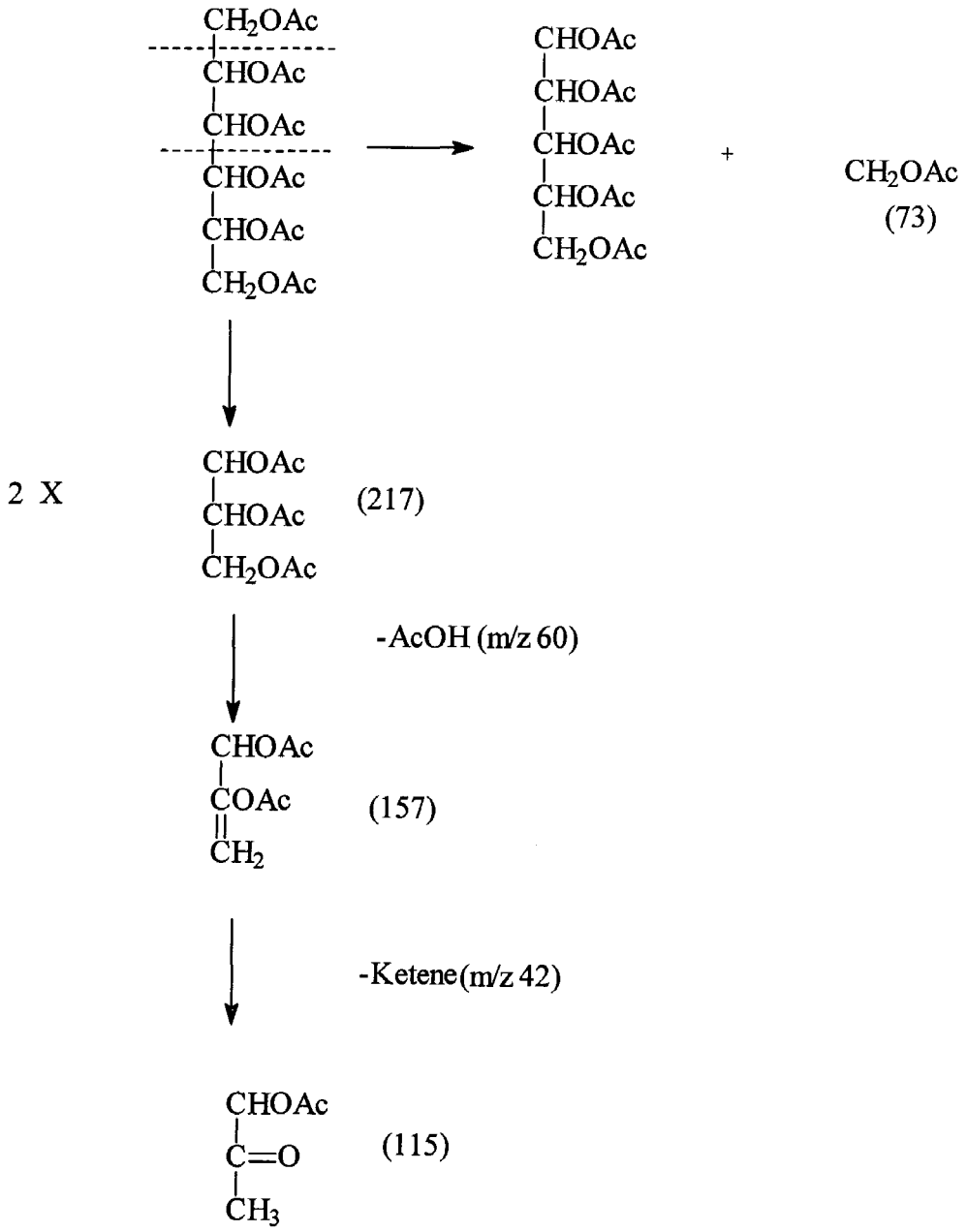
Individual peaks from gas chromatography were analysed by mass spectrometry and the representative spectra are given in appendix E. The spectra of pentose sugars (alditol acetate derivatives) were found to be the same, as were those of hexose sugars. This result supported previous reports which established that stereoisomeric substances are practically indistinguishable by mass spectrometry (Björndal *et al.*, 1970a; Seymour *et al.*, 1979). The mass spectra of alditol acetate derivatives of *Dunaliella* monosaccharides are listed in table 3.7. Various mass fragments were produced as demonstrated in schemes 1-3. Fragmentation can arise from either end of the molecule, with a generally equal probability of cleavage between any two carbon atoms, except for scheme 3 where cleavage between methoxylated and acetoxylated carbons is preferred.

Comparison of spectra with those of known sugars confirmed the presence of pentose and hexose sugars as well as the presence of a 2(5)-O-methyl hexose. The mass fragment m/z 73 arise from cleavage of C_1 or C_6 from the rest of the molecule whereas cleavage between C_3 and C_4 produces mass fragments of m/z 217. The loss of an acetoxyl group from this fragment gives rise to another fragment of m/z 157 (217-60).

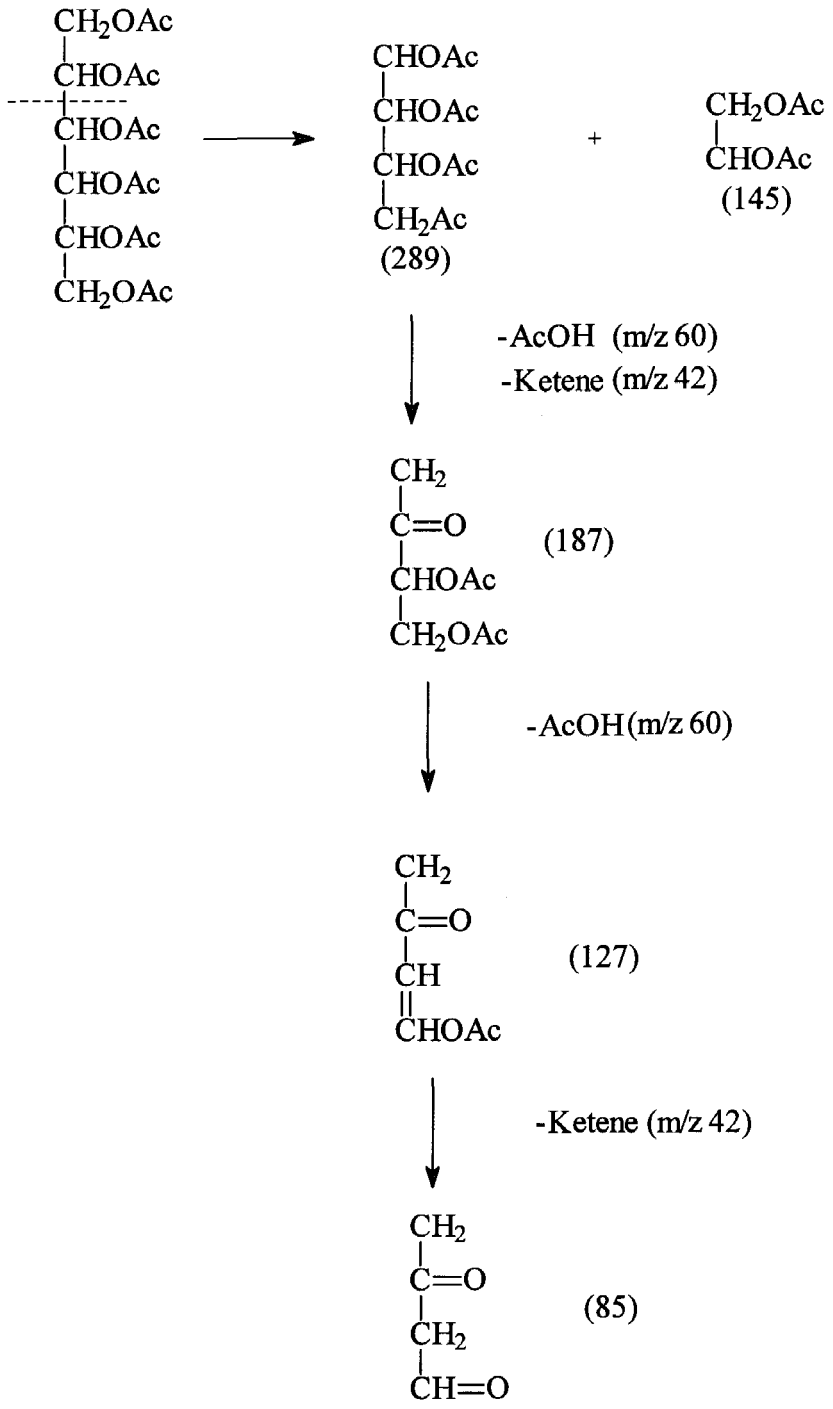
Table 3.7 Primary fragments in the mass spectra of *Dunaliella* monosaccharides (alditol acetate derivatives).

Mass fragments m/z	Pentose	Hexose	2(5)-Omethyl hexose
73	x	x	
85	x	x	
87			x
97		x	x
103	x	x	
115	x	x	
117			x
127	x	x	
129			x
139		x	x
145	x	x	
157		x	x
158	x		
187	x	x	
217	x	x	
289	x	x	

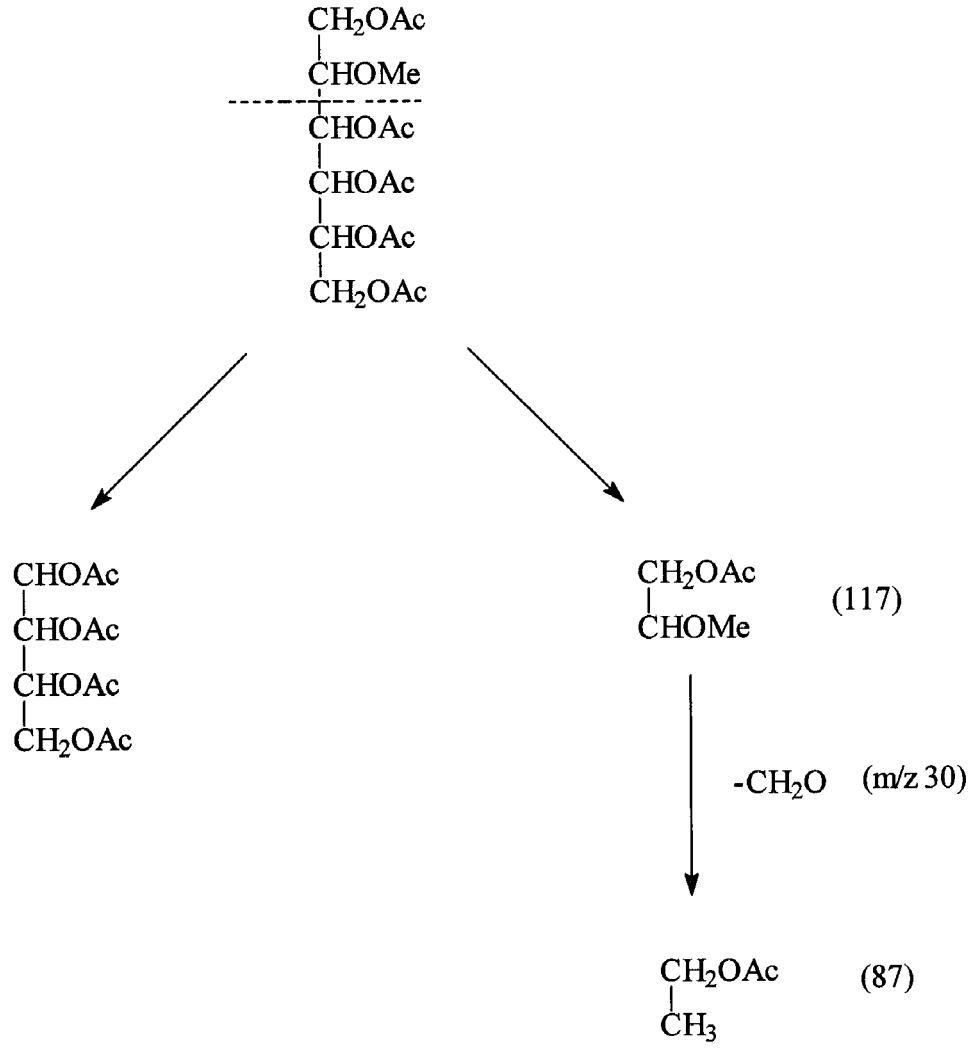
Primary fragments having higher m/z values are of low intensity and not included in the table



Scheme 1: Typical fragmentation pathway for a hexose sugar (alditol acetate derivative).



Scheme 2: Alternative fragmentation pathway for a hexose sugar (alditol acetate derivative).



Scheme 3: Fragmentation pathway for an O-methyl hexose sugar (alditol acetate derivative) methylated at C_2 or C_5 position.

Following the loss of a ketene (m/z 42), a mass fragment of m/z 115 is produced. Scheme 2 shows another fragmentation pathway and how the various fragments were produced. Pentose sugars also follow the same fragmentation pathways as hexose sugars. Similar mass fragments are produced for pentose and hexose sugars, except for larger mass fragments which are only possible with sugars that have longer carbon chains (Seymour *et al.*, 1979).

The primary fragment m/z 117 (scheme 3) is obtained from alditol acetate derivatives having an acetoxyl group at C_1 or C_6 and a methoxyl group at C_2 or C_5 respectively (Björndal *et al.*, 1970b). This mass fragment is a distinguishing feature of 2(5)-O-methyl pentose or hexose.

3.3.5 Functional group analysis of *Dunaliella* exopolysaccharides by infrared spectrometer

Further characterization of *Dunaliella* EPS involved the determination of functional groups found in the polymer by infrared spectrometry. Two mulling agents were employed to investigate functional groups found in *Dunaliella* EPS. The absorption bands of both mulling agents are marked nujol and H (hexachlorobutadiene) in figures 3.7a and 3.7b respectively. Figure 3.7b shows a strong, broad absorption band at 3391.1 cm^{-1}. Such a band, which occurs near 3400 cm^{-1}, is characteristic of O-H stretching vibrations. The N-H stretching vibrations also give a characteristic absorption band in the 3300-3500 cm^{-1} range, however, an N-H absorption band is much sharper and less intense than the O-H band.

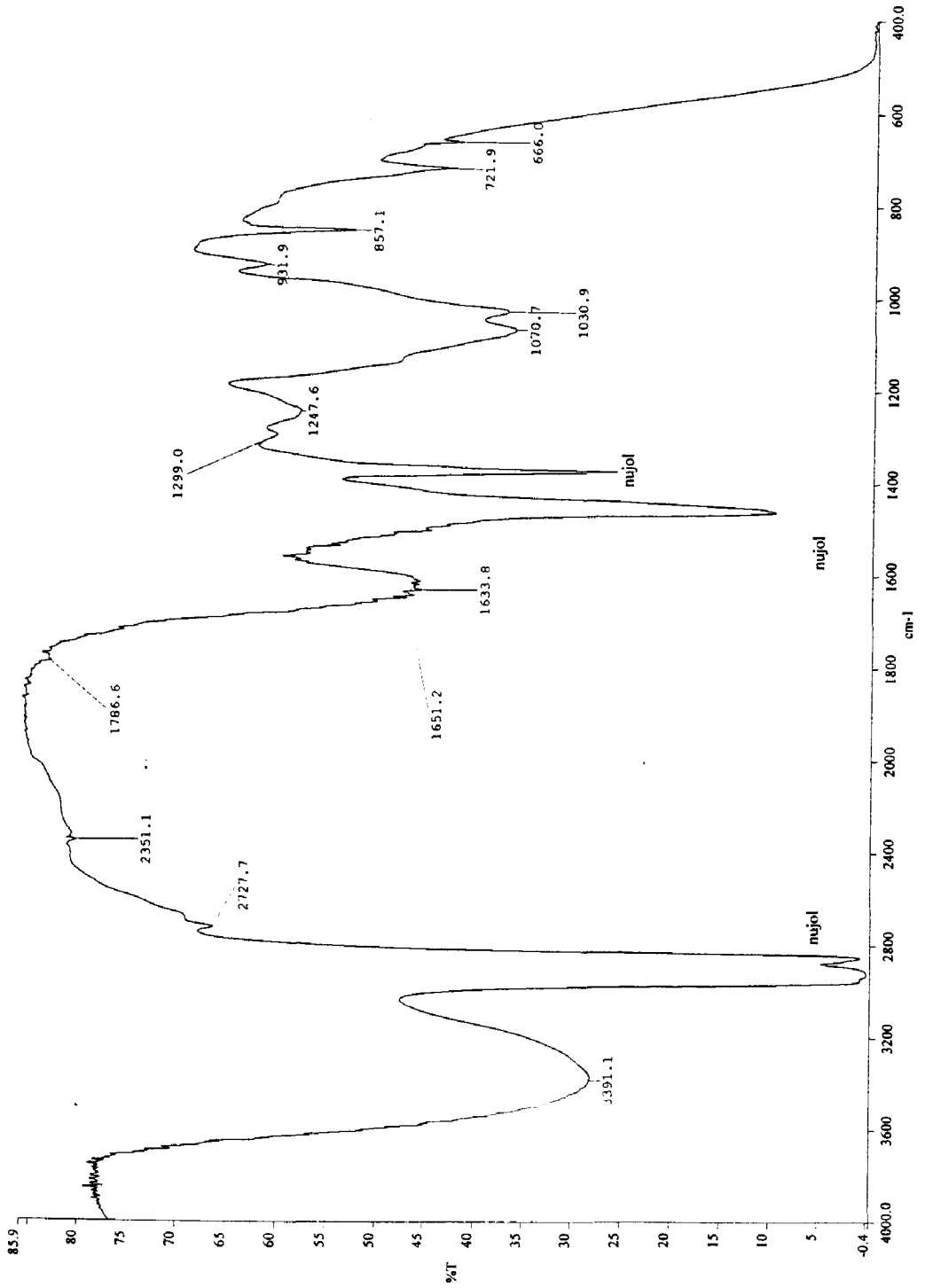


Figure 3.6(a) Functional group analysis of *Dunaliella* exopolysaccharide by infrared spectrometry. Exopolysaccharide was prepared in nujol mulling agent, and scanned at MidIR region.

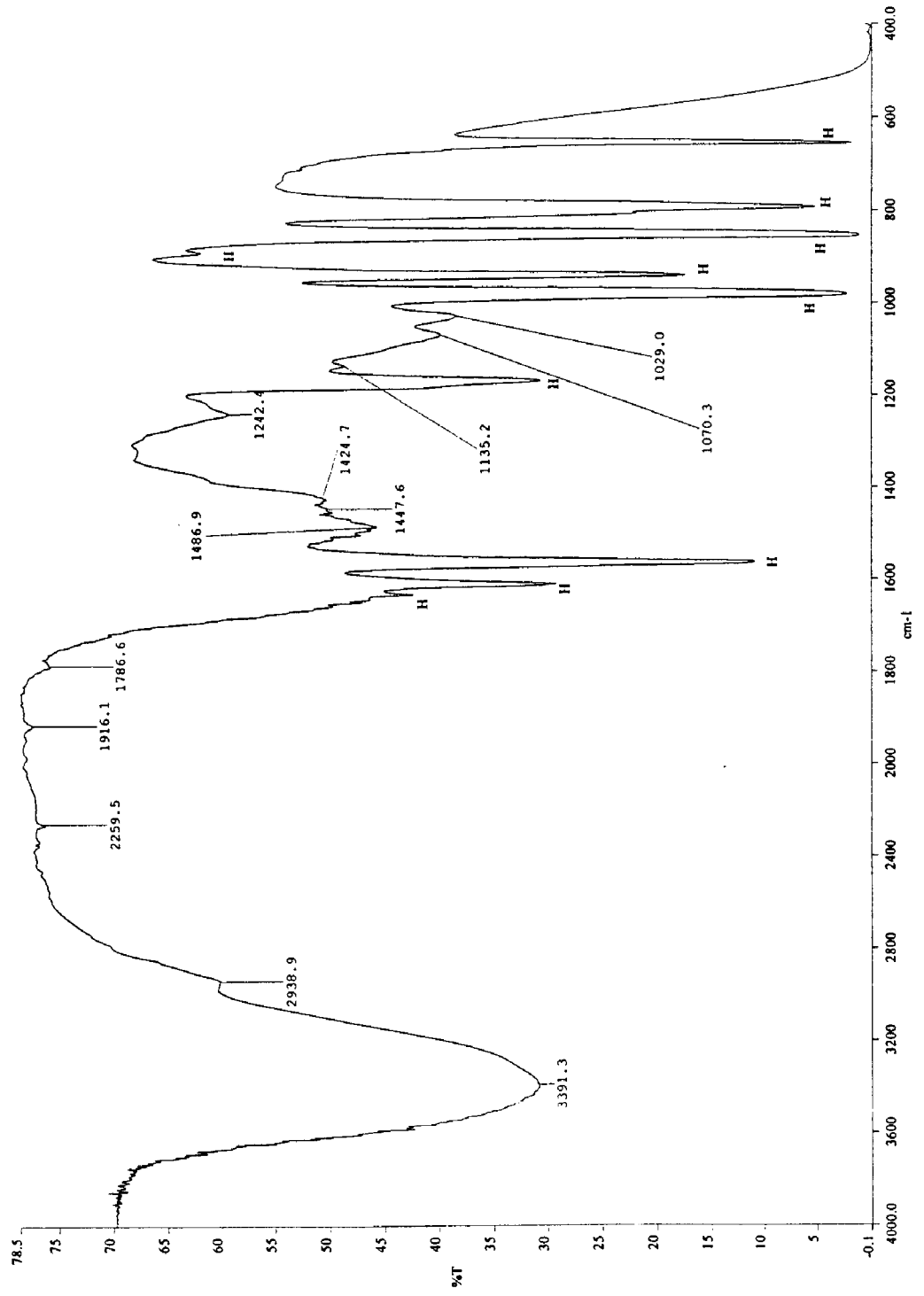


Figure 3.6(b) Functional group analysis of *Dunaliella* exopolysaccharide by infrared spectrometry. Exopolysaccharide was prepared in hexachlorobutadiene mulling agent and scanned at MidIR region.

Another strong, broad band was observed at 1633.8 cm^{-1}, indicating the presence of O-H bend or N-H in plane deformation vibrations. The CH_2 scissor, in plane deformation bands and C-N in plane deformation bands were found at frequencies between 1300-1240 cm^{-1}. The presence of S=O (symmetrical) stretching vibrations were shown by a characteristic absorption band at 1075-1010 cm^{-1}. The bands appeared in the form of a pair. Also occurring in the same range is the absorption band for C-O stretching vibrations. Strong to medium but sharp bands were also found at 940-850 cm^{-1} and 725-660 cm^{-1}, which were associated with the O-H out of plane deformation vibrations and C-S stretching vibrations respectively.

In order to establish if any absorption bands due to the sample were masked by the nujol, hexachlorobutadiene mulling agent was also used. The spectrum for EPS prepared in hexachlorobutadiene is shown in figure 3.7b. A somewhat weak shoulder was observed near 2940 cm^{-1}, which suggests the presence of C-H stretching vibrations. In addition, a cluster of strong to medium bands were observed in the 1500-1420 cm^{-1} range. This range is typical of CH_2 scissor in plane bending motions. Similarly to figure 3.7a, the O-H stretching vibrations occurring near 3400 cm^{-1} as well as a pair of bands in the 1075-1010 cm^{-1} range were observed. In brief, these results suggest the presence of the following functional groups in *Dunaliella* exopolysaccharides, namely: methyl, hydroxyl, amino, and sulphate groups. In addition, the absence of a carboxyl group was also demonstrated. These functional groups have been shown to occur in microbial/algal exopolysaccharides (Kuyucak and Volesky, 1988).

3.4 Discussion

The method of Terho and Hartiala (1971) was employed to assay for the presence of sulphate groups. The procedure is essentially quantitative, and various dilutions of the sample showed a proportional decrease in sulphate content. The presence of sulphate groups in algal exopolysaccharide has been demonstrated in other studies (Nunn *et al.*, 1973). *Dunaliella* exopolysaccharide was also found to contain a small amount of protein when subjected to the Lowry method. The significance of this test was to establish if, like many microbial/algal exopolysaccharides, *Dunaliella* exopolysaccharides have any nonpolysaccharidic material attached to them. Several studies have indicated that microbial/algal exopolysaccharides can have protein and/or pyruvic acid attached to them as prosthetic groups (Sloneker and Orentas, 1962; Hellebust, 1974).

Accordingly, it was expected that infrared studies would show the presence of both amino and carboxyl functional groups to confirm the presence of protein in *Dunaliella* exopolysaccharides. Contrary to this expectation, the carboxyl group was not detected by infrared analysis, which suggests that the colour formation observed when exopolysaccharides were subjected to the Folin-Lowry method may have been due to some interference. This was most probably sulphate, which has been shown to interfere at concentrations above 1 % (Lowry *et al.*, 1951). Shekharam *et al.* (1987) reported sulphate content of 2.3 % in polysaccharides of *Spirulina platensis*. Some polysaccharides from red algae, such as carrageenan, have also been found to contain 22-35 % of sulphate groups (Güven *et al.*, 1990). *Dunaliella* exopolysaccharide contain sulphate groups, probably at a concentration that interferes with the Folin-Lowry

reagents.

Separation of monosaccharide constituents on gas chromatography was achieved, with each sugar derivative giving only one peak. This result indicates that the column used had good separation properties for carbohydrates and that column condition were also suitable to achieve good resolution.

Seymour *et al.*, (1979) reported that the order of emergence for various PAAN derivatives is essentially the same for all columns. The order of emergence of PAAN derivatives reported in this study supports the findings by Dmitriev *et al.*, (1971) and Seymour *et al.* (1979). The order was as follows, rhamnose, ribose, fucose, arabinose, xylose, mannose, glucose and galactose for commercial standards. The order of emergence of standards on such different columns, as observed by Seymour *et al.* (1979) suggests that the primary reason for the separation of compounds is an inherent feature of the structure of the sugar.

The same order of emergence was observed for alditol acetate derivatives except for glucose and galactose whose order of emergence swopped compared to that of PAAN derivatives. This finding supports a report by McGinnis (1982) who reported similar observations using both PAAN and alditol acetate derivatives on OV-225 column. The reason for this observation cannot be explained.

The GC retention time may be considered as a function of the linearity of a compound,

and this linearity being dependent on the number of cis-hydroxyl groups in the parent sugar. The dominating factor relating sugar structure to GC retention times is the number of hydroxyl groups or acetoxyl groups for the PAAN derivatives. This supports the concept that PAAN derivatives are retained on the column by interaction of the moderately polar acetyl groups with the stationary phase. The greater the number of acetyl groups, the stronger the interaction (Seymour *et al.*, 1979). The above mentioned factors apply even for alditol acetate derivatives because the difference between PAAN and alditol acetate derivatives is the CH_2OAc and $C{\equiv}N$ for alditol acetate and PAAN derivatives respectively on C_1 of the sugar molecule. The rest of the molecule has -OAc groups for both derivatives.

Not surprisingly, pentose sugars eluted faster than hexose sugars. In addition, 2-O-methyl mannose also eluted faster than unsubstituted hexose sugars because of the presence of a nonpolar functional group (methyl group). This finding is consistent with a report by Seymour *et al.* (1979) which suggests that with PAAN derivatives, functional groups can either be more polar than the acetate group, and thus lengthen the retention time (e.g. replacement of an O-acetyl by an N-acetyl group), or less polar, and so shorten the retention time (e.g. replacement of an acetoxyl by deoxy group). It is very likely that the above factors are true for alditol acetate derivatives as well. The similarities between PAAN and alditol acetate derivatives have been described above.

The preliminary determination of monosaccharide composition of *Dunaliella* EPS showed the presence of arabinose, xylose, sugar Y, mannose, glucose, and galactose. The

monosaccharides identified in this study are frequently found in exopolysaccharides of different algal groups (Augusto *et al.*, 1986; Shekharam *et al.*, 1987). Sugar Y was identified as 2-O-Methyl mannose and was also found to be the major constituent of *Dunaliella* exopolysaccharide, showing a relative peak area of approximately 160 % relative to xylose, whereas arabinose and galactose were present in very small amounts. The monosaccharides (PAAN derivatives) were found to occur in the following ratio respectively, arabinose, xylose, 2-O-methyl mannose, mannose, glucose, galactose (0.15:1.0:1.66:0.64:0.48:0.15).

The ratio was, however, found to be slightly different for alditol acetate derivatives. The ratio was found to be 0.18:1.0:1.41:0.86:0.22:0.85 for arabinose, xylose, 2-O-methyl mannose, mannose, galactose, glucose respectively. The ratio of arabinose and galactose relative to xylose remains fairly low but that of glucose increased almost two fold. The trend was almost similar for both derivatives.

Several other peaks were also observed whose retention times could not be correlated with any of the standards. It is not clear which compound these peaks represented. However, it should be borne in mind that extracted soluble polymers, obtained in the supernatant or filtrate will be contaminated to some degree, by residual components of the culture medium and by intracellular material from ruptured cells. Whilst low molecular weight contaminants may be removed by dialysis against several changes of ddH_2O for 24 h, the removal of high molecular weight contaminants is more difficult (Lester *et al.*, 1984). Hence, it is possible that the exopolysaccharide was not completely

pure, resulting in the appearance of the extra peaks that have been referred/alluded to.

The mass spectral analysis of various peaks (alditol acetate derivatives) indicated the presence of pentoses and hexoses. The spectra of pentoses were similar as were those of hexoses. This finding supports reports that mass spectra of isomeric alditol acatates having the same structure but different configurations are practically indistinguishable (Björndal *et al.*, 1970a). This is reported to be the same even for various glycosides and other stereoisomeric substances. This observation further confirms a report (Seymour *et al.*, 1975) that mass spectra of D-mannose derivatives is identical to those of D-glucose methylated in the same position.

The fragmentation pathways presented in schemes 1-3 accounted for most of the fragments that were produced. However, some major fragment ions in the spectra were displaced by +1 or +2 (e.g. m/z 129 and 158) mass units. These increments were attributed to the fact that the fragments could contain deuterium isotopes. Previous studies (Björndal *et al.*, 1970b) have shown that when a deuterium atom is introduced in a substance of m/z 101, the m/z value shifted to 102. A mass fragment of m/z 103 could have probably resulted from a diacetyl oxonium ion through the process of rearrangement which occurs due to ionization (Rose and Johnstone, 1982). Alternatively, it could have been formed by elimination of a ketene group from a mass fragment of 145 (145-42). The former is more likely to occur because a ketene is never eliminated directly from a primary fragment (Björndal *et al.*, (1970b). Its elimination is preceded by an elimination of either acetic acid or methanol and must also leave behind an acetoxyl group attached

to an unsaturated carbon atom. A satisfactory explanation cannot be offered for the origin of some fragments such as m/z 97, 129 and 139. Nonetheless, some of these fragments have been reported in mass spectra of simple sugars (alditol acetate derivatives) (Seymour *et al.*, 1979).

A comparison of the mass spectrum of sugar Y with that of authentic standards confirmed that sugar Y is 2-O-methyl mannose (Björndal *et al.*, 1970a; 1970b; Jansson *et al.*, 1976). Surprisingly, the presence of amino and sulphate groups was not detected by mass spectrometry. However, sulphate groups are reported to be acid-labile (Painter, 1983) suggesting that the functional group was lost during the derivatisation and hydrolysis processes. It is not clear whether the amino group is also acid-labile or not, but it is possible that it was also lost during the process. These groups were, however, detected by infrared analysis. Infrared analysis of polysaccharide functional group did not expose the polysaccharide to harsh conditions that could result in loss of some important functional groups. The removal of substituent groups during hydrolysis was also reported by Aspinall (1982), who further reported that the acid-stable substituent groups, such as ethers are retained on hydrolysis and give rise to "new" sugars.

The full interpretation of an IR spectrum is difficult because most organic molecules are so large that they have dozens of different bond stretching and bending motions. Thus an IR spectrum will contain dozens of absorption bands. In one sense, this complexity is valuable because an IR spectrum serves as a unique fingerprint of a specific compound. Both the presence and absence of certain functional groups are easy to detect on an IR

spectrum (McMurry, 1995). In this study, the presence of hydroxyl groups was demonstrated by a strong, broad absorption band in the region 3300-3500 cm^{-1}. Apart from the frequency, both intensity and shape of absorption band are also crucial for the identification of a particular functional group (Bellamy, 1975).

Although probably masked by the broad nature of the O-H absorption band in the region 3300-3500 cm^{-1} (McMurry, 1995), the presence of amino groups was demonstrated by absorption bands that were observed elsewhere in the spectrum, e.g. 1633.8 cm^{-1} (Brown *et al.*, 1988). Another functional group found to exist in the EPS is the sulphate group. Its presence was shown by the absorption bands at 725-660 cm^{-1} and 1075-1010 cm^{-1} (Weast and Astle, 1980). Ketones and aldehydes show a strong C=O bond absorption in the IR region from 1660-1770 cm^{-1}. The exact position of the C=O absorption band varies slightly from compound to compound but is highly diagnostic of the exact nature of the carbonyl group. The band is usually strong and sharp, and clearly absent from spectra presented in figures 3.7a and 3.7b. This finding suggests that the polymer is not an acidic polysaccharide, which explains why the pH of the solution increases markedly (from 6.62 to 10.04) when the polysaccharide is dissolved in ddH_2O.

3.5 Conclusion

Dunaliella EPS is composed of arabinose, xylose, 2-O-methyl mannose, mannose, glucose, and galactose. 2-O-Methyl mannose constitutes the major component of the exopolysaccharide whereas arabinose and galactose are present at relatively low levels. The presence of sulphate groups was established by the sodium rhodizonate method

(Terho and Hartiala, 1971) and confirmed by the infrared spectrum. Whether *Dunaliella* exopolysaccharide has protein component attached to it or not remains inconclusive. It is, however, assumed that the colour formation observed when the polysaccharide was subjected to the Lowry method was due to interference, and thus, the *Dunaliella* exopolysaccharide probably has no protein attached to it. Pyruvate is sometimes found associated with algal exopolysaccharides, but its presence was not investigated in this study.

Both hydroxyl and amino groups were found using infrared spectrometry. It is presumed that the amino group detected by infrared analysis is a substituent group of some monosaccharides. Further analysis such as elemental microanalysis is necessary to confirm the presence of these functional groups. However, what is important in terms of the present study is the presence of these functional groups (hydroxyl, amino and sulphate) which renders this polymer a potential biosorbent for removal of heavy metals from solution.

CHAPTER 4

REMOVAL OF HEAVY METAL FROM SYNTHETIC SOLUTIONS USING EPS

4.1 Introduction

4.1.1 Removal of metals from single metal solutions

The use of biopolymers as sorbents for hazardous metals or strategically important metals has been a topic of intense research in recent years. Due to the excellent selectivity for certain metals by biopolymers and the low cost of producing biopolymers under mild conditions, the biopolymer approach can offer alternative to conventional methods of metal recovery (Jang *et al.*, 1991). Jang and coworkers also demonstrated that the metal binding affinity of alginate gel was not significantly affected by the ionic strength of the solution.

A number of studies have demonstrated an increase in metal binding capacity as a result of the chemical modification of functional groups (Delben *et al.*, 1989; Muzzarelli, 1985; Delben and Muzzarelli, 1989; Eiden *et al.*, 1980; Guibal *et al.*, 1992; Saucedo *et al.*, 1992). For example, the compound NDTC collects about 700mg/g of uranium at an equilibrium concentration of 20mg/l of uranium (Muzzarelli, 1985). Studies on the metal mass balance through the activated sludge process have reported substantial metal

removal (Brown *et al.*, 1973) and the process is probably the best documented example of a biological heavy metals removal system. In the process, biomass population grown under aerobic conditions, oxidises dissolved or suspended organic matter present in wastewater. Metal removal takes place by adsorption of dissolved metals onto the activated sludge floc (Wase and Forster, 1997). The role of surface microbial polysaccharides has been proposed as a possible mechanism of metal removal in this system (Brown and Lester, 1979; 1982). Indeed, specific metal ion removal processes have been suggested in which polysaccharide polymer production is stimulated and then used in a separate stage for heavy metal removal from solution (Dugan and Pickrum, 1972).

The possibility of using algal polysaccharides as biosorbents for the removal of heavy metals from industrial solutions and wastewater has been demonstrated (Volesky and Holan, 1995). Resins and activated charcoals currently used become ineffective when the metal concentration is below certain levels or when the trace metals are accompanied by major quantities of common salts (Muzzarelli *et al.*, 1989). Chitoplex is a chitin-based polysaccharide in the form of a rigid granular gel, and is reportedly apt for the chelation of metal ions and metal complexes from aqueous solutions (Muzzarelli *et al.*, 1989). Its irregularly shaped particles exhibit porous and accessible structure which favours chelation from aqueous solutions.

One of the problems encountered with chitosan is that it forms viscous, water soluble salts in acidic solutions. In the case of metal binding, this would preclude the use of acids

to elute the metals in any regeneration step (Wase and Forster, 1997). However, the problem can be overcome by cross-linking with gluteraldehyde without affecting the native structure and metal binding capability of chitosan.

Depending on the type of metal and its solution chemistry as well as on the type of biomass and the prevailing sorption conditions, some materials can sequester the metallic ions selectively while others bind them without distinguishing (Volesky, 1992). Two key aspects which have to be taken into consideration in conjunction with the metal uptake capacity of the sorbent for a specific sorbate are the characteristics of the solution system and the characteristics of the sorbent. The chemical properties of the sorbent material include the types of chemical functional groups which are present in the material binding the metallic ions, the degree of ionization of the sorbent surface and ash content.

It is generally believed that metal cations are complexed by negatively charged sugar units of a polysaccharide chain. The carboxyl groups, present abundantly in galacturonic, glucuronic, and mannuronic acids, as well as the sulphate groups could be considered as ligands mainly responsible for the bulk of metal sorption (Bar-Or and Shilo, 1987; Kaplan *et al.*, 1987; Holan and Volesky, 1994). The uronic acid residues made up 30 to 40 %, by weight, of the EPS of mesotaenium. Strong metal binding ability was found associated with the uronic acid fraction whereas neutral sugars (xylose, rhamnose and mannose) did not have metal binding abilities (Mangi and Schumacher, 1979).

The ion exchange properties of sulfated polysaccharides from a number of brown algae have also been reported (Holan and Volesky, 1994). These sulfate esters of the cellular polysaccharides include the following :

1. Fucoidans in division phaeophyta contain $O\text{-}SO_3^-$ groups at C_2 and C_3 or contain disulfate esters at C_2 and C_3 in α-1,4-linked L-fucopyranosyl residues.
2. Carrageenan, agars, porphyrans, furcellarans, furonans, etc in division Rhodophyta contain sulfated galactans at C_2 or C_4 or C_6. Disulfate esters at C_2 and C_6 were identified in λ-carrageenas.
3. Sulfate esters exist in division Chlorophyta at C and C of ß-1,3-galactans (Holan and Volesky, 1994)

The amino groups found in some polysaccharides e.g. chitosan (Masri and Randall, 1978), also have complexing ability for metallic ions such as lead, copper or chromium (Aksu and Kutsal, 1991). Hauer (1978) reported that these functional groups are effective for those metals that form complexes with ammonia e.g. copper, zinc and mercury. The bond formation between these functional groups and metal ions was found to be accompanied by the displacement of protons, dependent, in part, on the extent of protonation as determined by the pH.

Since the pioneering work of Fogg and Westlake (1955), a large body of evidence regarding the release of metal-complexing organic materials by freshwater and marine algae has been reported (Kohn *et al.*, 1968; Dugan and Pickrum, 1972; Swallow *et al.*,

1978). All floc-forming microorganisms studied have either structurally or chemically different polymers. The chemical composition and structural linkages of monomeric units determines the physical and chemical properties of the extracellular polymers from individual species of organisms (Dugan and Pickrum, 1972).

Some extracellular polymers have been reported to slough off the cells and remain in colloidal suspension which results in increased viscosity of the surrounding solution when produced extensively by cells (Dugan and Pickrum, 1972). By comparison, the polymer properties may be such that the polymer remains as a loose slime layer in the vicinity of the cells, or it may remain as a well defined capsule or a zoogloeal matrix around the cells which synthesize it (Dugan and Pickrum. 1972).

The key feature in bioflocculation appears to be synthesis of relatively insoluble extracellular polymer strands so that they remain in the vicinity of cells and do not dissolve away. In this manner, cells and other particulate materials (e.g. metals) become entangled into a flocculent conglomerate on a microscopic level (Dugan and Pickrum, 1972). The free amino groups in chitosan have been shown to bind metallic ions and hydrogen ion by competitive interaction (Masri and Randall, 1978).

Lester and coworkers (1984) demonstrated a decrease in metal binding capacity by microbial biomass that has been stripped off membrane polymers as opposed to native biomass. In addition, extracted polymers were found to have even higher metal binding capacities which suggests that the role of polymers in the removal of metals in an

activated-sludge process may be more important than any other mechanism (Lester *et al.*, 1984). Contrary to this finding, Wehrheim and Wettern (1994) found that whole cells accumulate more metal ions than isolated cell walls. This discrepancy could be attributed to the fact that in the latter study, intact cell walls were used whereas in the former, various cell wall constituents (polysaccharides, proteins, etc) were isolated and used for metal uptake.

4.1.2 Competition studies

Although single toxic metallic species rarely exist in natural and wastewaters, a great deal of research has been focussed on the uptake of single species of metal ions (Fourest *et al.*, 1994; Sağ and Kutsal, 1996a; 1996b; 1997; Sağ *et al.*, 1998). More common are wastewaters containing many metal ions and a range of co-contaminants (Galun *et al.*, 1984). Biosorption of single metal species of heavy metal ions is affected by several factors. These factors include the specific binding properties of the biomass and physicochemical parameters of the solution such as pH, temperature, metal concentration and biomass concentration (Ting *et al.*, 1991). This investigations have been conducted to study the removal of many metals simultaneously.

The optimum initial pH for the biosorption of copper ions on *R. arrhizus* was found to be 4.0 (Sağ and Kutsal 1996a) whereas that of Cr(VI) and Fe(III) ions under similar experimental conditions was 2.0 (Sağ *et al.*, 1998). The different pH binding profiles for heavy metal ions was attributed to the nature of the chemical interactions of each metal with the biomass and are related to the isoelectric point of the biomass. At pHs above the

isoelectric point, there is a net negative charge on the biomass and the ionic state of ligands such as carboxyl, phosphate and amino groups will be such as to promote reaction with the metal cations (Sağ and Kutsal, 1996a). However, as the pH is lowered, the overall charge will become positive, which will inhibit the approach of positively charged metal cations.

Even though conditions of optimum adsorption of heavy metal ions to various biomasses are known, the presence of multiplicity of metals leads to interactive effects (Ting *et al.*, 1991). The mechanisms associated with metal biosorption are complex and dependent on the nature of the physicochemical interaction of both the metal ion in solution and the biomass biosorptive sites (Macaskie and Dean, 1989). Many other factors affect the capacity of biomass to bind more that one metal simultaneously. The combined effects of two or more metals on biomass also depend on the number of metals competing for binding sites, metal combination, levels of metal concentration, residence time etc (Sağ and Kutsal, 1996a; 1996b).

The interactive effects of a mixture of metals on biomass can be extremely complex and three types of responses may be produced by a particular type of biomass:

(i) Synergism - the effect of a mixture is greater than that of each of the individual effects of the constituents of the mixture.

(ii) Antagonism - the effects of a mixture is less than that of each of the individual effects of the constituents of the mixture.

(iii) Noninteraction - the effects of the mixture is equivalent to each of the

individual effects of the constituents in the mixture (Ting *et al.*, 1991).

Information on the study of biosorption in multi-metal systems is sketchy. The presence of a second metal ion has been reported to influence the adsorption capacity due to competition for the binding sites (Sağ *et al.*, 1998). Similar experimental results were also obtained in the selective biosorption of copper(II) and chromium(VI) ions from binary mixtures and previously reported (Sağ and Kutsal, 1996a). The competition between metal ions to bind to active components of the biomass can result in both the synergistic and antagonistic responses. For example, the equilibrium uptake of nickel ions in the presence of lead ions decreased substantially as compared with the removal of single nickel ions in solution under the same experimental conditions (Sağ and Kutsal, 1997). In addition to competition for adsorption sites on the biomass, the screening effect of a competing metal was also mentioned as a possible explanation for the observed antagonism. On the other hand, synergism has been attributed to the damage of cellular membrane structures.

Selective binding for certain metals by biomass has also been demonstrated (Sağ *et al.*, 1998). In single metal ion systems for chromium(VI) and iron(III), chromium ions were more effectively adsorped to the biomass (*R. Arrhizus*) at higher values of initial metal ion concentration than for iron. Similarly, the removal of chromium(VI) from binary metal mixtures by both *C. vulgaris* and *R. arrhizus* in the presence of iron(III) was always greater than the removal of iron(III) under the same experimental conditions, despite the

fact that the optimum pH for biosorption of both ions is 2.0 (Sağ *et al.*, 1998). Similar results were previously reported by Kuyucak and Volesky (1988) where the biomass of *S. natans* selectively sequestered gold from the solution containing other metallic ions such as lead, zinc , uranyl and silver. In another study, the surface adsorption of zinc appeared to be relatively constant (about 15 %) at a fixed zinc concentration but with increasing cadmium concentration. In other words, an increase in the concentration of cadmium could not displace zinc ions. This evidence supported the hypothesis that zinc and cadmium bind to different functional groups of the biomass because of the different affinities of the ligands for these metals on the biomass (Ting *et al.*, 1991). Competition for binding sites or mutual screening effect of the competing ions was not observed over the range of metal concentration studied.

This part of the study sought to investigate the possibility of using algal exopolysaccharides for bioremediation of water polluted with heavy metals using both single metal and binary metal solutions. The metal uptake capacity of the biomass was also studied and compared with other biosorbents.

4.2 Materials and Methods

4.2.1 Materials

Chemicals

Chemical of high (analytical) grade were supplied by the following companies:

Saarchem (Pty) Ltd., Johannesburg, S. Africa - Copper chloride and Lead nitrate

Equipment

The following equipment was used in carrying out experimental work:

Incubator shaker - Labcon

Atomic Absorption Spectrometer (GBC 909AA) - Varian Techtron Pvt Ltd.

4.2.2 Methods

4.2.2.1Preparation of EPS suspensions

Dunaliella exopolysaccharide was weighed into 100 m*l* flasks, resuspended in deionised water and allowed to dissolve as much as possible. The pH was adjusted using 1.0 M NaOH or 1.0 M HCl prior to addition of metal solution.

4.2.2.2Effect of pH on metal binding

The pH of EPS suspensions (400 μg/m*l*)was adjusted using 1.0 M NaOH or 1.0 M HCl prior to incubation with metal. The pH range evaluated was from 2.0 to 6.0. After addition of metal, the mixtures were incubated for 3 h in a shaking incubator (170 rpm). To establish the effect of pH on metal precipitation, control experiments were conducted without EPS, by adjusting the pH of deionised water between pH 2.0 and 9.0. Aliquots (2.5 m*l*) were sampled and filtered through 0.45 μm cellulose acetate filters (filters did not bind metals), followed by analysis for metal concentrations. The initial metal concentrations were 200 μmol/*l*.

4.2.2.3Effect of biomass concentration

Freeze dried EPS was weighed into 100 m*l* flasks, resuspended in deionised water at various concentrations (200 - 800 μg/m*l*) and allowed to dissolve as much as possible. The pH was adjusted using 1.0 M NaOH or 1.0 M HCl prior to addition of metal. A small volume of concentrated metal solution was then added to a final concentration of 200 μmol/*l* (see table 4.4). As a control, deionised water was made up with the metal solution, i.e. no biomass was added. The flasks were incubated for 3 h in a shaking incubator (170 rpm). Thereafter, 2.5 m*l* aliquots were sampled and filtered through 0.45 μm cellulose acetate filters. The filtrates were then analysed for metal concentrations. The initial metal concentrations were 200 μmol/*l*.

4.2.2.4 Effect of temperature on metal binding

EPS was weighed and resuspended in deionised water (400 μg/m*l*) and again allowed to dissolve as much as possible. The pH of the suspensions was adjusted to 5.0 using 1.0 M NaOH or 1.0 M HCl. After addition of metal, the flasks were incubated for 3 h with shaking at various temperatures (18; 25; 32 and 40 °C). Following filtration, the filtrates (2.5 m*l* aliquots) were analysed for metal concentrations. The initial metal concentrations were 200 μmol/*l*.

4.2.2.5 Adsorption kinetic studies

The EPS suspensions were prepared as described above, followed by the addition of metal solution. For determination of binding rates, samples were taken at the following

intervals (0; 2; 5; 10; 30; 60; 120 and 180) with shaking at pH 5.0 and 25 °C. Analyses for metal concentrations were done as described above. The initial metal concentrations were 200 μmol/*l*.

4.2.2.6 The effect of metal concentration

Analyses of metal removal by EPS were done over the range 100 to 400 μmol/*l*. Incubation was as described above with shaking for 3 h. Thereafter, 2.5 m*l* aliquots were sampled and filtered through 0.45 μm cellulose acetate filters. The filtrates were then analysed for metal concentrations. The pH was adjusted to 5.0 using 1.0 M NaOH or1.0 M HCl. The equilibrium binding isotherms were used for determination of metal binding capacity (Volesky, 1990).

4.2.2.7 Competition studies

To determine the adsorption characteristics of the competing metal in binary metal mixtures, the initial concentrations of the competing metal (Cu and Pb) were varied. Different ratios of metals were used, that is, 1:1; 1:2; 1:4; 2:1 and 4:1 (Cu:Pb). Experiments were performed at a pH of 5.0 and a temperature of 25 °C. A combination of these parameters allow metal removal of approximately 75 % in single metal solutions of copper and lead. An incubation period of 3 h was employed with stirring at 170 rpm. Samples were taken in triplicate assays, filtered through cellulose acetate filters (0.45 μm) and the filtrates analysed for metal concentration by atomic absorption spectrometer.

4.2.2.8Metal analysis

A GBC 909 atomic absorption spectrometer (AAS) was used for all metal analysis.

4.3 Results

4.3.1 The effect of pH on metal precipitation

The influence of pH on metal precipitation from solution was investigated. The precipitation profiles for copper and lead are presented in figures 4.1 and 4.2 respectively. Similar trends were observed for both metal ions, with slight precipitation (≤10 %) occurring at pH values below 6.0. At pH 2.0, copper ions did not precipitate at all whereas approximately 3 % lead precipitation was recorded under similar conditions.

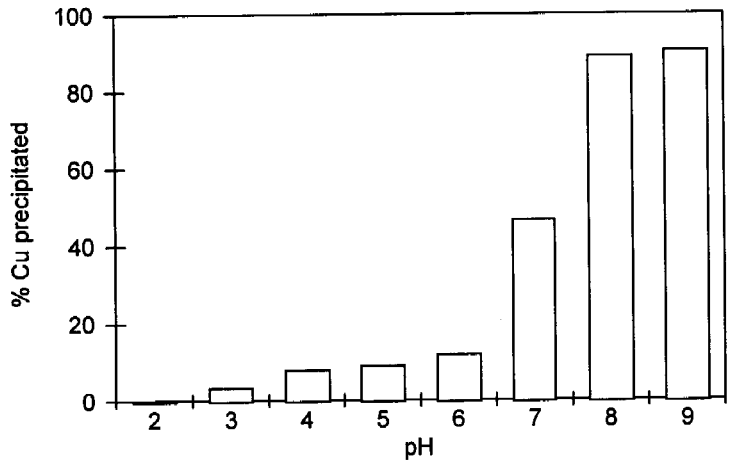


Figure 4.1: The effect of pH on precipitation of copper ions from aqueous solution. Initial copper concentration of 200 µmol/*l* and biomass concentration of 400 µg/m*l* was used. Contact time was 3 h at 25 °C with shaking (170 rpm). Results represent the mean of triplicate assays.

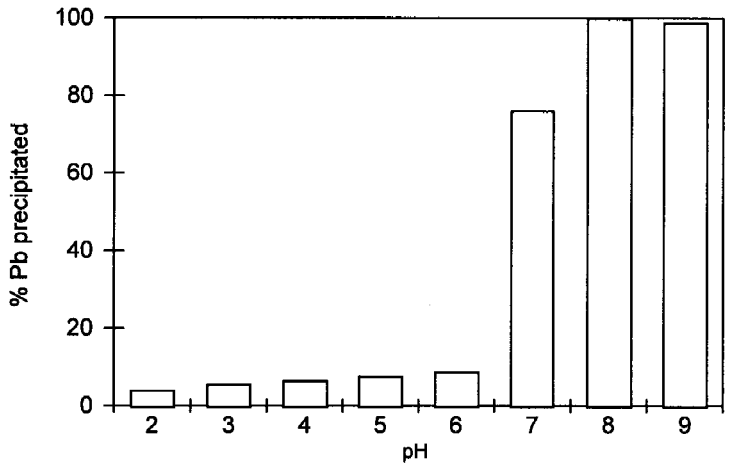


Figure 4.2: The effect of pH on precipitation of lead ions from aqueous solution. Conditions were as described above. Results represent the mean of triplicate assays.

Increasing the pH to 7.0 resulted in about 45 % and 75 % precipitation for copper and lead respectively. Almost 100 % lead precipitation was observed at pH 8.0 and 9.0, whilst copper precipitation was approximately 90 % at the same pH values.

4.3.2 The effect of pH on metal removal

The pH of *Dunaliella* EPS solution was adjusted prior to addition of metal solution and the pH values investigated ranged between 2.0 and 6.0. The removal of copper from solution by *Dunaliella* EPS was found to increase with an increase in pH (figure 4.3). The lowest percentage copper removal (15.4 %) was recorded at pH 2.0. This removal increased two fold to 32.7 % when the pH was raised to 3.0. At pH 4.0, 5.0 and 6.0, percentage copper removal increased to 51.8, 63.7 and 74.9 % respectively.

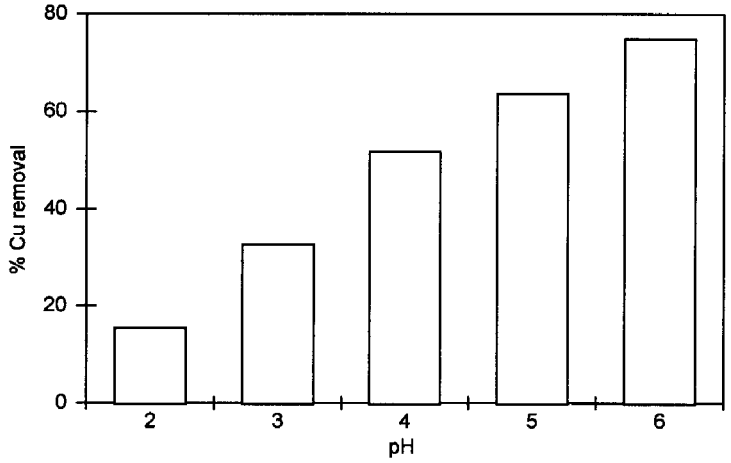


Figure 4.3: The effect of pH on copper removal from solution by extracellular polysaccharides. Results represent the mean of triplicate assays.

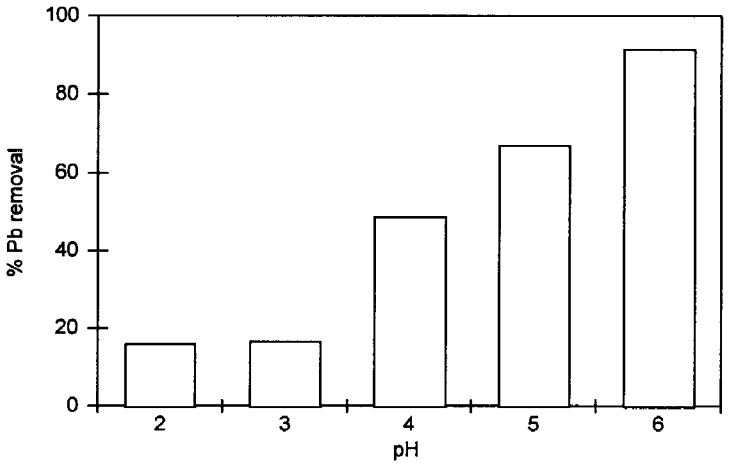


Figure 4.4: The effect of pH on lead removal from solution by *Dunaliella* extracellular polysaccharides. Results represent the mean of triplicate assays.

Table 4.1 Change in pH of lead solution occurring during metal-biomass interaction

Initial pH	Final pH
2.0	2.09
3.0	2.83
4.0	3.82
5.0	4.74
6.0	5.46

A similar trend was observed for lead removal except that the increase in percentage removal between pH 2.0 and 3.0 was very marginal (figure 4.4). The percentage lead removal for pH 2.0 and 3.0, was 15.8 and 16.5 % respectively. When the pH was raised to 4.0, removal increased to 48.6 %. At pH 5.0, removal of 66.8 % was recorded whereas at pH 6.0 it was 91.3 %. The percent removal for both metals was almost equal for the pH values investigated with the exception of pH 6.0 where lead removal was considerably higher (91.3 %) compared to that of copper (74.9 %). A decrease in pH of metal solution was observed when metals were removed from solution by the biomass (table 4.1), e.g. an initial pH of 5.0 decreased to 4.74 after 3 h incubation.

Table 4.2 Adsorption of copper and lead by *Dunaliella* exopolysaccharides

pH	Adsorption of copper (mg/g)	Adsorption of lead (mg/g)
2.0	14.8	12.0
3.0	31.5	12.3
4.0	44.1	42.4
5.0	54.8	59.4
6.0	63.2	82.7

The term adsorption was used to determine the exact amount (percentage) of metals bound to biomass. Adsorption was defined as the percentage metal removal minus percentage metal precipitated at that particular pH. Table 4.2 shows adsorption of copper and lead by *Dunaliella* exopolysaccharides. It can be seen from this table that at pH below pH 4.0, the adsorption of copper was higher than that of lead. However, as the pH was increased above 4.0, adsorption of lead was higher than that of copper. This finding could not be explained because different batches of exopolysaccharides were used for these metals.

4.3.3 Effect of biomass concentration on metal removal from solution

The effect of biomass concentration on copper removal from solution is presented in figure 4.5. The percentage of copper removed from solution increased with increasing biomass concentrations. The increase appears to reach a plateau at biomass concentrations above 600 $\mu g/ml$. Figure 4.6 shows the effect of biomass concentration on lead removal from solution. The percentage of lead removed from solution increased with increasing biomass concentrations. Contrary to observations for copper, no plateau was observed in the removal profiles for lead. However, the metal uptake capacity per gram of biomass decreased with increasing biomass concentrations (table 4.3).

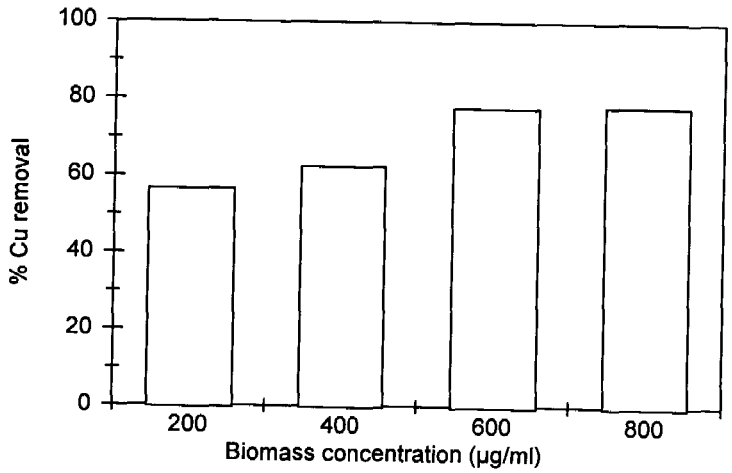


Figure 4.5: The effect of biomass concentration on copper removal from aqueous solution. The pH was adjusted to 5, and initial copper concentration was 200 µmol/*l*. Incubation period was 3 h at 25 °C with stirring (170 rpm). Results represent the mean of triplicate assays.

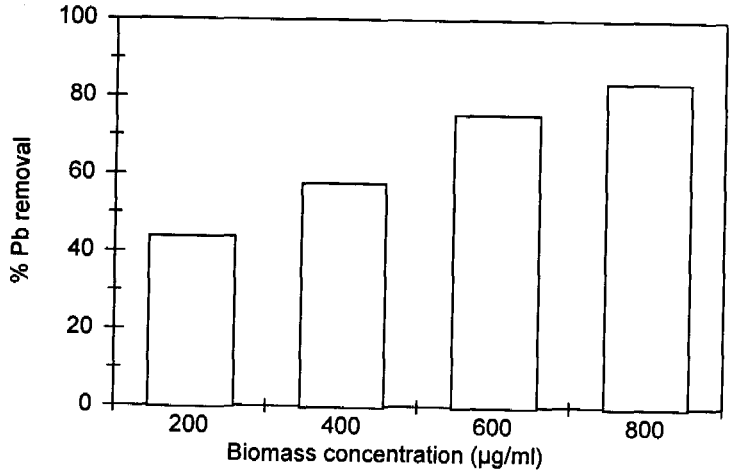


Figure 4.6: The effect of biomass concentration on lead removal from aqueous solution. Results represent the mean of triplicate assays.

Table 4.3 The effect of biomass concentration on metal uptake from solution

[Biomass] (μg/m*l*)	% copper removal	Copper uptake (mg/g)	% lead removal	Lead uptake (mg/g)
200	56.6	36.0	43.8	90.9
400	62.4	19.8	57.5	59.6
600	77.6	16.4	75.4	52.1
800	78.1	12.4	83.8	43.4

4.3.4 The effect of temperature on metal removal from solution

The effect of temperature on metal removal by *Dunaliella* EPS was studied at the following temperatures 18, 25, 32 and 40 °C. Figures 4.7 and 4.8 show the temperature

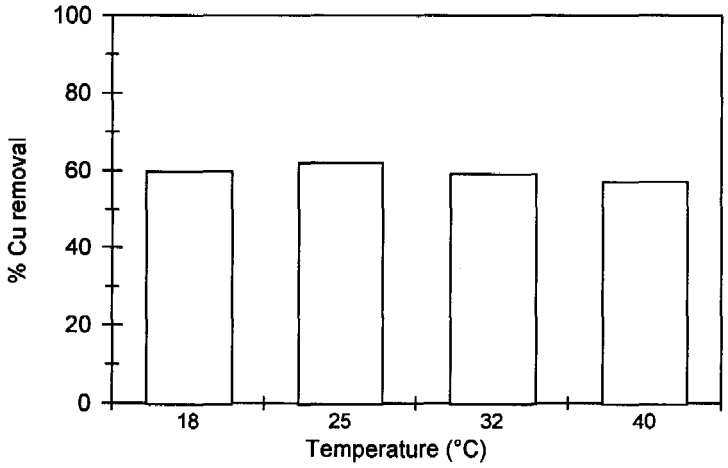


Figure 4.7: The effect of temperature on copper removal from solution by Dunaliella extracellular polysaccharides. Results represent the mean of triplicate assays.

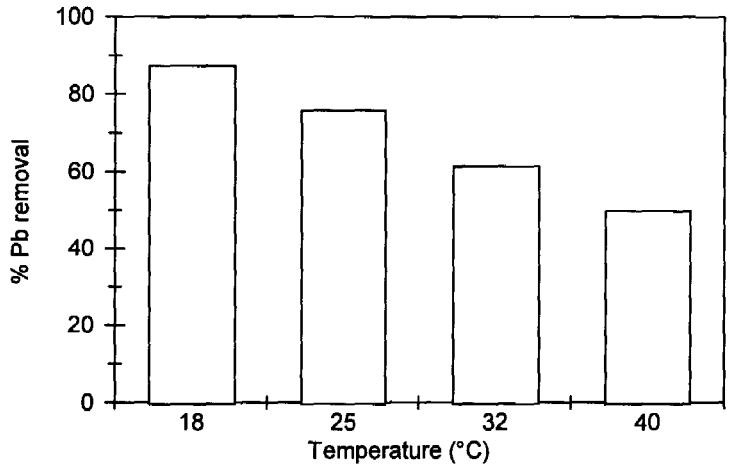


Figure 4.8: The effect of temperature on lead removal from solution by Dunaliella extracellular polysaccharides. Results represent the mean of triplicate assays.

profiles for copper and lead respectively. The removal of copper did not appear to be affected by increases in temperature. At the above mentioned temperatures, copper removal was found to vary slightly at 59.6, 61.9, 59.0 and 57.2 % respectively. However, a slightly different trend was observed for lead removal at the same temperatures. The removal of lead was found to be temperature dependent, and to decrease with an increase in temperature. Removal of 71.5 % was recorded at 18 °C, which is comparatively higher than that of copper at 18 °C. But at 25 °C, the percent lead removal (62.3 %) was almost the same as that of copper (61.9 %). As the temperature was increased to 32 and 40 °C, the percent lead removal decreased to 50.2 % and 40.7 % respectively. The percentage lead removal decreased by almost 40 % when the temperature was changed from 18 to 40 °C.

4.3.5 Kinetic study of metal removal from solution by *Dunaliella* EPS

The ability of *Dunaliella* exopolysaccharides to remove metals, copper and lead, from single metal solutions was investigated at pH 5.0 and a temperature of 25 °C. The kinetic profile of copper removal from solution is presented in figure 4.9 and that of lead removal in figure 4.10. The rate of metal removal was found to be fairly high reaching completion within 15 minutes. Continued incubation beyond 30 minutes did not result in further increase in

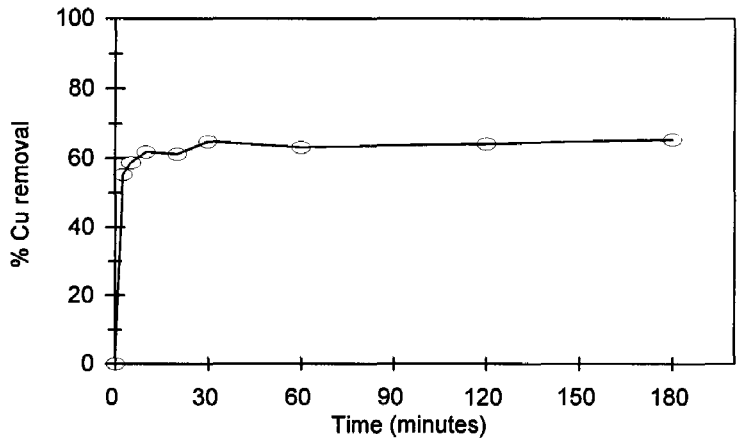


Figure 4.9: The rate of copper removal from solution by *Dunaliella* extracellular polysaccharides. Results represent the mean of triplicate assays.

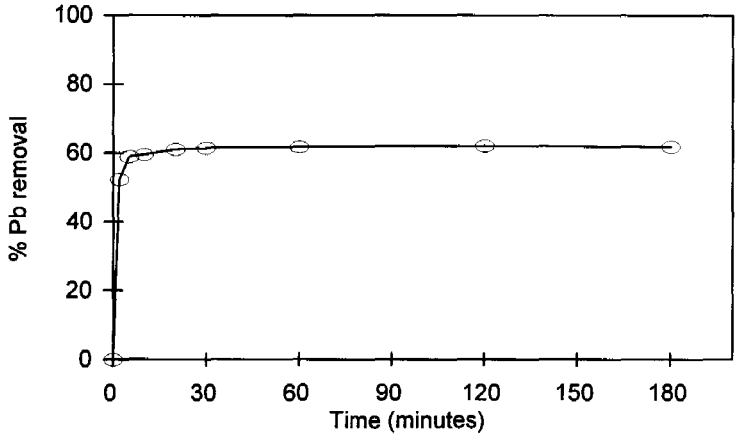


Figure 4.10: The rate of lead removal from solution by *Dunaliella* extracellular polysaccharides. Results represent the mean of triplicate assays.

percentage metal removal, indicating that the biomass is in equilibrium with the metal solution. It was also found that at 25 °C and pH 5.0, metal removal was almost the same (approximately 65 %) for copper and lead after 3 h incubation.

4.3.6 Biosorption isotherms

To establish the biomass binding capacity, 400 µg/m*l* of biomass was subjected to increasing concentrations of metals (copper and lead) in single metal solutions. After 3 h incubation, the equilibrium metal concentration was determined and plotted as a function of metal binding capacity (q). The binding isotherms were generated over a range of initial metal concentrations of 100 to 400 μmol/*l* (refer table 4.4 for conversion to mg/*l*).

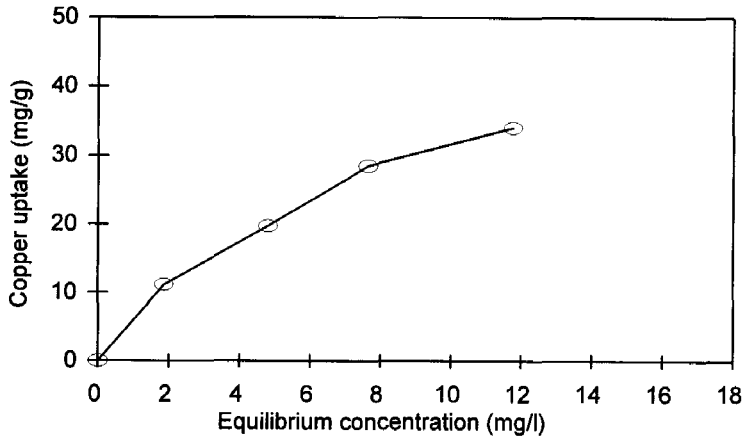


Figure 4.11: The equilibrium isotherm for copper. Results represent the mean of triplicate assays.

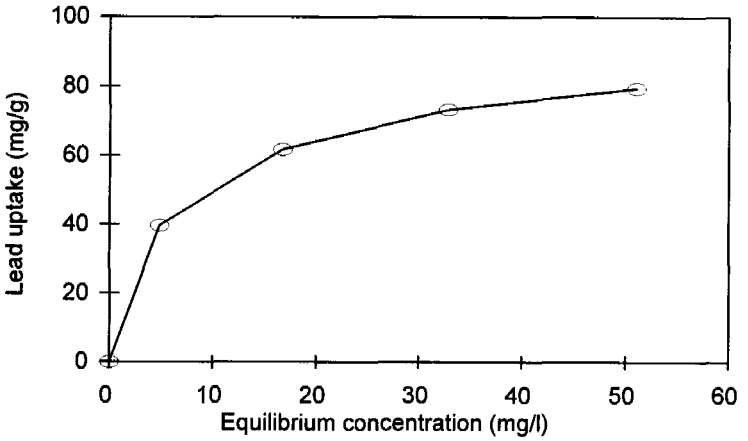


Figure 4.12: The equilibrium isotherm for lead. Results represent the mean of triplicate assays.

Table 4.4 Conversion of metal concentration from μmol/*l* to mg/*l* for copper and lead.

Cu (μmol/*l*)	Cu (mg/*l*)	Pb (μmol/*l*)	Pb (mg/*l*)
100	6.354	100	20.72
200	12.709	200	41.44
300	19.064	300	62.16
400	25.418	400	82.88

The uptake capacity was calculated from the following equation :

$q = V(Ci - Cf)/M$, where

q is uptake capacity (mg/g)

V - volume of metal solution (*l*)

C*i* - initial metal concentration (mg/*l*)

C*f* - final (equilibrium) metal concentration (mg/*l*)

M - weight of biomass (g)

From the data presented in this study, it was found that the uptake capacity of *Dunaliella* EPS for copper and lead was 35 and 79.8 mg/g biomass. However, it should be noted that the concentrations of copper and lead used were not sufficiently high to saturate the biomass, which would allow the graph to reach the plateau. This suggests that the capacity could actually be higher than values presented in this study.

4.3.7 Competition studies

The combined effects of copper and lead ions on *Dunaliella* EPS metal removal was investigated as a function of different combinations of initial metal ion concentrations at

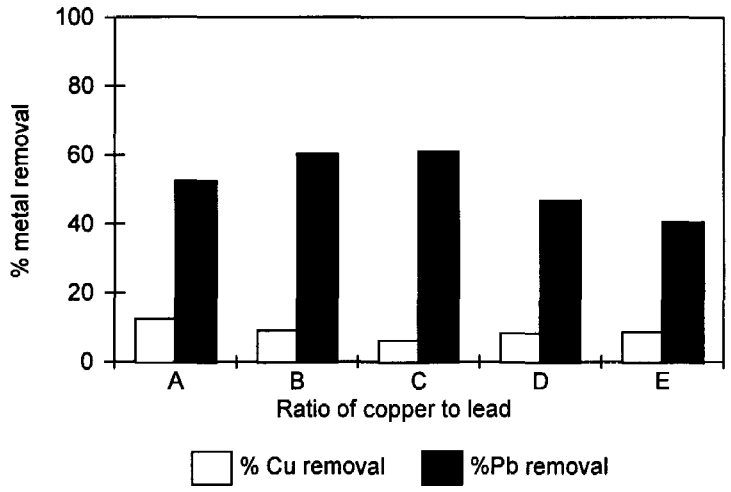


Figure 4.13: Metal competition for binding sites on *Dunaliella* extracellular polysaccharides. A = 1:1; B = 1:2; C = 1:4; D = 2:1 and E = 4:1 (Cu:Pb), where 1 is the initial metal concentration of 200 µmol/*l*. Results represent the mean of triplicate assays.

Table 4.5 The ratio of competing metals (copper and lead) in binary metal solution

Copper (µmol/*l*)	Lead (µmol/*l*)	Ratio of copper to lead	% Copper removal	% lead removal
200	200	A (1:1)	12.3	52.4
200	400	B (1:2)	9.0	60.2
200	800	C (1:4)	6.1	60.9
400	200	D (2:1)	8.2	46.8
800	200	E (4:1)	8.6	40.5

a pH value determined as optimum for both metals. The results were presented as percentage metal removal relative to initial metal concentration (figure 4.13). The initial metal concentrations are presented in table 4.5. At a ratio of 1:1 (Cu:Pb), it was found

to occur as a result of ionization of the biomass functional groups. Doyle *et al.* (1980) reported that when the hydrogen ion concentration is increased, the affinity of the biomass for metals decreased, but the numbers of metal binding sites remained constant, suggesting that cations and protons compete for the same sites. A study by Lee *et al.* (1998) has also demonstrated that hydrogen ions compete strongly with metal cations for binding sites on biomass. The type of metal is also crucial to biosorption. Some metals such as copper, nickel and lead are removed at higher pH values whereas certain others e.g. chromium and iron are adsorbed at lower pH (2.0).

It must be noted that the pH of the metal solution decreases during incubation with biomass. This phenomenon was also reported by Fourest and Roux, (1992) and was attributed to the release of protons into solution due to displacement by metal cations (Fourest and Roux, 1992; Nourbakhsh *et al.*, 1994). However, buffers could not be used because buffers have the ability to complex metal ions (Tobin *et al.*, 1984). Furthermore, the presence of buffer ions in metal solutions has been found to reduce metal biosorption (Volesky and May-Phillips, 1995).

Interestingly, *Dunaliella* EPS is capable of removal of metal ions from solution at pH 2.0, although not optimally. This observation suggests the involvement of a functional group which is ionized at pH 2.0. The characterization of EPS revealed the presence of sulphate, amino and hydroxyl groups. Of these three, sulphate group is the only one that ionizes at pH 1.5 (Christ *et al.*, 1992). From these results, it can be deduced that sulphate groups are responsible for metal removal at pH 2.0. The other functional groups found in

Dunaliella exopolysaccharides (amino and hydroxyl) have also been reported to remove metals from solution (Holan *et al.*, 1993; Hauer, 1978).

A biomass concentration of 400 μg/m*l* was used for metal removal. Higher biomass concentrations removed more metals from solution on a percentage basis. However, a concentration of 400 μg/m*l* was chosen because accumulation per gram of biomass substantially decreased as the biomass concentration was increased (table 4.2). This finding has been reported elsewhere (Fourest and Roux, 1992) and was attributed to the shortage of metal concentration in solution as biomass concentration was increased. A decrease in metal uptake from 52 mg/g to 8 mg/g was observed when the *Rhizopus arrhizus* concentration was increased from 5 g/l to 50 g/*l*. It was, therefore, not necessary to use *Dunaliella* EPS concentrations above 400 μg/m*l*. The data obtained from these simple experiments can be of great interest in scale-up process to optimise industrial effluent purification.

The effect of temperature on copper removal was not particularly pronounced over a range of temperatures investigated. This observation supported other reports which showed that metal removal is not considerably affected by increases in temperature (Kuyucak and Volesky, 1988; Brady and Duncan, 1994). The result was expected since nonviable biomass, which does not need metabolic activity, was used for metal removal. It is, therefore, unlikely for such biomass to be affected by temperature. However, the removal of lead was affected by temperature. A decrease in lead uptake was observed with increasing temperature. It should be mentioned that a different batch of *Dunaliella*

EPS was used for studies on lead uptake.

The second batch was different from the first in terms of texture and solubility in water. The first was less soluble whereas the second was more soluble and even formed a viscous solution in water. This unexpected behaviour regarding the temperature profiles for lead removal was ascribed to the difference biomasses used for copper and lead. It is suggested that the solubility of the biomass was responsible for the decrease in percentage lead removal with increasing temperature. At higher temperatures, the polysaccharide molecules will probably loose their conformation and the functional groups will thus be exposed. Consequently, those functional groups which are temperature sensitive will be destroyed, leading to a decrease in metal removal.

A kinetic study of metal removal showed that the rates of metal removal by *Dunaliella* EPS are rapid. Approximately 65 % metal removal was observed in the first 10 min. The rapid removal process is of importance because it allows for treatment of larger volumes of effluent within a short time.

The uptake capacity of metal by biomass showed that *Dunaliella* EPS has a higher uptake capacity for lead than for copper. The uptake capacity for lead was 80 mg/g compared to approximately 35 mg/g for copper. These results confirmed the hypothesis that metal ions with larger ionic radii are taken up to a larger extent compared to those with smaller ionic radii (Kuyucak and Volesky, 1988; Tobin *et al.*, 1984).

The effects of the presence of lead ions on the removal of copper and visa versa was investigated. Results showed that under constant experimental conditions, there appeared to be an inhibition of copper removal by the presence of lead. When both metals were present at equimolar ratios, the percentage copper removal was reduced to 12 % compared to 75 % in single solutions. The percentage copper removal was not significantly affected by an increase in the ratio of lead to copper. This could suggest a selective binding for copper on certain biomass functional groups, and for lead on certain others. An increase in the ratio of copper to lead did not improve the percentage copper removal. However, the removal of lead was considerably reduced from 60 % to 40 %. These results further confirm the preferential removal of lead ions by *Dunaliella* EPS.

When lead was present as a competing metal, the combined removal of both metals was slightly less than the removal for either metal in single metal solutions. This observation demonstrates that the effect of lead is non-interactive. When copper was present as a competing metal, the combined removal of both metals was significantly less than the removal for either metal in single metal solutions. Therefore, the response can be described as antagonistic (Ting *et al.*, 1991).

4.5 Conclusion

The removal of copper and lead by *Dunaliella* EPS was pH dependent. This biomass also has good metal removal capacity which was higher for lead (80 mg/g) than for copper (35 mg/g) and compares very well with others biosorbents. For example, sphagnum moss peat has an uptake capacity of 16.4 mg/g for copper and 30.7 mg/g for lead (Ho *et al.*,

1996). The uptake was fairly rapid with 75 % removal achieved in the first 10 min.

In all experiments, the removal of copper from the solution in the presence of lead was always less than the uptake of lead under the same conditions. Notwithstanding the ratio of copper to lead or visa versa, the surface adsorption for copper appears to be relatively constant (about 10 %). This evidence supports the hypothesis that copper and lead bind to different biomass binding sites.

Algal mass culture provides a source of cheap metal biosorbent in the form of extracellular polysaccharides. Immobilization and column studies of these types of biomass can improve the handling and separation of the biomass from effluent. It should be borne in mind that these metal recovery systems are not yet sufficiently developed to replace current technologies. However, they may be used as polishing step for removing trace concentrations of metals that ion exchange resins cannot deal with and with further development could replace some of the current technologies.

CHAPTER 5

GENERAL DISCUSSION

It is well established that South Africa is water deficient country with an annual rainfall well below the world annual rainfall. Water is essential to life, to social development and to economic progress. Population growth, urbanization and industrialization serve to increase the demands for water resources. Furthermore, it has been projected that even if all the potential viable water resources in South Africa are fully developed, the supply will not meet the demand of the year 2025. This scarcity of water has resulted in the management of water resources being entrenched in the new constitution. The National Water Bill was also recently (1998) promulgated, the purpose of which is to ensure protection, conservation and management of water resources.

The depletion and degradation of already limited water resources by pollutants from mining and industrial processes further exacerbates the problem. Conventional methods of wastewater treatment are capable of reducing metal pollutants to any desired degree, but the cost increases rapidly as the desired concentration becomes smaller. These methods, consequently, leave trace amounts of pollutants in wastewaters. The use of biological systems can provide an alternative, cost-effective and efficient wastewater purification system. This

study investigated the use of algal extracellular polysaccharides as a potential alternative to existing systems or as a polishing stage in the removal of trace concentrations of metals that the existing technology cannot deal with.

Algae are frequently subjected to fluctuating environmental conditions such as salinity, light intensity, temperature and nutrient depletion. Adaptation to these extreme environmental conditions has been found among others, to include the production of extracellular polysaccharides. Hence, initial studies focused on investigation of extracellular polysaccharide production by *Dunaliella* under various conditions effecting growth and polysaccharide release. These included light intensity and nutrient depletion. High light intensity did not induce the production of extracellular polysaccharides by *Dunaliella* cultures. Nitrogen depleting conditions were found to induce polysaccharide production. The production was enhanced by an increase in the ratio of carbon source.

The polysaccharides produced were then isolated, purified and characterized. Characterization involved the determination of monomeric composition as well as the functional groups comprising the polysaccharide. The following monosaccharides were found to constitute Dunaliella exopolysaccharide, namely: xylose, arabinose, 2-O-methyl mannose, mannose glucose and galactose. Infrared studies of Dunaliella polysaccharides revealed the presence of the following functional groups: amino, hydroxyl and sulphate groups. The presence of carboxyl groups in the polysaccharide could not be demonstrate by

infrared studies, which suggests that the polysaccharide did not have protein or pyruvate bound to it. Despite the lack of carboxyl group, the presence of amino, hydroxyl and sulphate groups are sufficient to render the polysaccharide a potential metal biosorbent.

The potential for *Dunaliella* exopolysaccharide to be used as a metal biosorbent was subsequently investigated. All metal binding studies were done in batch reactors, and the parameters affecting metal uptake were investigated. Metal uptake was rapid with 65 % removal observed in the first 10 min. Uptake was found to be pH dependent and the optimum pH for metal removal was 5.0. Both hydroxyl and sulphate groups will be ionized at pH 5.0, which suggests that these functional groups are responsible for metal removal at this pH. Temperature did not have a considerable effect of metal uptake. *Dunaliella* polysaccharide showed good metal binding capacity and efficiency. The uptake capacity for copper and lead was 35 mg/g and 80 mg/g respectively which compared well with other biosorbents.

While the uptake of single metal species of heavy metal ions has been extensively studied, little attention has been given to the study of multi-metal systems. The dependence of the bioremoval process upon pH, temperature and metal concentration has been optimized for only one metal at a time. The study of multi-metal systems could shed light into the understanding of industrial effluent treatment because industrial effluents typically contain more than one metal species and numerous other contaminants. The presence of multiple metal ions leads to interactive effects and affects the bioaccumulation process. The removal

of copper from aqueous solution was inhibited by the presence of lead. However, a four-fold concentration of lead could not displace or prevent copper from binding to the biomass at that marginal level. This result suggested that there was no competition for binding sites, meaning that copper was selectively bound to certain functional groups on the biomass. Most importantly, *Dunaliella* exopolysaccharides appeared to remove lead preferentially. For all the conditions studied, the percentage lead removal, in the presence of copper, as always was above 40 %, regardless of the ratio of copper to lead. Selective binding is very important from the point of view of industrial application, because it normally allows for selective recovery of the different metals from the biomass.

Although the prospects of bioremediation are good, it should be borne in mind that such metal removal systems are not yet sufficiently developed to replace the existing technologies. The use of *Dunaliella* exopolysaccharides in bioremediation needs further studies such as immobilization and column systems. Other studies should also involve further reduction of costs in the form of distillation, recovery and reuse of the 2-propanol used in precipitation of the polysaccharide.

APPENDICES

Appendix A : Preparation of algal growth media.

Reagents for preparation of Baam Medium.

Reagent	mass (g) per liter
NaCl	87.66
$NaHCO_3$	4.2
KNO_3	0.51
$MgSO_4.\ 7H_2O$	1.23
$CaCl_2.2H_2O$	0.044
KH_2PO_4	0.027
Trace element solution	2 m*l*

Reagents for preparation of artificial medium.

Reagent	Mass (g) per liter
Na_2CO_3	22.6
Na HCO_3	6.93
NaCl	123
Na_2SO_4	13.4
KCl	2.06
KNO_3	0.257
KH_2PO_4	0.499
Trace element solution	2 m*l*

Reagents for preparation of trace element solution.

Reagent	mass (g) per liter
$FeCl_3$	0.417
EDTA	2.233
H_3BO_3	11.453
$MnCl_2.4H_2O$	1.382
$ZnCl_2.2H_2O$	0.1681
$CoCl_2$	0.00259
$CaCl_2.2H_2O$	0.342×10^{-4}

Appendix B : Reagent preparation.

Folin Lowry reagents.

Reagent A : 2 % Sodium carbonate (Na_2CO_3) in 1.0 M NaOH, store at 4 °C.

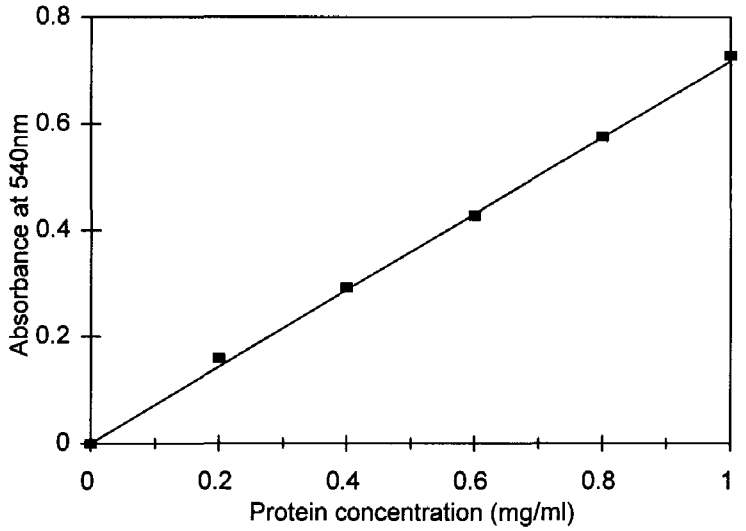
Reagent B_1 : 1 % Copper sulphate ($CuSO_4.5H_2O$) in distilled water, store at 4 °C.

Reagent B_2 : 2 % Sodium Potassium tartrate ($C_4H_4KNaO_6.4H_2O$) in distilled water, store at 4 °C.

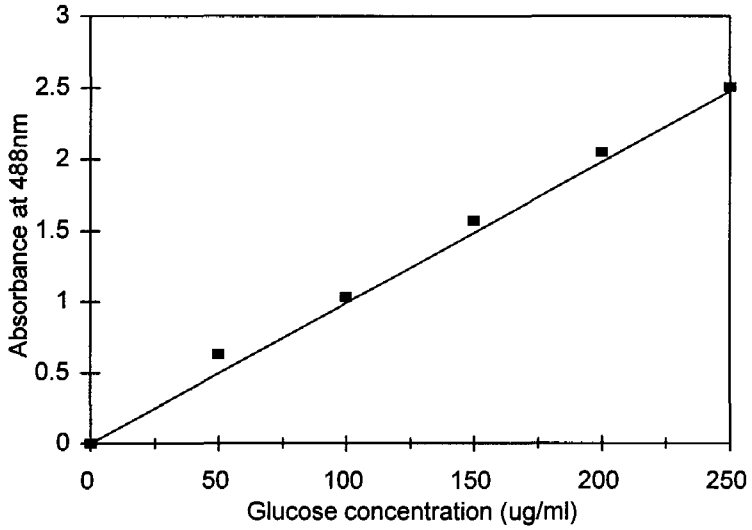
Reagent C : Prepare fresh on the day of experiment. Place 10 m*l* of reagent B_2 in a beaker and add 10 m*l* reagent B_1, with stirring. Then add with stirring, 1.0 *l* of reagent A. The order of addition must be adhered to. Store at 4 °C.

Reagent E : Prepare by diluting a commercial 2.0 N reagent (Folin reagent, phenol reagent) to 1.0 N with water. Store in a brown bottle or foil covered bottle at 4 °C.

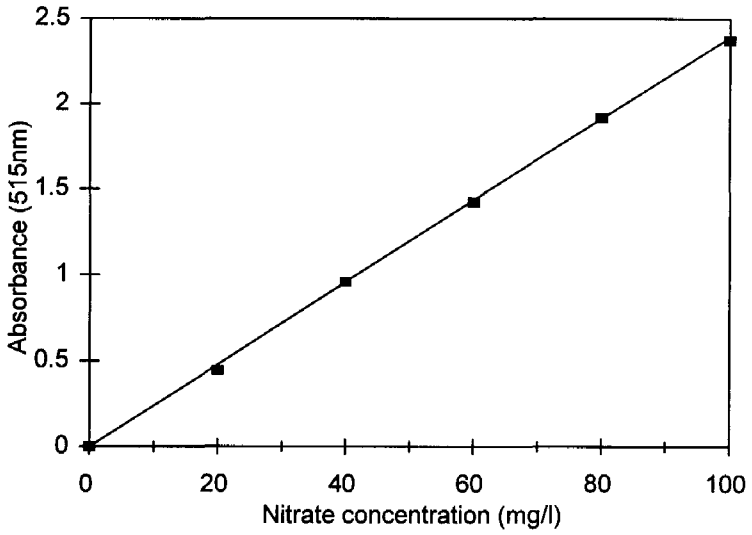
Appendix C : Standard curves for colorimetric assays.



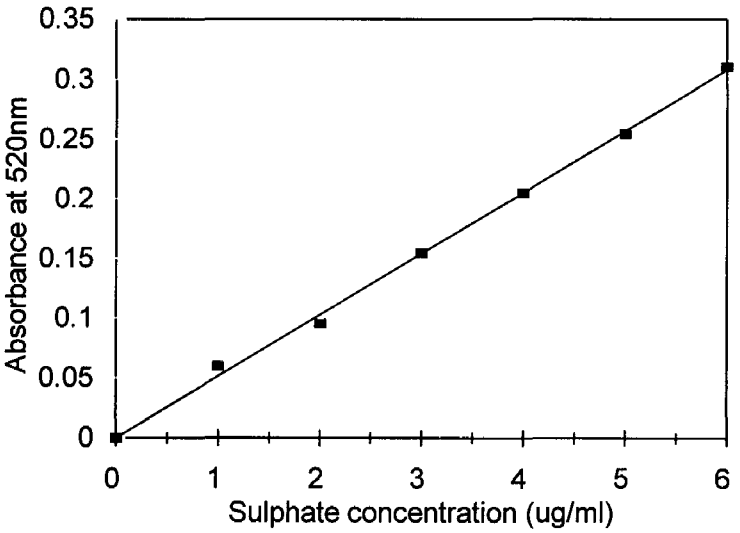
The Folin-Lowry protein standard curve.



The glucose standard curve.

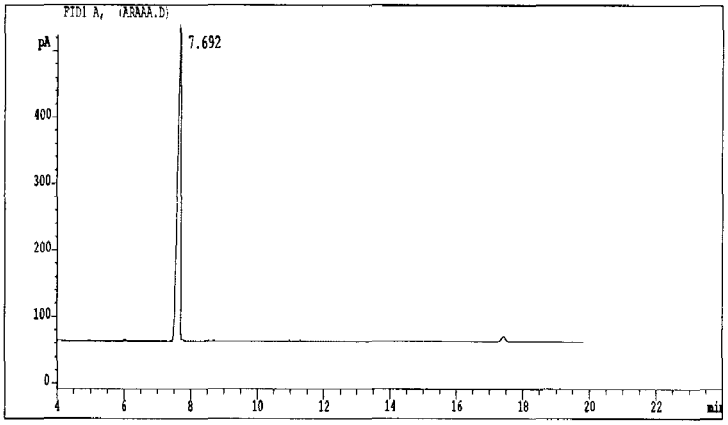


Standard curve for determination of residual nitrate concentration in culture media.

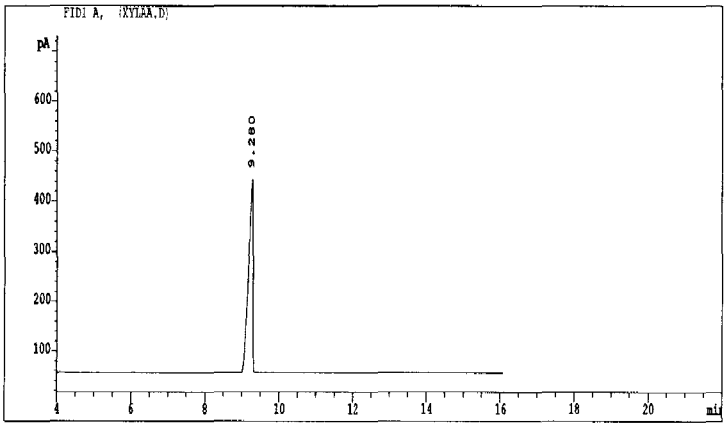


Standard curve for the determination of sulphate groups in *Dunaliella* exopolysaccharides.

Appendix D : GC standards for commercial monosaccharides.

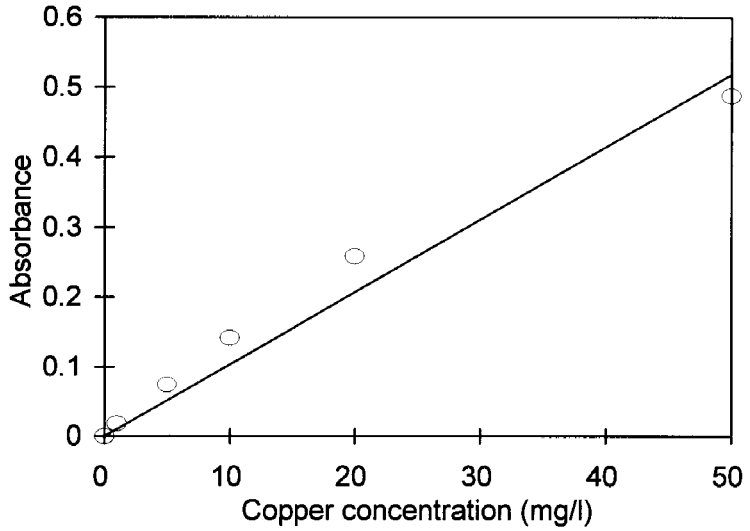


A chromatogram for arabinose (alditol acetate derivative) standard run on DB-225 column.

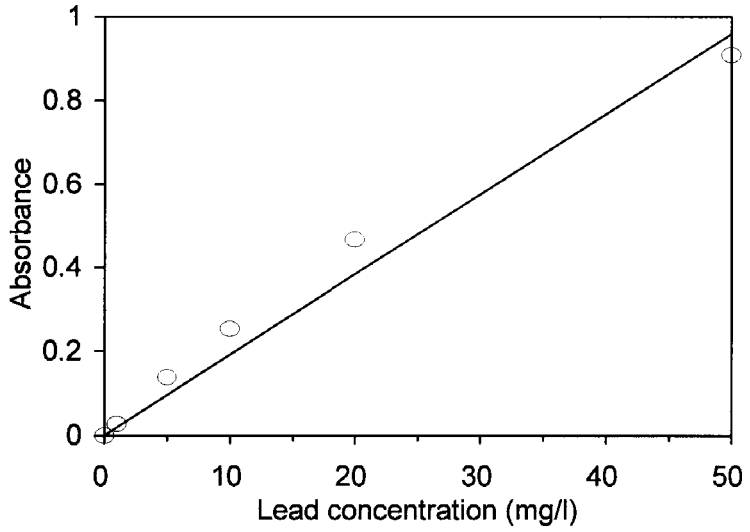


A chromatogram for xylitol acetate standard run on DB-225 column.

Appendix E : standard curves for metal determination (AAS)

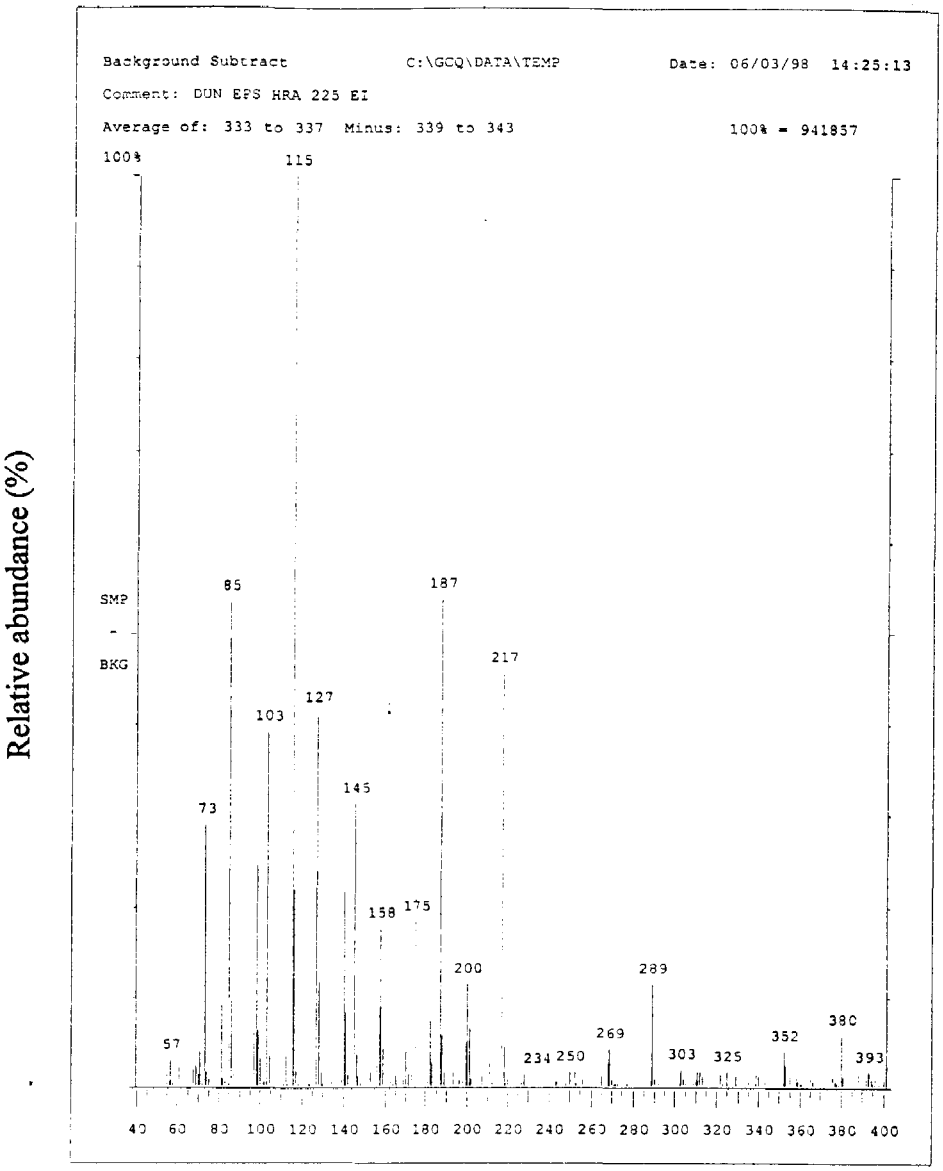


Standard curve for copper.

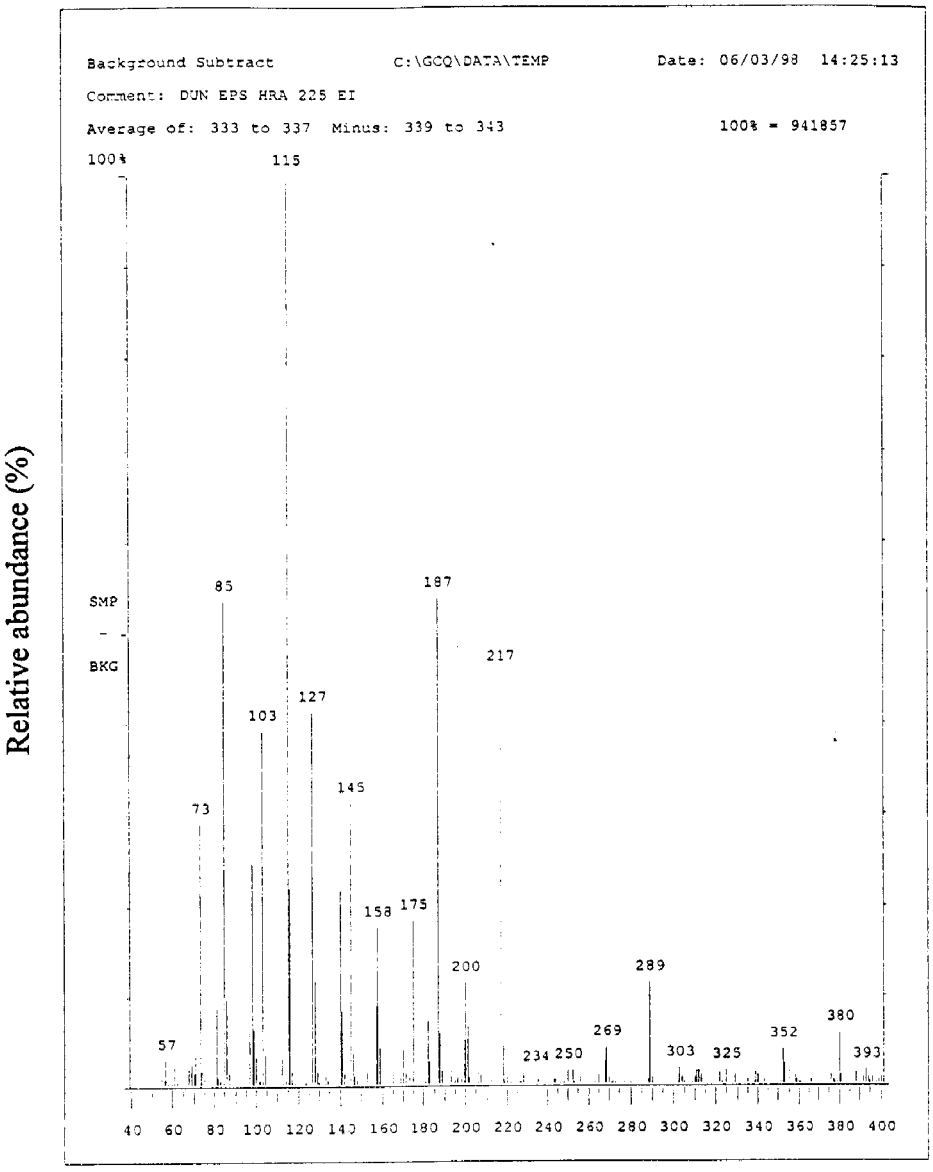


Standard curve for lead

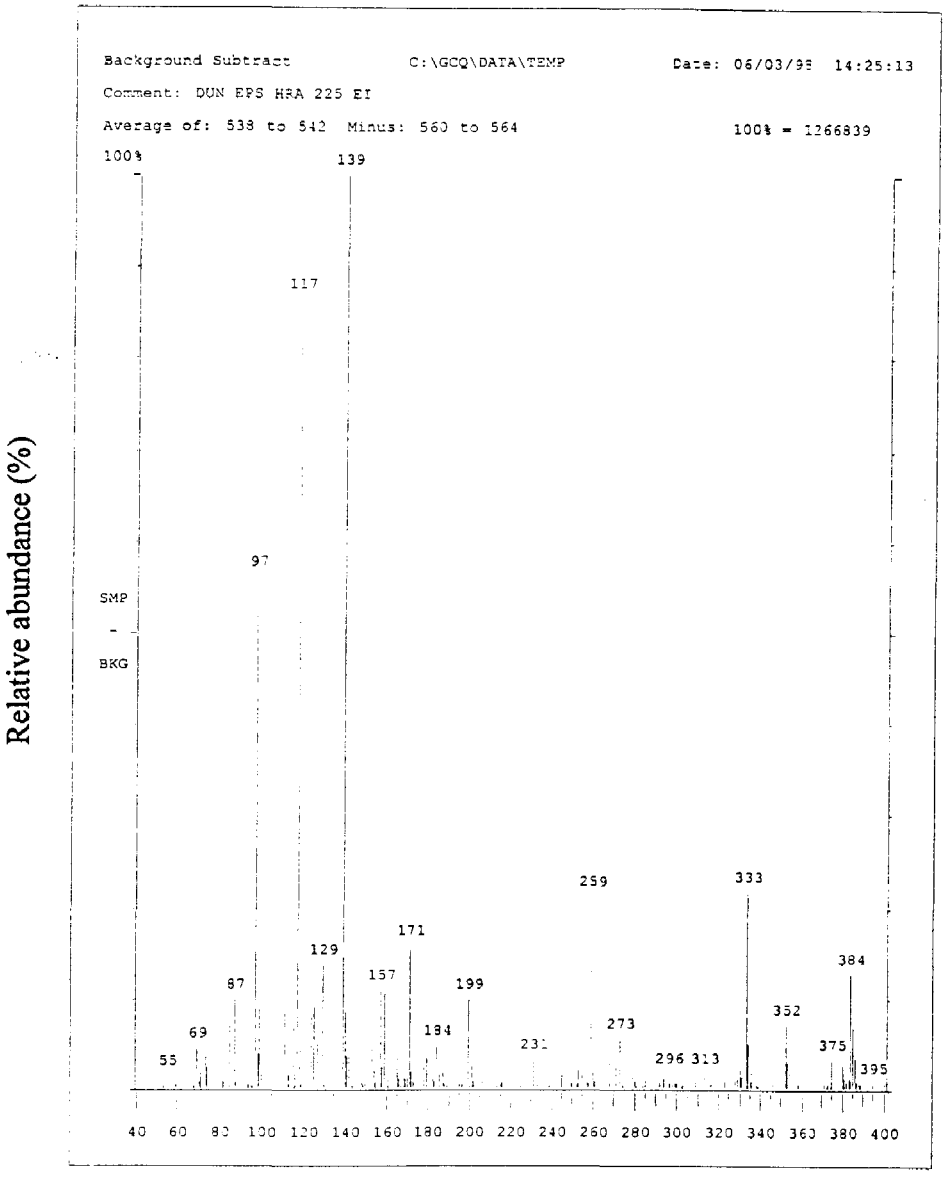
Appendix F : Mass spectra of Dunaliella exopolysaccharides



A typical mass spectrum of a pentose sugar (alditol acetate derivative) from *Dunaliella* exopolysaccharide.



A typical mass spectrum of a hexose sugar (alditol acetate derivative) from *Dunaliella* exopolysaccharide.



A typical mass spectrum of a 2-O-methyl hexose sugar (alditol acetate derivative) from *Dunaliella* exopolysaccharide.

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