STUDIES ON GELLAN GUM

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This thesis is dedicated to the memory
of James and Charlotte Curran.

Declaration

I declare that this thesis was composed by myself and that the work presented herein is my own.

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ABSTRACT

The composition of gellan gum was studied by gas chromatographic methods. The Hakamori method for GC/MS analysis revealed the presence of 33% 1,4 rha, 30% 1,3 glc and 31% 1,4 glc. Following carboxyreduction using the tetrahydrofuran method, the monosaccharide sugars present were, 24% 1,4 rha, 24% 1,3 glc, 26% 1,4 glc and 25% 1,4,6 glc (corresponding to glcA). This agreed with the findings of O'Neill *et al.* (1983) and Jansson *et al.* (1983) who simultaneously elucidated the structure of gellan gum.

The production of EPS by twenty mutant strains of *Sphingomonas paucimobilis* was compared. Large differences in the yield of polymer were evident. The chemical composition, viscosity, gelation and rheological properties of EPS from some of these strains were further investigated revealing differences in structure and physical properties.

The effect of varying nitrogen, carbon and magnesium concentration of the growth medium on the physiology and composition of gellan from *S. paucimobilis* WT was compared with two mucoid mutants, PA4 and SB10, and the growth of the non-mucoid, MJ8 strain. Decreased nitrogen concentration favoured the production of exopolysaccharide. EPS was also produced with carbon-limiting conditions.

Greater yields of EPS were obtained with reduced magnesium conditions although the polymer so produced contained up to 11% mannose in some strains. The culture medium viscosity was less than that obtained with non-limiting conditions, possibly indicating EPS of poorer rheological quality.

The wild type strain was believed to have undergone strain degeneration due to its low yield and altered composition compared with the findings obtained early in the course of this study.

ABBREVIATIONS

Ac	Acetyl
ATCC	American Type Culture Collection
c	Concentrated
CD	Circular Dichroism
CF	Cystic Fibrosis
CoA	Coenzyme A
CPS	Capsular Polysaccharide
dH_2O	Distilled Water
DM	Davis Mingioli Medium
DMSO	Dimethyl Sulphoxide
d O_2	Dissolved Oxygen
DSC	Differential Scanning Calorimetry
ECR	Effective Carbon Response
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl Methane Sulphonate
EPS	Extracellular Polysaccharide
EtOH	Ethanol
fuc	Fucose
gal	Galactose
GC/MS	Gas chromatography/Mass spectrometry
GDP	Guanosine Diphosphate
glc	Glucose
GLC	Gas Liquid Chromatography
glcA	Glucuronic acid
gulA	Guluronic acid
HPLC	High Performance Liquid Chromatography
ID	Internal Diameter
LPS	Lipopolysaccharide
man	Mannose
manA	Mannuronic acid
MeOH	Methanol
NCIB	National Collection of Industrial Bacteria
NCIMB	National Collection of Industrial and Marine Bacteria
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PEP	Phosphoenol pyruvate
PHB	Poly-ß-hydroxybutyrate
Pi	Inorganic Phosphate
PMAAs	Partially Methylated Alditol Acetates
psi	Pounds per square inch
pyr	Pyruvate
rha	Rhamnose
rpm	Revolutions per minute
TCA	Tricarboxylic acid
YE	Yeast Extract
η	Viscosity

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Water soluble polymers are frequently used as thickening, suspending and gelling agents. Starches, celluloses and polyacrylamides are used predominantly, along with substantial quantities of plant gums such as locust bean and guar. Despite costly collection and extraction, the algal polysaccharides, alginate, carrageenan and agar, are also used extensively (Pace & Righelato, 1980). Some uses are shown in Table 1.1.

Due to the increased cost of plant and algal gums, manufacturers have been encouraged to look more closely at the industrial production of gums. Microbial polysaccharides may be used as alternatives to synthetic or natural water-soluble polymers with properties almost identical to currently used gums or with improved rheological characteristics. They are not vulnerable to crop failure, climatic conditions or marine pollution like plant and algal gums (Sandford *et al.*, 1984).

In the late 1950's, the Northern Regional Research Labs in Peoria, Illinois studied the anionic heteropolysaccharide from *Xanthomonas campestris* - xanthan gum (figure 1.1). This polymer was remarkably stable to heat, acids, alkalis, and was shown to exhibit high viscosities at low concentrations and low shear rates. These remarkable thixotropic properties of xanthan have encouraged and stimulated work in many labs seeking improved methods of production and other microbial polysaccharides with novel properties (Pace & Righelato, 1980).

1.1 Differentiation of Exopolysaccharide

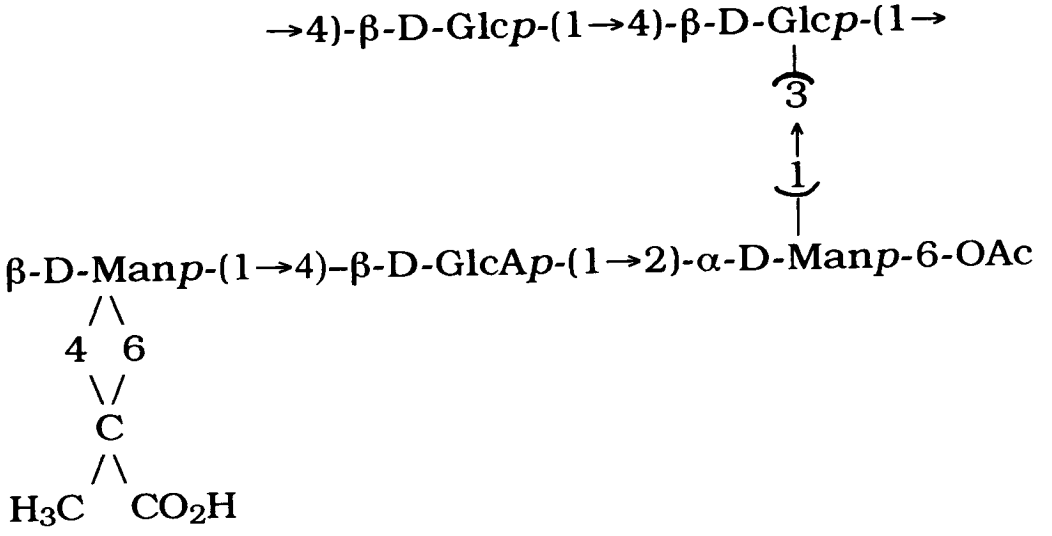
Extracellular polysaccharides (EPS) comprise capsule and slime secreted by many bacteria and form an interface between the bacterial cell and the environment (Morris & Miles, 1986). EPS can be CAPSULE which is firmly associated with the cell surface by covalent binding, or SLIME which is loosely associated with the cell surface. Differentiation between the two forms may be difficult, since cells producing large amounts of capsular polysaccharide may release some material at the periphery, giving the appearance of slime production (Whitfield, 1988). Mutations between capsule and

Table 1.1. General industrial applications of polysaccharides

Market (and application)	Properties	Polysaccharides
Adhesives (wallpaper)	Thickening	Alginate Starch
Agriculture (Flowable pesticides)	suspension drift control	Xanthan
Detergents	Antiredeposition	Carboxymethyl cellulose
Explosives (nitrate slurries)	Water resistance	Guar gum Xanthan
Fire fighting	Foam stabilisation	Guar gum guar derivatives
Ink	Rheology	Gum arabic
Oilfield (drilling muds)	Viscosity, suspension	Xanthan Cellulose ethers
(enhanced recovery)	Viscosity	
Paint (industrial coatings)	Rheology - suspension	Hydroxyethyl cellulose
Paper (coatings)	Rheology	Alginate
Photography	Antistatic coating	Sodium cellulose sulphate
Room deodorant gels	Stable gels	Carrageenan
Textiles (warp sizing)	Film forming	Starch, modified starch
Textiles (pigment printing)	Rheology - binder compatibility	Guar gum Locust bean gum

From Sandford *et al.* (1984).

Figure 1.1: Chemical repeat unit of xanthan from *Xanthomonas campestris* (Jansson *et al.*, 1975)



slime are known. Most bacteria have preference for one type or another; one *Klebsiella* strain can produce chemically identical slime and capsule (Wilkinson *et al.*, 1954).

Several bacteria, including strains of *Rhizobium* sp., *Agrobacterium* sp., and *Alcaligenes faecalis* var. *myxogenes* are able to synthesize more than one chemically distinct exopolymer (Sutherland, 1985).

Distinction between wall polymers and EPS can often be difficult although in capsular gram negative bacteria lipopolysaccharide (LPS) and EPS may be distinguished by immunoelectron microscopy (Bayer *et al.*, 1986). In the laboratory EPS is probably not essential, although it is widely found in newly isolated bacteria (Sutherland, 1989).

There has been much speculation about the function of EPS but few definitive answers. Chemical structure and the mode of biosynthesis of EPS have been widely studied (Kenne & Lindberg, 1983), but until recently less was known about genetics and regulation of polymer synthesis.

1.2 Biological functions of EPS

Little is known about the natural function of EPS and most proposed roles, such as a protective function or as virulence factors, are speculative. The number of studies on the molecular basis of functional properties *in situ* is limited. The ability of bacteria to produce a capsule may be a response to selective pressure in natural environments. The ability of microbes to surround themselves in a highly hydrated EPS layer may provide protection against desiccation and predation by protozoans. The physical properties of capsular polysaccharides (CPS) (hydrophilicity, charge and viscosity), may also increase protection. A layer of polysaccharide gel around a cell may also affect the diffusion properties of nutrients into and out of the cell (Dudman, 1977) and render the cell inaccessible to antibacterial agents (Costerton *et al.*, 1987). As many polymers are anionic they may also bind and affect the penetration to the cell surface of useful and toxic metal ions (Dudman, 1977).

1.3 Chemical composition of EPS

The carbohydrate components in microbial EPS comprise most of the sugars found in plant and animal polysaccharide e.g. neutral hexoses (D-glucose, D-galactose, D-mannose) and 6-deoxyhexoses (L-fucose, L-rhamnose), plus a number of previously unknown sugars (Sutherland, 1977). Acetylaminosugars are mainly restricted to certain bacteria e.g. *S. aureus*, and *S. pneumoniae* (Jansson *et al.*, 1981) and are not found in polymers from *Klebsiella, Rhizobium* and *Xanthomonas*. Uronic acids are common constituents of EPS especially D-glucuronic acid. D-galacturonic acid is less common. Only a small number of polymers possess D-mannuronic acid which may be found in bacterial alginates together with L-guluronic acid (Linker & Jones, 1964; 1966, Gorin & Spencer, 1966). There are also some unusual sugars previously thought to be found only in LPS i.e. ketodeoxyocturonic acid (KDO) (Jann *et al.*, 1980).

In addition to carbohydrate components, there are also ester-linked substituents and pyruvate ketals. Ester-linked substituents do not normally contribute to the overall charge on polymers, while pyruvate ketals contribute to the ionic nature of polymers (Sutherland, 1977).

The most common ester linked constituent is acetate but propionyl groups may be found in *E. coli* K14 (Jann & Jann, 1983) and ester linked glyceryl in *Pseudomonas elodea* (Kuo *et al.*, 1986). In each case these components are present in addition to acetyl groups. Ester-linked succinyl groups can also be found in addition to O-acetyl in certain *Agrobacterium* species and in the EPS from *R. trifolii,* the content varying from 0.4-7.4% (Hisamatsu *et al.*, 1978; Amemura & Harada, 1983). Also present in some bacterial polysaccharides, *R. trifolii* and *R. leguminosarum,* are ester linked residues of 3-OH butanoate attached to D-galactose (Hollingsworth *et al.*, 1984; 1987; Kuo & Mort, 1986).

1.4 Structure of EPS

Exopolysaccharide can be of two general types.

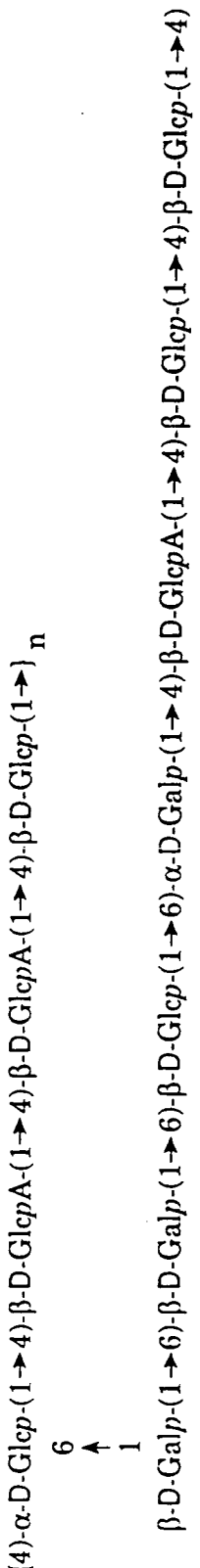
Homopolysaccharides are composed of a single monosaccharide e.g. cellulose, levans and dextrans (Jeanes, 1974), all of which are neutral homopolymers. Some bacterial alginates can also be composed solely of D-mannuronic acid (Haug *et al.*, 1966). Among

homopolysaccharides, some examples of repeat structure can be found. Scleroglucan, the fungal polymer from *Sclerotium* species, is a linear β 1→3 linked glucan with single glucosyl sidechains attached to every third main chain residue, effectively a tetrasaccharide repeat unit (Rodgers, 1973).

Heteropolysaccharides are almost without exception composed of regularly repeating units varying in size from disaccharides to repeat units containing 11 sugars. An example of the latter is the complicated repeat unit of *R. leguminosarum* pv. *phaseoli* 127K87 (figure1.2) (Morris, 1992). Usually heteropolysaccharides are charged. Repeat units may be linear, although it is more common to have a short sidechain, varying from 1 to 4 sugars in length. Very rarely sidechains are also branched. An example of this is found in *K. aerogenes* type 67 which is also composed of 5 different monosaccharides (Dutton & Karunaratne, 1983). A few polysaccharides have several sidechains each attached to monosaccharides on the main chain. *K. aerogenes* type 60 has 3 sidechains per repeat unit (Dutton & Di Fabio, 1980). Increasingly, microorganisms with the ability to synthesise more than one polymer have been reported. It is unclear how simultaneous synthesis of polysaccharide with common constituent sugars (and presumably common sugar nucleotide precursors) is regulated. Only in a few systems has there been an attempt to study regulation of polysaccharide synthesis and determine the nature of any control mechanisms in operation. *R. meliloti* strain 201 produces two extracellular acidic polysaccharides, one which contains glc, gal, man and pyr in the molar ratio 4:3:2:1 (Yu *et al.*, 1981) and the second polymer glc:man:glcA in the ratio 3:3:2. A marine pseudomonad synthesised two polymers of entirely different composition with no shared components (Christensen *et al.*, 1985).

Non-carbohydrate constituents of extracellular polysaccharides are frequently present in non stoichiometric amounts and may vary depending on either growth conditions or growth phase (Sherwood *et al.*, 1984; Sutherland, 1982). Most xanthan preparations contain approximately equimolar amounts of glucose and mannose, and half the amount of glucuronic acid and acetate. Pyruvate is only found on

Figure 1.2: Chemical repeat unit of *Rhizobium leguminosarum* pv. *phaseoli* 127K87 (after Morris, 1992).



about a third of the available terminal β-D-mannosyl residues (Jansson *et al.*, 1975). Perhaps because of its larger repeat unit and greater degree of complexity, xanthan shows considerable variability, especially when bacteria are grown under different physiological conditions. The pyruvate and acetyl content of products from a single strain may both vary (Tait *et al.*, 1986). Portions of the main chain may be deficient in side chain trisaccharide. This has been determined with enzymic studies using cellulases (Sutherland, 1984a) and from physical studies on xanthan solutions (Milas & Rinaudo, 1986).

1.5 Biosynthesis and Control of EPS

Most EPS are presumed to be synthesised by cellular mechanisms identical or similar to those involved in cell wall synthesis; only dextrans and levans are synthesised extracellularly (Pace & Righelato, 1980). Most EPS are synthesised by particulate enzyme systems which have as their precursors nucleotide diphosphate sugars or, more rarely, nucleotide monophosphate sugars, as activated glycosyl donors (Troy, 1979). The sugars of the repeating unit are added sequentially by specific transferases to a C55 isoprenoid alcohol phosphate. This is identical to the carrier lipid previously described in the biosynthesis of peptidoglycan and lipopolysaccharide O-antigen (Troy *et al.*, 1971). The isoprenoid alcohol is thought to be responsible for facilitating the accurate and ordered formation of the repeating unit structure (Sutherland, 1982; Troy, 1979); and in solubilisation in the hydrophilic membrane domain and transport across the membrane.

It has been suggested by Sutherland (1977) that the availability of isoprenoid alcohol carrier is a determinant of the rate of exopolysaccharide synthesis. The carrier is common to cell wall polysaccharide and EPS synthesis, therefore, lower rates of production of EPS in growing bacteria, could be due to competition from cell wall synthesis, for the carrier lipid.

1.5.1 Acetate and pyruvate substituents

Acetyl coenzyme A and phosphoenol pyruvate (PEP) serve as precursors for acetate and pyruvate and are added to the polymer at

the level of lipid intermediate. In *Klebsiella* type 8 EPS, the addition of pyruvate groups was necessary for the subsequent completion of repeating units (Sutherland, 1977). In EPS where the degree of substitution is not stoichiometric, and may vary with growth conditions (e.g. *Xanthomonas campestris*), substitution reactions do not appear to be prerequisites for assembly of the glycan structure (Ielpi *et al.*, 1981a; 1983).

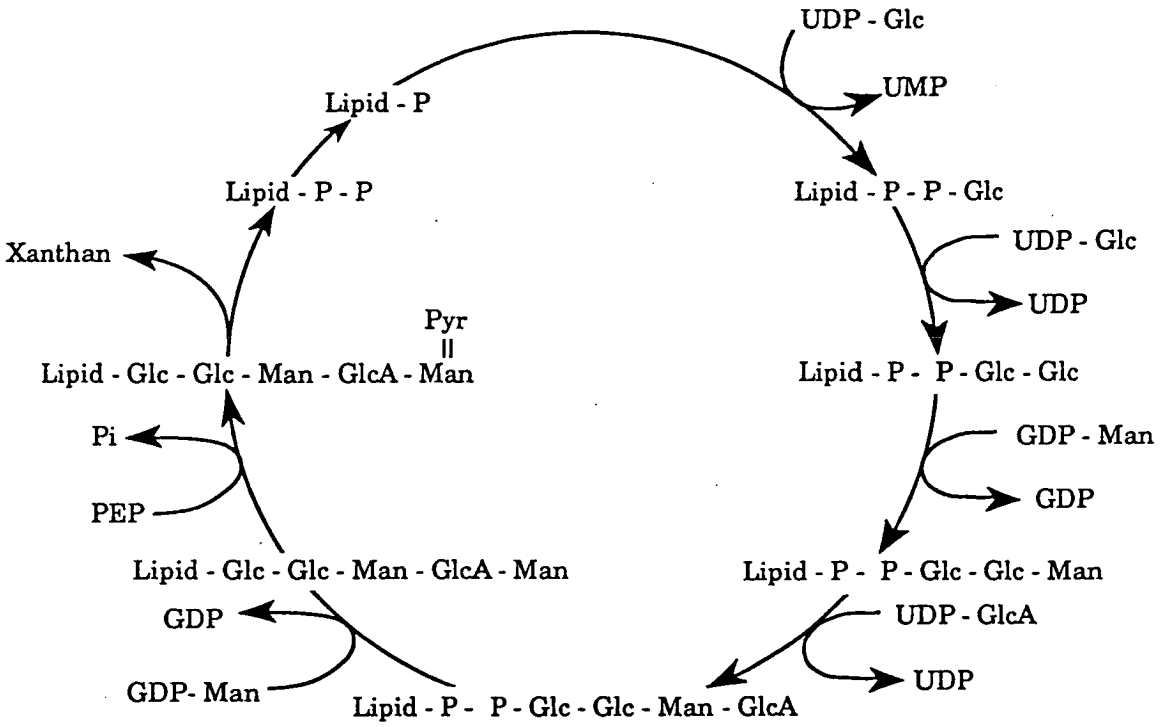
1.5.2 Role of acetate in alginate biosynthesis

In *A. vinelandii*, polymannuronic acid is synthesised first by the organism (Larsen & Haug, 1971). Subsequently, a C5 epimerisation of some mannuronate residues due to an extracellular epimerase occurs, converting mannuronic acid (manA) groups to guluronic acid (gulA) (Pindar & Bucke, 1975). O-acetyl groups on ManA may prevent this epimerisation reaction (Davidson *et al.*, 1977).
The ratio of ManA/GulA in *A. vinelandii*, and distribution of guluronic acid residues, may also depend on the calcium ion concentration which influences the epimerase activity. Molecular weights from *A. vinelandii* are polydisperse and an extracellular alginate lyase may play a part in determining the molecular weight distribution (Haug & Larsen, 1971).

1.5.3 Elucidation of xanthan biosynthetic pathway

Xanthan biosynthesis has been demonstrated using ethylene diamine tetraacetic acid (EDTA) treated bacteria (figure 1.3). The product was shown to be a pentasaccharide unit containing acetyl and pyruvate groups (Jansson *et al.*, 1975). Cellobiosyl-P-P-lipid was identified on addition of UDP-^{14}C glc to EDTA treated cells (Ielpi *et al.*, 1981a). A trisaccharide was then formed on addition of GDP-mannose and pentasaccharide produced if UDP-glucuronic acid and GDP-mannose were added. The polymer became acetylated using Acetyl CoA at the lipid intermediate stage (Ielpi *et al.*, 1983) while pyruvate was added to the terminal -mannosyl residue from phosphoenol pyruvate (PEP) (Ielpi *et al.*, 1981b).

Figure 1.3: A scheme for xanthan biosynthesis (after Ielpi *et al.*, 1981b)



1.6 Physiological Conditions for EPS Production

1.6.1 Medium composition

Most EPS producing microorganisms that have been studied require carbohydrates as their carbon and energy source. However, many different carbon sources can be converted into EPS e.g. amino acids, fatty acids, TCA cycle components and hydrocarbons(Sutherland, 1990). Ammonium salts or amino acids are the most common nitrogen sources used by bacteria yielding exopolymer (Sutherland, 1982). Various ions are known to be required either for substrate uptake or as cofactors in polysaccharide synthesis. Polysaccharide synthesis in washed suspensions of *Enterobacter aerogenes* was stimulated by Mg^{2+}, K^{+} and Ca^{2+} ions (Wilkinson & Stark, 1956). Other ions, amino acids and growth factors may also be required in small quantities and are often added to the culture medium in the form of yeast extract or casein hydrolysate. Phosphate is the major anionic requirement of microbial cells.

In the production of xanthan gum from sucrose, higher polymer yields were obtained following the addition of small amounts of pyruvate, succinate or α-ketoglutarate to the growth medium. This may lead to an improved metabolic balance between carbon flow from hexose substrate through the hexose monophosphate and Entner-Duodoroff pathways and oxidation through the TCA (Krebs) cycle (Souw & Demain, 1979).

Most EPS producing microorganisms are either aerobes or facultative anaerobes. In the latter, EPS synthesis generally occurs only when the organism is grown aerobically. Culture aeration is an important requirement for polymer production in both prokaryotes and eukaryotes (Sutherland, 1977). However, not all microbial cells require maximal aeration for good production of polysaccharides (Dudman, 1960). *K. aerogenes* requires vigorous aeration for maximal polysaccharide synthesis (Duguid & Wilkinson, 1953).

1.6.2 Effect of carbon substrate

Most studies on the effect of varying physiological conditions have been made with gram negative bacteria. However, physiological conditions have a profound effect on EPS production in all types of bacteria. In a study of *Lactobacillus casei* subspecies *rhamnosus*

Wicken *et al.*, (1983) observed that whereas wall polysaccharide remained constant under different culture conditions, capsular polysaccharide production in batch culture was dependent on the carbon substrate. In continuous culture, capsule synthesis was affected by the dilution rate and the nature of the limiting carbon substrate. There was no indication of changes in the composition of the capsular material.

Alginates are synthesised by several plant pathogenic *Pseudomonas* species, production being dependent on the availability of glucose or gluconate as carbon substrate. When sucrose was used as carbon substrate, some strains produced only alginate while others yielded a mixture of levans and alginate (Fett *et al.*, 1986).

The effect of altering the culture medium composition was studied to determine the effect on xanthan production by *X. campestris* (Souw & Demain, 1979). Sucrose was found to be a better carbon source than glucose for xanthan production. However, when the sucrose concentration was greater than 4%, polysaccharide yield decreased, an effect not found when glucose was used as carbon source.

1.6.3 Effect of limiting nutrient conditions

The amount of (carbon) substrate converted by the cell to EPS depends on the composition of the growth medium. Generally media which contain a high carbon to limiting nutrient ratio, often nitrogen, are favoured for polysaccharide production (Duguid & Wilkinson, 1953). The optimum C:N ratio for growth and polymer yield was found to be 10:1 in *Chromobacterium violaceum* (Corpe, 1964).

The type of growth limiting substrate has also been found to influence the composition and rheological properties of polysaccharide produced by *Xanthomonas* species. In a study by Evans *et al.*, (1979) different amounts of rhamnose were reported in EPS from *Xanthomonas juglandis* when the organism was grown on different limiting substrates. Davidson (1978) reported relatively slight differences in glucose:mannose ratios in xanthan grown under various nutrient limitations but found man, glcA and pyr concentrations decreased under magnesium limitation. This suggests there may be apparent defects in the side chains of xanthan gum. Similar effects were found with phosphate limitation.

When the carbohydrate composition of xanthan was studied (Tait *et al.*, 1986) under sulphate limitation, it was found to be relatively constant over a range of dilution rates although the acyl content decreased with increasing dilution rate as did the consistency index. In batch culture, acylation appeared to reach a maximum immediately prior to the termination of logarithmic growth. Large differences in maximum consistency indices were reported during or after log growth perhaps due to a lower relative molecular mass of polysaccharide as cells grew rapidly. The viscosity and the chemical composition of xanthan from *Xanthomonas juglandis* were also found to be dependent on physical conditions. (Evans *et al.*, 1979).

In a study on *R. trifolii* (DeHollander *et al.*, 1979) there was good polysaccharide synthesis under nutrient limiting conditions when nitrogen, sulphur or phosphate was used as the limiting nutrient and carbon source was in excess. However, it is now clear that a number of different bacteria produce appreciable amounts of polysaccharide even under carbon limitation.

Dicks & Tempest (1967) reported lower yields of polysaccharide produced under K limitation. This was later thought to be due to an inhibition in nutrient uptake where there was competition between K^+ and NH_4^+ for a common transport system. A study by Ferrala *et al.*, (1986) on the morphology of capsular polysaccharide (CPS) in *Azotobacter chroococcum* had similar findings. *A. chroococcum* is a nitrogen fixing microbe which produces an acidic heteropolysaccharide. Growth of these bacteria caused changes in the appearance of ruthenium red stained capsular material examined by electron microscopy. Care was taken to avoid changes that might otherwise be due to the encystment process. When grown under non limiting conditions the CPS appeared as an electron dense capsular material closely associated with the outermost layers of the wall/membrane complex. Under iron limitation the capsule appeared to be more extensive and diffuse. With molybdate limitation this change was less marked, whereas simultaneous limitation of both ions revealed little difference from samples obtained under iron limitation. By contrast, cells allowed to encyst differed little when grown under metal ion sufficiency or deficiency. In nitrogen-fixing species, such as *A. chroococcum*, a limitation in Fe/Mo is probably equivalent to an extreme form of nitrogen limitation (Sutherland, 1988) (Table 1.2 shows a selection of nutrient limitation studies).

Table 1.2: Nutrient limitation studies

Microorganism	Polymer Produced	Reference
X. campestris	xanthan	Moraine & Rogovin (1971) Davidson (1978) Tait, (1984)
K. aerogenes	EPS	Neijssel & Tempest (1975)
A. vinelandii	alginate	Deavin *et al.* (1977) Jarman *et al.* (1978)
Pseudomonas NCIB 11264	acetylated EPS containing glc, gal, rha, man	Williams & Wimpenny (1977; 1978)
P. aeruginosa	alginate	Main *et al.* (1978)
R. trifolii	EPS	DeHollander *et al.* (1979)
X. juglandis	xanthan	Evans *et al.* (1979)
Z. ramigera	EPS	Norberg & Enfors (1982)

1.6.4 Batch cultivation of EPS

The chemical composition and physical properties of EPS synthesised in batch cultures are now known to depend on the physiological conditions present at the time of synthesis. Unfortunately, many studies have used batch culture material that represents the total synthesis of the bacterium, i.e. a pool of macromolecular types that probably varies considerably in both carbohydrate composition and acylation (Sutherland, 1988).

Exopolysaccharide is produced at different stages of the growth phase, for different organisms. In their studies on EPS production by *Enterobacter aerogenes,* Duguid & Wilkinson (1953) noted that, under the conditions used, bacterial growth ceased after 24 hours. Much of the polysaccharide was found after bacterial growth had ceased, but, the rate of polymer production was greatest in the logarithmic growth phase. Thereafter, the production rate declined. With *Pseudomonas* NCIB 11264, EPS production was apparently a feature of the late logarithmic and early stationary phases of batch culture growth (Williams & Wimpenny, 1977). In *P. aeruginosa* the maximum rate of polysaccharide synthesis was noted during exponential growth and little more polymer was formed when growth ceased (Mian *et al.*, 1978). Alginate production in *A. vinelandii* was reported to continue throughout both the exponential and stationary phases (Horan *et al.*, 1981).

In batch culture, parameters like pH and optimum media composition can be rapidly determined. There is likely to be a rapid pH change unless the media is heavily buffered. There may also be some oxygen limitation, which is difficult to determine in batch culture in shaken flasks. The substrate level, concentration of ions and growth factors all fluctuate throughout growth.

1.6.5 Continuous cultivation of EPS

In continuous culture, growth is controlled through a single limiting nutrient. Instrumentation permits the continuous measurement of such growth parameters as pH and dissolved oxygen concentration (dO_2). Air flow and impeller speed may also be controlled. Using different substrates and nutrient limitations, growth rate may also be

varied to determine the effect on polysaccharide production and composition.

When *X. campestris* was grown in continuous culture the xanthan produced with Mg or P limitation contained 1% pyruvate; whereas under C, N, K or S limitation it had 5.5-8.5% pyruvate. Polysaccharide yield and viscosity also varied according to dilution rate (Davidson, 1978). In *X. juglandis* the greatest polymer yields were obtained from N or S limitation. The polysaccharide produced also had higher viscosity than that obtained from K, Mg or C limitation (Evans *et al.*, 1979).

Polysaccharide production by *Pseudomonas* NCIB 11264 species was found to be dependent on temperature, pH, medium composition and also growth rate in continuous culture (Williams & Wimpenny, 1978). Maximum EPS production was obtained under nitrogen limitation; phosphate limitation yielding lower amounts of polymer. Optimal conversion of substrate was 73% compared to 34% in batch culture. Differences in polysaccharide were found when bacteria were grown under various conditions. In continuous culture the conversion of substrate into alginate varied between 56-64% in *P. aeruginosa* (Mian *et al.*, 1978). When the dilution rate increased from 0.05-0.1 h^{-1} the yield of alginate fell slightly, but the specific rate of polysaccharide synthesis increased. This increase in specific rate of EPS synthesis with specific growth rate was different from other bacterial species (e.g. *A. vinelandii*, *X. campestris* and *Pseudomonas* NCIB 11264) where the rate of polysaccharide synthesis was essentially independent of growth rate.

One of the main problems associated with the continuous cultivation of polysaccharides is bacterial strain instability. Variants with reduced or altered EPS production may emerge. If the variant can grow more rapidly than the polymer producing strain, it will quickly outgrow the original strain and cause loss of EPS production. In *X. juglandis* poor stability of the microbial strain was noted under C or K limitation (Evans *et al.*, 1979). While in *P. aeruginosa* (Mian *et al.*, 1978) non polysaccharide producing variants appeared after 7 generations.

1.6.6 Industrial production of polysaccharides

Commercial production of EPS is currently by batch fermentation, where the microorganisms are cultured under conditions that are both optimal for polymer production and economical in terms of substrate utilisation and conversion to product. Various continuous processes have been described but none have been used for industrial EPS production.

The high viscosity of the final fluid of EPS fermentation creates some problems for the industrial producer. Margaritas & Pace (1984) have described possible problems associated with continuous culture for EPS production. These include mass transfer phenomena, mixing characteristics, power requirements and product recovery.

The industrial user does not utilise analytical grade substrates because of cost. Pettit (1979) described xanthan production for which 1-5% carbon source was required (e.g. glucose, sucrose, starch). Nitrogen was obtained from NH_4^+ ions. Phosphonate buffer, Mg^{2+} ions and trace elements were also required. Alkali was added to maintain pH at 6-7.5 and glucose was added gradually during the 136 hour process.

All commercial fermentation processes require the use of stable microbial strains. Much effort has gone into strain selection for the production of polysaccharides such as xanthan. In addition to obtaining strains yielding high amounts of EPS, efforts have been made to isolate *Xanthomonas* strains devoid of colour, cellulase etc. Pseudomonads may convert much of the substrate (up to 50%) into polyhydroxybutyrate (PHB). The producer using such an organism would need to select a strain incapable of forming PHB or other unwanted products under the conditions employed (Sutherland, 1990).

1.7 Physical Properties of EPS

1.7.1 Order to disorder transition

Many microbial polysaccharides are known to undergo a transition from ordered to disordered states as the temperature of aqueous solutions increases. This process can be studied using techniques such as Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimetry (DSC), viscometry and circular dichroism (CD). Most

bacterial polysaccharides in aqueous solution at ambient temperature are in ordered state. Usually the transition temperature is above 30°C, with completion generally at 50-90°C (Wolf *et al.*, 1978).

Normally bacterial polymers undergo a single transition, but in solutions of *Klebsiella* type 8 polysaccharide there are two steps at 30°C and 42°C respectively. The first step involves a change from the initial helical conformation derived from interaction between uronic acid and the main chain hexoses, to a cooperative reorientation of uronic acid. Subsequently the backbone reoriented in another helical conformation which then underwent transition to a less ordered state as temperature increased (Sutherland, 1988).

1.7.2 Effect of ions on EPS

The majority of EPS are anionic and interact with various cations. This affects solution properties and gel formation of polysaccharides. Some EPS resemble algal alginates, and form gels with specific cations; others may form gels with a wide range of cations. If gels are not formed, cations may still have a marked effect on the physical properties of a polysaccharide solution. Some polymers may yield viscous solutions in distilled water whilst having greatly decreased viscosity, due to altered configuration, when salts are present.

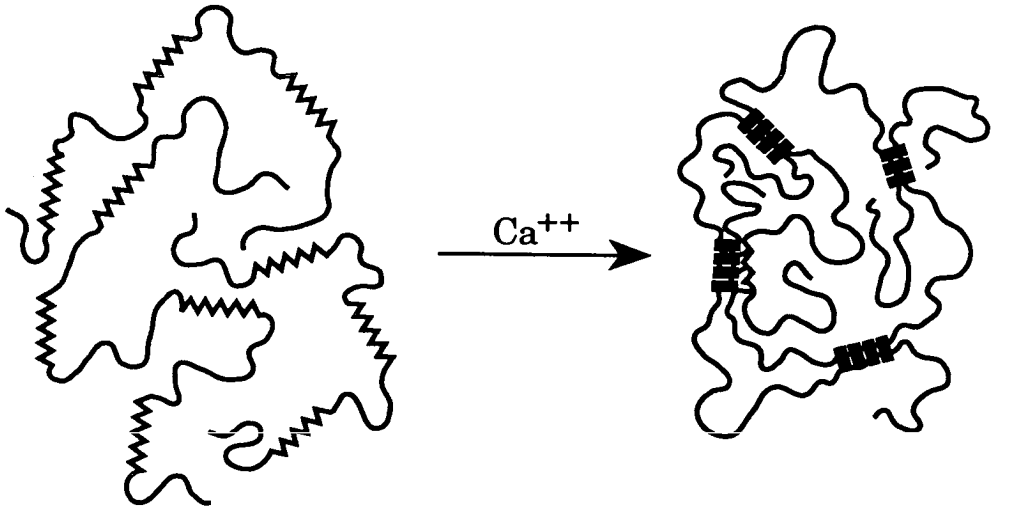
The effect of anions has also been studied (Clarke-Sturman *et al.*, 1986). Anions strongly influence order to disorder transition. High concentrations of Br^- or SCN^- decrease polymer stability by decreasing transition temperature whereas SO_4/PO_4 have the opposite effect if they do not cause polymer precipitation.

1.7.3 Gel formation in alginate

Alginate gelation has been studied using marine algal alginates. Gelation is promoted by the presence of Ca^{2+} and involves cooperative binding of the cations by polyguluronic acid sequences in polycrystalline junction zones (Rees, 1969; Bryce *et al.*, 1974).

An egg-box model was proposed by Grant *et al.* (1973) where there was regular packing and coordination of the cations in the interstices of associated extended sections of the polysaccharide chains (figure

Figure 1.4: The "egg-box" model for ion binding to alginates (after Grant *et al.*, 1973).



represents guluronate blocks

represents Ca^{++}

1.4). It was suggested that polyguluronate sequences were primarily responsible for interchain association and consequent gel formation. The polyguluronic acid, poly (-G-)$_n$, sequences possess a higher selectivity for Sr^{2+} than Ca^{2+} ions. Limited formation of junction zones occurs with Mg^{2+} and monovalent ions, although these do not cause gelation (Seale *et al.*, 1982). *Pseudomonas* alginates appear not to gel, due to lack of contiguous L-guluronic acid residues (Skjåk-Bræk *et al.*, 1986), although Ca^{2+} is strongly bound after deacetylation (Geddie, 1992).

1.7.4 Comparison of gelation in the polymers XM6 and K54

Comparative studies of EPS XM6 from *Enterobacter* NCIB 11870 (figure 1.5) and the capsular polysaccharide from *K. aerogenes* K54 (figure 1.6) show the importance of non-carbohydrate substituents in gelation. XM6 disperses in water to give a viscous liquid and at higher ionic strength produces thermoreversible gels (Nisbet et al., 1984). XM6 may be regarded (O'Neill *et al.*, 1986) as a naturally occurring, deacetylated form of the capsular polysaccharide of K54 (Dutton & Merrifield, 1982). K54 disperses in water as a viscous liquid but does not gel at a higher ionic strength. Deacetylation of K54 results in a polysaccharide which disperses in water and does gel at higher ionic strength. Therefore, the presence of a single O-acetyl group on the fucosyl residue of every alternative tetrasaccharide is sufficient to inhibit gelation (O'Neill *et al.*, 1986; Dutton & Merrifield, 1982).

Comparative X-ray fibre diffraction studies (Atkins, 1986) have been used to assess the helical conformations and the nature of any intermolecular association of XM6 and K54. XM6 yields highly crystalline X-ray diffraction patterns consistent with strong polymer-polymer interactions and gelation. K54 yields diffraction patterns with diffuse layer lines that are poorly crystalline, consistent with weak intermolecular interactions (Atkins *et al.*, 1979). This indicates poor polymer-polymer association and an inability to gel. Deacetylation of K54 produces the highly crystalline diffraction patterns characteristic of XM6. This suggests a change in helical conformation on deacetylation. However, meridional spacings in K54 and XM6 patterns remain unchanged and it is the layer line spacing

Figure 1.5: Chemical repeat unit of the polysaccharide XM6 from *Enterobacter* NCIB 11870 (after O'Neill *et al.*, 1986).

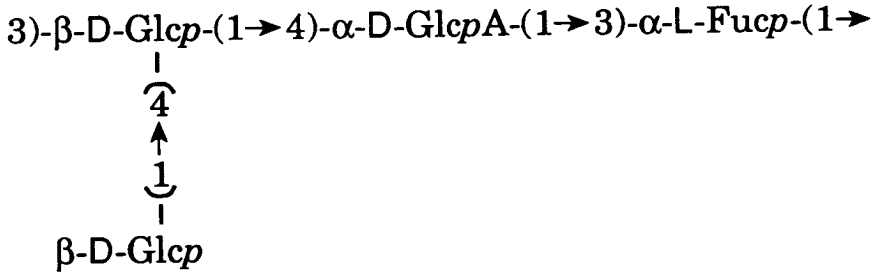
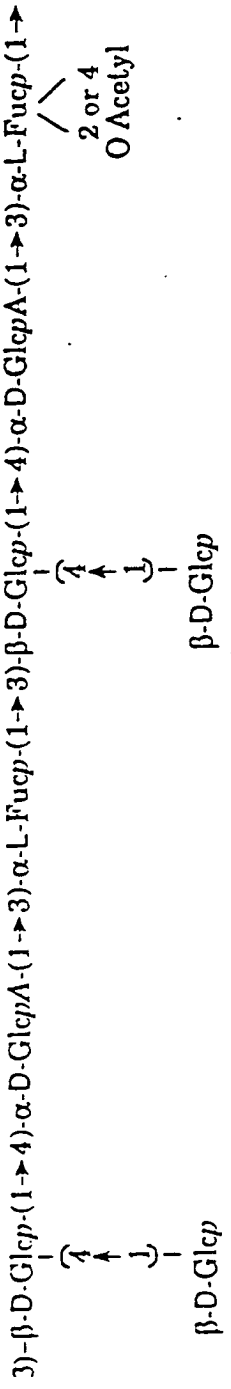


Figure 1.6: Chemical repeat unit of the polysaccharide K54 from *Klebsiella aerogenes* serotype K54 (Dutton & Merrifield, 1982).



which appears to change on deacetylation. It is thought that both K54 and XM6 adopt a relatively rigid double helical structure (Atkins 1986). The K54 diffraction pattern is considered to be a poorly resolved pattern of the characteristic XM6 pattern. Acetylation does not appear to alter the helical conformation but controls the intermolecular association and crystallisation of polysaccharide chains and, hence, gelation.

1.8 Gellan gum

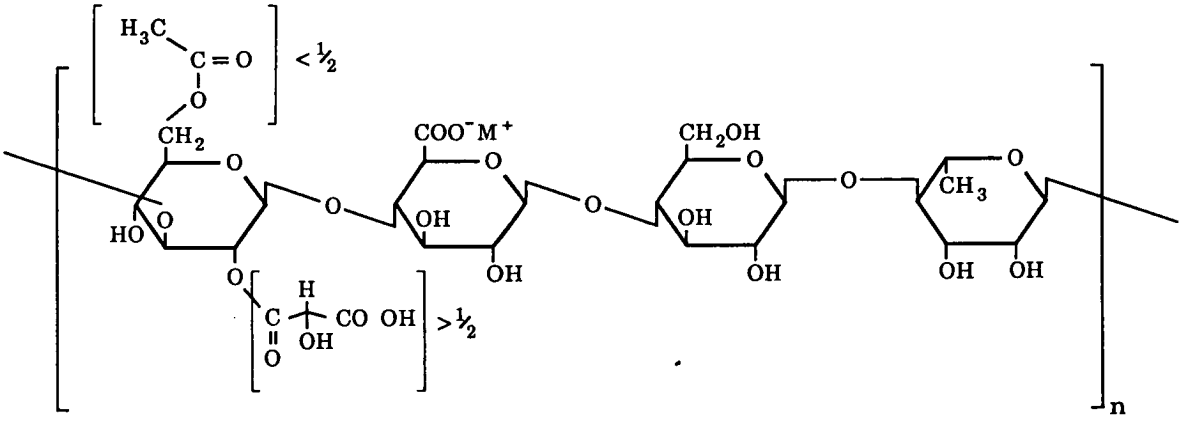
1.8.1 Elucidation of the chemical structure of gellan gum

The Kelco Division of Merck have discovered and commercialised gellan gum and a series of structurally related biopolymers. Gellan gum, known also as PS-60 or S-60 (Kang *et al.*, 1982) is the linear, anionic heteropolysaccharide produced by *Pseudomonas elodea* by aerobic fermentation (figure 1.7). *P. elodea* is a gram-negative, rod-shaped, non capsulated bacteria approximately 2.5 x 0.7 μm long. It produces poly-β-hydroxybutyrate and is motile by polar multitrichous flagella. *Pseudomonas elodea* has recently been reclassified as *Sphingomonas paucimobilis*, along with related species producing polymers similar to gellan gum (Pollock, 1993). *P. elodea* was called *Auromonas elodea* previous to this (Moorehouse, 1987). Biochemical data and microbiological characteristics of both *P. elodea* and *S. paucimobilis* have been described in detail (Kang *et al.*, 1982; Lobas *et al.*, 1992; Pollock, 1993).

Elucidation of the structure of gellan gum was achieved simultaneously by Jansson *et al.* (1983) and O'Neill *et al.* (1983). It was found to be a linear tetrasaccharide repeating structure of D-glucose, D-glucuronic acid and L-rhamnose in the ratio 2:1:1. Methylation analysis revealed the four sugars in equimolar amounts. while NMR studies showed that three of the sugars were in the β-configuration with only L-rhamnose in the α-configuration.

The polymer was believed to contain 6% O-acetyl groups (Brownsey *et al.*, 1984) found to be O-6 linked at the 1→3 glucose residue (Carroll *et al.*, 1983). Later L-glycerate was also found to be present in the gellan gum. The glyceric acid moiety was O-2 linked to the same molecule bearing the acetyl ester. Acetate was previously thought to

Figure 1.7: Chemical repeat unit of gellan gum elaborated by *Pseudomonas elodea* NCIMB 12171 (O'Neill *et al.*, 1983; Jansson *et al.*, 1983).



be the only alkali labile substituent present on one in three of the tetrasaccharide repeats (Kuo *et al.*, 1986). It has recently been suggested that polyhydroxybutyrate may also be a substituent of the gellan repeat unit (Giammatteo *et al.*, 1990).

1.8.2 Commercial interest in gellan gum as a food polymer

Gellan has created much interest as a potential food hydrocolloid due to its wide range of gel textures and its many diverse applications. Successful toxicity trials have been completed and gellan has received food approval in Japan and limited food approval in the USA. Food approval is being sought in the UK and Europe (Morris, 1992). The prospect of replacing many of the currently used food polymers with one "universal gelling agent" is a welcome idea for many food manufacturers (Sanderson *et al.*, 1983). The potential food uses of gellan are outlined in Table 1.3.

The exact response a hydrocolloid will have to a range of adverse conditions (heat, pressure, shear, time, change in ion concentration) must all be taken into account when considering its suitability as a food gelling agent. The response of the polymer to these conditions will influence the processing techniques to which a product is subjected (Attwool, 1987). The temperature change in transit, and on display, may alter the product and lessen its appeal to the customer. Gellan gum is suitable as a food gelling agent due to its stability when subjected to a wide range of adverse conditions.

1.8.3 Gellan gum as an agar replacement in growth media

Gellan gum may be used as an agar replacement in microbiological media. It is sold under the tradename GELRITE. A large range of gel strengths are available by adjusting the concentration of gellan and the ion concentration used for gelation. Generally media is of strength 200-400g/cm^2. The gels are stable to multiple autoclavings, melt/set reversibly like agar, and are chemically inert to most biological growth media additives (Kang *et al.*, 1982b). The two main applications in this area are tissue culture media and the culturing of sensitive microbes which are sometimes inhibited by the impurities in agar.

Table 1.3. Some potential food applications of gellan gum (after Baird *et al.*, 1983)

Major food area	Typical products	Current gels/thickeners
Confectionery	Starch jellies, Pectin jellies, marshmallows	Pectin, starch, gelatin, agar xanthan, locust bean gum
Jams and Jellies	Low cal. jams imitation jams, bakery fillings, jellies	Pectin, alginate, carrageenan
Fabricated foods	fabricated fruits, vegetables & meats	alginate, carrageenan, locust bean gum
Water based gels	Dessert gels, Aspics	Gelatin, alginate, carrageenan
Pie fillings & puddings	Instant desserts, canned puddings, pie fillings	starch, carrageenan, alginate
Pet foods	canned and gelled pet foods	alginate, carrageenan, locust bean gum
Icings and frostings	Bakery icings, canned frostings	agar, starch, pectin, xanthan/guar gum
Dairy products	yoghurt, gelled milk, ice cream, milk shakes	carrageenan, gelatin, alginate, guar gum, locust bean gum

With asexual reproduction of plants from small pieces of plant tissue or single plant cells, new plants may be produced in a manner which bypasses the normal seed or spore stage. This eliminates genetic variations producing plants with identical characteristics to the donor plant. Time is saved in not having to produce a seed crop. Greater control can be obtained over the quality and uniformity of the desired final crop (Ichi *et al.*, 1986). Gelling agents form a support matrix on which the plant develops. Nutrients and appropriate growth regulators are placed into a container with the gelling agents under sterile conditions. The plant tissue or single cell is placed on the gel and several weeks later the plant has developed to a point where it can be transplanted and allowed to develop further (Baird *et al.*, 1983). The gelrite matrix is free from the plant impurities or sulphates associated with agar. The plant develops rapidly, not because gellan stimulates growth but because it has less of an inhibitory effect than some other gelled media (Colegrove, 1983).
Gelrite compared favourably with agar for the growth of clinically important (Shungu *et al.*, 1983) and thermophilic (Lin & Casida, 1984) eubacteria. A study was also carried out to determine the value of gellan gum as an agar substitute in the cultivation of mesophilic *Methanobacterium* and *Methanobrevibacter* species (Harris, 1985). Gelrite was found to be optically clearer and drier than agar at an equivalent concentration; this reduced smearing of colonies by condensing water, which, in turn, made colony counting more accurate. Preparation time of plates was reduced due to the higher gelling temperature of GELRITE. The greater gel strength facilitated spreading of inocula and prevented the formation of gas cavities. Replica plating was also improved using GELRITE. For these reasons, it was considered that GELRITE was superior to agar in many applications (Harris, 1985).

1.8.4 Other uses of gellan gum

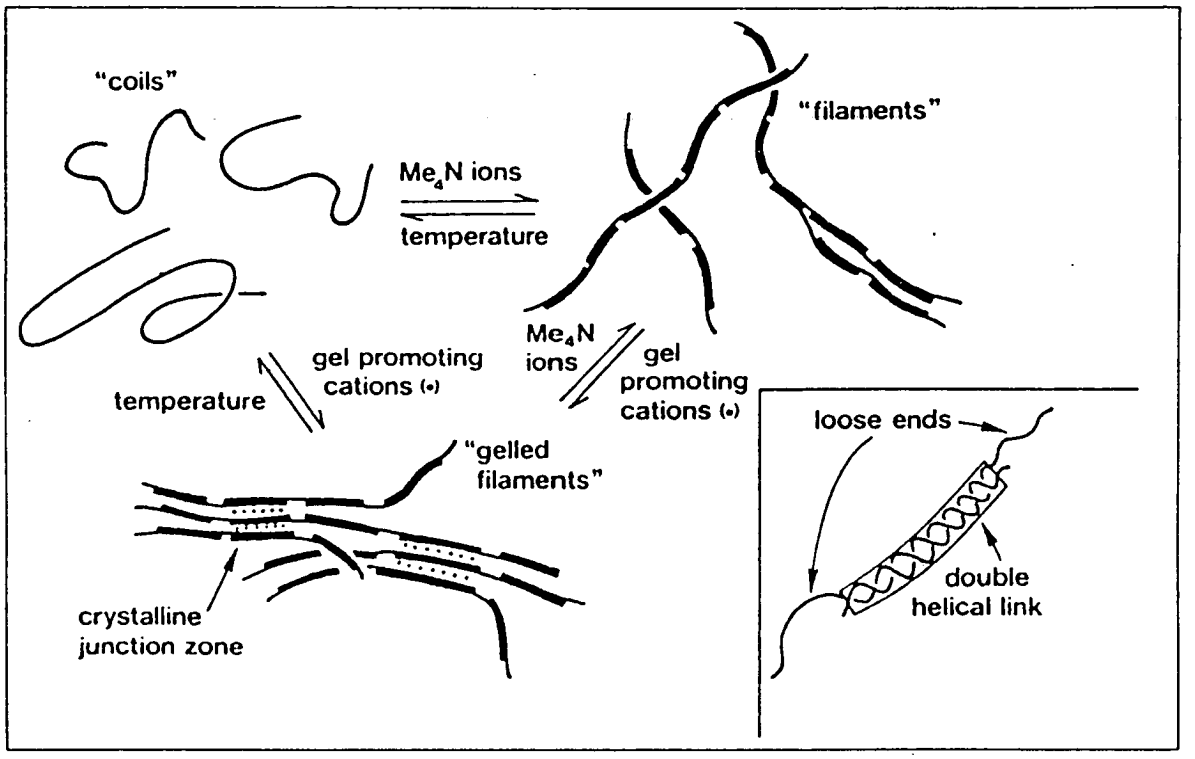
Gellan can be used in conjunction with gelatin to form coacervate gels useful in microencapsulation (Chilvers & Morris, 1987). The synergistic reactions of gellan and gelatin have been studied previously for the formation of high strength, rigid coacervate gels (Shim, 1985). Coacervates of a particular texture are formed by

heating the gellan and gelatin mixtures to the required temperature and cooling. This can be used to encapsulate both solid and liquid particles. Multicomponent mixtures of gellan, xanthan and galactomannans provide the basis for varying hardness, elasticity and cohesiveness of the gels. The effect of pH on gel formation at low polymer concentrations has also been studied to distinguish between simple and complex coacervation (Chilvers & Morris, 1987).
Gellan can also be used with high levels of glycols in industrial gels (Sandford *et al.*, 1984), These gels can provide the controlled release of fragrances, insecticides, herbicides and pheromones etc. Their rate of evaporation can be varied with glycol content (Baird *et al.*, 1983).

1.8.5 Gel formation

Gellan forms gels in the presence of either monovalent or divalent cations (figure 1.8). The setting temperature and the strength of the gel depend on the nature and concentration of the cation as well as on the polysaccharide concentration (Sanderson & Clark, 1983). In the native high acetyl form, gellan dissolves in deionised water to produce viscous solutions. In the presence of ions, thermoreversible gels are formed which are elastic and cohesive. These gels are unable to support their own weight and are similar to xanthan-locust bean gum gels (Sanderson & Clark, 1983). The unique gels prepared from low acetyl gellan gum are made by cooling hot solutions of gum in the presence of added cations. It is thought that gellan gelation may occur in two steps : chain ordering, followed by chain association. In the reverse direction (heating) disaggregation occurs first followed by melting of the individual helices.
Brittle gellan gum gels are similar to carrageenan gels which also require cations for gelation. Kappa-carrageenan creates problems in dispensing since it may start to thicken at temperatures greater than its setting temperature. Gellan presents no similar problems since gellan has no significant viscosity above its gelling temperature (Kang *et al.*, 1982a)

Figure 1.8: Schematic model for gellan gum gelation (after Morris, 1992).



POLYMER	GEL NATURE	MELTING POINT	SETTING POINT	HYSTERESIS
GELLAN (Ca^{2+} gel)	brittle	90°C	45-50°C	45-50°C
KAPPA-CARRAGEENAN	brittle	40-95°C	25-75°C	15-20°C
AGAR	brittle	60-97°C	32-39°C	60°C

Like agar, brittle gellan gum gels exhibit a very marked hysteresis; both are similar in their thermal stability, but gellan differs from agar in its speed of gelation (Kang *et al.*, 1982b).

The melting temperature of gellan is 90°C and the setting temperature is approximately 40°C depending on the cation used for gelation. In the absence of salt, the transition shows no detectable thermal hysteresis when studied by differential scanning calorimetry. There is a simple conformational transition from a double helix at low temperature to a single coil structure at high temperature. Gels formed with K^+ set at lower temperatures than those with Ca^{2+}. The concentration of divalent cation needed is much lower (Kang *et al.*, 1982b). This is similar to gelation of the polymer XM6 with Na^+ and Ca^{2+}, in which higher concentrations of monovalent ions are also required; the gel formed with XM6 shows a very sharp transition at 30°C which is independent of the ions used.

Gellan differs from alginate in its lack of specificity towards the ion bound. Monovalent ions induce gels in which the strength increases with atomic number. There is no selectivity within the alkali metal or alkaline earth cations : Mg^{2+} or Ca^{2+} have almost the same effect. The transition elements are more potent gel formers than the alkali earth ions and gel strength increases in the order $Zn^{2+} < Cu^{2+} < Pb^{2+}$ (Grasdalen & Smidsrød, 1987). Although virtually any salt causes gelation, gellan cannot gel with borates which react with rhamnose to form a complex (Colegrove, 1983).

1.8.6 Factors affecting gelation

As ionic strength increases gels become more brittle. Gel melting and setting temperatures are influenced by ion levels; with higher salt levels it is possible to prepare gels which do not remelt even after

autoclaving. For a given gum concentration, hardness increases through a maximum as ionic strength is increased. However, the divalent ions, calcium and magnesium, provide maximum gel hardness at molar concentrations, about 1/25 of those required with monovalent ions (Sanderson & Clark, 1983).

Low acetyl gellan gum gels are reported to be fairly stable to pH in the range 3.5-8. This is important as many foods are in the range pH 3.5-7. Changes in pH do not alter the setting points of the gel but there does appear to be an effect on melting temperatures in some cases. At pH 3.5 the melting temperature is slightly increased.

Some gellan gum gels show signs of syneresis. In these situations another gum may be added as a water control agent (e.g. xanthan). When syneresis is due to protein interaction a protective hydrocolloid (carboxymethyl cellulose (CMC), pectin, carrageenan) is necessary.

Low molecular weight hydrophilic molecules prevent gelation of gellan gum at levels in excess of 50%. This property can be useful to modify gel texture. Below optimal ion levels, low levels of sucrose increase gel hardness. Sucrose makes gels slightly less brittle and more plastic (Sanderson & Clark, 1983).

1.8.7 Effect of primary structure on gelation

The primary structure of microbial exopolysaccharides has a great effect on their capacity to form gels. This can be seen in a number of polysaccharides which possess closely related structures. For example, curdlan forms gels, whereas, the structurally similar scleroglucan, differing only in the presence of glycosyl sidechains, does not (Rodgers, 1973).

Gellan can form gels whilst other polymers, structurally related to it and bearing sidechains do not. In welan gum, one of the gellan series, the presence of a sidechain stabilises the ordered structure, but also inhibits the aggregation process necessary for gelation. Welan and these other polymers are all capable of forming highly viscous solutions. It is apparent that very minor structural differences can affect the ability of the polymers to form gels (Jansson *et al.*, 1985).

It is likely that the gelling mechanism of gellan is based on the formation of double-helical junction-zones, followed by aggregation of

the double helical segments, which lead to a three dimensional network, as proposed for other gel-forming polysaccharides (Rees, 1969).

It has been suggested that acetylation of 1→3 linked glucose in native gellan will not disrupt double helix formation, but that the aggregation of the molecules will be lessened, compared to deacetylated material. Therefore acetylation would be expected to lessen, rather than completely abolish, gel-forming properties, resulting in a weaker gel (Chandrasekaran *et al.*, 1988a). The only alkali-labile substituent originally reported in gellan was acetyl which was situated on approximately one in three of the repeating tetrasaccharide units. However, it was not expected that removal of acetate could cause such a major change in physical properties. Kuo *et al.* (1968) discovered the presence of about one mole of L-glycerate also situated on the 1→3 linked glucose residue. Although the glycerate normally encountered in metabolism is D-glycerate, both D-glycerate (Saier & Ballou, 1968) and L-glycerate (Kondo *et al.*, 1980) have been found as constituents of bacterial polysaccharides. It was considered that the glyceric ester is the major cause of differences in physical properties between the native and deacylated polymer, rather than the acetic ester. This is concluded because glycerate is:

(1) bigger than acetate,
(2) more abundant than acetate in the polymer and,
(3) in a crowded location, namely, between C-3 linked to the preceding rhamnose and C-1 linked to the following glucuronic acid (Kuo *et al.*, 1986).

1.8.8 Fibre diffraction studies on gellan gum

Native gellan gum contains approximately 6% O-acetylation and forms weak, elastic gels. Upon deesterification gellan gum forms highly viscous aqueous solutions which even at concentrations as low as 0.04% can form hard, brittle gels (Moorehouse *et al.*, 1981). Cations promote intermolecular association and gelation in gellan gum (Chapman *et al.*, 1988). X-ray fibre diffraction studies were used to examine the differences in structure of the two types of gel (Carroll *et al.*, 1982; 1983). Acetylated (5.6% acetyl) and partially

deacetylated (1.7%) gellan gum were compared. In the former sample, diffuse equatorial reflections imply only poor lateral packing of the aligned chains. The layer line spacing corresponded to a repeat in the backbone of 2.82 nm. Meridional reflections occurred only on every third layer line. The simplest explanation was a three-fold single helix with an axial projection of the chemical repeat unit of 0.94 nm. In the deacetylated (1.7%) sample the molecular structure appears unchanged but the equatorial reflections are resolved more clearly. The five spots index onto a trigonal lattice (a = b = 1.65 nm) with certain systematic absences. Thus, deacetylation enhances the packing of the polymers. This increase in crystallinity is consistent with a transition to a harder more brittle gel. Acetyl groups inhibit crystallisation, reducing the size and strength of the junction zones (Miles *et al.*, 1984). The importance of water in the formation of crystalline structures has been noted in studies of the polysaccharide from Klebsiella K9 (Isaac *et al.*, 1981). Dehydration of the gellan fibres does not alter the molecular structure or alignment but does disrupt the lateral packing by blurring the equatorial reflections (Carroll *et al.*, 1982; 1983).

The quality of these early diffraction patterns was inadequate for a detailed structural analysis. High quality diffraction patterns from well oriented, polycrystalline specimens of the lithium salt were produced (Upstill *et al.*, 1986). The axial projection of the polysaccharide 2.82 / 3 = 0.94 nm, was thought to be unusually short for an unbranched tetrasaccharide repeat. It was about half the theoretical maximum of 1.92 nm obtained by summing the lengths of virtual bonds between oxygen atoms in the glycosidic linkages. In addition to very contracted single helices, a number of intertwined double helix structures were studied. This study provided no structural model consistent with the X-ray data.

Reexamination of the crystal structure of gellan indicated that the gellan chains had different backbone conformations from those previously considered. (Chandrasekaran *et al.*, 1988a). Gellan formed a parallel, half-staggered, double helix similar to that reported for the gel-forming polysaccharide ι-carrageenan (Arnott *et al.*, 1974). Each chain had left handed 3 fold helix symmetry, and displayed an extended cellulose-like conformation. The carboxylate group of D-

glucuronate and the 1→4 linked D-glucose and L-rhamnose residues were engaged in interchain interactions which stabilised the double helix. The free hydroxyl groups of L-rhamnose and the hydroxymethyl groups of 1→3 linked D-glucose residues are involved in hydrogen bonds between the double helical molecules (Chandrasekaran *et al.*, 1988b).

1.8.9 Gellan family of bacterial polysaccharides

Following the discovery of gellan, a number of related polysaccharides, also with novel functionality, were obtained from other bacterial strains (figure 1.9). The most recently isolated polymer, also has a linear tetrasaccharide repeat equivalent to gellan, but with L-mannose replacing L-rhamnose. (O'Neill *et al.*, 1990). The five related polymers have the same main chain sequence as gellan, but also carry a sidechain (figure 1.10). Two polymers, S-657 and S-194, have disaccharide sidechains of L-rhamnose (Chowdhury *et al.*, 1987b) and D-glucose (gentibiosyl groups) respectively (Jansson *et al.*, 1986). The pentasaccharide repeat of S-130 has a variable side chain of either L-rhamnose or L-mannose. This is unusual, both in containing L-mannose and in having a variable side chain (Jansson *et al.*, 1985). Two other polymers, S-88 and S-198 (Chowdhury *et al.*, 1987a), have an L-rhamnose side chain, although, S-198 has the side chain present in only 50% of repeat units at the 1→3 linked glucose (Jansson *et al.*, 1986). In both of these products the main chain rhamnose may be replaced by L-mannose. It is not known whether these sugars are found at random or whether two separate polymers are produced one containing L-rhamnose, the other L-mannose.

1.8.10 Applications of gellan related polymers

Rhamsan (S-194), shows high viscosity at low concentrations and low shear rates, with no significant viscosity loss at temperatures up to 100°C (Baird *et al.*, 1983). It is also compatible with high levels of salt and is therefore being exploited for its ability to suspend components of suspension fertilisers (Colegrove, 1983). Suspension fertilisers have become more popular due to the lower production costs achieved by the use of lower grade materials. A higher percentage of

Figure 1.9: Structures of the gellan family of bacterial polysaccharides

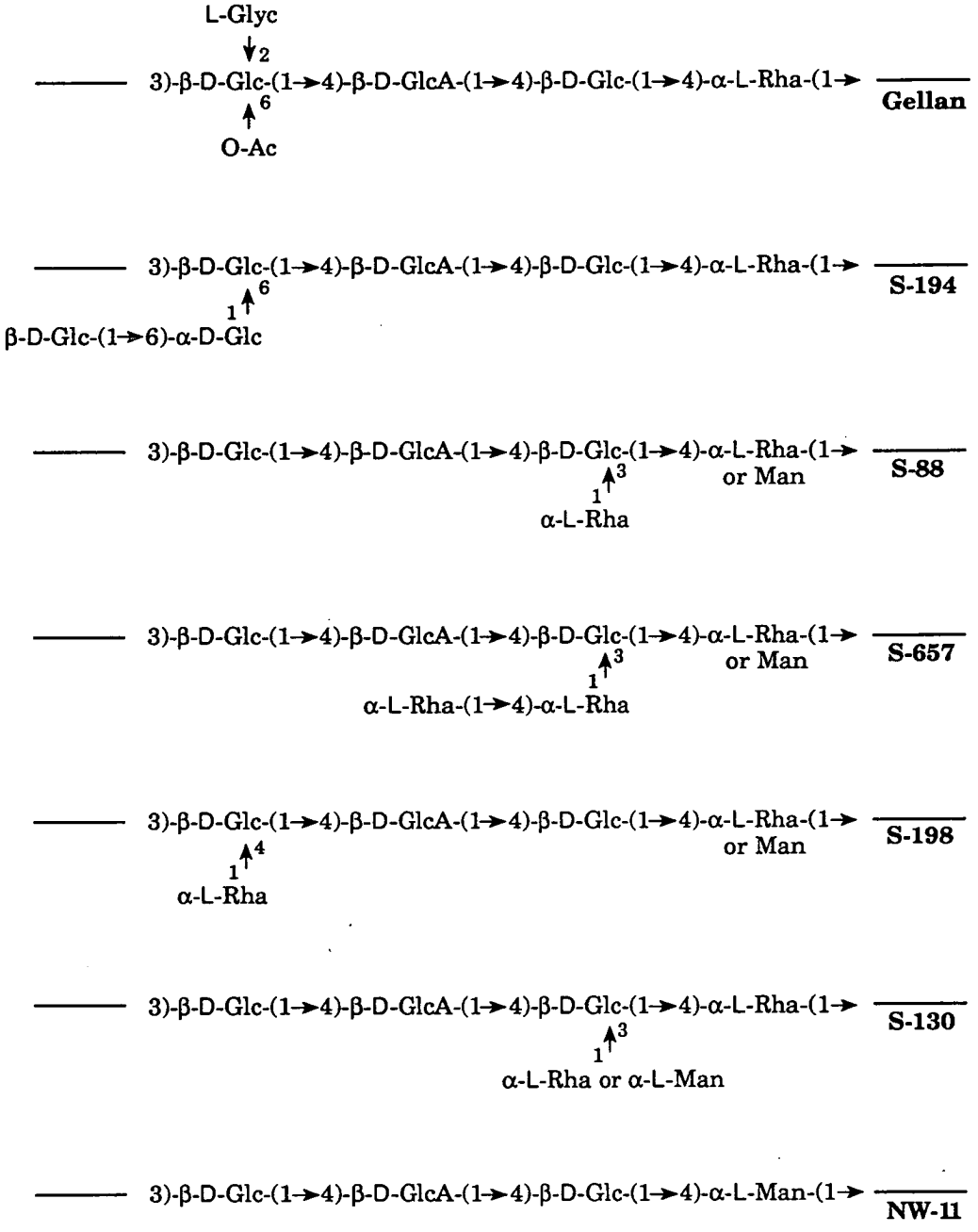
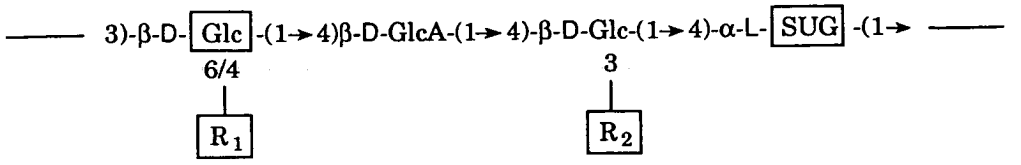


Figure 1.10: Backbone repeating unit in common to the gellan family of bacterial polysaccharides



	R_1	R_2	SUG
Gellan	H	H	Rha
S-130	H	α-L-Rha-(1→/α-L-Man-(1→	Rha
S-194	β-D-Glc-(1→6)-α-D-Glc-(1→6)	H	Rha
S-88	H	α-L-Rha-(1→	Rha/Man
S-198	α-L-Rha-(1→4)	H	Rha/Man
S-657	H	α-L-Rha-(1→4)-α-L-Rha-(1→	Rha/Man
NW-11	H	H	Rha

potassium and macronutrients can be used and uniformly distributed. Most polysaccharide suspending agents are incompatible with the high concentrations of ammonium polyphosphate or orthophosphate used in suspension fertilisers and "salting out" occurs. Due to its salt compatibility, S-194, may be used in a wide variety of suspension fertilisers (Baird *et al.*, 1983). Due to its shear stability, S-194 may also be used in flowable pesticides where it is standard practice to grind the active ingredient to a fine particle size during its preparation. This produces a more stable suspension. This intense shear generally results in viscosity loss from most other polymer solutions.

Welan gum (S-130), produces high viscosity solutions at low concentrations which have excellent suspending properties. Viscosity is retained at temperatures of 150°C which means S-130 has proved useful in drilling fluids, to drill into deep and therefore, very hot oilwells. It can develop high viscosity in most saline waters and shows no significant viscosity loss until the pH exceeds 11.

The polymer produced by *Alcaligenes* ATCC 31853 was named S-198. It was found to have excellent stability to shear and temperature and may be potentially useful in the developing market of water based lubricants replacing the currently used petroleum oils which present problems with pollution and disposal (Baird *et al.*, 1983).

1.9 Aims of this Study

The aim of this work was to examine the effect of nitrogen, carbon and magnesium limiting conditions on the physiology and composition of gellan gum from the wild type strain of *Sphingomonas paucimobilis*. This was compared to EPS production in two mucoid mutant strains, PA4 and SB10, and to the non-mucoid mutant, MJ8. In addition, the difference in the production of EPS and its final composition was compared in twenty mutant strains of *S. paucimobilis*. Differences in physiology, chemical composition and rheological properties were examined further. The main objectives of this work were to determine any change in the structure of EPS due to the altered growth conditions used and ultimately to produce gellan gum of maximum yield and optimum quality.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The microorganisms used in this study are listed in Table 2.1.

2.2 Culture Media

For the production of polysaccharides and growth experiments, all strains of *Sphingomonas paucimobilis* were grown in Davis and Mingioli (DM) medium (Table 2.2).

For nutrient limitation experiments the organisms were grown in Davis and Mingioli media modified by alteration of the C, N or Mg^{2+} content.

All cultures were preserved by lyophilisation and were maintained on agar slopes of Yeast extract agar (Table 2.3).

2.3 Culture Conditions

Liquid cultures were grown in 250 ml or 2 l Erlenmeyer flasks containing 100 ml or 800 ml of DM media respectively. The flasks were shaken at 180 rpm at 30°C for 4 days.

2.4 Growth Experiments

During growth experiments 50 ml samples of culture media were taken at various times. Growth was measured by the absorbance of culture samples at 600 nm in a Pye Unicam 8800 spectrophotometer.

2.5 Cell Dry Weight Determination

Cells were recovered by centrifugation at 18 000 g for 60 min, washed in distilled water, centrifuged again and lyophilised.

2.6 Culture Medium Viscosity

The viscosity of the culture medium was determined using a Brookfield digital viscometer. A range of shear rates from 0.3-60 s^{-1} was available.

Table 2.1a: Strains of *S. paucimobilis* used to study the effect of nutrient limitation on growth and EPS production.

Organism	Source
Pseudomonas elodea Wild Type strain (NCIMB 12171)	Dr. V.J. Morris, Institute of Food Research, Norwich
Sphingomonas paucimobilis SB10 PA4 MJ8 (non mucoid)	Dr. I. Sâ-Correia, Instituto Superior Tecnico, Lisbon

Table 2.1b: Mutant strains of *s. paucimobilis* also studied in the course of this work (described in detail in Table 3.7).

S. paucimobilis	Source
WI, WA	derived from wild type stain, Edinburgh
12171 mut 2, 12171 gel$^-$, mut 1 spont	spontaneous non mucoid variants, Edinburgh
MJ8, MJ16, MJ21, M118, MJ200, MJ216	EMS mutants, Lisbon
R40, Rif40, Rif60	Rifampicin resistant mutants, Lisbon
Pe B4, NM, HM2, E315	Colony morphology variant, Lisbon
ER10	spontaneous mutant, Lisbon

Table 2.2: The composition of Davis and Mingioli medium

Component	**g l^{-1}**
K_2HPO_4	7
KH_2PO_4	3
$(NH_4)_2SO_4$	1
$MgSO_4.7H_2O$	0.1
glucose	20

The glucose and MgSO4 were sterilised separately. Solutions were sterilised at 15 p.s.i. for 20 min.

Table 2.3: The composition of Yeast Extract Agar

Component	**g l^{-1}**
Na_2HPO_4	10
KH_2PO_4	3
K_2SO_4	1
NaCl	1
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.01
$FeSO_4$	0.001
Casein Hydrolysate	1
Yeast Extract	1
Glucose	20
Agar	20

Solutions of glucose and MgSO4 were sterilised separately. Solutions were sterilised at 15 p.s.i. for 20 min.

2.7 Residual Glucose Assay

The amount of glucose in the culture medium was determined by the glucose oxidase method as used by Brivonese (1985).

Reagents

Glucose oxidase : (1 mg ml^{-1} in Tris / HCl; pH 7.0)
Peroxidase : (2 mg ml^{-1} in Tris / HCl; pH 7.0)
ADTS : (10 mg ml^{-1} in Tris / HCl; pH 7.0)
(2,2'-azino-di-(3-ethylbenzthiazidine sulfonic acid)
Tris / HCl Buffer (pH 7.0)

Standard : 50 µg ml^{-1} Analar glucose

Method

50 µl glucose oxidase, 10 µl peroxidase, 150 µl ADTS, 450 µl Tris/HCl and 100 µl sample, containing 0-3 µg glucose, were combined sequentially in a 1 ml microcuvette. The samples were incubated at 37°C for 1 hour before the absorbance at 415 nm was measured.

2.8 Polysaccharide Production

Polysaccharide was recovered from the culture medium by diluting with an equal volume of distilled water and centrifugation at 18 000 g for 1 h. After removal of the cells, polysaccharide was precipitated by the addition of 2 volumes of cold acetone. The resulting precipitate was left for 48 h, then washed with fresh acetone. After dissolving in distilled water, the polysaccharide was dialysed against running water at 4°C for 48 h, lyophilised and weighed.

2.9 Colorimetric Assays

All assays were performed at least in duplicate and the results were expressed as a percentage of the total carbohydrate, as determined by the phenol-sulphuric acid assay (Dubois *et al.*, 1956).

2.10 Acetyl Determination

O-acetyl content was determined by the method of Hestrin (1949).

Reagents

2M Hydroxylamine HCl
3.5M NaOH
c HCl, diluted 1:2 in H_2O
0.37M $FeCl_3.6H_2O$ in 0.1N HCl

Standard

0.04M Acetylcholine chloride in 1mM Sodium Acetate (pH 4.5)

Method

400 µl of a 1:1 mixture of hydroxylamine HCl and NaOH were added to 200 µl of a 0.2% polysaccharide solution. After 2 min incubation at room temperature, 200 µl of HCl and 200 µl $FeCl_3$ were added. The solutions were mixed at each stage and the absorbance at 540 nm was recorded.

2.11 Phenol-sulphuric Acid Assay

Total carbohydrate content was determined using the method of Dubois *et al.* (1956).

Reagents

5% aqueous phenol
c H_2SO_4 (A.R. grade)

Method

200 µl of phenol was added to 200µl of test solution containing 1-20 µg carbohydrate. After mixing, 1 ml of c H_2SO_4 was added. The solutions were left for 15 min at room temperature before the absorbance at 490 nm was measured.

2.12 Sugar Analysis

2.12.1 Saeman Hydrolysis

0.5 ml of 72% ($^{w}/_{w}$) sulphuric acid was added to a pyrex tube containing 5 mg polysaccharide then left for 3 h at room

temperature. The slurry was then diluted with 5.5 ml distilled water. The samples were mixed then heated in a hot block at 100°C for 2.5 h. After 1 h the tubes were cooled in a water bath at 20°C and 1 ml of sample was removed for uronic acid analysis. After 2.5 h, the tubes were cooled in an ice water bath. The remaining sample was neutralised with concentrated $Ba(OH)_2$ to pH 5-6. 200 µl of 2 deoxy-D-glucose solution (1 mg ml^{-1}) was added as an internal standard. The solution was filtered through Whatman No. 42 fluted filter paper into a 250 ml round bottomed flask. The filter paper residue was washed with distilled water. The combined filtrates were evaporated to dryness under vacuum at 35-40°C, redissolved in 2 ml of distilled water and transferred to a glass tube.

Reduction

1 ml of a freshly prepared solution of $NaBH_4$ (8 mg ml^{-1}) was added to the sample tube which was sealed with parafilm and stored for 3 h at room temperature or overnight in the cold (2°C). The excess borohydride was removed by adding 6-8 ml of a Dowex-50W-X8 resin solution (20-50 US mesh(H)). After 30 min the solution was filtered through a Whatman No. 1 filter into a 50 ml round bottom flask. The resin was washed with 10 ml of distilled water. The combined filtrates were evaporated to dryness in a rotary evaporator at 35-40°C, 2 ml of dry methanol was added and the sample evaporated to dryness in a rotary evaporator. This procedure was repeated three times. 0.5 ml of water was then added, the solution transferred to a pyrex tube and dried. Next, 0.5 ml of dry methanol was added and evaporated with a rotary evaporator. This process was repeated three times.

Acetylation

0.1 ml pyridine (dried with KOH) and 0.1 ml of acetic anhydride were added to the sample. This was heated in a sealed tube at 120°C for 20 min. Excess pyridine and acetic anhydride were removed by co-evaporation with toluene, (repeated four times). Finally, the sample was dissolved in 0.2 ml of acetone and injected into the GLC.

GLC Analysis

This was performed on a Carbo Erba Strumentazione 4100 GC with 3% OV-225 packed column. Temperature 200°C, injection 1 µl, integrator : Trivector Trio.

2.12.2 Stones Method

200 µl of 72% ($^{w}/_{w}$) sulphuric acid was added to a pyrex tube containing 5 mg of polysaccharide then left at room temperature for 3 h. 2.2 ml of distilled water was added, mixed and incubated for a further 2.5 h at 100°C in a hot block. After 1 h the tubes were cooled in a water bath at 20°C and 0.5 ml of hydrolysate was removed for uronic acid analysis. After 2.5 h the samples were cooled by placing in a water bath at 20°C. 200 µl of 2-deoxy-D-glucose (1 mg ml^{-1}) was added to each tube as an internal standard. 1 ml of hydrolysate was transferred to a fresh tube. 0.2 ml 25% NH_3 and 0.1 ml of a freshly prepared solution of 3M NH_3 containing 150 mg ml^{-1} $NaBH_4$, were added. The samples were mixed and incubated at 30°C for 1 h. The samples were again cooled in a water bath at 30°C. Once cool, 100 µl of glacial acetic acid was added. 0.3 ml of solution was transferred to a fresh tube and 0.45 ml of 1-methyl-imidazole and 3 ml of acetic anhydride were added. The tube contents were mixed and incubated at 30°C for 30 min, then cooled on ice.

3 ml of distilled water and 3 ml of dichloromethane were added to each tube. After mixing, the tubes were centrifuged at low speed for 1 min. The upper aqueous layer was removed using a pasteur pipette and vacuum pump. 3 ml of distilled water and 2 ml of dichloromethane were added to each tube. After mixing the tubes were centrifuged and the upper layer removed. 3 ml of distilled water was added to each tube. The samples were mixed, centrifuged and the upper layer removed. This was repeated twice more.

The organic layer was transferred to a clean pyrex tube. The dichloromethane was evaporated under a stream of Argon at 40°C, 1 ml of acetone was added and evaporated. This was repeated twice. The samples were dissolved in 80 µl of acetone ready for GLC Analysis.

2.13 Uronic Acid Analysis

Uronic Acid was determined by the method of Blumenkrantz & Asboe-Hansen (1973).

Reagents

25 mM Sodium Borate in c H_2SO_4

0.15% *m*-phenyl phenol in 0.5% NaOH

Standard

Glucuronic Acid in saturated benzoic acid (1 mg ml^{-1})

(Range 1-40 µg ml^{-1})

1 ml of sulphuric acid was added to pyrex tubes and placed in the freezer for 20 min. The tubes were then placed in an ice water bath; 0.2 ml of sample was added slowly to each tube and mixed gently. The tubes were heated for 10 min in a hot block at 100°C, then cooled quickly in an ice water bath for 2 min, before adding 100 µl of *m*-phenyl phenol. The tubes were mixed and left for 20 min before the absorbance was read at 524 nm.

2.14 Methylation Analysis of Neutral Sugars in Polysaccharides

2.14.1 Hakamori Method

(A)Preparation of Dimsyl Anion (methyl sulphinyl methanide anion)

2.1g of Sodium Hydride (80% suspension in oil) was weighed into a 150 ml three-necked flask. To wash the Sodium Hydride, 5 ml of pentane (dried over molecular sieve, 0.4nm) was added and the liquid was decanted, this procedure was repeated four times. The decanted pentane was poured into ethanol to neutralise any NaH present. The washed Sodium Hydride was dried by flushing Argon through the flask via two injection needles. 30 ml DMSO (dried over molecular sieve) was added carefully to the flask with a glass syringe under a stream of Argon. The flask was placed in an ultrasonic bath for a few minutes then heated at 50-60°C for 5 h, under a stream of Argon. The result was a greenish-grey opalescent solution of dimsyl anion. This reagent could be stored in a freezer and was stable for at least one month (stored with dry P_2O_5).

(B)Methylation

1-6 mg of sample in a serum vial was dried for 24 h in a vacuum oven at 40°C with P_2O_5. The sample was dissolved in DMSO (dried over molecular sieve, 0.4nm), 1 ml/5 mg sample. The serum vial was closed with a rubber septum and flushed with Argon via two injection needles. Sample dissolution was facilitated by degassing and treatment in an ultrasonic bath. Dimsyl anion (2M, 1 ml/5 mg sample) was added dropwise using a syringe and hypodermic needle. Excess pressure was released through a second needle. The sample was agitated in an ultrasonic bath for 3 h at approximately 40°C. The tube was put in an ice bath and methyl iodide (1 ml/5 mg sample) was added dropwise as above. Excess pressure was released via a second needle. After ultrasonic agitation at room temperature for 2 h the vial was opened and the excess methyl iodide removed by flushing with a stream of Argon.

(C) Purification of partially methylated polysaccharides

The reaction mixture was poured into a dialysis bag (pre-boiled with distilled water) and dialysed against running tap water overnight. If any insoluble material was present after methylation, an equal volume of $CHCl_3$/MeOH (50%) was added after opening the vial. The solution was filtered through glass fibre paper (Whatman GF/C) and the filtrate was poured into a dialysis bag and dialysed against a mixture of 50% ethanol/distilled water. Three changes of EtOH/d H_2O were made over 48 h. The contents of the bag were then concentrated to dryness in a 50 ml round bottom flask using a rotary evaporator. The sample was resuspended in a known volume of chloroform :methanol and $^1/_3$ used for PMAA's (e.g. 3-5 mg) and $^2/_3$ used for carboxyreduction (i.e. dried down and frozen until required).

(D) Making Alditol Acetates

The Saemen Hydrolysis method from 2.12.1. was then used to make Alditol Acetates with the following exceptions:

Hydrolysis: 2 h at 100°C with 1 ml concentrated formic acid. The formic acid was then evaporated and 1 ml 0.25 M sulphuric acid was added and heated for 12 h at 100°C. No internal standard was added.

Reduction: NaB^2H_4 (10 mg added as solid) was used instead of a $NaBH_4$ solution.

(E) GLC Analysis

Carbo Erba Strumentazione HRGC 5160 with a 15 m capillary OV-225 column (0.53 mm internal diameter (ID), film thickness 1 μm). Temperature programme : 1 min @ 150°C, 150-220°C with 1°C/min, 10 min @ 220°C, injection 0.4 μl. Integrator : Spectra Physics SP 4290 with IBM computer system.
Isothermal (for calculation of relative retention times) : 80 min @ 170°C, 170-220°C with 20°C/min, 10 min @ 220°C.

(F) Calibration of peak positions and measurement of peak areas

The peaks from the GC-Analysis were determined with GC-MS measurements. The mass spectra of the partially methylated alditol acetates were compared with well known mass spectra from the Department of Organic Chemistry, University of Stockholm to give linkage positions (Jansson *et al.*, 1976). The relative retention times of each peak were used for determination of the derivatized sugar. Quantitative determination of the glycosidic linkage isomers present in the sample was accomplished through integration of GC peak areas. The peak areas were divided by the e.c.r. (effective carbon response) factors (Sweet *et al.*, 1975) to make a correction for the different molecular weights.

2.14.2 Sodium Hydroxide Method

3-6 mg of sample was dried for 24 h in a pyrex tube in a vacuum oven at 40°C with P_2O_5. The sample was dissolved in 1 ml of DMSO (dried over molecular sieve, 0.4 nm). Approximately 0.25g of powdered NaOH was added to the solutions, which were then degassed and flushed with Argon. The samples were agitated in an ultrasonic bath for 3 h then cooled on ice. 3 ml of methyl iodide was added to the samples. The samples were replaced in an ultrasonic bath and agitated for a further 2 h, after which the excess methyl iodide was blown off with Argon. The derivitisation of the methylated samples was as described in 2.14.1.(C) onwards.

2.15 Methylation Analysis of Uronic Acid Containing EPS

2.15.1 Redgwell Method (Redgwell & Selvendran, 1986).

If uronic acids were present in a sample it was necessary to reduce the carboxyl group prior to the hydrolysis step used in the preparation of alditol acetates. This was achieved by treatment with a refluxing solution of $LiAl^2H_4$.

Samples were used as prepared in 2.14.1.(C).

The samples and apparatus were dried for 24 h in a vacuum oven at 40°C with P_2O_5 prior to use. 10 ml of dichloromethane:ether mixture (1:4) (both dried with molecular sieve) was added and mixed well. A large excess of $LiAl^2H_4$ (40-50 mg / 5 mg methylated material) was added and the solution was agitated for a few seconds in an ultrasonic bath to disperse the reagent. The solution was refluxed for 4 h. At the end of the reaction, the excess reagent was destroyed by adding carefully a few drops of ethyl acetate followed by 10 ml of distilled water. The mixture was titrated with a solution of 1M phosphoric acid to pH 5-6 on a magnetic stirrer. The copious precipitate of hydroxide was removed by filtration on a sintered filter (porosity 3) and washed thoroughly with 5 ml of $CHCl_3$ / MeOH (50%) to remove adsorbed polymer. This was repeated 5 times.

The combined solutions of polymer were collected and evaporated to dryness. The residue was redissolved in 50% $CHCl_3$/MeOH and filtered through glass fibre paper (Whatman GF/C) and again evaporated to dryness prior to hydrolysis.

The method from 2.14.1.(D) was followed for the preparation of alditol acetates.

2.15.2 Tetrahydrofuran (THF) Method

The samples were those prepared in 2.14.1.(C).

The pyrex tubes containing sample were dried over P_2O_5 in a vacuum oven at 40°C for 24 h. 40 mg of Lithium Aluminium Deuteride was added to the tubes. 1 ml of THF / mg sample was also added. The samples were placed in a hot block at 65°C for 4 h in sealed tubes. The tubes were then cooled on ice. Ethanol was added to the samples dropwise until the fizzing stopped. Distilled water was added dropwise to the samples until the reaction ceased. 2-3 ml of

distilled water was added to the samples which were then transferred to 25 ml beakers; 1M phosphoric acid was then used to neutralise the solutions to pH 5-6. An equal volume of THF was added to the tubes which were then centrifuged for 1 min at low speed (1500 rpm) on a bench top centrifuge. The supernatant was decanted into a separate flask. The pellet was washed three times with THF (5 ml). The combined supernatants were dried in a rotary evaporator.
The method from 2.14.1.(D) was followed for the preparation of alditol acetates.

2.16 Neutral Sugar Analysis by HPLC

Hydrolysis

10 mg of polysaccharide was hydrolysed in the presence of 0.5 N H_2SO_4 for 18 hours at 100°C in a sealed ampoule. The hydrolysate was neutralised by the addition of the bicarbonate form of Amberlite IR410 resin (prepared from Cl^- form) and then Amberlite MB1 mixed bed resin. The neutralised solution was dried under vacuum and resuspended in 100 µl deionised water (Elga Ltd., High Wycombe, England). The sample was filtered through a 0.45 µm filter (Millipore (UK) Ltd., Watford, England) prior to analysis.

Standards

0.1 M solutions of glucose, galactose, mannose and rhamnose were prepared for the determination of retention times.

Analysis

Analysis was carried out using a Gilson High Performance Liquid Chromatograph (HPLC) connected to a Knaur refractive index monitor. The monosaccharides were separated using a Biorad HP-X87P PB^{2+} (cation exchange) column (30 cm x 7.8 mm internal diameter (ID) (Bio-Rad Laboratories, Richmond, California, USA) at 85°C. The mobile phase was deionised water and the flow rate 0.2 ml min^{-1}. The mobile phase was filtered and degassed prior to use.

2.17 Polymer Deacylation

Deacylation was achieved by treating a 0.1% solution of purified polysaccharide with 0.01M NH_4OH at 60°C for 1h. The solution was

then neutralised with H_2SO_4, dialysed against distilled water for 48h, purified and lyophilised.

2.18 Gel Strength Testing

Gels (0.3%) were prepared in plastic petri dishes, covered with cling film and allowed to set for 24h. Gel strength measurements were taken using an Instron 3250 Mechanical Spectrometer. Gel strengths were measured between parallel plates using oscillatory shear (between 1-50% deformation). The gels in the petri dishes were mounted onto the bottom plate by wetting the bottom of the petri dish with chloroform in order to stick it to the plate. It is essential that the polysaccharide samples have the same thermal history.

CHAPTER 3

RESULTS

3. PRELIMINARY STUDIES

3.1 Chemical Analysis of Gellan Gum

To determine the monosaccharide chemical composition of gellan gum elaborated by *Sphingomonas paucimobilis* wild type strain several methods were used.

(1) GC analysis of neutral sugars,

(2) GC/MS methylation analysis of neutral sugars

(3) GC/MS analysis of carboxyreduced samples.

3.1.1 GC analysis of neutral sugars

Two methods of derivitisation were used to prepare samples for GC analysis as outlined in Chapter 2.12. The first method used was Saemen hydrolysis followed later by Stones method. During both procedures, samples of hydrolysate were removed for uronic acid analysis by colorimetric assay, as these would not be detected by the method GC analysis for neutral sugars. The original method used for neutral sugar analysis Saeman hydrolysis, involved time consuming neutralisation steps. Latterly, Stones method proved to be a simple and relatively quick technique for the preparation of sugars for GC analysis as the results obtained by both methods were comparable, most sugar analyses were performed using Stones method.

With both methods, monosaccharide sugars were analysed by gas chromatography following their conversion to alditol acetates. Figure 3.1. shows the chemical reactions involved in the conversion of an aldose to an alditol acetate using the conversion of glucosamine of glucosaminitol as an example (taken from Fox *et al.*, 1990).

Prior to GC analysis of unknown samples, known sugar standards were used to identify sample peaks. Sugar standards were prepared which contained 1 mg ml^{-1} of glucose, galactose, mannose, rhamnose, arabinose, xylose and fucose. The internal standard 2-deoxy-glucose was also added to this mixture at a concentration of 200 $\mu g\ ml^{-1}$. The standard was then analysed by GC to determine retention times and to calculate correction factors for each sugar in relation to the internal standard. Sample sugars were then identified by retention times relative to sugar standards. Figure 3.2. shows a

Figure 3.1: Example of the alditol acetate derivitization reaction. Glucosamine, an aminoaldose which exists predominantly in the ring form, is reduced with Sodium Borohydride to glucosaminitol, which exists entirely as a linear molecule. After drying the sample, hydroxyl groups are acetylated with acetic anhydride to produce glucosaminitol (after Fox *et al.*, 1990).

SUMMARY OF THE ORIGINAL ALDITOL ACETATE METHOD

Step 1 Reduction of aldose to alditol using sodium borohydride
Step 2 Removal of borate generated in first step by multiple evaporations with acidic methanol
Step 3 Acetylation with acetic anhydride using a catalyst such as pyridine
Step 4 Gas chromatographic analysis

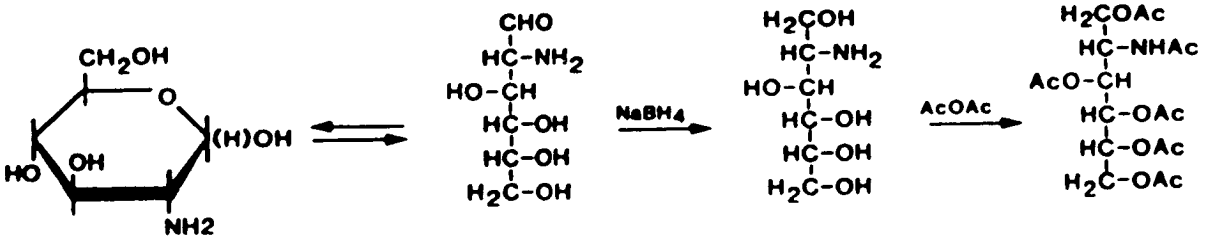


Figure 3.2: Gas chromatogram of a sugar standard solution containing 1 mg ml^{-1} each of glucose, galactose, mannose, rhamnose, arabinose, fucose and xylose and 2-deoxy-glucose at 200 μg ml^{-1}.

The sugar standard solution was used for the calculation of retention times and correction factors for each sugar relative to internal standard, 2-deoxy-glucose.

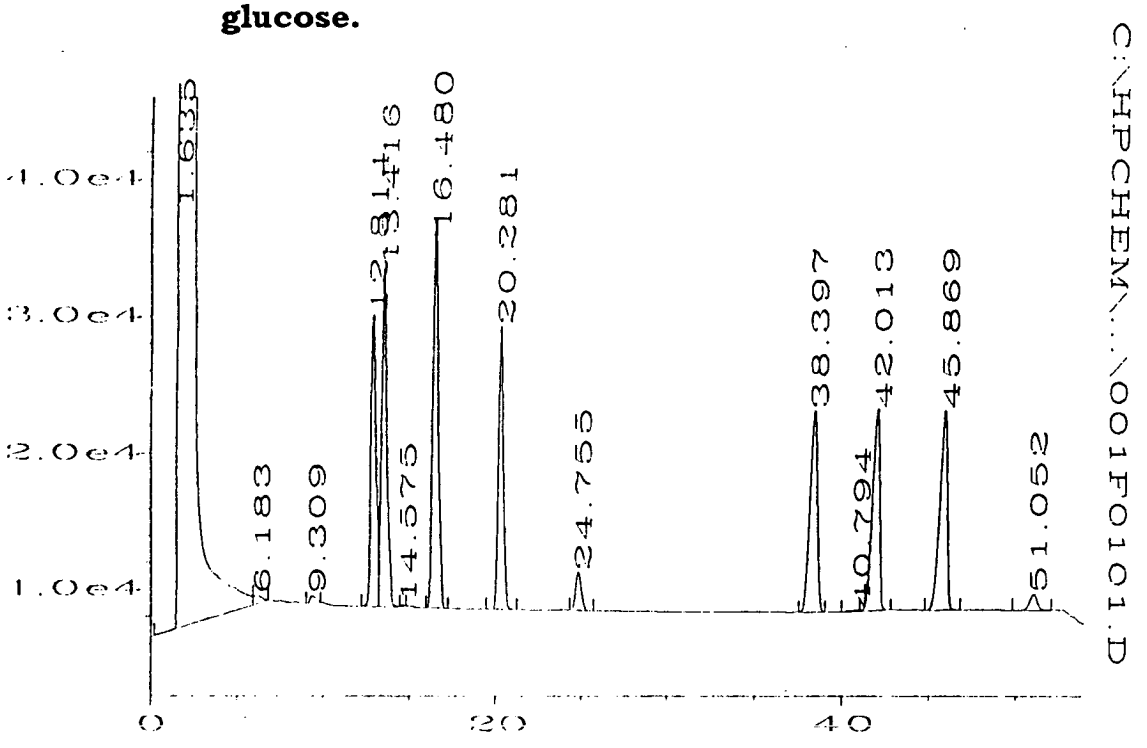


Table 3.1: Retention times and correction factors of the monosaccharides contained in a sugar standard solution relative to 2-deoxy-glucose internal standard.

Monosaccharide	Retention Time (min)	Correction Factor
Rhamnose	12.5 ± 0.2	1.15 ± 0.02
Fucose	13.1 ± 0.3	0.92 ± 0.07
Arabinose	16.0 ± 0.4	0.97 ± 0.01
Xylose	19.6 ± 0.4	1.01 ± 0.02
2-deoxy-glucose	**23.9 ± 0.7**	**1.00 ± 0**
Mannose	37.2 ± 0.8	1.14 ± 0.05
Galactose	40.8 ± 0.8	1.14 ± 0.07
Glucose	44.5 ± 1.0	1.17 ± 0.05

typical GC trace of a sugar standard solution, whilst Table 3.1 shows the monosaccharide components of the standard solution in addition to retention times and correction factors for each sugar.

Three batches of gellan gum from wild type *Sphingomonas paucimobilis* were analysed by Stones method and the results are presented in Table 3.2. The concentrations of rhamnose and glucose were calculated from the GC chromatogram whilst glucuronic acid was determined by colorimetric assay.

3.1.2 GC/MS methylation analysis of neutral sugars

Neutral sugars were studied by GC/MS methylation analysis, by preparation of permethylated alditol acetates (PMAAs). The schematic diagram of figure 3.3. outlines the steps involved in the preparation of PMAAs. Two methods were used for preparation of PMAA's (as outlined in Chapter 2.14.). Initially the Hakamori method was used, which involved the preparation of di-methyl sulphinyl anion (dimsyl anion). This procedure depended entirely on the activity of each batch of dimsyl anion. If the dimsyl anion was inactive, samples would be undermethylated and results were inconclusive. The sodium hydroxide method was easier to execute and was used later in the course of this work. A comparison of the two methods using gellan gum produced from *S. paucimobilis* wild type grown in Davis Mingioli medium with 2% glucose, is shown in Table 3.3.

With the Hakamori method a total of 32.6% 1,4 rhamnose was detected. This was calculated by combining the percentages obtained for the three rhamnose peaks e.g. 1,4 rha (30.5%), 1,3,4 rha (1.5%) and 1,2,4 rha (0.6%). The fragments 1,3,4 rha and 1,2,4 rha result from undermethylation of 1,4 rhamnose at carbon atoms 3 and 2 respectively.

Although only 22.7% 1,3 glucose was detected, this figure increases to 30.4% if we include the values for t- glc (0.4%), 1,3,4 glc (6.9%) and 1,2,3 glc (0.4%). In total, 31% 1,4 glc was found by adding together the two peaks 1,4 glc (29.6%) and 1,4,6 glc (1.4%). Again, for the glucose fragments, the minor peaks detected result from under methylation of 1,3 glc and 1,4 glc respectively. Using the

Table 3.2: Chemical analysis of monosaccharide components of gellan gum. The concentrations of the neutral sugars glucose and rhamnose were determined by gas chromatography, whilst the glucuronic acid was measured by colorimetric assay.

Data shown are expressed as mean ± standard deviation of one analysis of three separate batches of gellan gum for *S. paucimobilis* wild type strain.

Monosaccharide	Mole %
Rhamnose	32.6 ± 7.2
Glucose	38.6 ± 3.9
Glucuronic acid	28.1 ± 3.7

Figure 3.3: Schematic diagram showing the production of Partially Methylated Alditol Acetates (PMAAs).

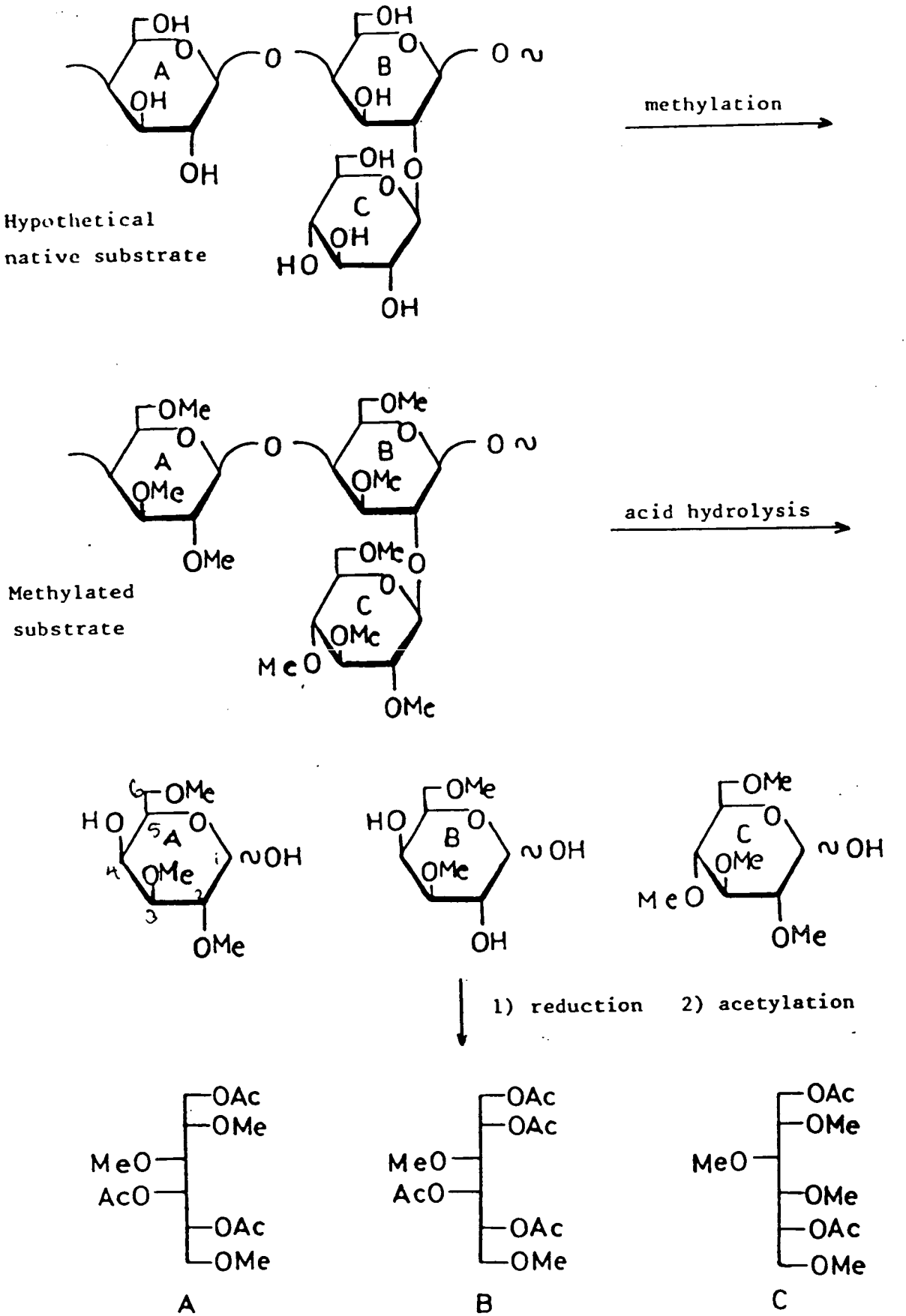


Table 3.3: Comparison of the Hakamori Method and Sodium Hydroxide (NaOH) Method for the methylation analyses of gellan gum elaborated by *Sphingomonas paucimobilis*. Values shown are the results expressed as a percentage of each sugar component from one analysis by each method of methylation.

Sugar Linkage	Hakamori Method (% monosaccharide)	NaOH Method (% monosaccharide)
1,4 Rha	30.5	38.1
t-Glc *	0.4	0
1,3,4 Rha	1.5	0
1,2,4 Rha	0.6	0
1,3 Glc	22.7	13.4
1,4 Glc	29.6	44.7
1,6 Gal	2.0	0
1,3,4 Glc	6.9	0
1,2,3 Glc	0.4	0
1,2,4 Man	0.9	0
1,4,6 Glc	1.4	0
1,3,6 Man	0.7	0
1,4,6 Gal	0.3	0
Hexitol hexaacetate	2.3	3.7

* t-glc indicates terminal glucose

Hakamori method, 2.3% galactose, 1.6% mannose and 2.3% hexitol hexaacetates were also present.
With the sodium hydroxide method 38.1% 1,4 rhamnose, 13.4% 1,3 glucose and 44.7% 1,4 glucose was found. Although there was an absence of minor peaks, 3.7% hexitol hexaacetates were also present.
Figure 3.4 shows the GC trace from the GC/MS methylation analysis of gellan gum from *S. paucimobilis* wild type strain prepared by the Hakamori method. Figures 3.5, 6 and 7 show the mass spectra produced from 1,4 rhamnose, 1,3 glucose and 1,4 glucose respectively. Sugar concentrations were calculated using peak areas from the GC analysis of PMAAs prior to GC/MS. The peak areas were then divided by the effective carbon response (e.c.r.) values outlined by Sweet *et al.* (1975). The peak area must be divided by these response factors to determine the relative moles required for quantitative analysis of sugars. The e.c.r. values used are shown in Table 3.4.

3.1.3 GC/MS analysis of carboxyreduced samples

Carboxyreduction permits the analysis of EPS containing uronic acid components. When uronic acids are present in polysaccharide it is first necessary to reduce the carboxyl group prior to hydrolysis. The methylation and purification steps of the Hakamori method used with neutral sugars were followed throughout this study. Two methods were used for the carboxyreduction. The Redgwell method involved refluxing with a solution containing lithium aluminium deuteride, to reduce the carboxyl group. The tetrahydrofuran method (THF), was simpler and faster to execute, and was generally used in this work.
Table 3.5 shows the results from the GC/MS analysis of gellan gum elaborated by *S. paucimobilis* wild type strain following carboxyreduction. Samples were prepared by both the Hakamori and NaOH methods, and the percentages of monosaccharide components detected by both methods were compared. Carboxyreduction using the THF method was carried out on both samples.
Sugar concentrations were calculated using the peak areas from GC analysis prior to GC/MS. The peak areas were then divided by the

Figure 3.4: Gas chromatogram showing the three major peaks produced from gellan gum elaborated by S. *paucimobilis*. The peaks correspond to the monosaccharides 1,4 rhamnose (29.03 min), 1,3 glucose (36.00 min) and 1,4 glucose (38.37 min).

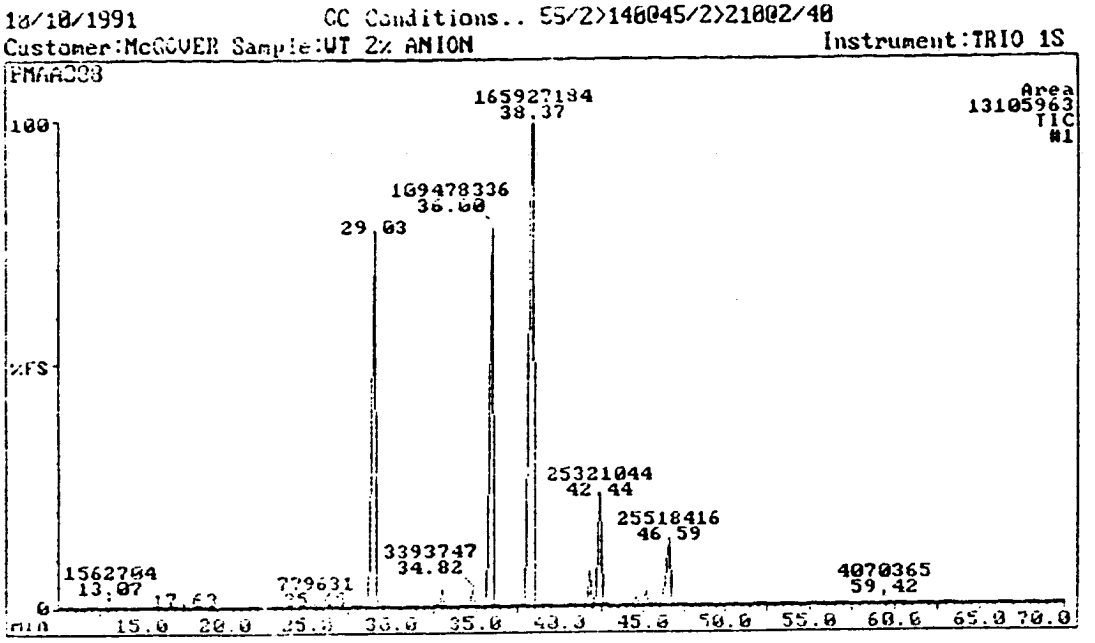


Figure 3.5: GC/MS methylation analysis spectrum corresponding to 1,4 rhamnose.

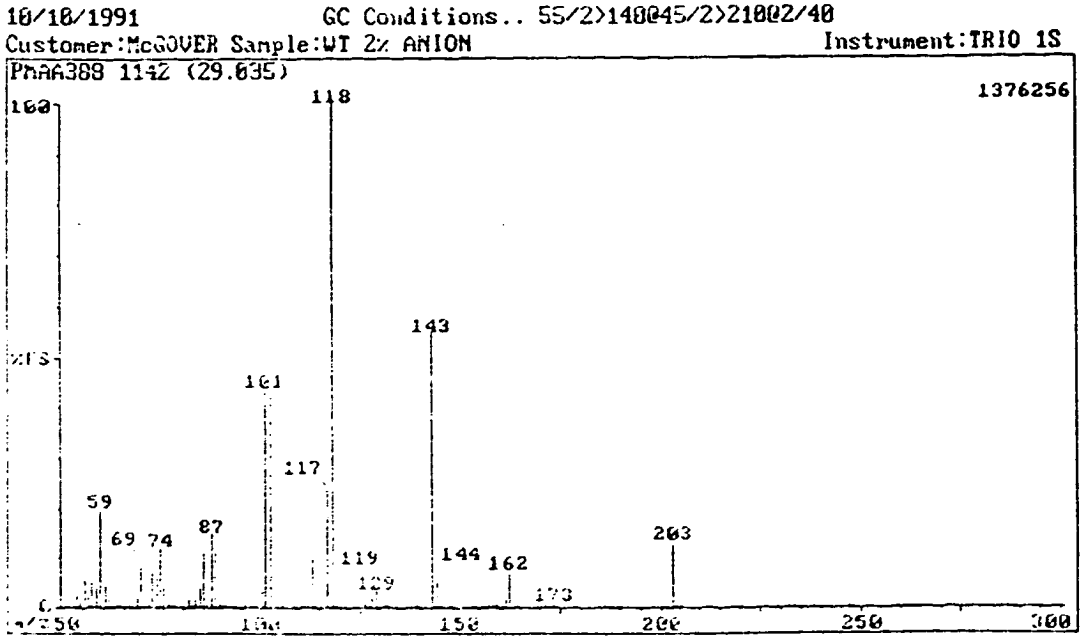


Figure 3.6: GC/MS methylation analysis spectrum corresponding to 1,3 glucose.

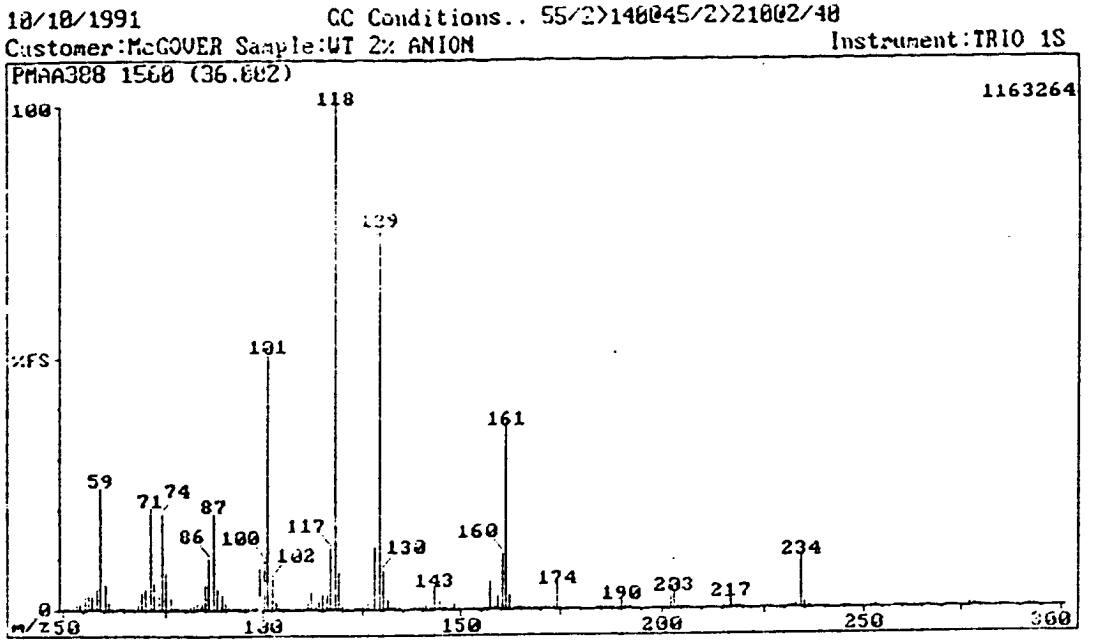


Figure 3.7: GC/MS methylation analysis spectrum corresponding to 1,4 glucose.

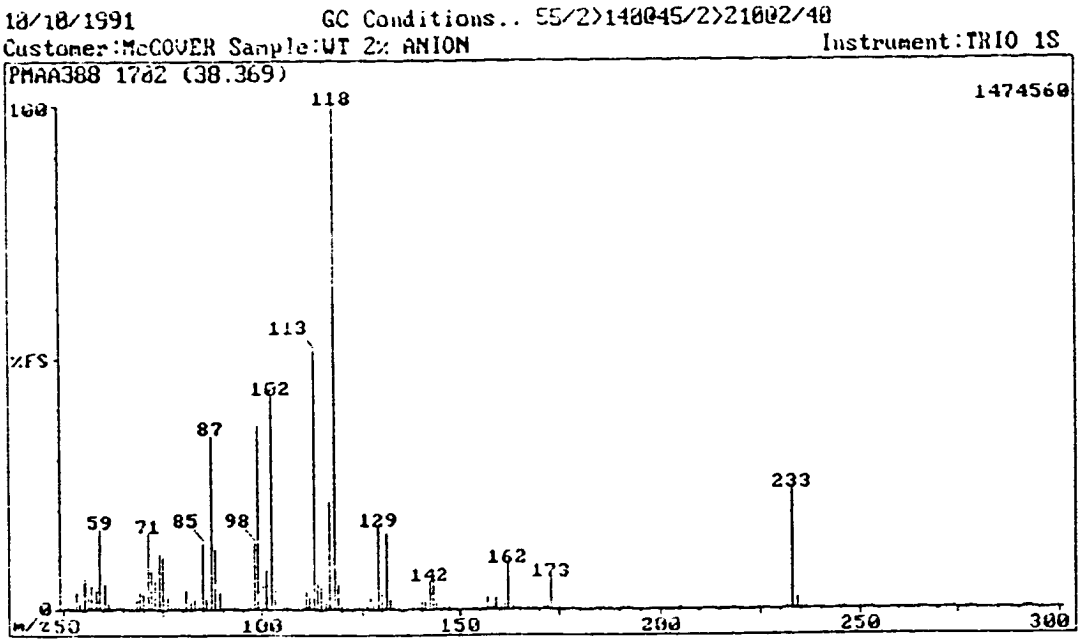


Table 3.4: Molar response factors[a] for partially methylated alditol acetates according to the Effective Carbon Response (ECR) Theory (after Sweet *et al.*, 1975).

Glycosidic linkage isomer	ECR theory factor for methylated samples
HEXOSE	
terminal	0.70
2- or 3- or 4-	0.74
6-	0.75
2,3- 3,4- or 2,4-	0.79
2,6- 3,6- or 4,6-	0.80
2,3,4-	0.84
2,3,6-,2,4, 6- or 3,4,6-	0.84
2,3,4,6-	0.89
PENTOSE	
terminal-(*f*)	0.60
terminal-(*p*)	0.61
2,3- or 4- (*p*) or 5-(*f*)	0.66
2- or 3-(*f*)	0.65
2,3-,3,4- or 2,4-(*p*) 2,5- or 3,5-(*p*)	0.70
2,3-(*f*)	0.69
2,3,4-(*p*) or 2,3,5-(*f*)	0.75
6-DEOXYHEXOSE	
terminal	0.70
2- or 3- or 4-	0.75
2,3-2,4- or 3,4-	0.79
2,3,4-	0.84

[a] Peak area ÷ response factor = relative moles.

Table 3.5: Comparison of carboxyreduced gellan gum prepared using the Hakamori Method and the Sodium Hydroxide (NaOH) Method of methylation analysis.

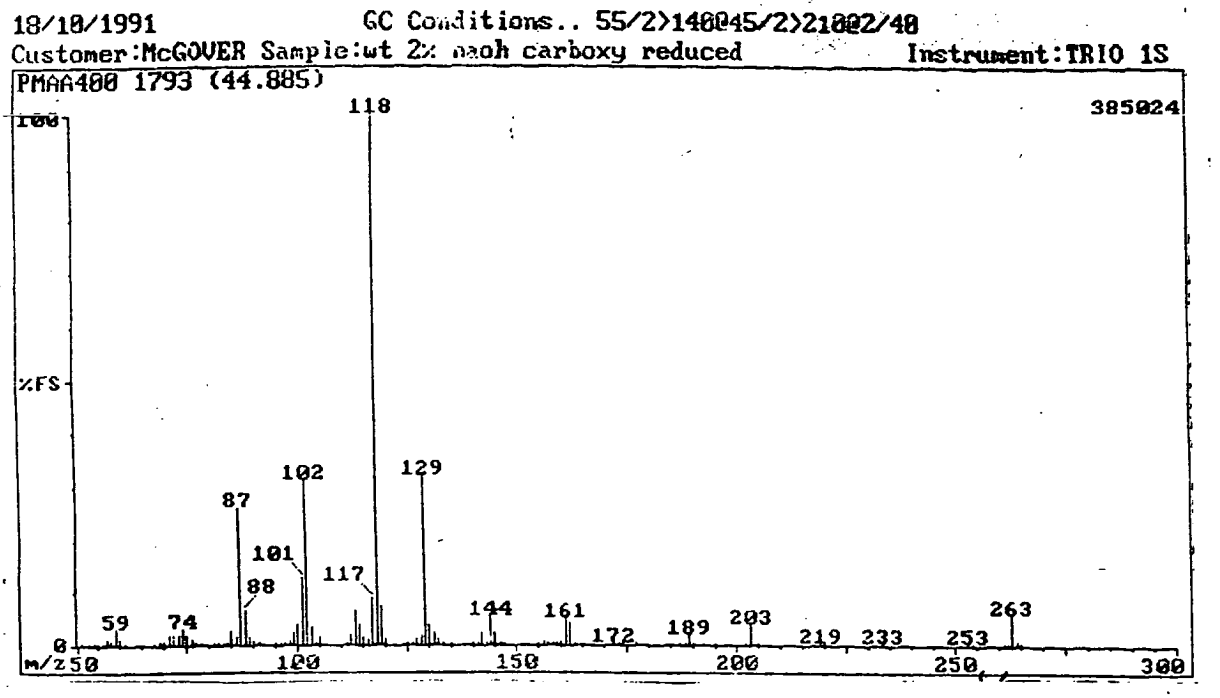
Constituent sugars are expressed as a percentage of total sugar identified.

Sugar Linkage	Hakamori Method (% monosaccharide)	NaOH Method (% monosaccharide)
t-Arabinose *	0.4	5.5
1,4 Rha	21.4	18.7
1,3,4 Rha	1.0	0.0
1,2,4 Rha	1.1	0.0
1,3 Glc	16.2	21.1
1,4 Man	0.0	2.9
1,4 Glc	25.9	25.2
1,3,4 Glc	1.6	0.0
1,2,3 Glc	4.7	0.0
1,3,6 Glc	0.8	0.0
1,4,6 Glc	25.0	18.2
1,2,3,4 Glc	0.5	0.0
6-deoxy-pentaacetate	0.5	0.0
Hexitol hexaacetate	0.8	8.3

* t-Arabinose means terminal arabinose.

ECR values outlined in Table 3.4. With the Hakamori method, 23.5% 1,4 rhamnose was detected (obtained by combining the three rhamnose fragments). Although only 16.2% 1,3 glucose was detected, this value increased to 23.8% when the minor fragments 1,3,4 glc (1.6%), 1,2,3 glc (4.7%), 1,3,6 glc (0.8%) and 1,2,3,4 glc (0.5%) were taken into consideration. There was also 25.9% 1,4 glucose and 25.0% 1,4,6 glucose (corresponding to glucuronic acid) detected by this method. Some of the main peaks were fragmented as already described. Arabinose (0.4%), 6-deoxy pentaacetate (0.5%) and hexitol hexaacetate (0.8%) were all present in small amounts.
With the NaOH method 18.7% 1,4 Rha, 21.1% 1,3 Glc, 25.2% 1,4 Glc and 18.2% 1,4,6 Glc (corresponding to glucuronic acid) was detected. In addition, 5.5% arabinose, 2.9% mannose and 8.3% hexitol hexaacetate was present. The spectrum obtained for 1,4,6 glucose is shown in figure 3.8.

Figure 3.8: GC/MS methylation analysis spectrum of carboxy-reduced 1,4 glcA corresponding to deuterated 1,4,6 glucose.



3.2 Gelation of Gellan Gum

The gelation of native and deacylated gellan gum was investigated. Polymer concentrations of 0.05-1.0% gellan were used. There appeared to be no difference in gelation in 0.1% $CaCl_2$ or 0.1% $MgSO_4$ for both native and deacylated gellan. Deacylated gellan gum formed rigid gels in $MgSO_4$ or $CaCl_2$ at levels greater than 0.2%. At lower gellan concentrations textures ranging from thick viscous solutions to a softer gel were formed. Native gellan gum, at concentrations less than 0.4% formed a solution. At 0.4% and 0.6% the gellan appeared to be in a viscous solution. At 0.8% and 1% a soft gel was formed, not a rigid gel as with the deacylated gellan (Table 3.6).

Table 3.6: Gelation of native and deacylated gellan gum in 0.1% $CaCl_2$.

Gellan (%)	Native	Deacylated
0.05	+	++
0.2	+	+++
0.4	++	++++
0.6	++	++++
0.8	+++	++++
1.0	+++	++++

Extent of gelation:-

+	solution
++	viscous solution
+++	soft gel
++++	rigid gel

3.3 Studies on Mutant Strains of *Sphingomonas paucimobilis*

Physiological studies were carried out on twenty strains of *S. paucimobilis*, including the wild type strain. Exopolysaccharide elaborated by the twenty isolates was precipitated and analysed. The strains studied in the course of this work are outlined in Table 3.7.

Fourteen of the isolates used were kindly provided by Professor Isabel Sà-Correia, Instituto Superior Tecnico, Lisbon. Six of these strains, MJ8, MJ16, MJ21, M118, MJ200 and MJ216 were produced following treatment with the alkylating agent ethyl methane sulphonate (EMS). Three strains, R40, Rif 40 and Rif 60, were mutants resistant to the antibiotic rifampicin. R40 produced high yields of expolysaccharide which was similar in structure to gellan gum. Strains Rif 40 and Rif 60 had lower yields of a polymer which was found to contain mannose. The four strains B4, HM2, NM and E3 IS, were chosen for study due to their altered colonial morphology from the wild type strain. Strain B4 produced EPS which was similar to that produced by the wild type strain, while strains HM2, NM and E3 IS produced EPS with varying amounts of mannose.

Strain ER10, was a spontaneous mutant of the wild type strain isolated at the lab of Professor Sà-Correia. The strain was believed to be a stable non-mucoid revertant.

The six other strains used during the course of this study, excluding the wild type strain, originated from the lab of Professor I.W. Sutherland. *P. elodea* strains WA and WI behaved physiologically in a similar manner to the wild type strain. However, the EPS produced by these strains was different from gellan gum produced by WT. Strains 12171 mut 2, 12171 gel- and mut 1 spont were non mucoid mutants which arose spontaneously from the wild type strain.

The twenty strains were grown in 250ml conical flasks containing 100ml of Davis Mingioli medium for 96 hours. Growth was measured at 600nm. Samples were also taken for cell dry weight measurements and viscosity of the culture broth was determined using a bench-top viscometer. EPS was estimated using the Dubois phenol/sulphuric acid assay on dialysed culture medium. Glucose utilisation was studied by the glucose oxidase assay on cell free culture medium.

Table 3.7: List of twenty strains of *Sphingomonas paucimobilis* studied for physiological differences and changes in EPS composition.

Strain	Origin
P. elodea WT	Wild Type strain, V.J. Morris, Norwich
P. elodea WI	Derived from WT (EPS found to contain traces of mannose)
P. elodea WA	Derived from WT (EPS found to contain traces of mannose)
12171 mut 2	Spontaneous non mucoid variant, Edinburgh
12171 gel-mut 1 spont	Spontaneous non mucoid variant, Edinburgh
MJ8	EMS mutant, Lisbon
MJ16	EMS mutant, Lisbon
MJ21	EMS mutant, Lisbon
M118	EMS mutant, Lisbon
MJ200	EMS mutant, Lisbon
MJ216	EMS mutant, Lisbon
R40	Rifampicin resistant mutant (high yield of EPS produced of similar composition to WT), Lisbon
Rif 40	Rifampicin resistant mutant (lower yields of mannose containing EPS), Lisbon
Rif 60	Rifampicin resistant mutant (lower yields of mannose containing EPS), Lisbon
Pe B4	Colonial morphology variant, Lisbon
HM2	Colonial morphology variant, Lisbon
NM	Colonial morphology variant, Lisbon
E3 IS	Colonial morphology variant, Lisbon
ER10	"stable" spontaneous mutant, Lisbon

Maximum values obtained for each test during the 96 hour incubation period are presented in Table 3.8. Each assay was carried out in duplicate on bacterial cultures which were set up in duplicate except for cell dry weight measurements where only one value was obtained from each flask. The results are expressed as mean values ± standard deviation.

3.3.1 Physiological Studies on *S. paucimobilis* strains

From Table 3.8 it is apparent that the twenty strains studied vary widely in their growth patterns.

The seven strains marked by an asterisk produced a fine precipitate material when the cell-free culture fluid was extracted with cold acetone at the end of the 96 hour growth period. This material was very different from the polymer, gellan gum, normally produced by *Sphingomonas paucimobilis* both in behaviour on acetone precipitation and in monosaccharide composition, as will be shown later in this chapter.

Growth curves were obtained by measuring the absorbance of culture fluid throughout the 96 hour incubation period. The culture fluid was diluted tenfold when the absorbance readings were greater than 1.0, which were generally between 12-24 hours of growth. The maximum absorbance values reached during the 96 hour period varied between 4.09 and 8.40. Cell dry weight were found to be within the range 1.36 - 1.88 gl^{-1} for those strains which were studied. Two of the strains with the largest cell yields, MJ8 and MJ216, were also strains that produced fine precipitate on acetone precipitation of cell free culture fluid.

The greatest EPS yield was 2.66 g l^{-1} for strain R40, a rifampicin-resistant mutant found to produce higher yields of gellan gum than the wild type stain. The wild type strain produced 1.98 g l^{-1} EPS. Three stains producing fine precipitate material yielded low EPS values 0.12 g l^{-1}, 0.16 g l^{-1} and 0.20 g l^{-1} for strains 12171 mut 2, 12171 gel- and mut 1 spont respectively. Strain MJ216, which also produced fine precipitate, on the other hand, produced 1.49 g l^{-1} EPS.

The greatest viscosity value was detected from wild type *Sphingomonas paucimobilis* culture broth. The viscosity readings

Table 3.8: **Table showing absorbance values (600nm), cell and EPS yields (gl $^{-1}$), culture medium viscosity (x10 $^{-3}$ Pa s) and glucose utilisation (g l $^{-1}$) for twenty strains of *Sphingomonas paucimobilis*. The figures shown are the maximum values found during the 96 hour incubation period of *S. paucimobilis* strains growing in Davis Mingioli medium at 30°C. EPS yields were calculated using the Dubois phenol sulphuric acid assay on dialysed culture medium. Glucose figures show the glucose remaining in the culture broth following incubation.**

Strain	Absorbance (600nm)	Cell dry wt. (g l $^{-1}$)	EPS wt. (g l $^{-1}$)	Viscosity (x10^{-3} Pa s)	Glucose Remaining (g l $^{-1}$)
WT	4.09 ± 0.10	1.36 ± 0.18	1.98 ± 0.63	35.2 ± 1.63	8.6 ± 0.41
WI	7.15 ± 0.50	ND	0.98 ± 0.10	22.4 ± 0.88	ND
WA	7.38 ± 0.09	ND	0.86 ± 0.03	23.0 ± 0.71	ND
12171 mut 2 *	5.18 ± 0.10	ND	0.12 ± 0.02	2.10 ± 0.31	ND
12171 gel- *	5.32 ± 0.27	ND	0.16 ± 0.03	1.46 ± 0.10	ND
mut 1 spont *	4.99 ± 0.27	ND	0.20 ± 0.02	2.32 ± 0.09	ND
MJ8 *	6.23 ± 0.07	1.82 ± 0.10	0.89 ± 0.07	1.02 ± 0.03	7.0 ± 0.05
MJ16 *	4.88 ± 0.16	1.46 ± 0.10	0.89 ± 0.10	ND	8.4 ± 0.50
MJ21	4.47 ± 0.57	1.46 ± 0.14	0.93 ± 0.08	ND	9.6 ± 0.60
M118	6.26 ± 0.18	ND	0.18 ± 0.07	17.0 ± 0.37	ND
MJ200	4.94 ± 0.24	1.40 ± 0.03	0.93 ± 0.05	ND	9.8 ± 0.37
MJ216 *	4.22 ± 0.05	1.88 ± 0.08	1.49 ± 0.23	ND	9.8 ± 0.18
R40	4.37 ± 0.38	1.56 ± 0.20	2.66 ± 0.18	ND	9.2 ± 0.18
Rif 40	7.12 ± 0.08	ND	0.65 ± 0.04	14.6 ± 2.45	ND
Rif 60	5.74 ± 0.22	ND	0.29 ± 0.04	17.4 ± 0.75	ND
Pe B4	7.62 ± 0.18	ND	0.19 ± 0.01	3.84 ± 0.08	ND
HM2	8.40 ± 0.78	ND	0.19 ± 0.04	11.20 ± 0.54	ND
NM	7.13 ± 0.30	ND	0.53 ± 0.21	13.10 ± 0.41	ND
E3 IS	6.59 ± 0.11	ND	0.56 ± 0.16	26.20 ± 0.74	ND
ER10 *	4.57 ± 0.14	1.52 ± 0.11	0.84 ± 0.08	1.02 ± 0.03	7.0 ± 0.52

Each culture was set up in duplicate flasks and each assay carried out in duplicate. Cell dry weight values are the result of only one extraction per flask. The results are expressed as mean ± standard deviation.

*** Strains produced a fine precipitate of polymeric material.**

varied between 1.02 and 35.2 mPa s. The readings were low for those strains producing fine precipitate.

Although, glucose assays were not performed for all isolates, it can be noted that between 7-10 g l^{-1} glucose was still present in the culture medium at the end of the growth period indicating that the cultures were not carbon-limited. The starting glucose concentration was 2% (20 g l^{-1}) and glucose utilisation was between 50-65% for the strains studied. Growth curves, cell and EPS yields and glucose utilisation graphs for strains R40 and ER10 are presented in figures 3.9 and 3.10 respectively.

3.3.2 EPS yields from *S. paucimobilis* strains

Sixteen of the strains studied were grown in Davis Mingioli medium in 100ml volumes in 250ml conical flasks for 96 hours. The cells were removed from the culture broth by centrifugation and three volumes of cold acetone were then added to the supernatant to precipitate the polysaccharide material. The EPS precipitated was freeze-dried and weighed. The values for one such experiment are shown in Table 3.9. The samples are listed in descending yield of EPS.

The six strains at the foot of the table, marked by an asterisk, produced a fine precipitate on acetone precipitation. This material was like powder and fell to the bottom of the flask on addition of acetone. Normally, exopolysaccharide wraps itself around the glass rod used to stir the mixture. When the EPS yields of Table 3.9 are compared to the EPS values obtained by the carbohydrate assay (Table 3.8), no correlation can be found between the two methods. Strain MJ216, which had a high reading with the carbohydrate assay of 1.49 gl^{-1} EPS, only yielded 1.6gl^{-1} EPS on acetone precipitation. Although the EPS yields measured by the two methods are very similar, the amount of EPS measured by acetone precipitation for strain MJ216 was the lowest yield of the sixteen strains, whereas the EPS yield measured by the carbohydrate assay was the third highest yield of the sixteen strains studied. Quite a strong relationship can be noticed between high EPS yields and high viscosity readings, especially the fine precipitate samples which produced only a small amount of EPS by acetone precipitation.

Figure 3.9: Growth (as measured by absorbance at 600nm), glucose uptake, cell and EPS dry weights of S. *paucimobilis* strain R40 grown in Davis Mingioli medium for 96 hours at 30°C.

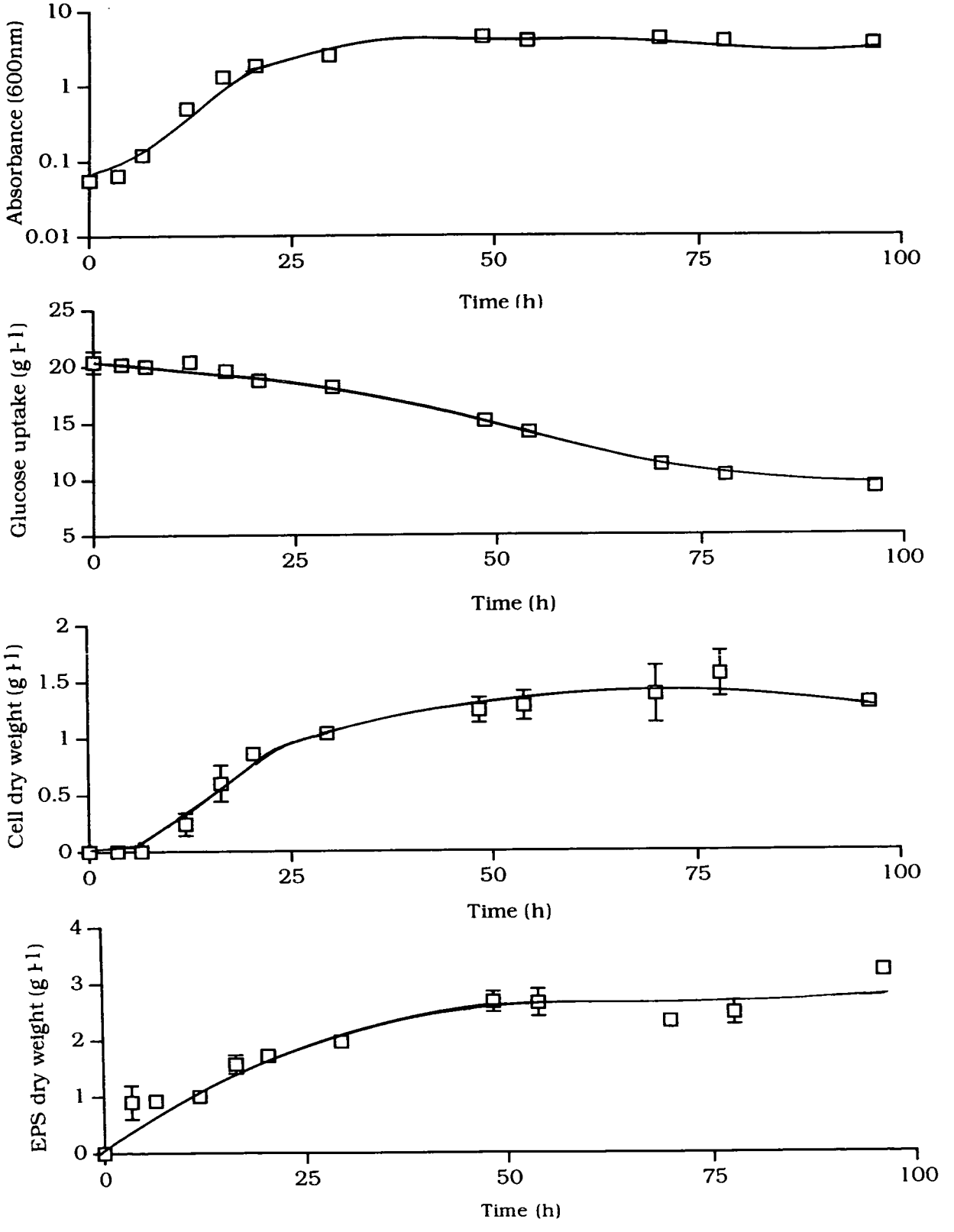


Figure 3.10: Growth (as measured by absorbance at 600nm), glucose uptake, cell and EPS dry weights of S. *paucimobilis* strain ER10 grown in Davis Mingioli medium for 96 hours at 30°C.

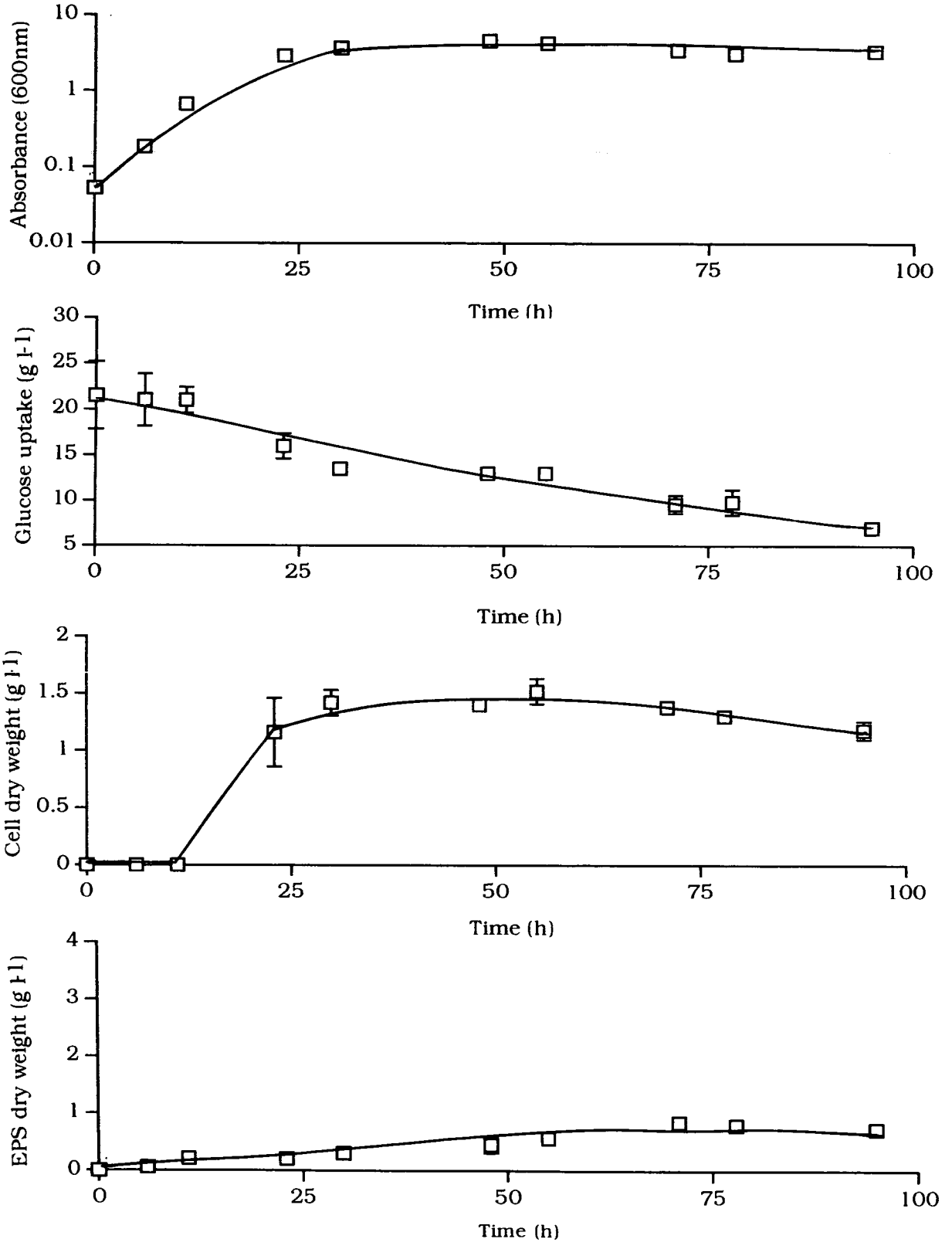


Table 3.9: Exopolysaccharide yields from *Sphingomonas paucimobilis* strains grown in Davis Mingioli medium (100ml volumes in 250ml conical flasks) for 96 hours at 30°C.

Strain	EPS Yield (gl^{-1})	Conversion of Glucose to EPS (%)
WT	8.0	40.0
WI	6.6	33.0
E3 IS	6.0	30.0
Rif 40	5.9	29.5
M118	5.8	29.0
NM	5.5	27.5
Rif 60	5.4	27.0
HM2	3.9	19.5
MJ200	3.8	19.0
MJ16 *	3.1	15.5
12171 mut 2 *	2.8	14.0
mut 1 spont *	2.7	13.5
MJ8 *	2.6	13.0
B4	2.5	12.5
12171 gel- *	2.1	10.5
MJ216 *	1.6	8.0

Values shown are the results from one extraction

* Strains producing fine precipitate EPS.

Strain B4, which had a low viscosity reading was also found to have a low yield of EPS.

Strains were also grown in volumes of 800ml in 21 conical flasks. When this exopolysaccharide material was precipitated, again using 3 volumes of cold acetone, the amount of EPS obtained was less. Yields of EPS from strains WT, MJ200 and MJ216 are compared below:-

Strain	Amount of EPS extracted (gl^{-1})	
	800ml medium in 21 flask	100ml medium in 250ml flask
WT	0.73	8.00
MJ200	0.41	3.80
MJ216	0.17	1.62

The polymer extracted from the larger volume was approximately ten times less than the amount obtained from the smaller volume.

3.3.3 Chemical composition of EPS produced by strains of *Sphingomonas paucimobilis*

Following acetone precipitation of exopolymers produced by the *S. paucimobilis* strains, the samples were analysed by gas chromatography. Monosaccharide components were quantified as a percentage of sugars detected. The results are shown in Table 3.10. The polymers analysed could be broadly based into two main categories:-

① EPS with a similar composition to gellan gum.
② Material which precipitated as a fine powder.

Table 3.10 has been subdivided into 5 sections. The first section shows three strains, WT, MJ21 and R40, producing EPS containing the same constituents as gellan gum, in varying ratios. The second,

Table 3.10: Table of analyses of exopolysaccharide elaborated by *Sphingomonas paucimobilis*. Monosaccharide components were analysed by gas chromatography. Acetyl constituents were assayed by colorimetric assay. Values are expressed as mean ± standard deviation of 2-4 analyses.

	GLUCOSE (%)	RHAMNOSE (%)	MANNOSE (%)	GALACTOSE (%)	ACETYL (%)
WT	57.7 ± 0.6	42.3 ± 0.6	-	-	NT
MJ21	54.1 ± 1.8	45.9 ± 1.8	-	-	NT
R40	53.6 ± 1.8	46.4 ± 1.8	-	-	NT
MJ200	55.8 ± 3.8	41.7 ± 2.1	2.4 ± 2.4	-	NT
B4	54.4 ± 0.4	41.1 ± 6.1	4.6 ± 6.4	-	4.3 ± 0.2
WA	53.6 ± 0.2	44.4 ± 0.2	1.9 ± 0.4	-	4.6 ± 0.6
Rif 60	53.5 ± 0.0	33.9 ± 0.0	12.7 ± 0.0	-	3.8 ± 0.9
HM2	53.1 ± 0.8	41.0 ± 2.9	6.0 ± 3.7	-	2.3 ± 1.1
E3 IS	51.3 ± 1.8	40.8 ± 3.0	7.9 ± 4.8	-	7.0 ± 0.2
WI	50.8 ± 0.5	43.2 ± 3.6	6.2 ± 3.2	-	3.7 ± 1.3
NM	50.6 ± 2.9	41.8 ± 1.1	7.6 ± 1.8	-	5.6 ± 0.8
M118	50.0 ± 5.0	39.3 ± 1.1	10.6 ± 3.9	-	5.0 ± 1.2
Rif 40	47.8 ± 5.6	42.8 ± 4.3	9.3 ± 9.9	-	7.7 ± 0.1
MJ216A	86.6 ± 3.5	13.4 ± 3.5	-	-	NT
MJ16	83.8 ± 0.4	5.2 ± 0.4	11.0 ± 0.1	-	NT
ER10	81.0 ± 2.6	4.7 ± 2.2	15.0 ± 1.0	-	NT
mut 2	15.7 ± 2.0	0.6 ± 1.1	83.7 ± 3.0	-	1.7 ± 0.4
mut 1	14.0 ± 4.1	5.6 ± 2.9	80.4 ± 7.0	-	1.4 ± 0.5
gel-	43.2 ± 2.1	4.4 ± 0.8	15.7 ± 3.8	36.7 ± 1.0	8.5 ± 0.2
MJ216B	25.0 ± 11.4	40.2 ± 1.8	16.8 ± 1.8	18.0 ± 14.9	NT

and largest section of Table 3.10, reports information on the EPS from 10 strains of *S. paucimobilis* which produce exopolymer which has a similar glc:rha ratio as gellan but contains also 1.9-12.7% mannose. The bottom section of the table is subdivided into 3 parts. Exopolymeric material elaborated by these bacteria did not precipitate from cell free supernatant in the usual manner to EPS.

The powder-like material from the culture broth fell to the base of the flask on addition of acetone and has been called fine precipitate. This material was of three different kinds. Section 3 of Table 3.10 shows fine precipitate elaborated by MJ216A, MJ16 and ER10 which was composed mainly of glucose with rhamnose and mannose, except for MJ216A which contained only glc and rha. Section 4 shows fine precipitate composed mostly of mannose, but also containing glucose and only a small amount of rhamnose. The fifth and final section of Table 3.10, shows EPS which contain galactose in addition to glc, rha and man.

Three batches of EPS were produced from strain MJ216 and analysed in duplicate. Two batches were found to contain the sugars shown by MJ216A, whilst the third batch contained the sugars shown by MJ216B. On one occasion when precipitating EPS from the culture medium of MJ200 using acetone, the polymer separated into two distinct fractions. Some polymer wrapped itself around the glass rod used to stir the acetone/culture broth mixture, this was called 200 ROD. The remainder fell to the bottom of the flask as a fine precipitate and was called 200 PPT. Sugar analyses of the two fractions revealed that 200 ROD had a similar sugar composition to wild type gellan gum (as shown in Table 3.10), whilst 200 PPT was composed mainly of glucose. This was the only strain, and the only occasion, where two separate fractions originated from the same culture.

Acetyl concentrations were also determined for some of the strains and were found to vary between 1.4-7.7% of EPS tested. The average acetyl content of the polymers studied was 4.7%. The neutral sugars contained in these polymers were also determined by HPLC and GC/MS. The ratios of glucose:rhamnose:mannose determined using these techniques were compared to the ratios found using GC. This data is shown in Table 3.11.

Table 3.11: Table showing ratios of glucose:rhamnose:mannose in extracellular material elaborated by strains of *Sphingomonas paucimobilis* determined by GC, HPLC and GC/MS.

Strain	GC	HPLC	GC/MS
MJ21	1.2 : 1.0 : 0.0	2.5 : 1.0 : 0.3	NT
MJ200	1.3 : 1.0 : 0.1	3.6 : 1.0 : 0.3	5.0 : 0.0 : 1.0
B4	1.3 : 1.0 : 0.1	1.5 : 1.0 : 0.1	2.2 : 1.0 : 0.3
WA	1.2 : 1.0 : 0.04	0.7 : 1.0 : 0.2	2.5 : 1.0 : 0.0
HM2	1.3 : 1.0 : 0.1	1.1 : 1.0 : 0.1	2.4 : 1.0 : 0.0
E3 IS	1.3 : 1.0 : 0.2	1.4 : 1.0 : 0.5	2.2 : 1.0 : 1
WI	1.2 : 1.0 : 0.1	0.9 : 1.0 : 0.0	NT
NM	1.2 : 1.0 : 0.2	1.2 : 1.0 : 0.2	2.6 : 1.0 : 0.0
M118	1.3 : 1.0 : 0.3	1.0 : 1.0 : 0.2	2.5 : 1.0 : 0.0
Rif 40	1.1 : 1.0 : 0.2	1.6 : 1.0 : 0.6	2.0 : 1.0 : 0.0
mut 2	1.0 : 0.04 : 5.3	1.0 : 0.9 : 3.6	NT
mut 1	1.0 : 0.4 : 5.7	1.0 : 1.3 : 2.5	NT
gel- *	2.7:0.28:1.0:2.3	0.7:0:1.0:1.3	1.5:0.1:1.0:2.0

NT Not Tested

Ratios shown are mean values of 2-3 repeat analyses

* Strain gel- results are ratios of glc:rha:man:gal.

3.3.4 Gelation of polysaccharides produced by certain strains of *S. paucimobilis*

The gelation of six polysaccharides were compared. Five were from *Sphingomonas paucimobilis* strains MJ11, MJ21, MJ200 ROD, MJ200 PPT and MJ210. The other polymer studied was S-130, produced by an *Alcaligenes* species also now regarded as *Sphingomonas*. Its structure is outlined in figure 1.9. One batch of S-130 polysaccharide was found to have the same sugar composition as gellan gum rather than the six sugar repeat expected. The samples were deacylated and gels prepared at 1% concentration. Four of the polysaccharides - MJ11, MJ21, MJ200 ROD and MJ210 formed gels at 1% concentration. The polymers from S-130 and MJ200 PPT did not gel.

3.3.5 Rheological measurements

Rheological measurements were attempted on eight polysaccharide samples. Certain strains could not be used, as the polysaccharide yield was very low. Gels were prepared as described in Chapter 2.18. The gels were initially assessed in petri dishes (Table 3.12). Where possible gel strength was tested using a mechanical rheometer. When no gelation occurred and the samples remained liquid, a viscosity measurement was taken instead. Gel strength plots were obtained for each sample. Two examples of a strong gel (WA) and weak gel (B4) were studied. Graphs obtained from samples WA and B4 are shown in figures 3.11 and 3.12 respectively. Sample WA formed a strong rigid gel whereas B4 remained in a liquid-like state and did not form a true gel. For sample WA (figure 3.11), G' >> G" by two orders of magnitude. Both G' and G" were independent of frequency, characteristic of an elastic gel. G' was large and the gel was free standing. For sample B4 (figure 3.12), G' ~ G" and both were frequency dependent with G" > G' at a sufficiently low frequency the curve would cross over. This suggested a weak viscoelastic gel, incapable of withstanding its own weight.

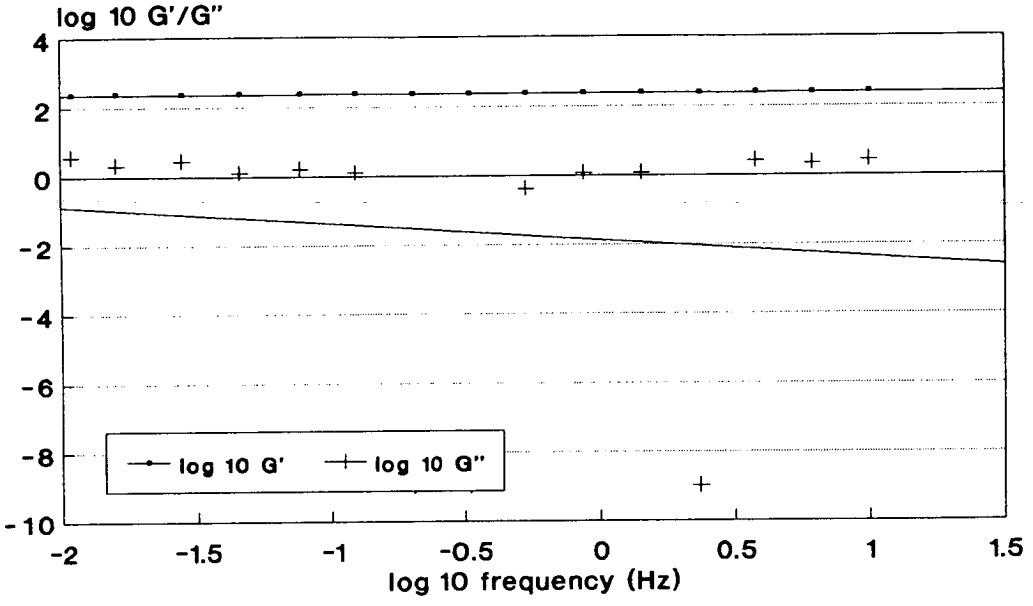
Table 3.12: Visual assessment of 1% polysaccharide gels prepared in petri dishes for rheological measurements.

Strain	Gel classification
WA	++++
NM	+++
M118	+++
Rif 40	+++
Rif 60	++
WI	++
E3 IS	+
B4	+

Code for gel classification:-

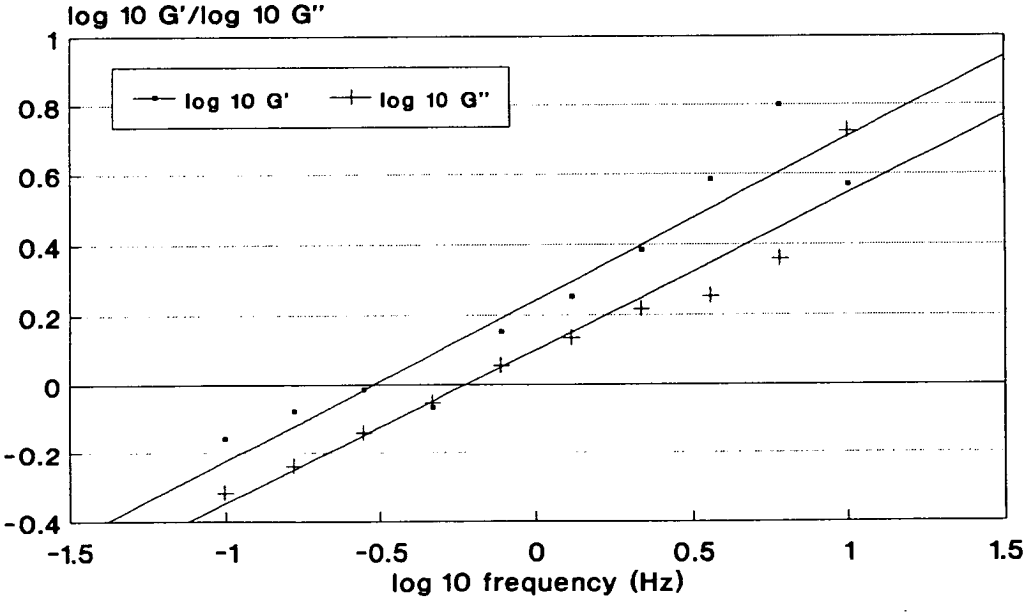
++++ rigid gel
+++ soft gel
++ viscous solution
+ solution

Figure 3.11: Automatic frequency sweep (controlled strain) for *Sphingomonas paucimobilis* strain WA.



1% DEFORMATION

Figure 3.12: Automatic frequency sweep (controlled strain) for *Sphingomonas paucimobilis* strain B4.



50% DEFORMATION

CHAPTER 4

RESULTS

4. THE EFFECTS OF NITROGEN LIMITATION ON *SPHINGOMONAS PAUCIMOBILIS*

4.1 Preliminary studies with *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with reduced levels of $(NH_4)_2SO_4$

The effects of nitrogen concentration on the growth and EPS production of *S. paucimobilis* was investigated. *S. paucimobilis* wild type strain was grown in Davis Mingioli medium (100ml in 250 ml conical flasks) containing 0-0.1% $(NH_4)_2SO_4$ over a 42 hour period (figure 4.1 and Table 4.1). Samples of culture broth were removed periodically and growth was measured by absorbance at 600 nm. Duplicate flasks were used for each condition and absorbance values were also taken in duplicate. The greatest optical density value of 2.65 was achieved when the culture medium contained 0.1% $(NH_4)_2SO_4$. Specific growth rates were calculated for *S. paucimobilis* wild type strain growing in Davis Mingioli medium with decreasing concentrations of $(NH_4)_2SO_4$. Specific growth rates throughout this chapter were calculated from $\log_{10}$ values and are, therefore, expressed as μ' values (Tempest, 1978) At 0.1% $(NH_4)_2SO_4$ the specific growth rate (μ') was 0.095. The specific growth rate decreased with decreasing $(NH_4)_2SO_4$ concentration to a value of 0.019 when no $(NH_4)_2SO_4$ was present in the culture medium.

Viscosity measurements were also recovered for samples of culture broth using a Brookfield viscometer. The shear rate was altered between 0.3-30 s^{-1}. Viscosity, at a shear rate of 6 s^{-1} decreased with increasing concentrations of $(NH_4)_2SO_4$ in a linear fashion (Table 4.1). The greatest viscosity value was recorded for culture broth containing the highest amount of $(NH_4)_2SO_4$ at 0.1%. Flasks containing less than 0.005% $(NH_4)_2SO_2$ had viscosity similar to the flasks with no $(NH_4)_2SO_4$. Exopolysaccharide yield was assessed by acetone precipitation of cell free culture broth for duplicate flasks following growth for 48 hours. EPS production increased with increasing $(NH_4)_2SO_4$ concentration of the culture medium (figure 4.2).

Figure 4.1: Growth curve for *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with varying concentrations of $(NH_4)_2SO_4$. Growth was recorded as absorbance readings at 600nm and plotted against time (h). Results are expressed as mean ± standard deviation of duplicate flasks with two readings from each flask.

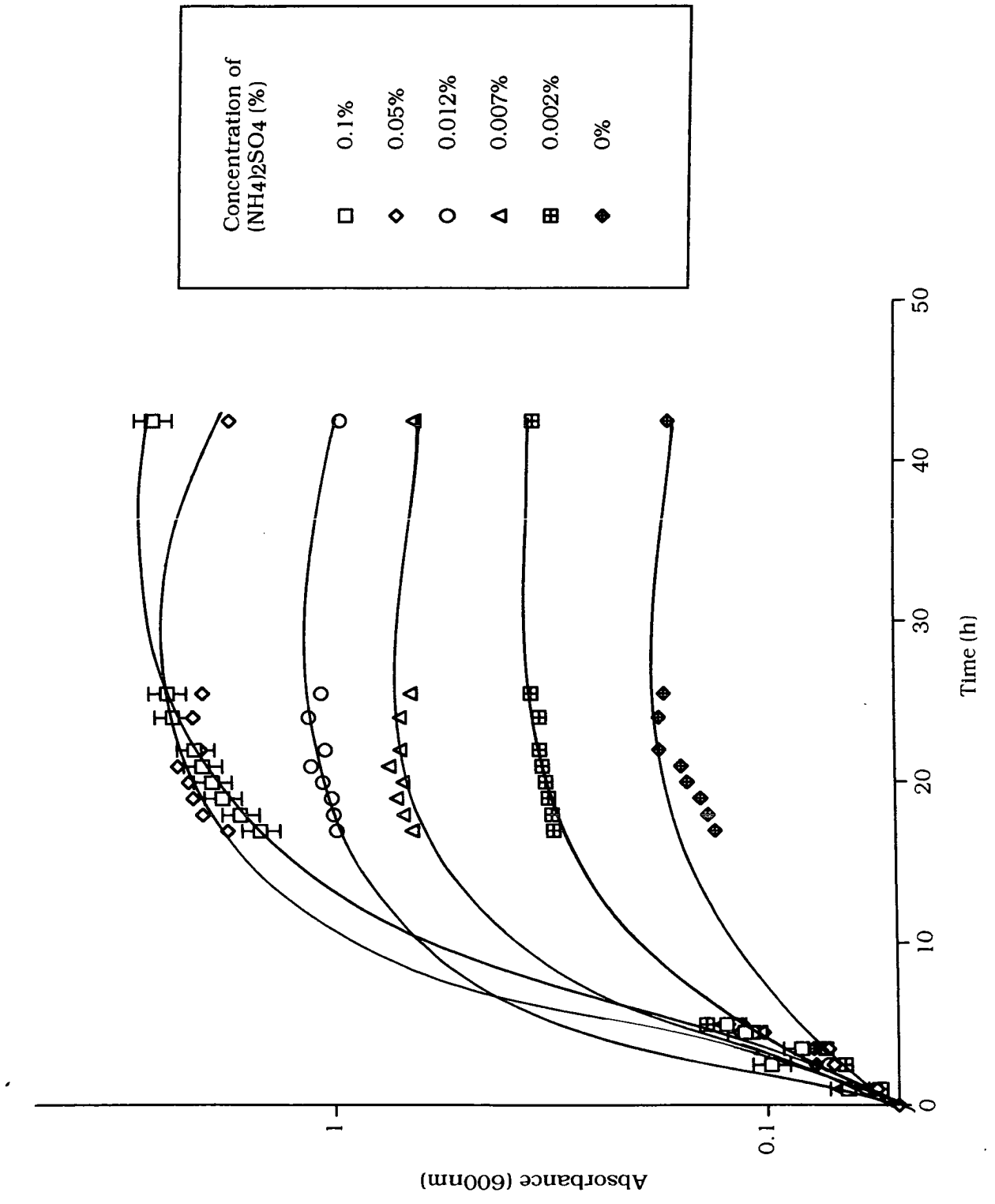


Table 4.1: Table showing specific growth rates, culture broth viscosity measurements and EPS yield for *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with different concentrations of $(NH_4)_2SO_4$. Each value is expressed as mean ± standard deviation. Each culture was grown in duplicate flasks and duplicate values were taken for both absorbance and viscosity readings. EPS yield is expressed as acetone precipitated material from each of the duplicate flasks following 48 hours growth.

CONCENTRATION OF $(NH_4)_2SO_4$ (%)	SPECIFIC GROWTH RATE (μ^1)	VISCOSITY OF CULTURE BROTH (SHEAR RATE 6 s^{-1})	EPS YIELD ($g\ l^{-1}$)
0.1	0.0953	28.6 ± 1.33	2.77 ± 0.40
0.05	0.088	17.7 ± 0.92	1.72 ± 0.27
0.012	0.0715	6.0 ± 0.37	ND
0.01	0.0635	4.4 ± 0.24	0.594 ± 0.04
0.0067	0.0602	3.5 ± 0.08	ND
0.005	0.0545	2.3 ± 0.39	0.489 ± 0.07
0.0029	0.044	1.9 ± 0.14	0.335 ± 0.01
0.002	0.044	1.73 ± 0.15	0.265 ± 0.01
0	0.019	2.3 ± 0.39	ND

It was necessary to examine the effect of reduced nitrogen levels on the growth of *S. paucimobilis* and the production of gellan gum in greater detail.

An experiment was set up to investigate whether cultures growing with reduced $(NH_4)_2SO_4$, would continue to grow, and produce more EPS, when the $(NH_4)_2SO_4$ concentration was restored to 0.1% following growth for 48 hours (Table 4.2). Duplicate flasks containing 100ml of Davis Mingioli medium were prepared containing 0.012% and 0.025% $(NH_4)_2SO_4$. One flask containing 0.1% $(NH_4)_2SO_4$ was used as a control representing the maximum nitrogen concentration studied. One flask with no $(NH_4)_2SO_4$ was also prepared. Samples of culture broth were removed daily for absorbance readings, culture medium viscosity measurements and carbohydrate assays. The carbohydrate assay was used to determine the amount of EPS in the culture broth. The carbohydrate assay was performed on dialysed cell free culture medium. All tests were carried out in duplicate. After 48 hours, when the cultures were believed to have reached stationary phase, the $(NH_4)_2SO_4$ concentration was restored to 0.1% in one of each of the flasks containing 0.012% and 0.025% $(NH_4)_2SO_4$ and in the flask with no nitrogen initially. The flasks were then incubated for a further 96 hours. The flask containing 0.1% $(NH_4)_2SO_4$ initially and one flask each containing 0.012% and 0.025% $(NH_4)_2SO_4$ were unsupplemented. After 48 hours, the flask initially containing 0.1% and 0.025% $(NH_4)_2SO_4$ had similar absorbance values of 1.41-1.50. The flasks with only 0.012% $(NH_4)_2SO_4$ had reached absorbances of 0.89 and 0.90. The optical density reading in the flask with no nitrogen was zero. After 144 hours, the OD had increased to 1.73 in the flask with 0.1% $(NH_4)_2SO_4$. In the flasks which had nitrogen added, the OD value was the same at 144 hours for the 0.025% concentration, but had increased to 1.38 and 1.52 for 0.012% and 0% $(NH_4)_2SO_4$ respectively. In the unsupplemented flasks at 0.025% and 0.012% $(NH_4)_2SO_4$ the absorbance had decreased at 144 hours. The viscosity readings for the 0.1% and 0.025% $(NH_4)_2SO_4$ concentrations were very similar after 48 hours. At 144 hours the viscosity had increased in the 0.1% $(NH_4)_2SO_4$. In the other flasks the viscosity stayed about

Figure 4.2: Exopolysaccharide production by *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium in 100ml amounts in 250ml conical flasks with different amounts of $(NH_4)_2SO_4$. EPS yield was expressed as mean ± standard deviation of duplicate flasks by acetone precipitation following growth for 48 hours.

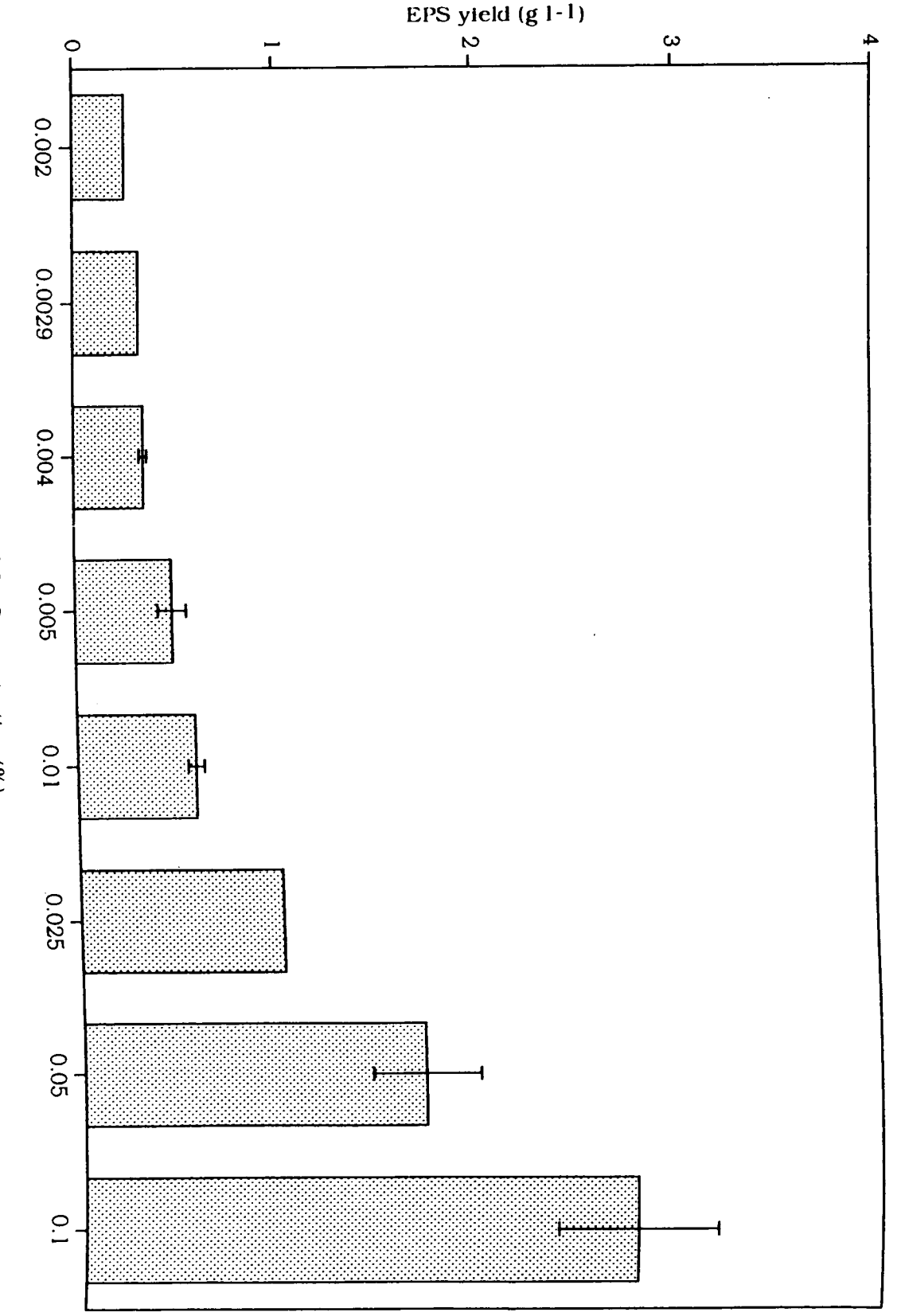


Table 4.2: Table showing change in absorbance (600nm), culture broth viscosity and EPS yield after 48 and 144 hours for cultures of *Sphingomonas paucimobilis* wild type strain growing in Davis Mingioli medium with reduced levels of $(NH_4)_2SO_4$. Flasks containing 0.1% 0.025%, 0.012% and no $(NH_4)_2SO_4$ were prepared and the effect of supplementing certain flasks with extra $(NH_4)_2SO_4$ after 48 hours was compared to unsupplemented flasks.

$(NH_4)_2SO_4$ CONCENTRATION	AFTER 48 HOURS			AFTER 144 HOURS		
	OD (600nm)	VISCOSITY ($x10^{-3}$ Pa s)	EPS ($g\ l^{-1}$)	OD (600nm)	VISCOSITY ($x10^{-3}$ Pa s)	EPS ($g\ l^{-1}$)
0.1% (CONTROL)	1.41 ± 0.08	16.0 ± 3.11	2.73 ± 0.21	1.73 ± 0.03	27.9 ± 0.71	3.25 ± 0.06
0.025%	1.50 ± 0.11	18.4 ± 0.28	1.94 ± 0.11	1.08 ± 0.10	18.9 ± 0.71	2.1 ± 0.18
0.025% (SUPPLEMENTED)	1.45 ± 0.11	16.7 ± 1.56	1.80 ± 0.08	1.51 ± 0.07	35.7 ± 0.28	3.98 ± 0.23
0.012%	0.89 ± 0.24	9.2 ± 0.85	1.54 ± 0.11	0.70 ± 0.16	10.3 ± 0.71	1.38 ± 0.07
0.012% (SUPPLEMENTED)	0.90 ± 0.11	9.8 ± 0.28	1.45 ± 0.17	1.38 ± 0.11	29.9 ± 1.98	4.61 ± 0.10
0%	0.01 ± 0.0	1.6 ± 0.03	0.24 ± 0.03	1.52 ± 0.14	26.7 ± 1.84	3.51 ± 0.34

Results are expressed as mean ± standard deviation for duplicate tests for each flask.

the same in the unsupplemented flasks but had greatly increased in the supplementary flasks. A similar pattern was noted with the EPS yields. The EPS yield was greater in the supplemented flasks at 144 hours than in the flask originally containing 0.1% $(NH_4)_2SO_4$.

4.2 Growth studies with *Sphingomonas paucimobilis* strains WT, SB10, PA4 and MJ8 in Davis Mingioli medium containing 0.1% $(NH_4)_2SO_4$

S. paucimobilis strains WT, SB10, PA4 and MJ8 were grown in Davis Mingioli medium (800ml in 2 l flasks) to determine differences between the three mutant strains compared to the wild type strain. The strains were grown initially in Davis Mingioli (DM) medium which contained 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. This composition of DM medium will be referred to throughout the following chapters as the "control" condition. In later experiments, the concentrations of glucose, $(NH_4)_2SO_4$ and $MgSO_4$ were decreased and the growth and EPS production from each strain compared to the control condition described in this section. For every growth experiment each culture was set up in duplicate. Samples of culture broth were removed periodically throughout the 96 hour growth period and measurements were made. Growth was assessed by the absorbance of culture broth at 600nm. Cell dry weights were made by centrifuging cells from a known volume of culture medium. EPS dry weight was then measured by acetone precipitation of the same cell-free culture fluid. Viscosity measurements were made directly on culture medium and the pH was also recorded. Residual glucose was calculated using a colorimetric assay on cell-free culture medium.

Growth and EPS production of *S. paucimobilis* strains WT, PA4, SB10 and MJ8 in DM medium (control condition) are shown in figures 4.3-4.6. Results are expressed as mean ± standard deviation for two tests from each duplicate flask except for cell and EPS dry weights where only one measurement was made for each flask. The maximum values achieved for each strain with different nitrogen concentrations are shown in Table 4.3. Broadly speaking, the data were very similar for the four strains. The greatest absorbance reading of 7.73 was found with strain MJ8 which was non mucoid (figure 4.6). Most strains reached stationary phase at about 25

Table 4.3: Summary table showing maximum values obtained for *S. paucimobilis* strains WT, SB10, PA4 and MJ8 grown in Davis Mingioli medium with reduced levels of $(NH_4)_2SO_4$. Values shown are expressed as mean ± standard deviation of duplicate assays carried out on duplicate flasks (except cell and EPS dry weight where only one measurement was made for each flask).

STRAIN PA4 $(NH_4)_2SO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.1	0.080	5.58 ± 0.68	1.12 ± 0.10	2.06 ± 0.28	5.90 ± 0.42	34.15 ± 2.89	5.74 ± 0.13
0.02	0.045	1.8 ± 0.3	0.42 ± 0.01	1.48 ± 0.20	15.43 ± 0.20	26.3 ± 1.62	6.77 ± 0.01
0.01	0.048	1.6 ± 0.6	0.19 ± 0.01	1.23 ± 0.25	14.65 ± 2.42	15.6 ± 0.32	6.86 ± 0.01
0.004	0.054	0.9 ± 0.03	0.15 ± 0.02	0.96 ± 0.26	18.62 ± 1.36	8.12 ± 0.04	6.93 ± 0.01

STRAIN WT $(NH_4)_2SO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.1	0.068	5.31 ± 0.67	0.88 ± 0.07	0.75 ± 0.10	3.68 ± 2.33	35.22 ± 2.15	5.86 ± 0.04
0.02	0.145	2.44 ± 0.10	0.72 ± 0.02	1.91 ± 0.17	10.82 ± 0.72	27.2 ± 0.72	6.72 ± 0.01
0.01	0.157	1.18 ± 0.02	0.39 ± 0.06	0.85 ± 0.11	16.59 ± 0.63	12.2 ± 0.16	6.82 ± 0.01
0.004	0.111	1.82 ± 0.28	0.19 ± 0.004	0.69 ± 0.007	18.03 ± 0.59	5.74 ± 0.06	6.91 ± 0.01

STRAIN SB10 $(NH_4)_2SO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.1	0.073	6.70 ± 2.09	0.83 ± 0.04	0.83 ± 0.23	5.30 ± 2.90	30.40 ± 5.43	5.98 ± 0.01
0.02	0.101	2.94 ± 0.33	0.68 ± 0.05	2.01 ± 0.68	10.60 ± 0.42	30.8 ± 1.13	6.91 ± 0.01
0.01	0.120	1.99 ± 0.04	0.29 ± 0.014	1.46 ± 0.06	12.06 ± 1.63	15.02 ± 0.13	6.82 ± 0.01
0.004	0.131	1.84 ± 0.17	0.27 ± 0.01	0.78 ± 0.00	18.83 ± 1.94	5.90 ± 0.03	6.91 ± 0.01

STRAIN MJ8 $(NH_4)_2SO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.1	0.067	7.73 ± 2.06	1.95 ± 0.70	0	4.35 ± 2.86	1.08 ± 0.02	6.20 ± 0.14
0.02	0.101	2.13 ± 0.25	0.54 ± 0.1	0	ND	1.06 ± 0.00	6.82 ± 0.00
0.01	0.089	1.42 ± 0.11	0.34 ± 0.05	0	ND	1.06 ± 0.00	6.88 ± 0.04
0.004	0	0.63 ± 0.10	0.14 ± 0.10	0	ND	1.03 ± 0.04	6.96 ± 0.014

Table 4.4: Table showing maximum cell and EPS weights, EPS/cell values, glucose utilised and EPS/glc values for *S. paucimobilis* strains WT, SB10, PA4 and MJ8. Cultures were set up in duplicate and grown in Davis Mingioli medium with varying amounts of $(NH_4)_2SO_4$ for 96 hours at 30°C.

STRAIN / $(NH_4)_2SO_4$ CONC. (%)		CELL DRY WT ($g\ l^{-1}$)	EPS DRY WT ($g\ l^{-1}$)	EPS/ CELL ($g\ g^{-1}$)	GLUCOSE USED $g\ l^{-1}$ OR (%)		EPS/ glc (%)
PA4	0.1	1.12	2.06	1.8	14.1	70	14.6
	0.02	0.42	1.48	3.5	4.6	23	32.2
	0.01	0.19	1.23	8.2	5.4	27	22.8
	0.004	0.15	0.96	6.4	1.4	7	68.6
WT	0.1	0.88	0.75	0.9	16.3	82	4.6
	0.02	0.72	1.91	2.7	9.2	46	20.8
	0.01	0.39	0.85	2.2	3.4	17	25.0
	0.004	0.19	0.69	3.6	2.0	10	34.5
SB10	0.1	0.83	0.85	1.0	14.7	74	5.8
	0.02	0.68	2.01	3.0	9.4	47	21.4
	0.01	0.29	1.46	5.0	7.9	40	18.5
	0.004	0.27	0.78	2.9	1.2	6	65.0
MJ8	0.1	1.95	-	-	15.6	78	-
	0.02	0.54	-	-	ND	ND	-
	0.01	0.34	-	-	ND	ND	-
	0.004	0.14	-	-	ND	ND	-

Results shown for cell and EPS dry weights are average values of one test from duplicate flasks. Glucose values are the mean of 2 tests from each duplicate flask. EPS/cell and EPS/glc values are calculated from the average values.

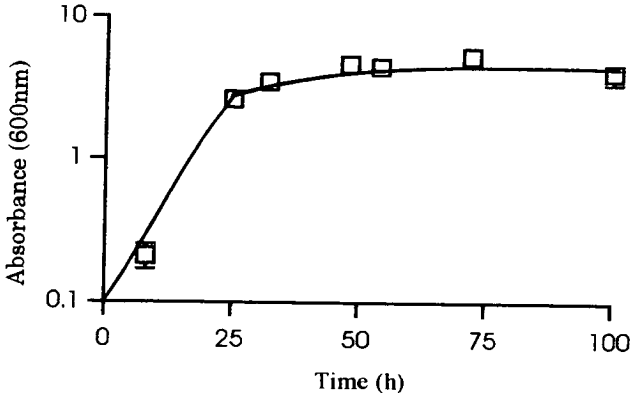
ND - Not Detected

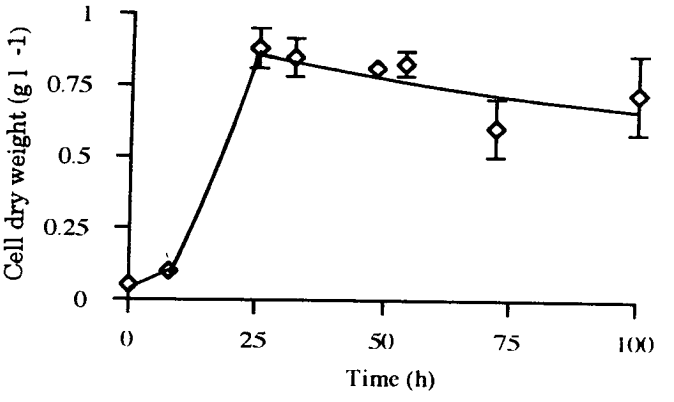
hours. Strain PA4 appeared to enter stationary phase after a slightly longer period (figure 4.4). The maximum specific growth rates for the four strains (Table 4.3) were very similar ranging from 0.063 to 0.080 h^{-1} for strains MJ8 and PA4 respectively.

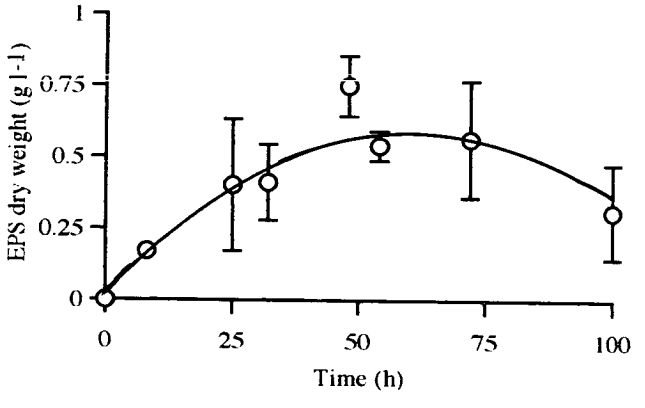
There was a large difference in maximum cell yield with 1.95 g l^{-1} produced from the non mucoid strain MJ8 (figure 4.6) and only 0.83 g l^{-1} for strain SB10 (figure 4.5). For the three EPS producing strains, the yield of polysaccharide was also different for each strain. The wild type strain produced 0.75 g l^{-1} EPS at 48 hours (figure 4.3) whilst strain PA4 produced 2.06 g l^{-1} EPS at 96 hours. The yield was also low for strain SB10 at only 0.85 g l^{-1} at 25 hours. Strains SB10 and WT produced the maximum amount of EPS in the first 48 hours of growth; following this there was a decrease in EPS yield as determined by the acetone precipitation method. Strain PA4 produced 1.8 g EPS g^{-1} cells whilst strains WT and SB10 produced only 0.9 g EPS g^{-1} cells and 1.0g EPS g^{-1} cells respectively (table 4.4). For the three mucoid strains, the culture medium became too viscous to measure after 48 hours (54 hours for strain SB10). For strain MJ8 the viscosity was about 1.00 for each time point measurement (figure 4.6). The amount of glucose consumed by each strain varied with strain (Tables 4.3 and 4.4). The wild type strain used 82% of the available glucose in the culture broth. This strain converted almost 5% of the carbon substrate used into EPS. Strain PA4 was more efficient than this. It used 70% of the initial glucose available but converted 14.6% of glucose utilised into exopolysaccharide. The pH of the culture broth did not alter dramatically during the 96 hour growth period. It decreased to between 5.74-5.98 for the mucoid strains and to 6.20 for strain MJ8.

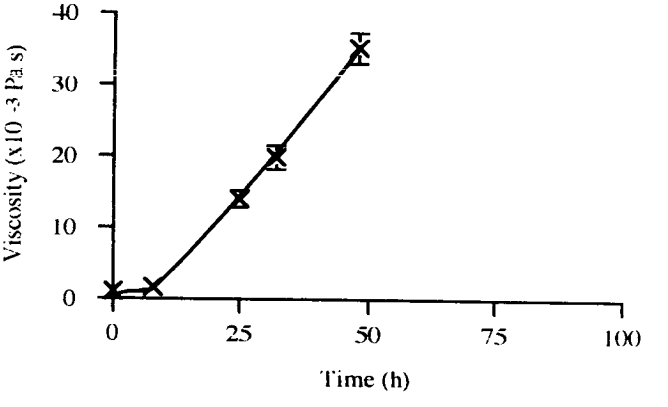
Figure 4.3

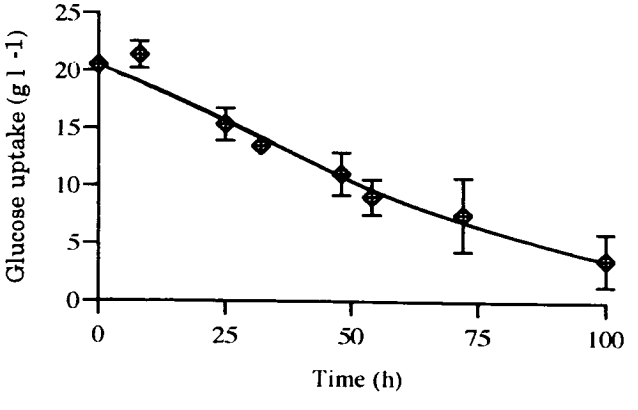
Growth and exopolysaccharide production for *S. paucimobilis* strain WT grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











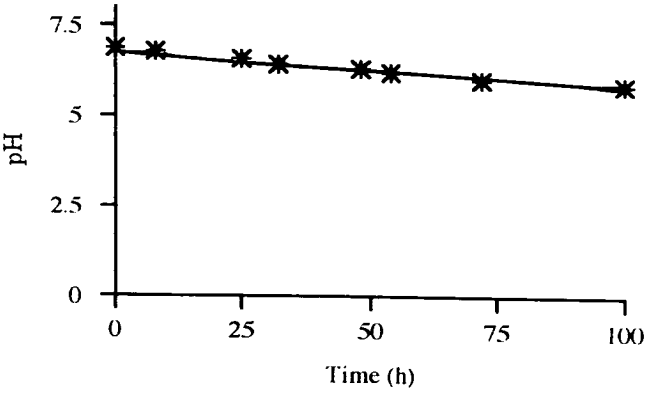
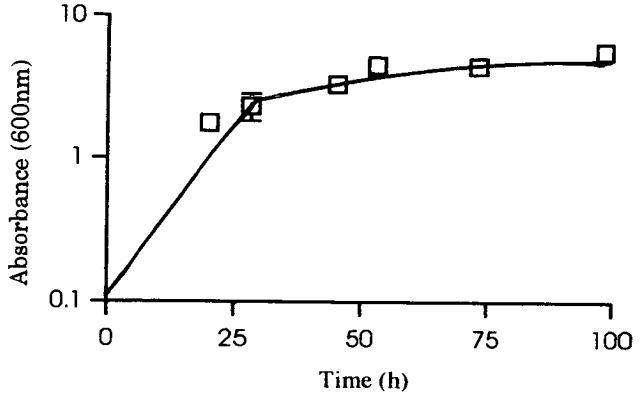
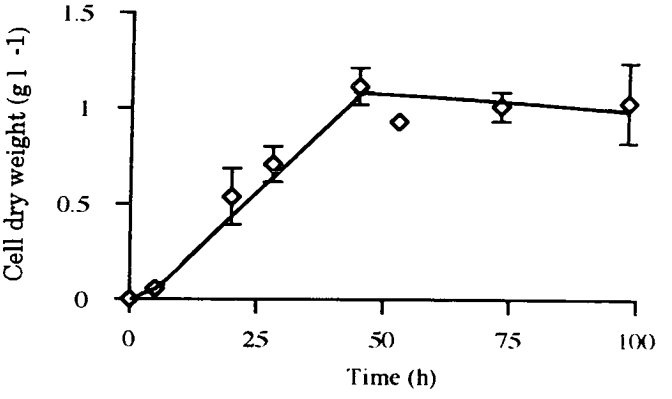
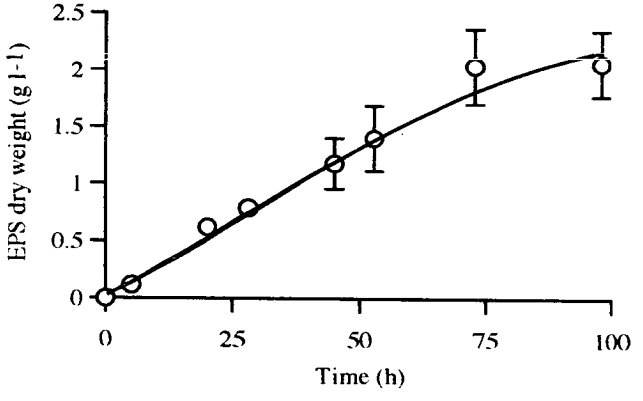


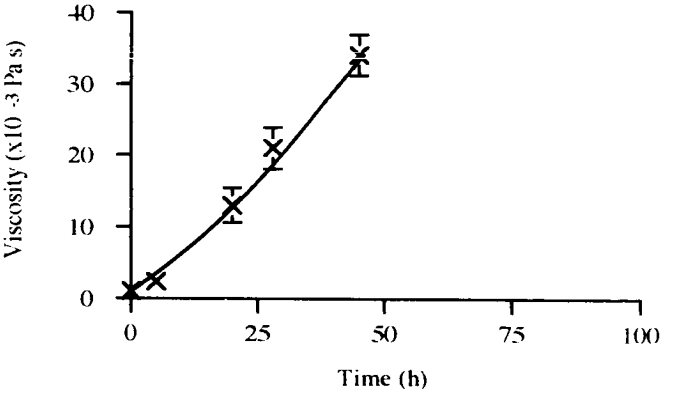
Figure 4.4

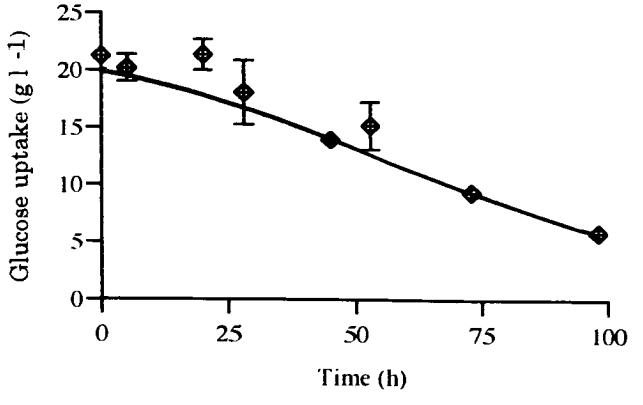
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











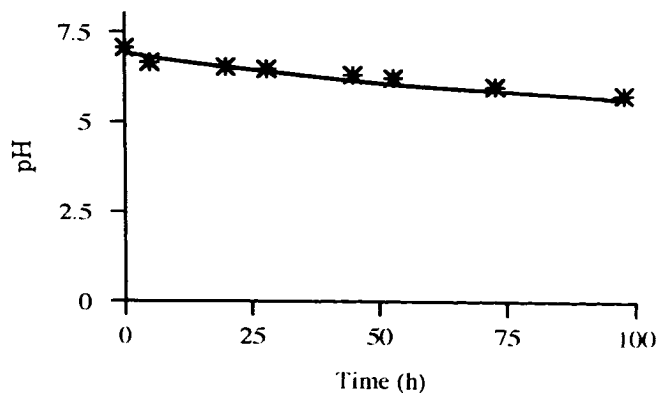
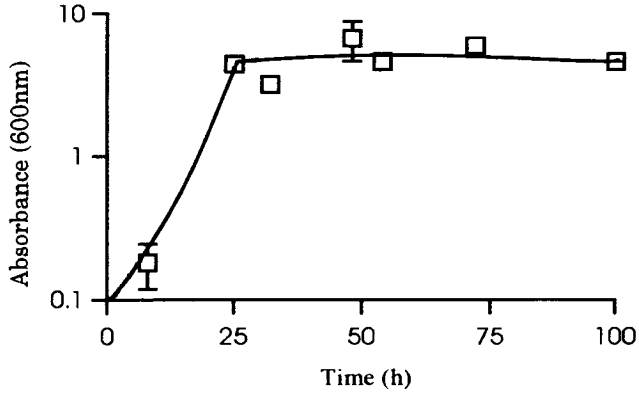
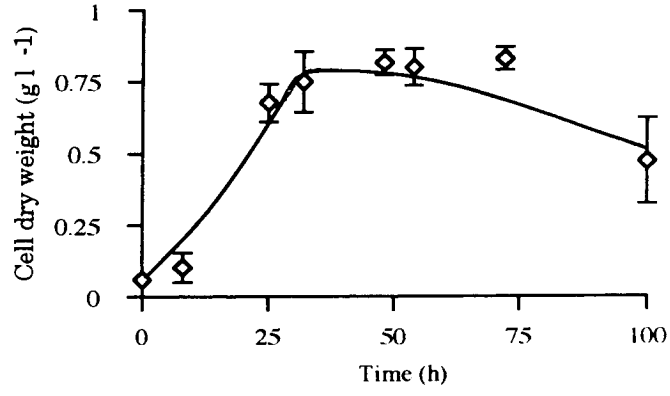
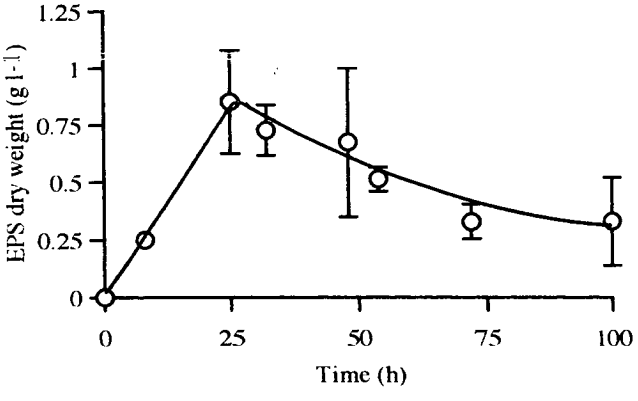


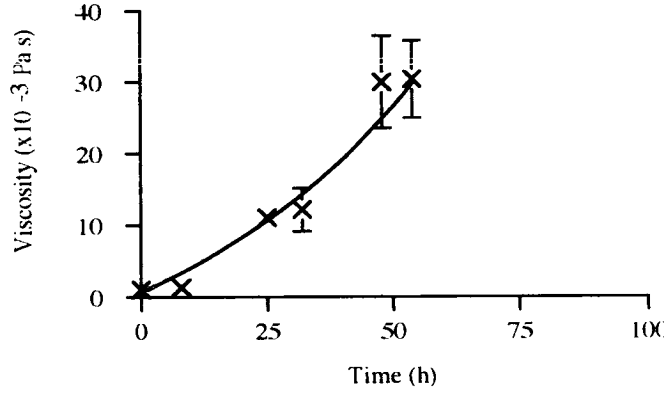
Figure 4.5

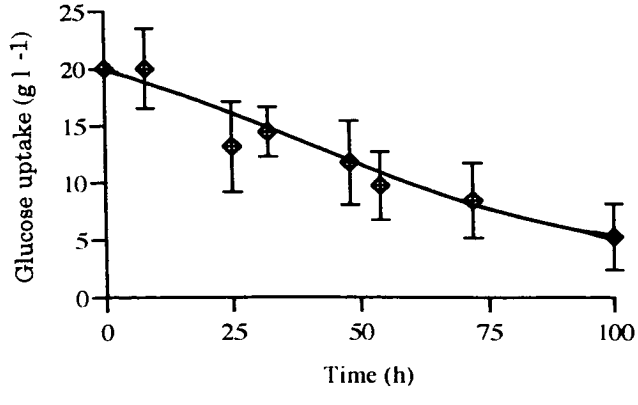
Growth and exopolysaccharide production for *S. paucimobilis* strain SB10 when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











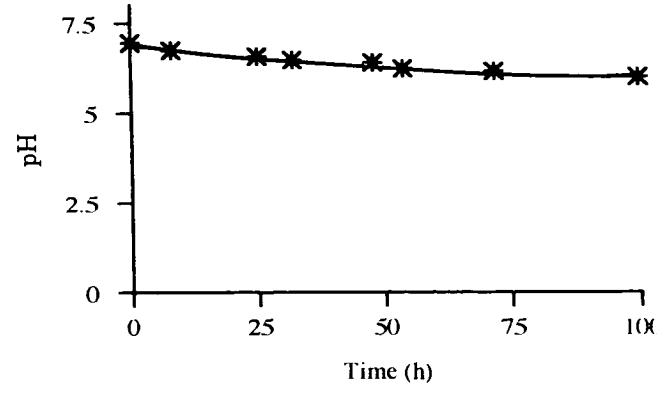
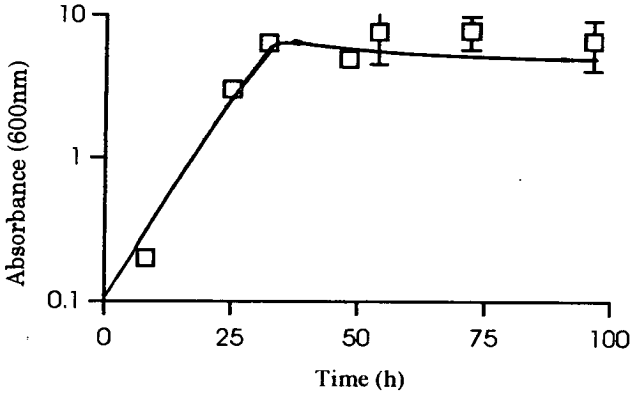
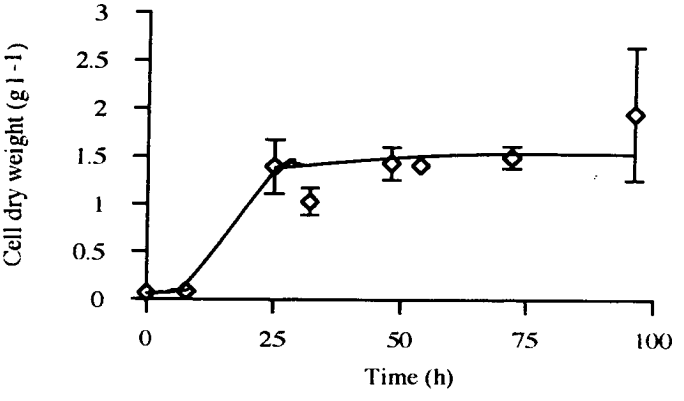
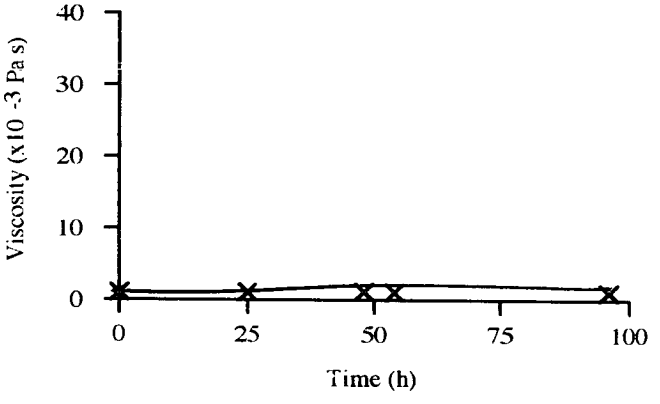


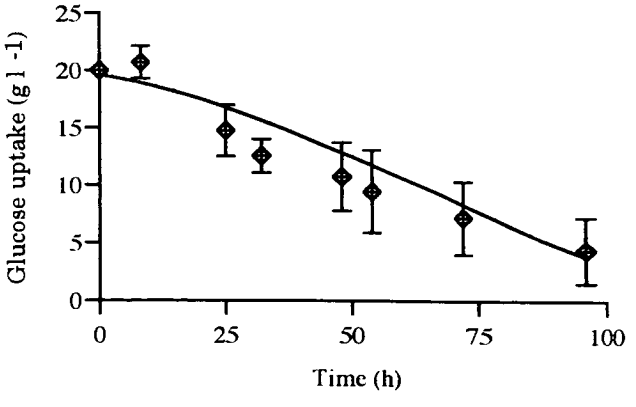
Figure 4.6

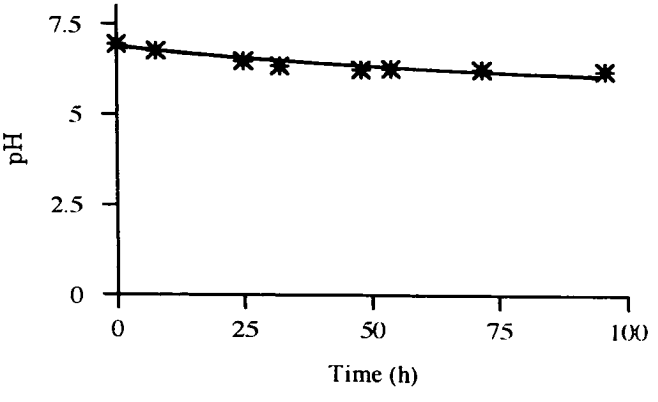
Growth and exopolysaccharide production for *S. paucimobilis* strain MJ8 when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











4.3 Growth studies with *Sphingomonas paucimobilis* strains WT, SB10, PA4 and MJ8 in Davis Mingioli medium with reduced levels of $(NH_4)_2SO_4$

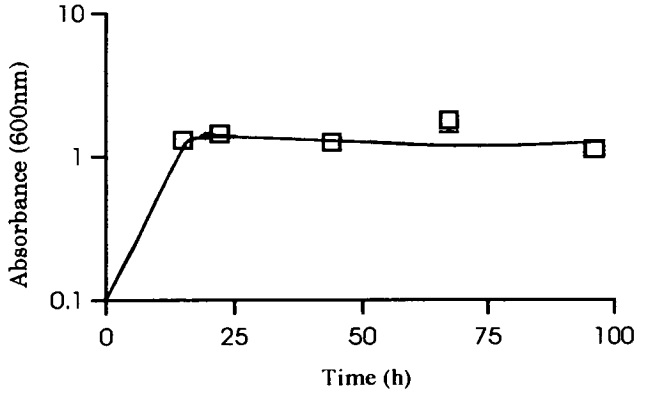
Growth and exopolysaccharide production was studied for *Sphingomonas paucimobilis* strains WT, SB10, PA4 and MJ8, in Davis Mingioli medium with reduced nitrogen levels. Growth and EPS production for the strains in DM medium containing 0.1% $(NH_4)_2SO_4$ have already been described. In these experiments, the nitrogen concentrations used were 0.02%, 0.01% and 0.004% $(NH_4)_2SO_4$. As the graphs for the different strains are very similar, only representative strains and nitrogen concentrations have been presented. Strain PA4 grown with 0.02%, 0.01% and 0.004% $(NH_4)_2SO_4$ shows the typical changes in the different parameters studied in figures 4.7-4.9. *S. paucimobilis* wild type strain grown in 0.01% $(NH_4)_2SO_4$ is also shown in figure 4.10. Maximum values of absorbance, cell and EPS dry weights, viscosity, glucose uptake and pH have been summarised in Table 4.3. Data for EPS produced per cell and glucose consumed are shown in Table 4.4. For all strains, the maximum absorbance values and cell dry weights, decreased with decreasing concentrations of $(NH_4)_2SO_4$ (Table 4.3). There was only one exception; strain WT had a greater maximum absorbance at 0.004% $(NH_4)_2SO_4$ compared to that found at 0.01% $(NH_4)_2SO_4$.

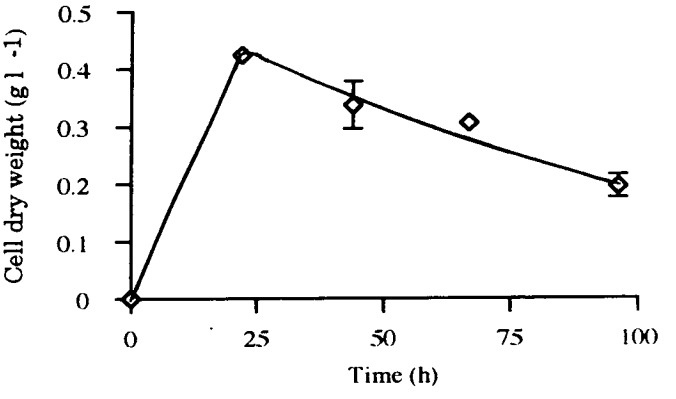
Both strains WT and SB10 produced a greater amount of EPS at 0.02% $(NH_4)_2SO_4$ than at 0.1% $(NH_4)_2SO_4$. The EPS yield decreased after that and the lowest yield was found with the lowest nitrogen concentration. In contrast, the amount of EPS produced per cell increased with increasing $(NH_4)_2SO_4$ concentrations. For strains PA4 and SB10 the EPS/cell value was less at the lowest nitrogen concentration that at 0.01% $(NH_4)_2SO_4$. For strain WT, 0.02% $(NH_4)_2SO_4$ had a greater EPS/cell value than 0.01% $(NH_4)_2SO_4$.

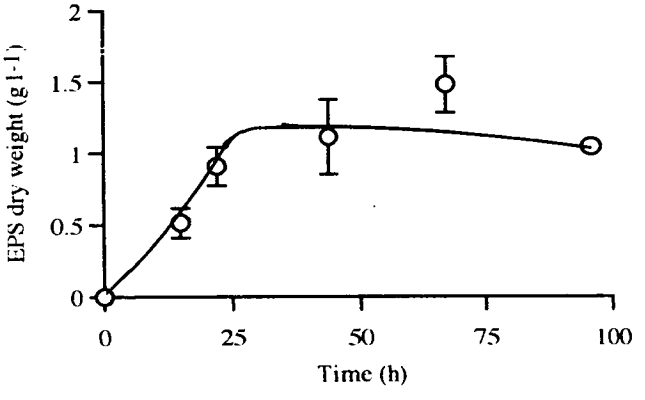
Generally speaking, EPS produced per glucose used became more efficient with decreasing nitrogen concentration. For strain SB10, 5.8% glucose used was converted into EPS at 0.1% $(NH_4)_2SO_4$ concentration whilst 65% glucose was used to make EPS at 0.004% $(NH_4)_2SO_4$ concentration. Maximum values for culture medium viscosity were found to decrease with decreasing concentration of $(NH_4)_2SO_4$. Less glucose was consumed with decreasing nitrogen

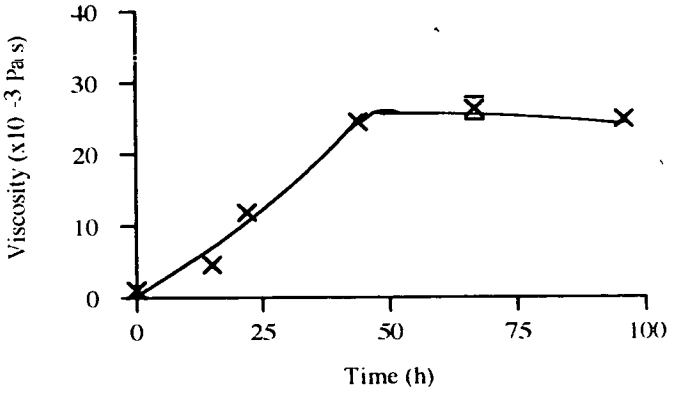
Figure 4.7

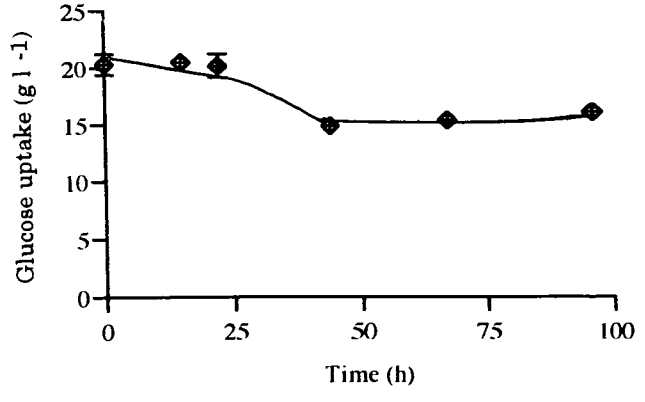
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 2% glucose, 0.02% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











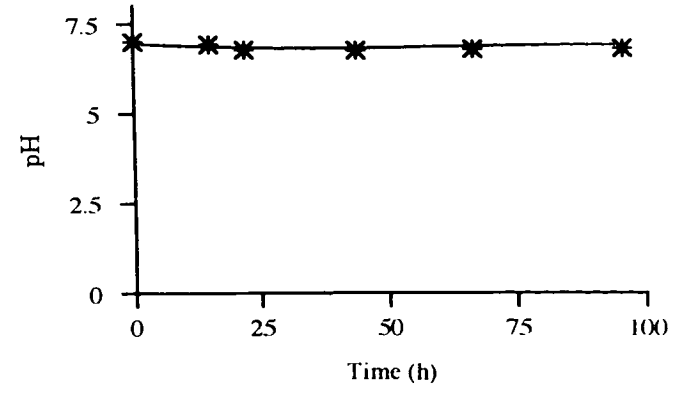
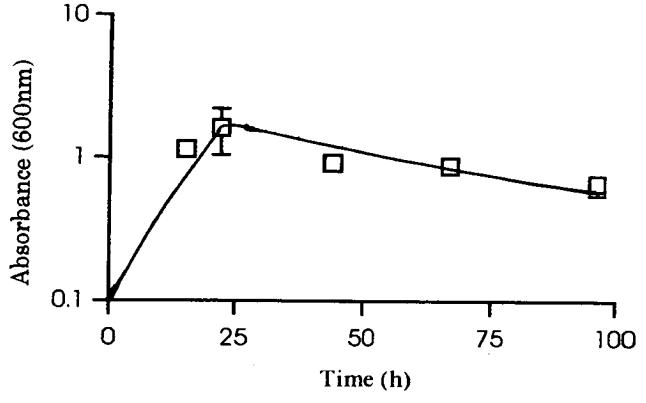
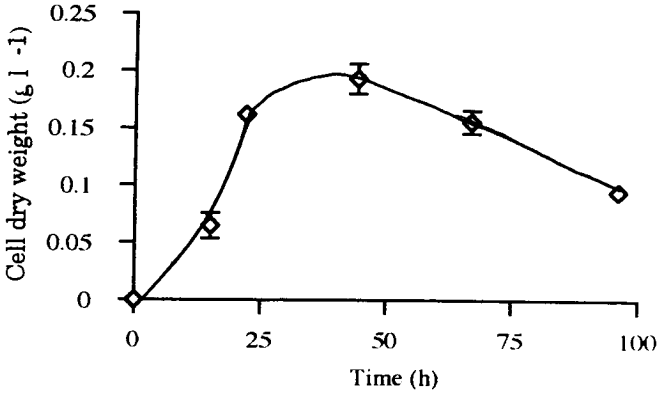
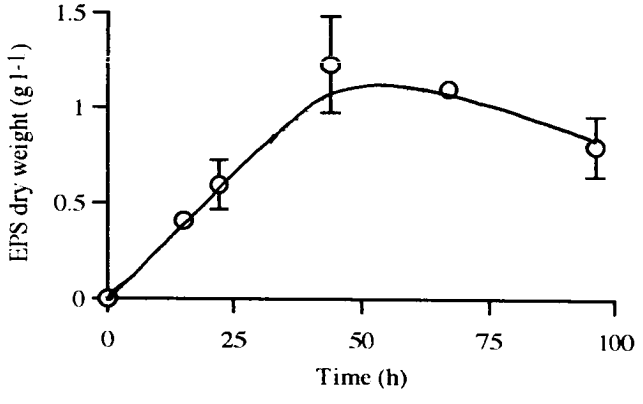


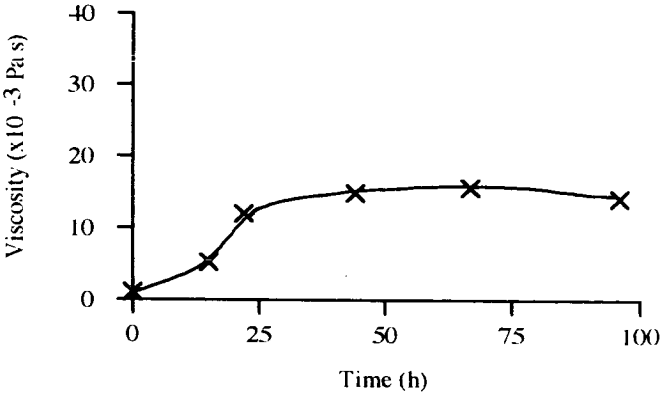
Figure 4.8

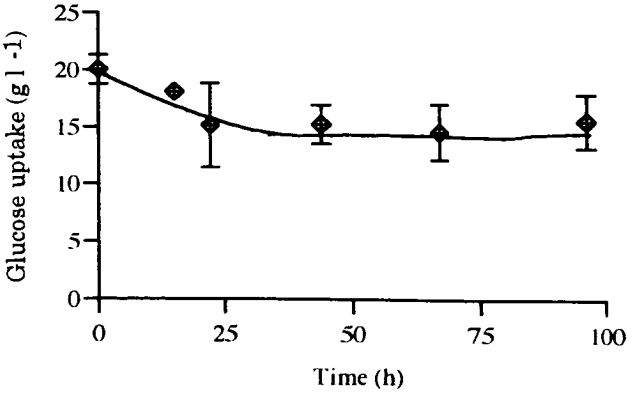
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











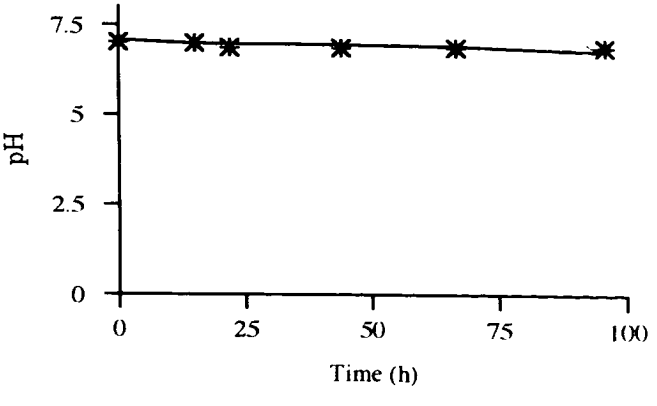
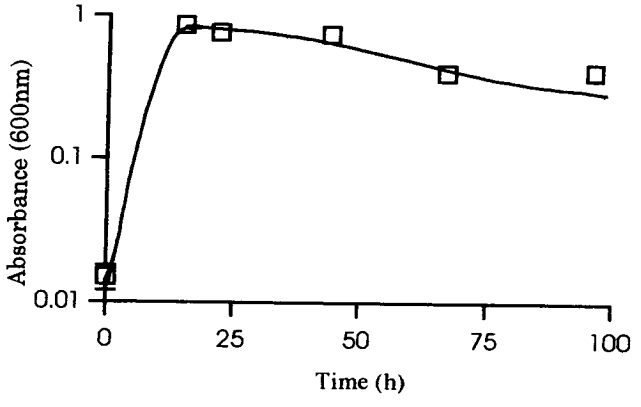
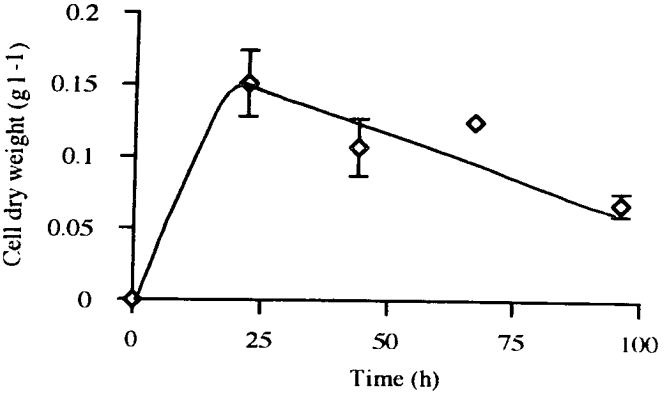
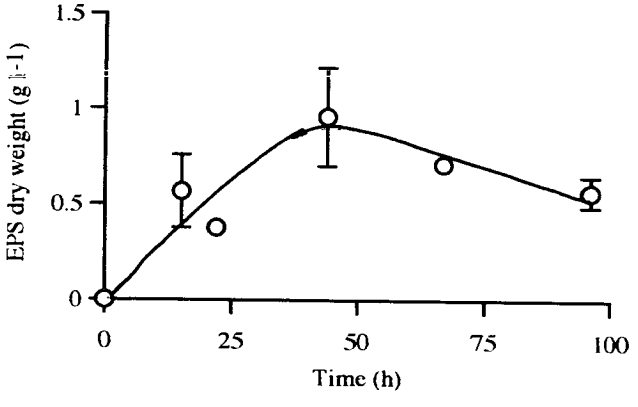


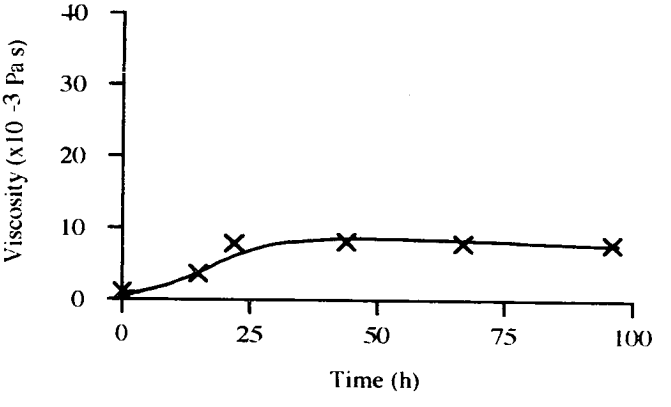
Figure 4.9

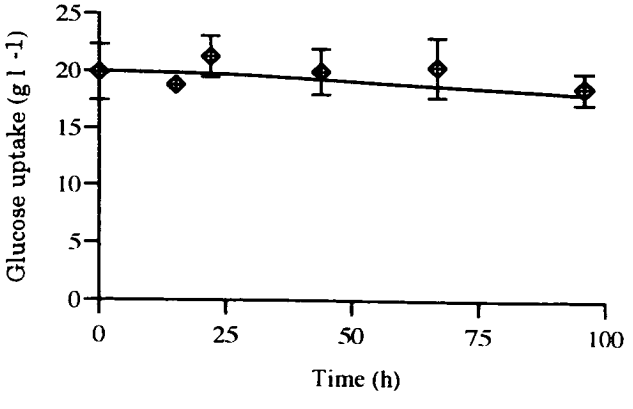
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 2% glucose, 0.004% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











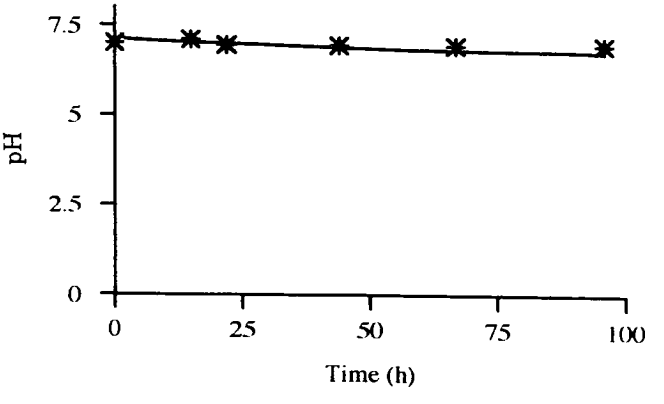
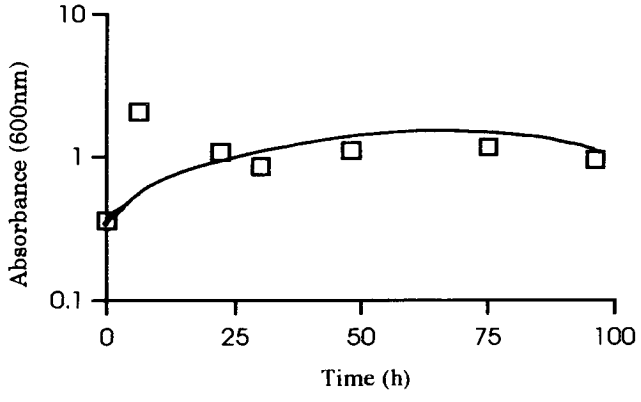
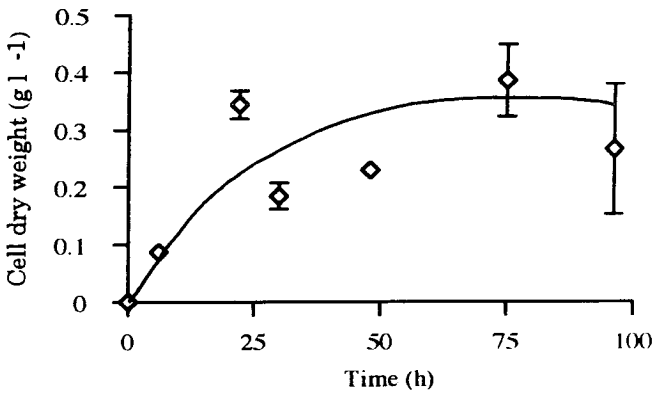
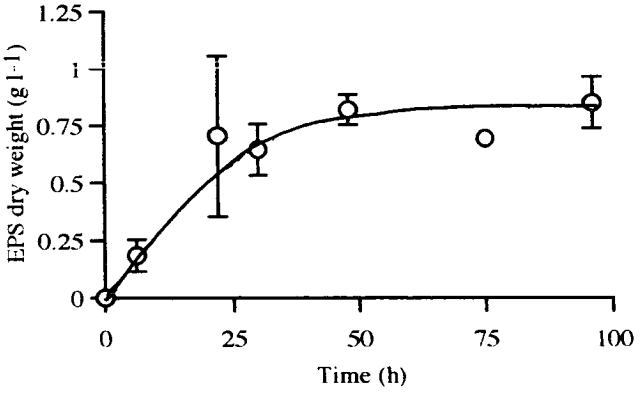


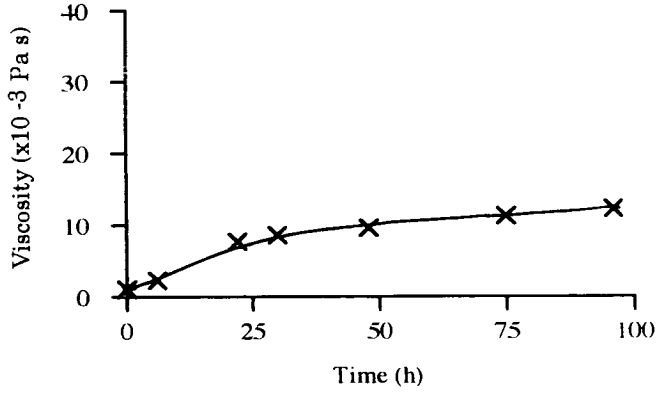
Figure 4.10

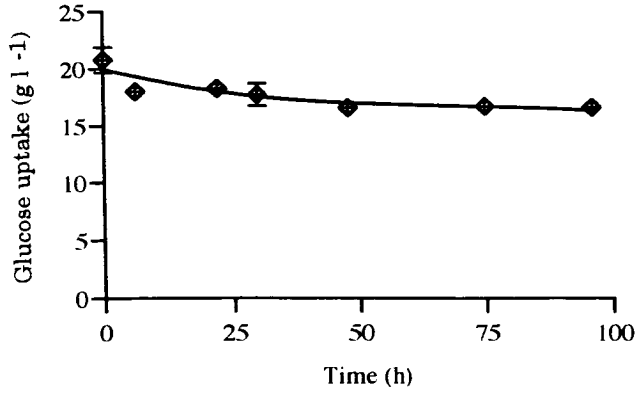
Growth and exopolysaccharide production for *S. paucimobilis* strain WT when grown in Davis Mingioli medium containing 2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.

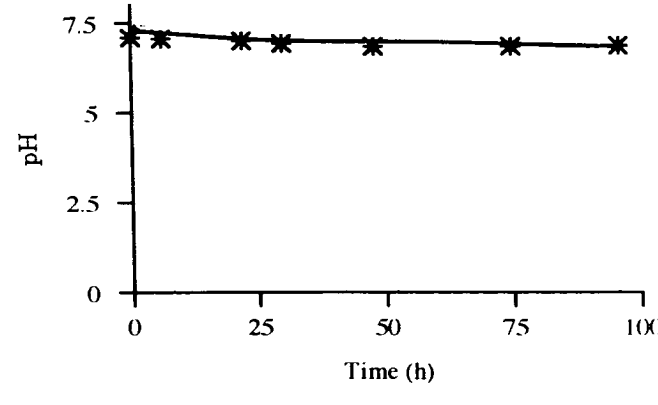












content of the culture broth and the pH change in the culture fluid was less with lower nitrogen concentrations.

The changes in the parameters described are shown graphically for strain PA4 in figures 4.7-4.9. At all concentrations of $(NH_4)_2SO_4$, there was a decrease in absorbance and cell and EPS yield with time in stationary phase. Maximum values were reached in the first 48 hours of growth and declined following this. The culture fluid viscosity was relatively stable in the last 48 hours of growth and did not show the decrease found with EPS yield. Although, the results are not shown for strain SB10, a similar pattern was noted. For the wild type strain, there was some decrease in absorbance and cell yield in the latter stages of growth as shown with 0.01% $(NH_4)_2SO_4$ concentration (figure 4.10). Interestingly, the EPS yield was more stable and seemed to remain constant in stationary phase with no decrease as described for strains PA4 and SB10.

4.4 Chemical composition of EPS elaborated by *Sphingomonas paucimobilis* strains WT, SB10 and PA4 in Davis Mingioli medium containing 0.1% $(NH_4)_2SO_4$

The neutral sugar composition of the polysaccharides elaborated by *S. paucimobilis* strains WT, SB10 and PA4 were determined by gas chromatography. The strains were grown in duplicate flasks of Davis Mingioli medium containing 0.1% $(NH_4)_2SO_4$ concentration (control condition). The results are expressed as mean values of two separate analyses (one from each flask). The data are presented as % (w/w) of total EPS analysed and as mole % of identified sugars. Values for *S. paucimobilis* strains WT, PA4 and SB10 are shown in Tables 4.5, 4.6 and 4.7 respectively.

For all samples the total sugar content (i.e. % of total EPS analysed) increased with time. The amounts of EPS varied with strain. The maximum total sugar contents were 31.9%, 12.0% and 8.6% for strains PA4, SB10 and WT respectively. In all samples studied, it was apparent from the gc chromatograms that there were often 1-3 unidentified peaks representing a considerable amount of material being studied. This material could not be quantified properly as no response factor was known for it relative to the internal standard. To calculate total sugar content, these peaks termed X_1-X_3, were given correction factors of 1.0. It was not included in the mole % of identified sugars as no molecular weights were known for the

unidentified compounds. In all of the samples mannose was present at levels of 1.8-4.4% of total identified sugars. In many cases this amount seemed insignificant as the quantities of unidentified sugar, or other sugars like fucose, were greater in weight, and in mole %, than the mannose content. Strain PA4 was the only sample where EPS was studied for the 4.5 hour time point. The amount of glucose:rhamnose identified was higher at this point than at any other point during growth in both weight and mole % value.

The importance of using weights of sugars can be noted for strain PA4 at 53 and 73 hours. At these time points, the mole % of rhamnose in identified sugars was the same. However, the amount of rhamnose actually identified was greater at 73 hours than 53 hours. For strain PA4, at all times, the amount of glucose was greater than the amount of rhamnose. This was not the case for strain WT in which the amount of rhamnose was always greater than the amount of glucose. For strain SB10, the % (w/w) of glucose and rhamnose as total EPS were identical for all time points.

Table 4.5: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* WT strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
22	1.0	1.2	nd	nd	0.04	nd	nd	0.01	0.3	nd	2.5
48	2.6	3.1	0.1	0.02	0.1	nd	nd	nd	0.3	nd	6.3
75	3.1	3.8	0.2	0.03	0.2	0.02	nd	nd	0.3	nd	7.7
96	3.5	4.3	0.2	0.06	0.1	0.01	nd	nd	0.3	0.02	8.6

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	42.2	55.5	nd	nd	2.4	nd	nd
48	41.4	54.5	2.2	0.2	1.6	nd	nd
75	40.8	53.9	2.0	0.3	2.8	0.3	nd
96	40.4	54.6	2.8	0.8	1.4	0.2	nd

nd = not detected

Table 4.6: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
4.5	5.4	2.0	0.3	0.1	0.2	nd	nd	nd	0.4	nd	8.4
20	10.8	7.9	0.5	0.1	0.3	nd	0.04	0.1	0.4	nd	20.2
28	13.8	12.4	0.6	nd	0.3	nd	nd	0.1	0.5	0.04	27.7
45	14.5	13.6	0.7	nd	0.4	nd	nd	0.1	0.5	0.04	29.8
53	15.2	12.7	0.9	0.06	0.3	nd	nd	0.06	0.4	nd	29.6
73	15.4	13.3	1.4	0.06	0.3	nd	nd	0.1	0.5	0.1	31.1
98	16.4	13.7	0.7	0.2	0.2	nd	0.05	0.1	0.5	0.08	31.9

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
4.5	65.8	26.8	3.6	1.5	2.3	nd	nd
20	52.8	42.5	2.4	0.5	1.6	nd	0.2
28	48.4	47.9	2.3	nd	1.4	nd	nd
45	47.3	49.0	2.4	nd	1.2	nd	nd
53	50.0	46.0	2.8	0.2	1.0	nd	nd
73	48.7	46.0	4.4	0.2	0.9	nd	nd
98	50.2	46.1	2.2	0.6	0.6	nd	0.2

nd = not detected

Table 4.7: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* SB10 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS								TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	
22	0.7	0.7	nd	nd	0.1	nd	nd	0.3	1.8
48	3.9	3.9	0.1	nd	0.3	nd	nd	0.3	8.6
75	4.3	4.3	0.2	0.03	0.3	nd	0.04	0.3	9.6
96	5.4	5.4	0.5	0.04	0.3	nd	nd	0.4	12.0

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	44.4	47.7	nd	nd	7.8	nd	nd
48	44.7	50.0	1.8	nd	3.5	nd	nd
75	44.6	48.9	2.3	0.2	3.5	nd	0.4
96	44.3	48.6	3.8	0.4	2.9	nd	nd

nd = not detected

4.5 Chemical composition of EPS elaborated by *Sphingomonas paucimobilis* strains WT, SB10 and PA4 in Davis Mingioli medium with reduced levels of $(NH_4)_2SO_4$

The neutral sugar composition of *S. paucimobilis* strains WT, PA4 and SB10 were studied following growth in Davis Mingioli medium containing 0.02%, 0.01% and 0.004% $(NH_4)_2SO_4$ concentrations. The EPS were similar to each other and to the EPS described in Section 4.4. For this reason only one table of EPS composition is shown for each strain. These strains were grown at 0.02% $(NH_4)_2SO_4$ (Tables 4.8-4.10). When strains WT, SB10 and PA4 were grown at reduced nitrogen levels, the amount of EPS accounted for following analysis, was less the lower the $(NH_4)_2SO_4$ concentration. The total sugar content (%) figures were on average 20.8%, 15.9% and 9.0% EPS for strains SB10, WT and PA4 respectively. For strain SB10, the amount of glucose was always greater than the amount of rhamnose at all time points. The amount of glucose identified at 6 hours was again much greater than the glucose found at any other time point. At the other nitrogen concentrations (data not shown), the glucose amount was always greater than the amount of rhamnose identified. The greatest amount of mannose detected was at 0.02% $(NH_4)_2SO_4$ concentration of 6%. For strain PA4, the amount of rhamnose and glucose identified were very similar.

However, when these values were expressed as mole % the rhamnose concentration was 48.0-50.4% as opposed to 43.8-47.0% for glucose. A similar situation was found for strain PA4 grown at other reduced nitrogen levels. For strain WT, the glucose and rhamnose values varied. At some points there was more rhamnose than glucose in both weight % and mole %. Rhamnose values varied between 39.6-57.3% (mole %) while glucose values were 39.6-56.6%.

Table 4.8: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* WT strain growing in Davis Mingioli medium containing 2% glucose, 0.02% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS									TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	
6	5.1	3.6	0.3	nd	nd	nd	nd	0.06	0.2	9.3
22	7.6	10.1	0.2	nd	0.3	nd	nd	nd	0.2	18.5
30	8.8	10.6	0.4	nd	0.3	nd	nd	nd	0.3	20.4
48	7.4	9.0	0.4	nd	0.2	nd	nd	nd	0.2	17.2
75	7.3	8.8	0.5	nd	0.3	nd	nd	nd	0.3	17.3
96	6.6	5.1	0.5	0.07	0.2	0.07	nd	nd	0.3	12.8

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
6	56.6	39.6	3.8	nd	nd	nd	nd
22	39.6	57.3	1.2	nd	1.8	nd	nd
30	41.5	55.2	1.8	nd	1.5	nd	nd
48	41.4	55.2	2.4	nd	1.0	nd	nd
75	41.0	54.2	2.8	nd	1.9	nd	nd
96	51.1	42.0	3.6	0.6	1.8	0.8	nd

nd = not detected

Table 4.9: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.02% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
15	2.7	2.6	0.08	0.06	0.08	0.03	0.04	0.04	0.12	nd	5.8
23	3.7	3.6	0.2	0.02	0.09	0.01	nd	0.04	0.2	0.02	7.8
44	5.0	5.0	0.4	nd	0.1	0.01	nd	0.04	0.2	0.06	10.7
67	4.5	4.7	0.5	0.02	0.1	0.01	nd	0.03	0.1	0.02	10.1
96	4.8	5.0	0.5	nd	0.1	nd	nd	0.02	0.2	0.05	10.6

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
15	46.2	48.0	1.4	1.2	1.7	0.7	1.0
22	47.0	49.2	2.1	0.2	1.3	0.2	nd
44	45.2	50.3	3.4	nd	1.0	0.1	nd
67	43.8	50.0	4.8	0.2	1.2	0.1	nd
96	44.1	50.4	4.1	nd	1.4	nd	nd

nd = not detected

Table 4.10: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* SB10 strain growing in Davis Mingioli medium containing 2% glucose, 0.02% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
6	4.1	1.5	0.2	0.1	0.2	0.2	0.1	0.3	0.2	1.9	8.9
22	12.3	7.1	0.5	0.02	0.2	0.04	nd	0.2	0.1	1.9	22.4
30	13.4	9.3	0.7	0.02	0.1	0.02	nd	0.2	0.1	2.1	26.2
48	13.9	9.7	1.0	0.03	0.1	0.01	nd	0.3	0.2	1.7	27.0
75	7.5	5.5	0.5	nd	0.2	nd	nd	0.2	0.1	1.9	16.0
96	12.4	8.2	1.4	0.03	0.2	0.02	nd	0.3	0.2	1.8	24.6

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
6	61.2	24.6	3.0	1.8	3.0	4.4	1.9
22	59.3	37.0	2.6	0.05	0.8	0.2	nd
30	54.6	41.5	3.0	0.1	0.6	0.1	nd
48	54.0	41.4	3.8	0.2	0.6	0.05	nd
75	52.7	42.6	3.5	nd	1.2	nd	nd
96	53.8	39.0	6.0	0.2	0.8	0.2	nd

nd = not detected

CHAPTER 5

RESULTS

5. THE EFFECTS OF GLUCOSE LIMITATION ON *SPHINGOMONAS PAUCIMOBILIS*

5.1 Growth studies with *Sphingomonas paucimobilis* strains WT, SB10, PA4 and MJ8 in Davis Mingioli medium with reduced levels of glucose

The effect of glucose limitation on growth and EPS production from *S. paucimobilis* strains WT, PA4, SB10 and the non mucoid MJ8 in duplicate flasks of Davis Mingioli medium was investigated. Each culture was set up in duplicate flasks of DM medium (800ml culture broth in 21 flasks) containing either 2%, 0.2% or 0.02% glucose. The control condition of 2% glucose was described in Chapter 4.2. Samples of culture broth were removed during the 96 hour growth period to measure absorbance (600nm), cell and EPS yield, culture medium viscosity, pH and glucose utilisation. As the graphs show certain similarities, only the results for the strains growing at 0.2% glucose will be presented (figures 5.1-5.4). A summary table showing the maximum values for all tests performed is shown in Table 5.1. For all strains studied, absorbance readings decreased with decreasing amounts of glucose in the culture medium. The greatest absorbance value was found for strain MJ8 at 2% glucose (Table 5.1). Absorbance values were greatest for all strains at about 24 hours. There was then a gradual decline in optical density. Cell and EPS yields also decreased with decreasing amounts of carbon substrate. There was also a decrease in cell yield after the first 24 hours. Cell yield varied considerably with the different strains. At 0.2% glucose, the maximum cell yield for strain PA4 was 0.28 g l^{-1}. This maximum value was found at 24 hours and was followed by a very sharp decline in cell yield thereafter (figure 5.2). The cell yield for the non-mucoid strain MJ8 was much greater at 0.60 g l^{-1} (figure 5.4). Although, there was still a decline in cell yield following this maximum value at 24 hours, it was much less severe than that found with strain PA4.

EPS yields varied between 0.41-0.66 g l^{-1} for the three polymer producing strains. There was a slight decrease in EPS yield towards the end of the 96 hour growth period, as can be shown by strain PA4 (figure 5.2). Viscosity values also decreased with decreasing glucose content of the culture broth. There was a slight decrease in viscosity

in strain PA4 growing at 0.2% glucose concentration.
The starting glucose concentration was only 2 g l^{-1} for the examples shown graphically in this chapter. This was rapidly used up in the first 25 hours with the exception of the wild type strain which appeared to totally exhaust the glucose supply by about 50 hours. There was not a great difference in pH for any of the strains studied.
It appears from Table 5.2 that EPS/cell values (g g^{-1}) increase for strain PA4, although not a large increase. For strain SB10 the values for the two conditions are the same at 1 g EPS g^{-1} cells. EPS formed from glucose utilised seems to become more efficient with decreasing glucose concentration.

Table 5.1: Summary table showing maximum values obtained for *S. paucimobilis* strains WT, SB10, PA4 and MJ8 grown in Davis Mingioli medium with reduced levels of glucose. Values shown are expressed as mean ± standard deviation of duplicate assays carried out on duplicate flasks (except cell and EPS dry weight where only one measurement was made for each flask).

STRAIN PA4 GLUCOSE CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
2	0.080	5.58 ± 0.68	1.12 ± 0.10	2.06 ± 0.28	5.90 ± 0.42	34.15 ± 2.89	5.74 ± 0.13
0.2	0.114	1.57 ± 0.02	0.28 ± 0.02	0.66 ± 0.05	0	8.26 ± 0.09	6.60 ± 0.01
0.002	0.097	0.34 ± 0.05	0.17 ± 0.01	0.43 ± 0.01	0	2.31 ± 0.18	6.64 ± 0

STRAIN WT GLUCOSE CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
2	0.068	5.31 ± 0.67	0.88 ± 0.07	0.75 ± 0.10	3.68 ± 2.33	35.22 ± 2.15	5.86 ± 0.04
0.2	0.143	1.99 ± 0.07	0.47 ± 0.12	0.49 ± 0.02	0	8.84 ± 0.32	6.69 ± 0.07
0.002	0.059	0.28 ± 0.02	0.102 ± 0.04	ND	0	2.54 ± 0.4	6.80 ± 0

STRAIN SB10 GLUCOSE CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
2	0.073	6.70 ± 2.09	0.83 ± 0.04	0.85 ± 0.23	5.30 ± 2.90	30.40 ± 5.43	5.98 ± 0.01
0.2	0.125	1.84 ± 0.22	0.41 ± 0.14	0.41 ± 0.01	0	6.93 ± 0.03	6.58 ± 0.02
0.002	0.059	0.47 ± 0.17	0.112 ± 0.04	ND	0	1.72 ± 0.22	6.60 ± 0.01

STRAIN MJ8 GLUCOSE CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
2	0.067	7.73 ± 2.06	1.95 ± 0.70	0	4.35 ± 2.86	1.08 ± 0.02	6.20 ± 0.14
0.2	0.133	1.38 ± 0.07	0.60 ± 0.03	0	0	1.17 ± 0.03	6.60 ± 0.01
0.002	0.050	0.285 ± 0.05	0.12 ± 0.03	0	0	1.06 ± 0	6.55 ± 0.07

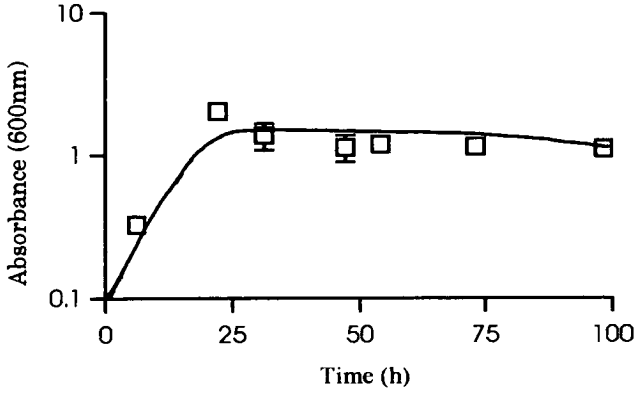
Table 5.2: Table showing maximum cell and EPS weights, EPS/cell values, glucose utilised and EPS/glc values for *S. paucimobilis* strains WT, SB10, PA4 and MJ8. Cultures were set up in duplicate and grown in Davis Mingioli medium with varying amounts of glucose for 96 hours at 30°C.

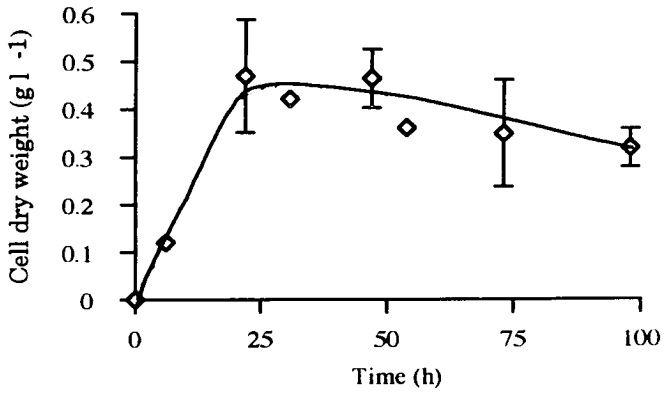
STRAIN / $(NH_4)_2SO_4$ CONC. (%)		CELL DRY WT ($g\ l^{-1}$)	EPS DRY WT ($g\ l^{-1}$)	EPS/ CELL ($g\ g^{-1}$)	GLUCOSE USED $g\ l^{-1}$ OR (%)		EPS/ glc (%)
PA4	2	1.12	2.06	1.8	14.1	70	14.6
	0.2	0.28	0.66	2.4	2.0	100	33.0
	0.02	0.17	0.43	2.5	0.2	100	21.5
SB10	2	0.83	0.85	1.0	14.7	74	5.8
	0.2	0.41	0.41	1.0	2.0	100	20.5
	0.02	0.11	-	-	0.2	100	-
WT	2	0.88	0.75	0.85	16.3	82	4.6
	0.2	0.47	0.49	1.0	2.0	100	24.5
	0.02	0.10	-	-	0.2	100	-
MJ8	2	1.95	-	-	15.65	78	-
	0.2	0.60	-	-	2.0	100	-
	0.02	0.12	-	-	0.2	100	-

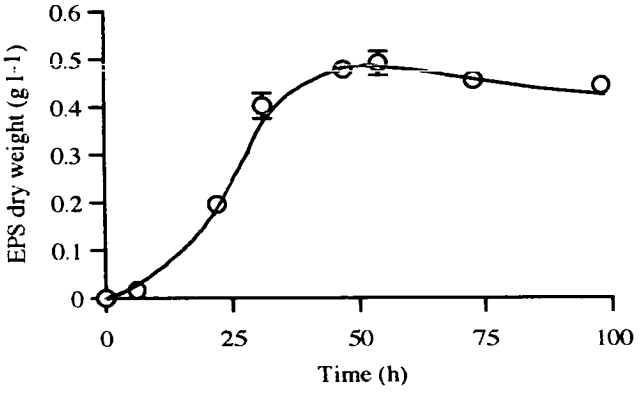
Results shown for cell and EPS dry weights are average values of one test from duplicate flasks. Glucose values are the mean of 2 tests from each duplicate flask. EPS/cell and EPS/glc values are calculated from the average values.

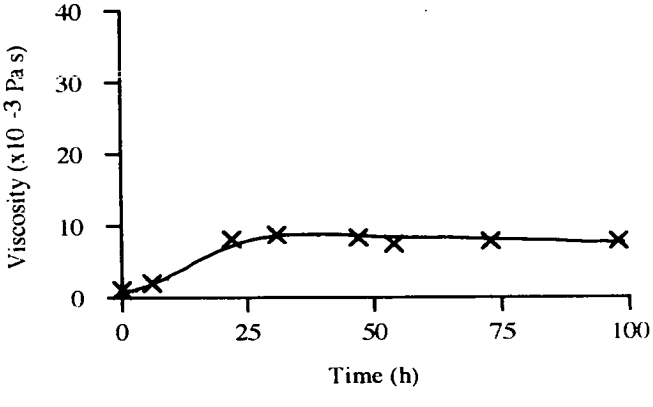
Figure 5.1

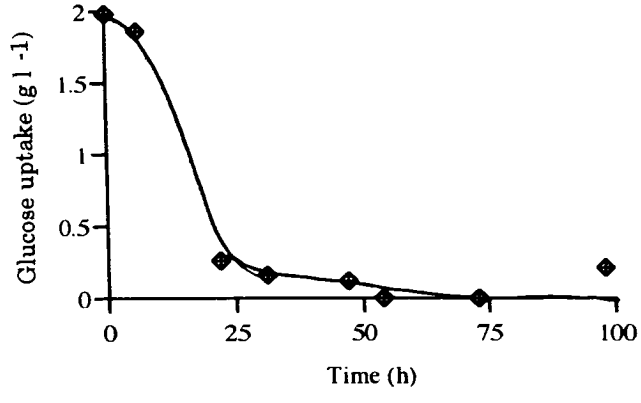
Growth and exopolysaccharide production for *S. paucimobilis* strain WT when grown in Davis Mingioli medium containing 0.2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











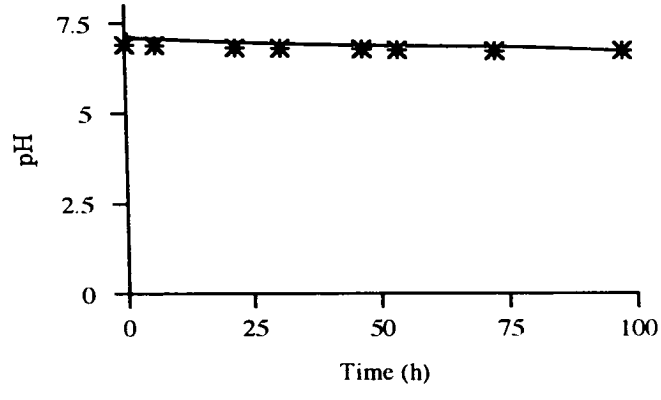
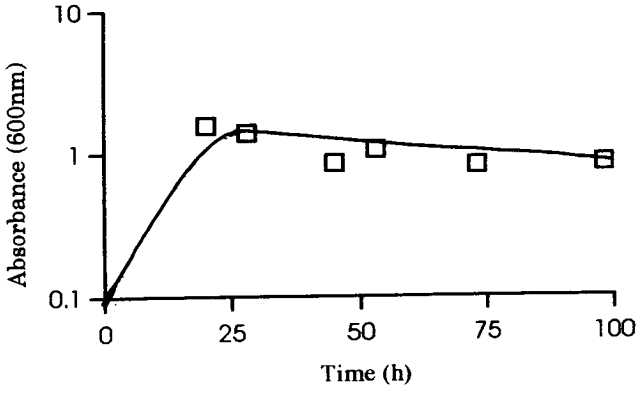
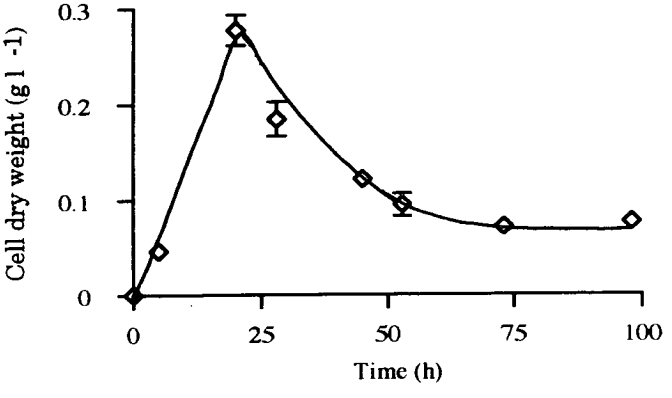
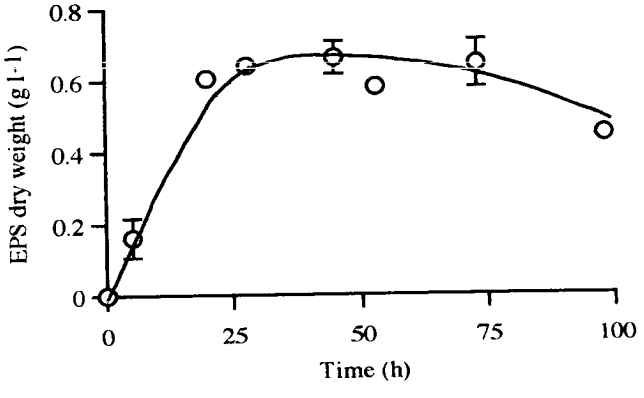


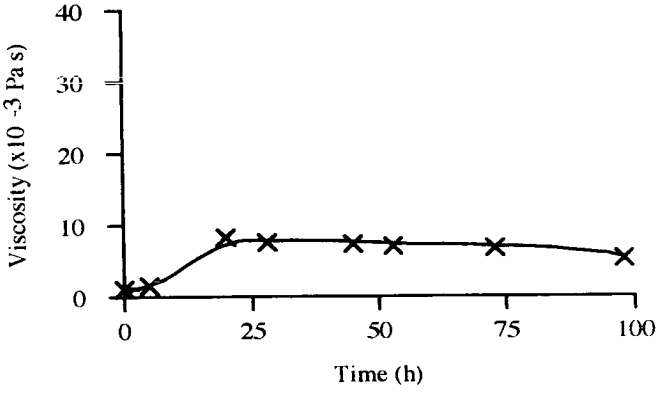
Figure 5.2

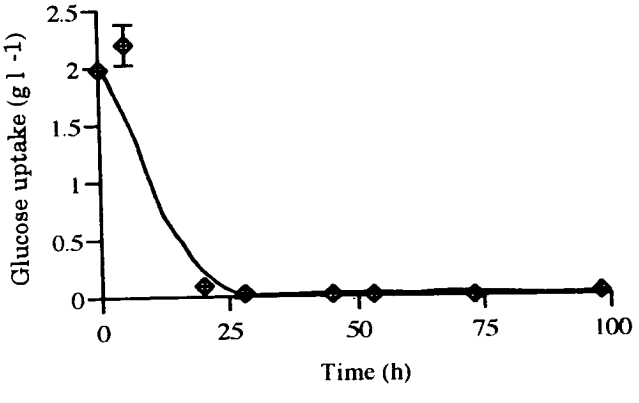
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 0.2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











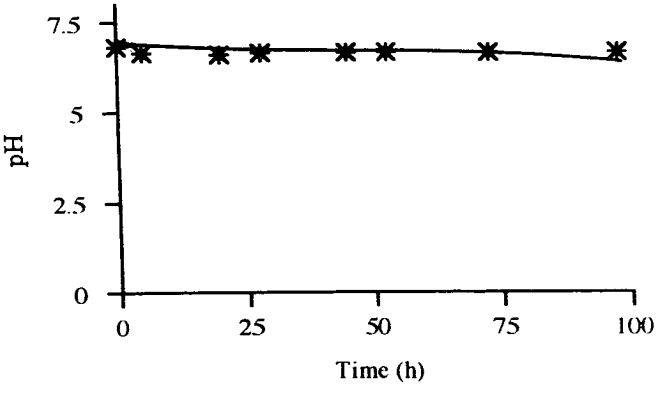
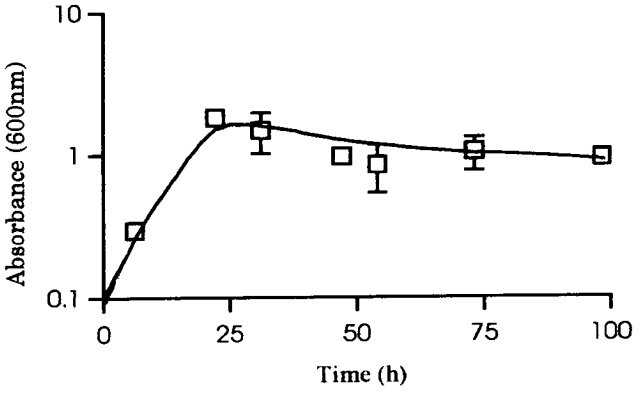
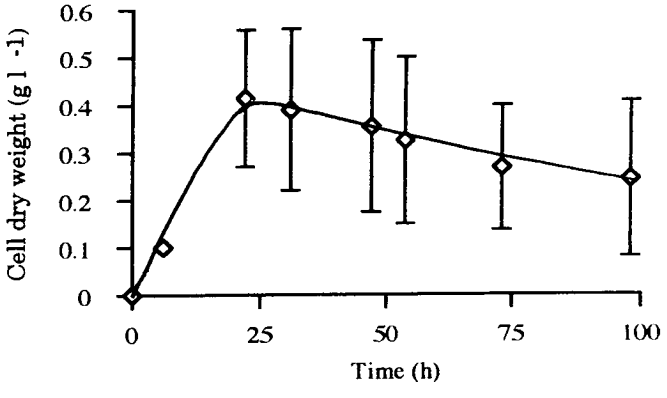
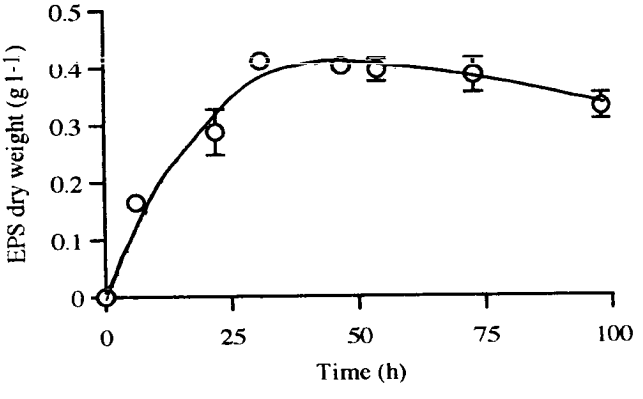
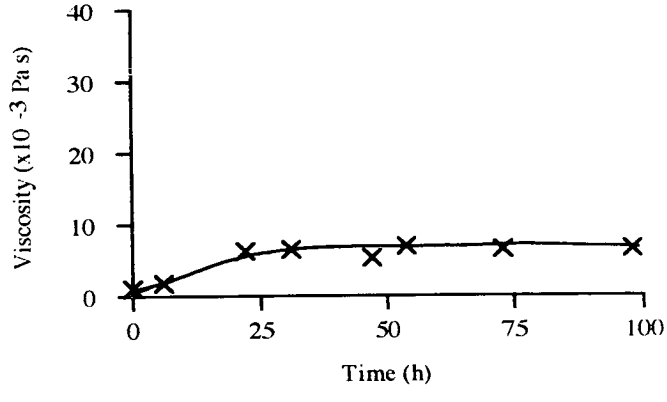


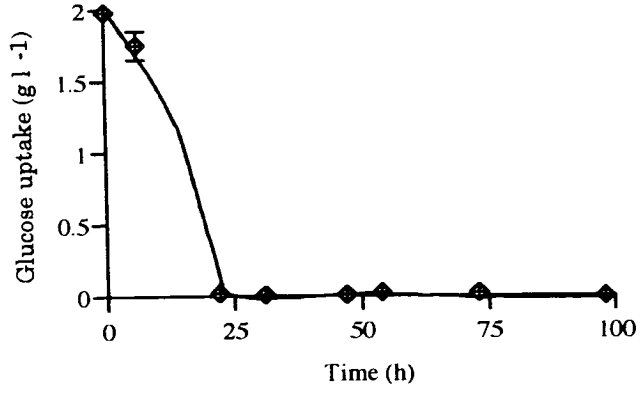
Figure 5.3
Growth and exopolysaccharide production for *S. paucimobilis* strain SB10 when grown in Davis Mingioli medium containing 0.2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











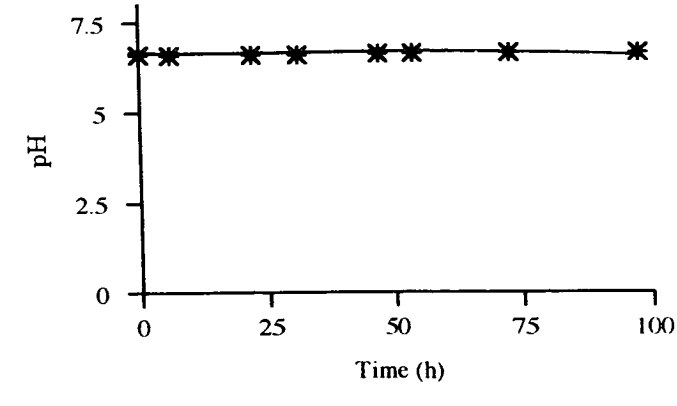
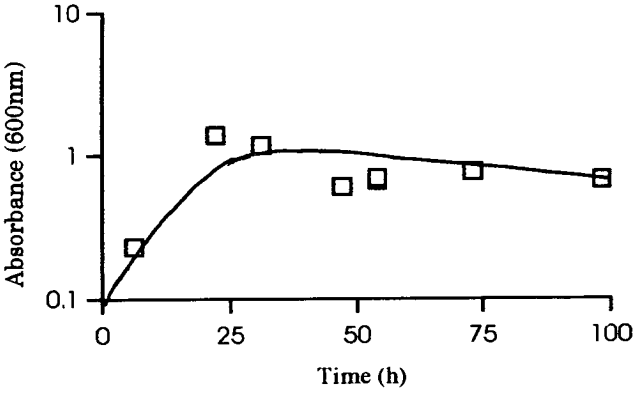
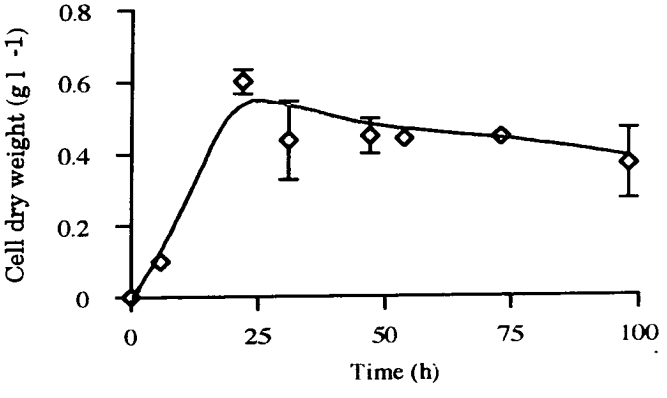
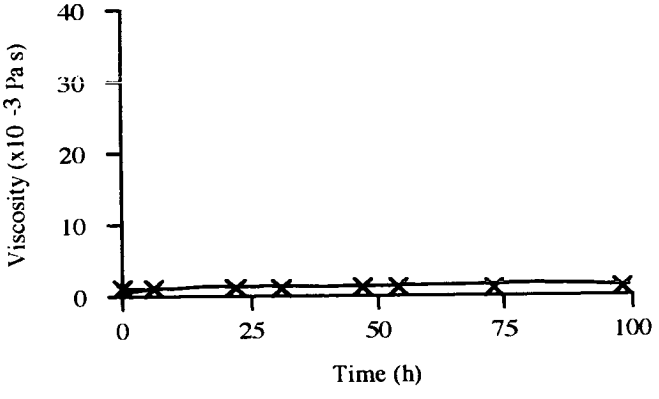


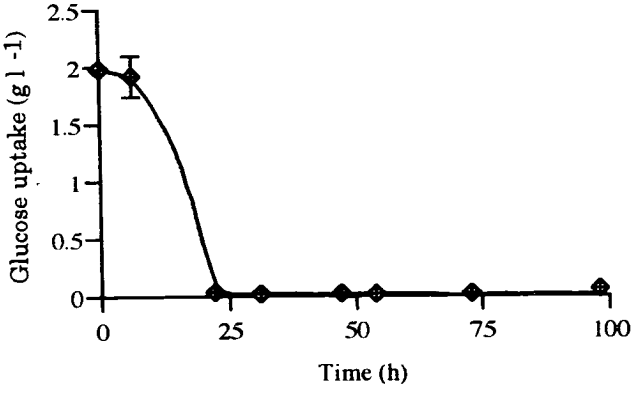
Figure 5.4

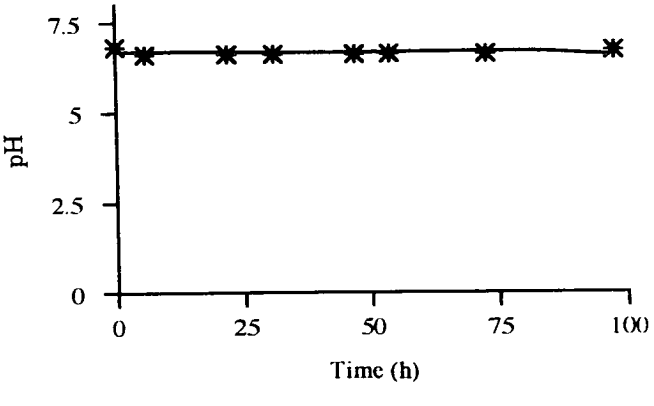
Growth and exopolysaccharide production for *S. paucimobilis* strain MJ8 when grown in Davis Mingioli medium containing 0.2% glucose, 0.01% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











5.2 Chemical composition of EPS elaborated by *Sphingomonas paucimobilis* strains WT, SB10 and PA4 in Davis Mingioli medium with reduced levels of glucose

The neutral sugar composition of *S. paucimobilis* strains WT, PA4 and SB10 was studied following growth in Davis Mingioli medium containing 0.2% and 0.02% glucose for 96 hours. The same strains were also grown in medium containing 2% glucose. Results for *S. paucimobilis* strain PA4 are shown in Tables 5.3 and 5.4. The composition of EPS from these control conditions have been described in detail in Chapter 4.4. At 0.2% glucose, the average amount of EPS accounted for during analysis was greater than at the lower concentration (0.02%) of glucose. At 0.2% glucose, the concentration of glucose was found to be greater than the rhamnose concentration except for the samples analysed at 73 hours when the rha:glc ratio was 46.0:48.2%. The concentration of mannose varied between 2.1-5.6%. However, the quantities of other sugars found were also present in similar amounts.

Table 5.3: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 0.2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
4.5	3.5	2.7	0.3	0.1	0.1	0.02	0.03	0.4	0.2	nd	7.4
20	9.6	7.6	0.4	0.1	0.5	nd	0.06	0.08	0.4	nd	18.7
28	10.4	8.9	0.5	0.1	0.5	nd	0.08	0.09	0.4	0.02	21.1
45	9.7	8.6	0.5	0.2	0.4	0.01	0.03	0.4	0.02	nd	19.9
53	10.3	9.3	0.4	0.1	0.5	0.04	nd	nd	0.04	nd	21.1
73	7.6	7.3	0.5	0.2	0.3	nd	nd	0.05	0.4	nd	16.3
98	9.2	6.5	0.5	0.2	0.2	0.02	0.08	0.05	0.2	nd	17.1

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
4.5	51.0	38.8	5.6	2.2	1.8	0.2	0.4
20	49.8	44.0	2.4	0.7	2.7	nd	0.4
28	48.6	45.6	2.2	0.6	2.5	nd	0.4
45	47.8	46.6	2.4	0.8	2.4	0.05	0.2
53	47.4	47.0	2.1	0.6	2.6	0.2	nd
73	46.0	48.2	2.8	1.0	2.0	nd	nd
98	51.7	42.6	2.8	1.2	1.2	0.2	0.4

nd = not detected

Table 5.4: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 0.02% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
4.5	2.0	1.3	0.4	0.06	0.1	0.1	nd	nd	0.03	0.3	4.3
20	4.1	4.1	0.3	0.07	0.2	nd	nd	nd	nd	0.3	9.0
28	3.8	3.7	0.5	nd	0.2	0.1	nd	nd	0.06	0.3	8.7
45	3.9	3.4	0.3	0.04	0.2	nd	nd	nd	0.03	0.3	8.4
53	3.1	2.7	0.4	0.04	0.2	0.06	nd	nd	0.04	0.3	6.8
73	3.8	2.8	0.4	0.1	0.1	0.1	nd	3.8	nd	0.3	7.8
98	4.1	3.9	0.3	0.06	0.08	0.1	nd	0.09	nd	0.3	8.9

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
4.5	47.8	34.2	9.6	1.8	3.6	3.0	nd
20	45.0	49.0	3.2	0.8	2.0	nd	nd
28	43.7	46.9	5.2	nd	2.8	1.4	nd
45	49.7	44.5	3.2	0.6	2.6	nd	nd
53	45.6	44.0	5.6	0.8	3.0	1.2	nd
73	48.6	39.8	5.7	1.7	2.0	2.1	nd
98	45.9	47.2	3.6	0.8	1.1	1.3	nd

nd = not detected

CHAPTER 6

RESULTS

6. THE EFFECTS OF MAGNESIUM LIMITATION ON *SPHINGOMONAS PAUCIMOBILIS*

6.1 Preliminary studies with *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with reduced levels of $MgSO_4$

The effect of magnesium concentration on the growth and exopolysaccharide production of *S. paucimobilis* was investigated. *S. paucimobilis* wild type strain was grown in Davis Mingioli medium containing 0-0.01% $MgSO_4$ concentrations over a 42 hour period (figure 6.1 and Table 6.1). Samples of culture broth were removed periodically and growth was measured by absorbance at 600nm. Duplicate flasks were used for each concentration of $MgSO_4$ and absorbance values were also taken in duplicate. The maximum concentration of magnesium used in these studies was 0.01%, however, the greatest absorbance values were observed from culture medium containing 0.0025% $MgSO_4$ (figure 6.1). The maximum specific growth rates were similar for cultures containing between 0.01-0.001% $MgSO_4$ (Table 6.1). At magnesium concentrations below 0.001%, specific growth rates were lower. Substantial growth was achieved in flasks containing culture broth to which no magnesium had been added (figure 6.1).

Viscosity measurements were also recorded for samples of culture broth using a Brookfield viscometer at shear rates between 0.3-30 s^{-1}. Viscosity values, at a shear rate of 6 s^{-1}, for all the $MgSO_4$ concentrations studied are shown in Table 6.1. Culture viscosity decreased with decreasing concentrations of $MgSO_4$, except for 0.005% and 0.0017% $MgSO_4$.

Figure 6.1: Growth curve for *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with varying concentrations of $MgSO_4$. Growth was recorded as absorbance readings at 600nm and plotted against time (h). Results are expressed as mean ± standard deviation of duplicate flasks with two readings from each flask.

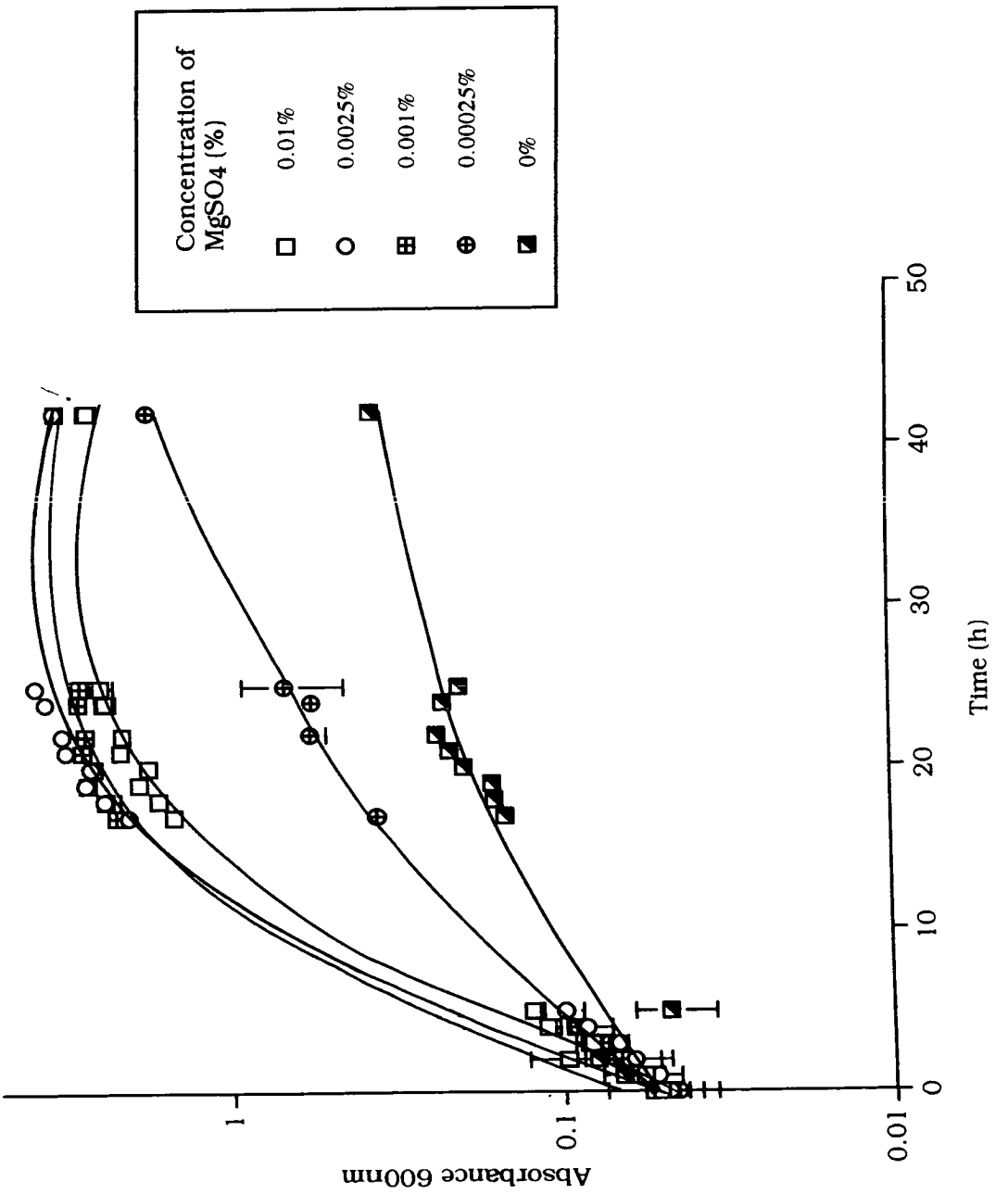


Table 6.1: Table showing specific growth rates and culture broth viscosity measurements for *Sphingomonas paucimobilis* wild type strain grown in Davis Mingioli medium with different concentrations of $MgSO_4$. Each value is expressed as mean ± standard deviation. Each culture was grown in duplicate flasks and duplicate tests were taken for both absorbance and viscosity measurements.

CONCENTRATION OF $MgSO_4$ (%)	MAXIMUM SPECIFIC GROWTH RATE (μ')	VISCOSITY OF CULTURE BROTH (SHEAR RATE s^{-1}) (mPa s)
0.01	0.088	28.6 ± 1.33
0.005	0.114	9.2 ± 0.10
0.0025	0.104	19.6 ± 1.45
0.0017	0.095	2.9 ± 0.84
0.0012	0.088	13.4 ± 1.66
0.001	0.104	5.0 ± 0.48
0.00067	0.095	4.2 ± 0.48
0.0005	0.067	3.1 ± 0.62
0.00025	0.046	2.3 ± 0.32
0	0.038	1.15 ± 0.08

6.2 Growth studies with *S. paucimobilis* strains WT, SB10, PA4 and MJ8 in Davis Mingioli medium with reduced levels of $MgSO_4$

Growth and EPS production were studied for *S. paucimobilis* strains WT, PA4, SB10 and MJ8, in Davis Mingioli medium with reduced magnesium levels. Growth and EPS production for the strains in DM medium containing 0.01% $MgSO_4$ have already been described (Chapter 4.2). In these experiments, the magnesium concentrations studied were 0.001%, 0.0005% and 0.0002% $MgSO_4$. The graphs for many of the strains and magnesium concentrations are very similar, therefore, only some of the data is shown graphically in this chapter. Maximum values of absorbance, cell and EPS dry weights, viscosity, glucose uptake and pH have been summarised in Table 6.2. Data for EPS produced per cell and glucose consumed are shown in Table 6.3. In general, maximum absorbance values for the four strains studied decreased with decreasing concentration of $MgSO_4$ in the culture broth (Table 6.2). However, strain SB10 yielded maximum absorbance values of 3.98 and 5.00 for 0.001% and 0.0005% $MgSO_4$ respectively. With magnesium limitation optical density values were still increasing at 96 hours for some strains. A typical example of this is strain PA4 growing in medium with 0.001% $MgSO_4$ (figure 6.2). For this strain, there was a slight decrease in cell weight towards the end of the growth period.

Maximum cell yields decreased with magnesium concentration only for strain PA4. For strains WT, SB10 and MJ8 the maximum cell yield was greater at 0.0005% $MgSO_4$ than at 0.001% $MgSO_4$. EPS yield was not affected by magnesium limitation in strain PA4. EPS yields were between 2.03-2.36 g l^{-1} for concentrations of $MgSO_4$ between 0.0002%-0.01% which represents a fifty-fold difference. For strains SB10 and WT growing at 0.01% $MgSO_4$ concentration, the EPS yield seemed very low at only 0.85 and 0.75 g l^{-1}. When these strains were grown with reduced levels of $MgSO_4$, the EPS yields were very similar at all magnesium concentrations. The greatest yield was obtained with the wild type strain growing at 0.0005% $MgSO_4$ with 3.57 g l^{-1} EPS (figure 6.4). Considering only 43% of the initial glucose was utilised, this represents 41% conversion of glucose into EPS (Table 6.3). The conversion of EPS

from glucose substrate, was higher at 0.0002% $MgSO_4$ (figure 6.5) where, since less glucose was used the EPS/glc value was 49.4%. With some strains EPS yield was also increasing at 96 hours as shown by PA4 at 0.0005% $MgSO_4$ (figure 6.3). Viscosity of the culture medium was also found to increase.

Glucose utilisation decreased with $MgSO_4$ concentration for strains WT and MJ8. The non-mucoid strain, MJ8, used less glucose than the mucoid strains at reduced levels of $MgSO_4$ (Table 6.3). For strains SB10 and PA4 the glucose uptake values for the concentrations 0.001% and 0.0005% $MgSO_4$ were rather similar. Culture viscosity was little affected by reduced magnesium levels and was greater than 20 mPa s, at the maximum values, for all the mucoid strains. Strain SB10, growing at 0.0002% $MgSO_4$ concentration, showed a decrease in viscosity towards the end of the 96 hour growth period. This coincided with a decrease in EPS yield, although maximum absorbance and cell yields stayed fairly constant (figure 6.6). With strain MJ8 the viscosity was about 1.00 mPa s at all $MgSO_4$ concentrations. The pH of the culture broth changed most at the greatest $MgSO_4$ concentration and was not radically different at the reduced magnesium levels.

Table 6.2: Summary table showing maximum values obtained for *S. paucimobilis* strains WT, SB10, PA4 and MJ8 grown in Davis Mingioli medium with reduced levels of $MgSO_4$. Values shown are expressed as mean ± standard deviation of duplicate assays carried out on duplicate flasks (except cell and EPS dry weight where only one measurement was made for each flask).

STRAIN PA4 $MgSO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.01	0.080	5.58 ± 0.68	1.12 ± 0.10	2.06 ± 0.28	5.90 ± 0.42	34.15 ± 2.89	5.74 ± 0.13
0.001	0.103	3.92 ± 0.34	0.54 ± 0.14	2.36 ± 0.37	9.46 ± 0.93	34.0 ± 1.86	6.60 ± 0.04
0.0005	0.085	3.41 ± 0.91	0.40 ± 0.25	2.28 ± 0.29	9.59 ± 0.48	24.6 ± 3.28	6.69 ± 0.02
0.0002	0.111	2.55 ± 0.53	0.32 ± 0.09	2.03 ± 0.37	7.59 ±0.86	23.1 ± 13.23	6.67 ± 0.02

STRAIN WT $MgSO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.01	0.068	5.31 ± 0.67	0.88 ± 0.07	0.75 ± 0.10	3.68 ± 2.33	35.22 ± 2.15	5.86 ± 0.04
0.001	0.131	2.91 ± 0.09	0.60 ± 0.28	3.24 ± 0.89	10.51 ± 1.09	27.9 ± 1.19	6.52 ± 0.01
0.0005	0.099	2.12 ± 0.07	0.72 ± 0.11	3.57 ± 0.14	11.38 ± 1.22	27.6 ± 1.03	6.58 ± 0.01
0.0002	0.068	1.12 ± 0.06	0.84 ± 0.02	2.93 ± 0.84	14.07 ± 1.78	21.4 ± 0.83	6.61 ± 0.04

STRAIN SB10 $MgSO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.01	0.073	6.70 ± 2.09	0.83 ± 0.04	0.85 ± 0.23	5.30 ± 2.90	30.40 ± 5.43	5.98 ± 0.01
0.001	0.103	3.98 ± 0.16	0.52 ± 0.13	2.70 ± 0.18	10.01 ± 0.88	23.0 ± 1.66	6.61 ± 0.04
0.0005	0.131	5.00 ± 0.63	0.65 ± 0.08	2.55 ± 0.99	9.50 ± 1.49	23.0 ± 1.10	6.63 ± 0.01
0.0002	0.099	2.84 ± 0.11	0.19 ± 0.10	2.89 ± 0.78	11.52 ± 0.85	24.4 ± 3.70	6.68 ± 0.01

STRAIN MJ8 $MgSO_4$ CONC. (%)	AVERAGE SPECIFIC GROWTH RATE	MAX ABSORBANCE (600 nm)	MAX. CELL WEIGHT ($g\ l^{-1}$)	MAX. EPS WEIGHT ($g\ l^{-1}$)	GLUCOSE REMAINING ($g\ l^{-1}$)	MAX. VISCOSITY ($x10^{-3}$ Pa s)	pH
0.01	0.067	7.73 ± 2.06	1.95 ± 0.70	0	4.35 ± 2.86	1.08 ± 0.02	6.20 ± 0.14
0.001	0.127	3.52 ± 0.51	0.85 ± 0.38	0	11.08 ± 1.10	1.10 ± 0.02	6.57 ± 0.01
0.0005	0.103	2.44 ± 0.18	1.06 ± 0.69	0	12.93 ± 0.67	1.06 ± 0.04	6.64 ± 0.02
0.0002	0.102	1.62 ± 0.61	0.68 ± 0.15	0	14.69 ± 1.40	1.08 ± 0.04	6.68 ± 0.01

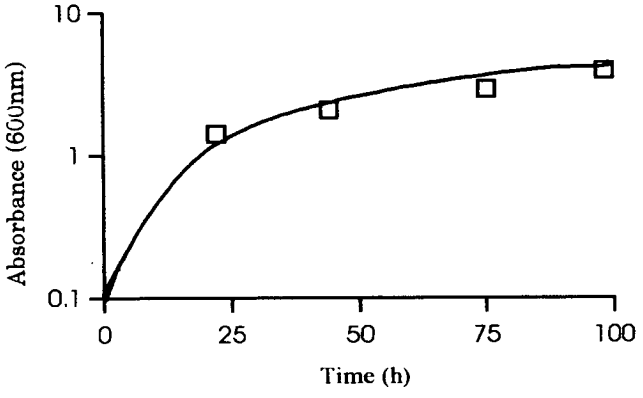
Table 6.3: Table showing maximum cell and EPS weights, EPS/cell values, glucose utilised and EPS/glc values for *S. paucimobilis* strains WT, SB10, PA4 and MJ8. Cultures were set up in duplicate and grown in Davis Mingioli medium with varying amounts of $MgSO_4$ for 96 hours at 30°C.

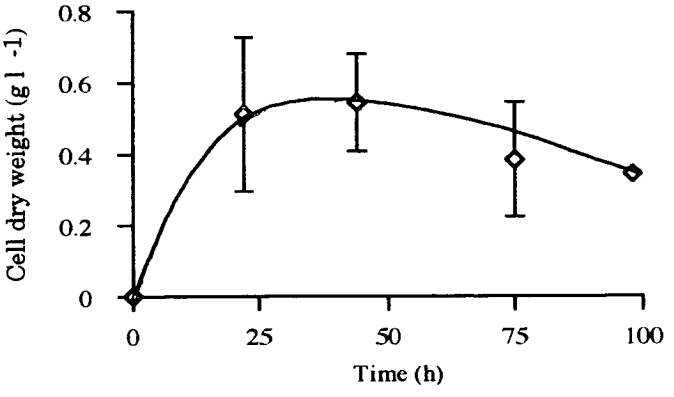
STRAIN / $(NH_4)_2SO_4$ CONC. (%)		CELL DRY WT ($g\ l^{-1}$)	EPS DRY WT ($g\ l^{-1}$)	EPS/ CELL ($g\ g^{-1}$)	GLUCOSE USED $g\ l^{-1}$ OR (%)		EPS/ glc (%)
PA4	0.01	1.12	2.06	1.84	14.1	70	14.6
	0.001	0.54	2.36	4.4	10.54	53	22.4
	0.0005	0.40	2.28	5.7	10.41	52	21.9
	0.0002	0.32	2.03	6.3	12.41	62	16.4
WT	0.01	0.88	0.75	0.85	16.3	82	4.6
	0.001	0.60	3.24	5.4	9.49	47	34.1
	0.0005	0.72	3.57	4.96	8.62	43	41.4
	0.0002	0.84	2.93	3.5	5.93	30	49.4
SB10	0.01	0.83	0.85	1.0	14.7	74	5.8
	0.001	0.52	2.70	5.2	9.99	50	27.0
	0.0005	0.65	2.55	3.9	10.5	52	24.3
	0.0002	0.19	2.89	15.2	8.48	42	34.1
MJ8	0.01	1.95	-	-	15.6	78	-
	0.001	0.85	-	-	8.92	45	-
	0.0005	1.06	-	-	7.07	35	-
	0.0002	0.68	-	-	5.31	27	-

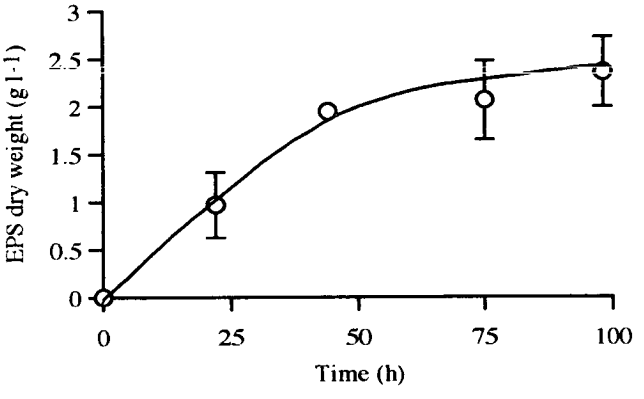
Results shown for cell and EPS dry weights are average values of one test from duplicate flasks. Glucose values are the mean of 2 tests from each duplicate flask. EPS/cell and EPS/glc values are calculated from the average values.

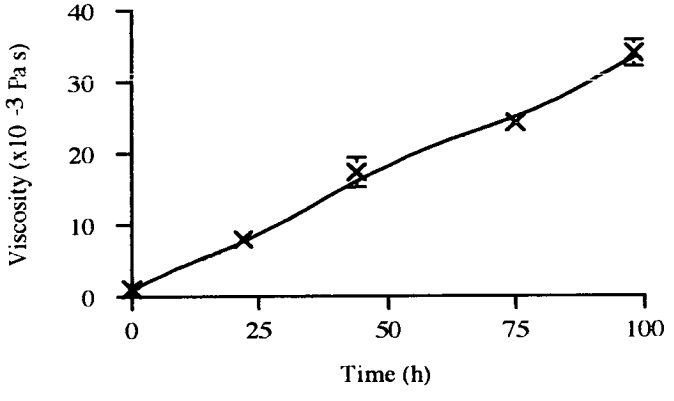
Figure 6.2

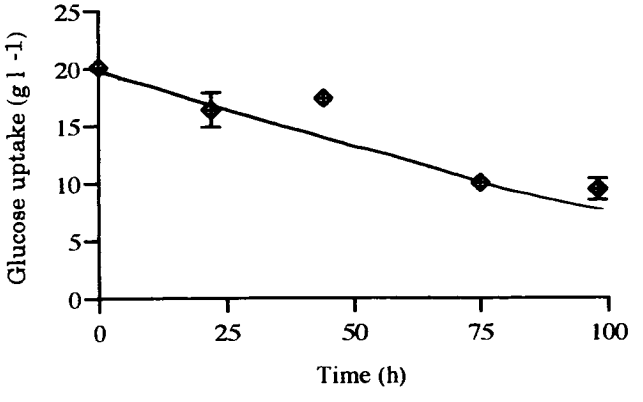
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.001% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











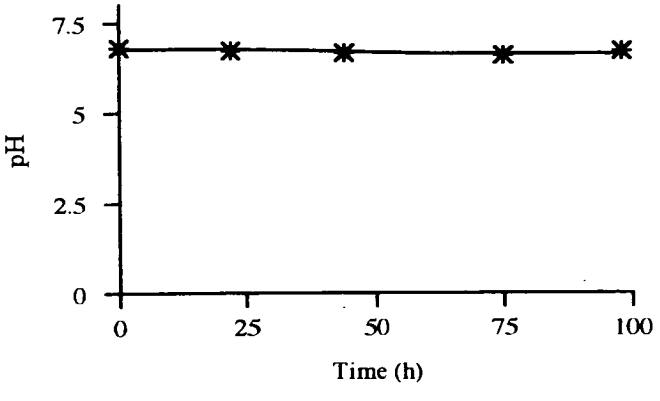
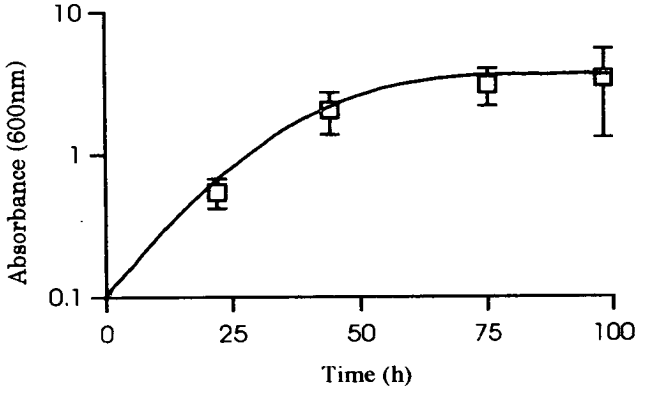
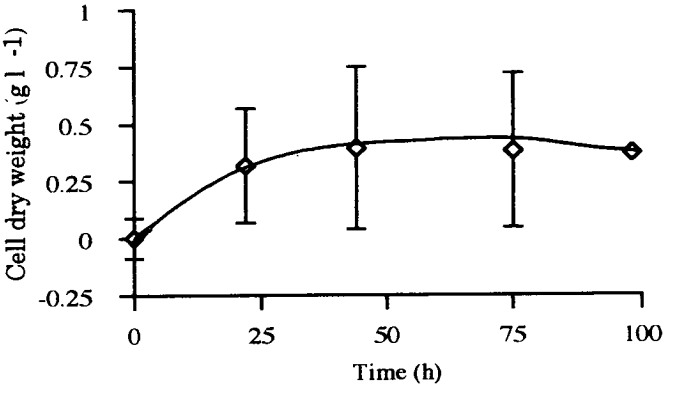
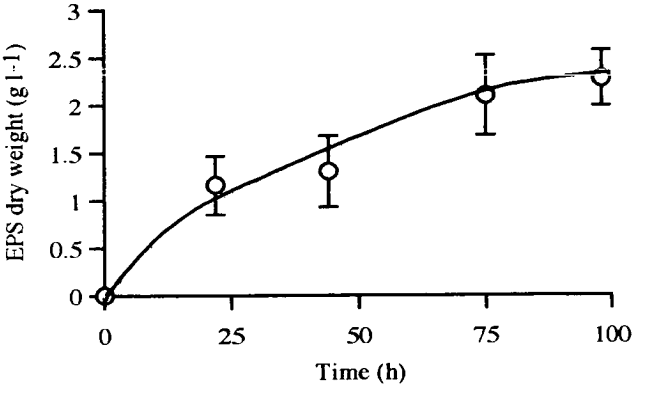


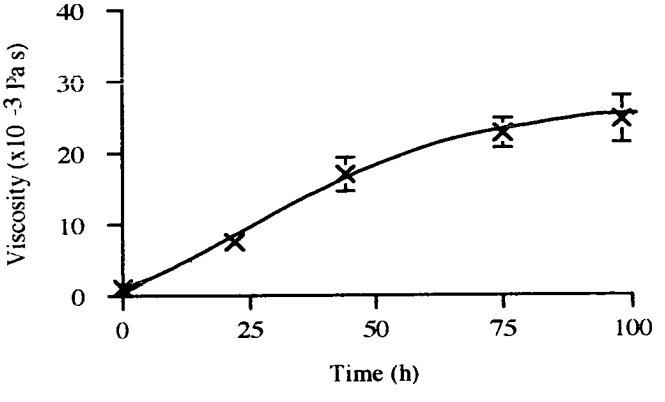
Figure 6.3

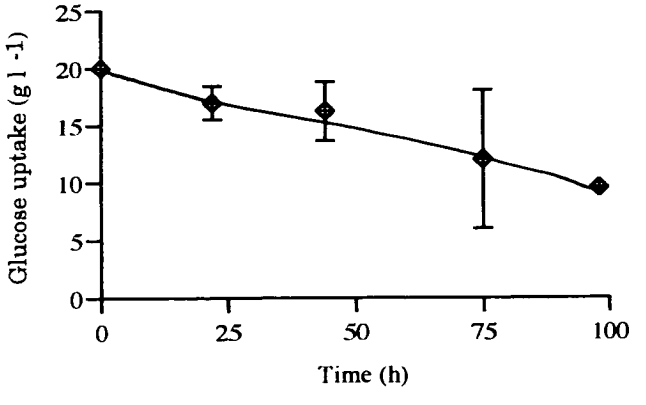
Growth and exopolysaccharide production for *S. paucimobilis* strain PA4 when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.0005% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











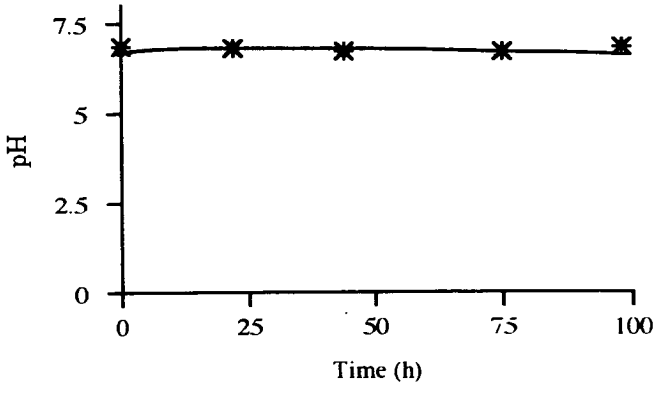
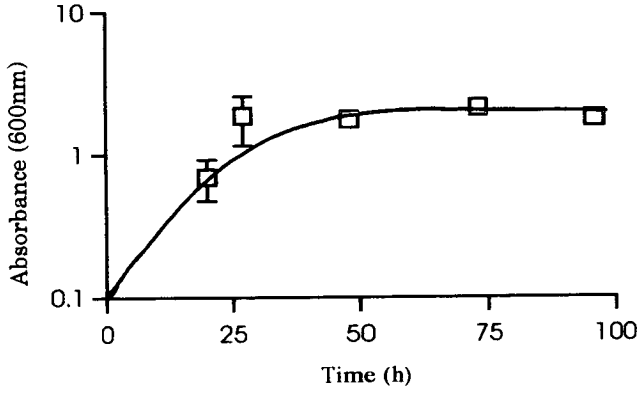
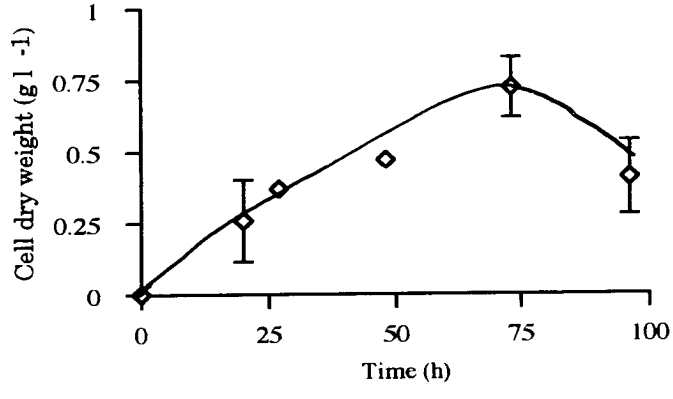
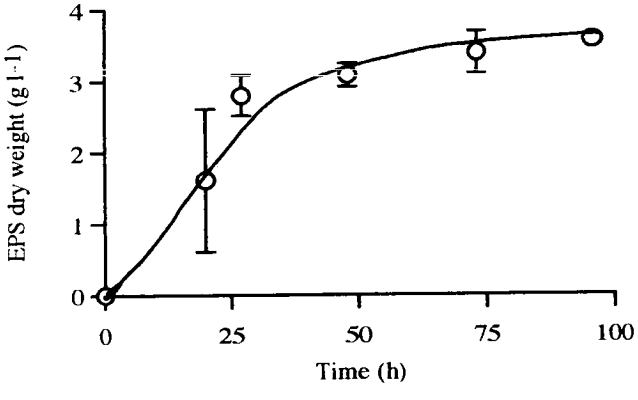


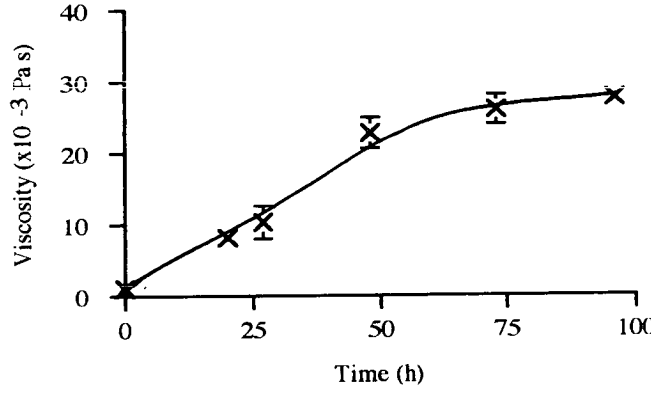
Figure 6.4

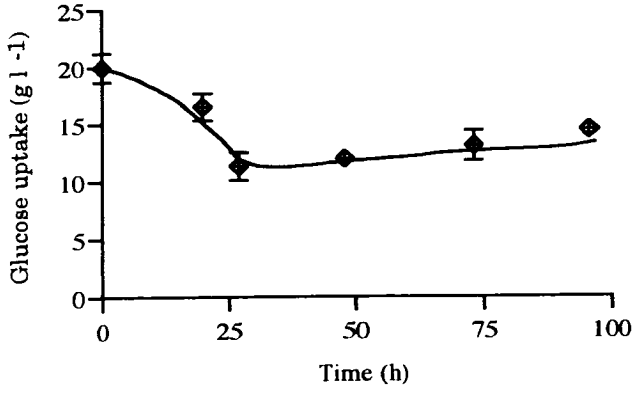
Growth and exopolysaccharide production for *S. paucimobilis* strain WT when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.0005% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











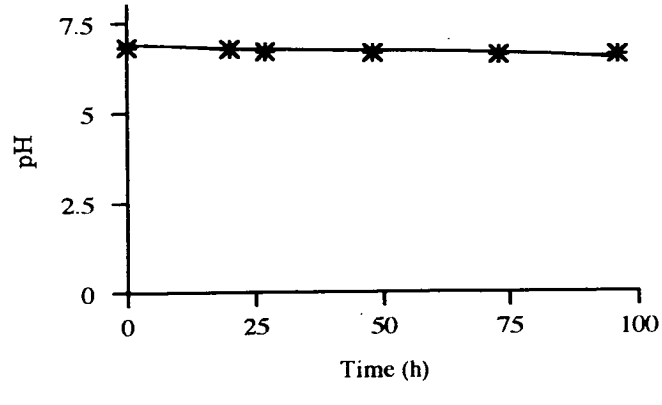
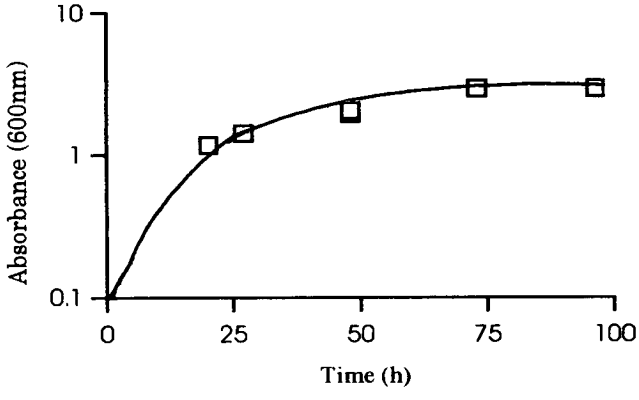
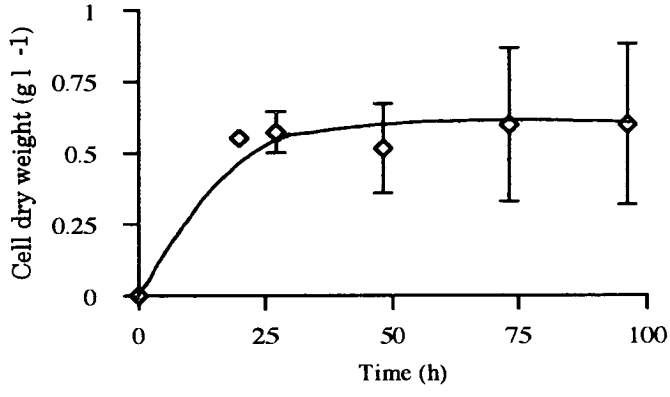
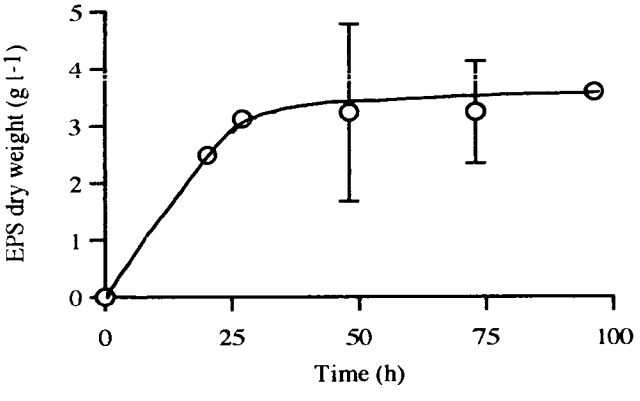


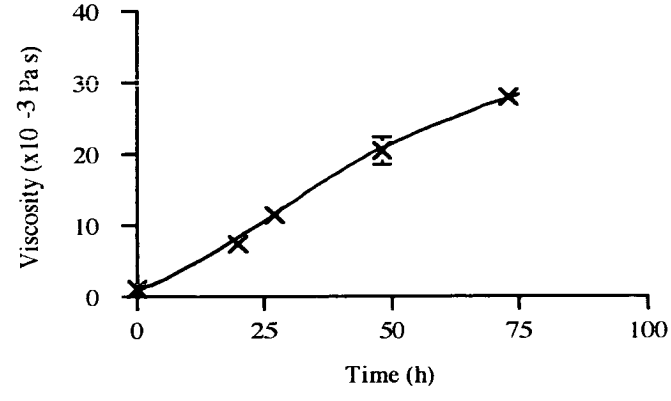
Figure 6.5

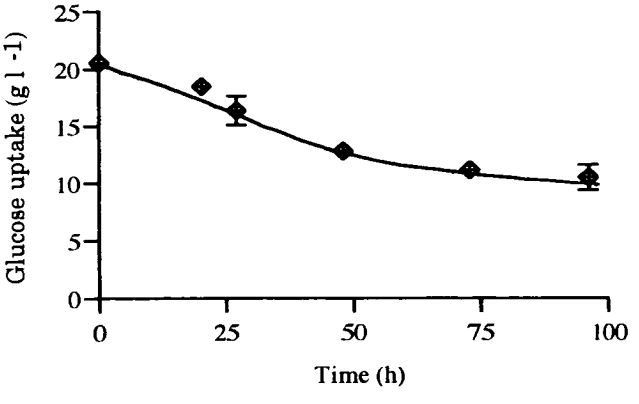
Growth and exopolysaccharide production for *S. paucimobilis* strain WT when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.001% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.











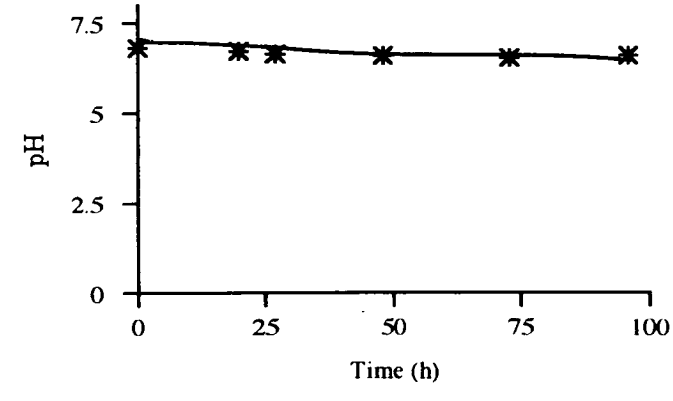
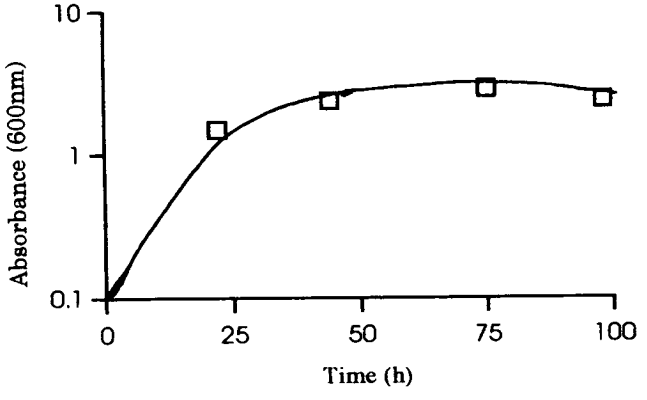
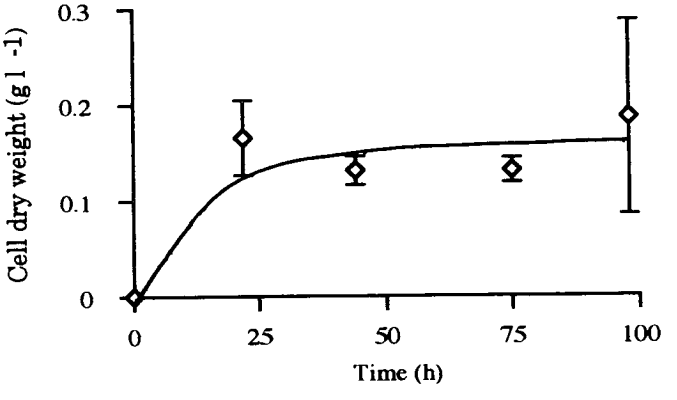
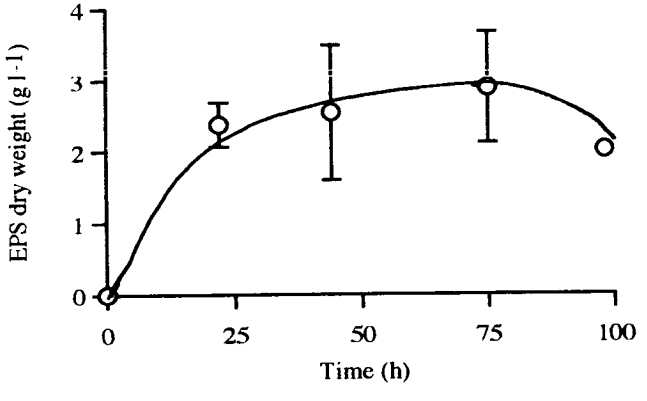
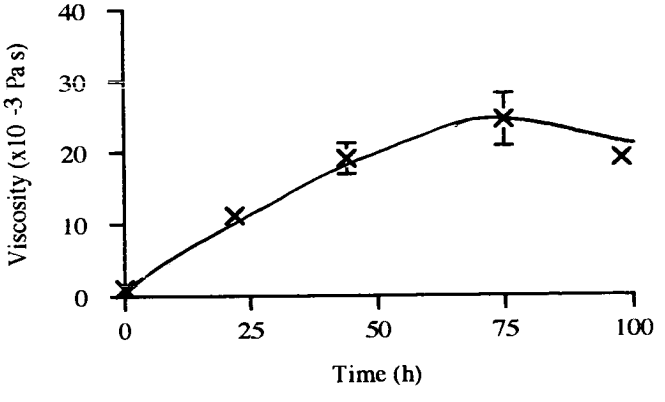


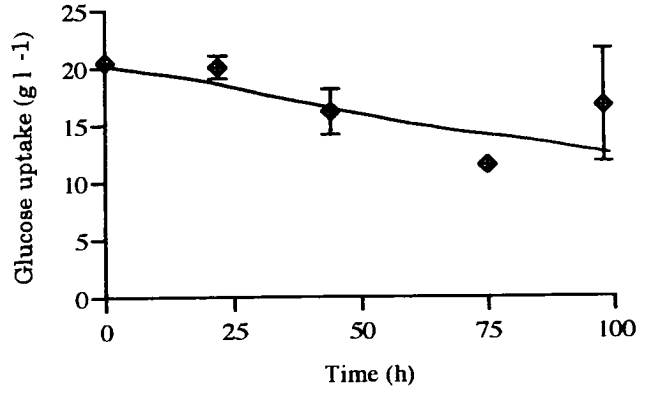
Figure 6.6
Growth and exopolysaccharide production for *S. paucimobilis* strain SB10 when grown in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.0002% $MgSO_4$ for 96 hours at 30°C. Growth was assessed as culture medium absorbance at 600nm. Cell dry weights (g l^{-1}) were measured following centrifugation of a known volume of culture medium. EPS dry weights (g l^{-1}) were determined by acetone precipitation. Viscosity measurements were taken directly on samples of culture fluid. Glucose concentration was determined by colorimetric assay and pH measurements were made directly in samples of culture broth removed periodically. Cultures were set up in duplicate and each assay was performed in duplicate except cell and EPS dry weights where only one measurement was made per flask. Results are expressed as mean ± standard deviation.

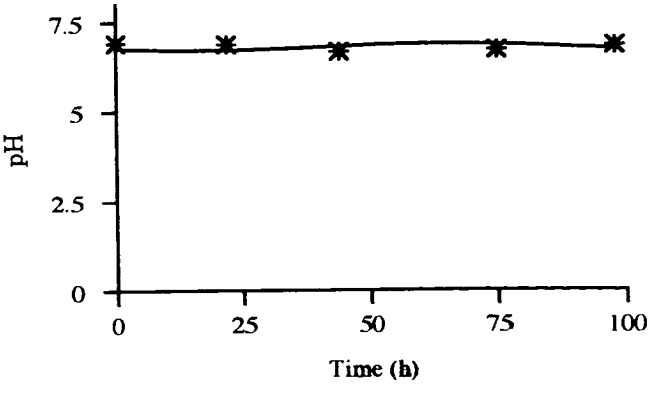












6.3 Chemical composition of EPS elaborated by *S. paucimobilis* strains WT, SB10, PA4 and MJ8 grown in Davis Mingioli medium with reduced levels of $MgSO_4$

The neutral sugar composition of the polysaccharide elaborated by *S. paucimobilis* strains WT, SB10 and PA4 were determined by gas chromatography. The strains were grown in duplicate flasks of Davis Mingioli medium containing 0.001%, 0.0005% and 0.0002% $MgSO_4$ concentrations. The EPS found were very similar to each other and to the EPS already described in previous chapters. For this reason, EPS composition for strain PA4 growing at the three reduced magnesium levels is shown in Tables 6.4-6.6. Also, EPS composition from strain SB10 grown in medium with 0.0005% $MgSO_4$ is described as it was found to contain a high mannose concentration (Table 6.7). With strain PA4, total sugar content (i.e. % of total EPS analysed) varied from 6.6-21.4% of hydrolysed sample (w/w). The low values of EPS % as (w/w) of total EPS, were always found early in the growth study, in this case 22 hours. For strain PA4, the glucose:rhamnose concentrations varied slightly with $MgSO_4$ concentration. However, mannose (mole %) decreased from 7.7% at 0.001% $MgSO_4$ to 5.7% at 0.0002% $MgSO_4$. The greatest mannose concentration noticed through all of these studies was strain SB10 growing at 0.0005% $MgSO_4$ where 10.3% of identified sugars was mannose. It seemed in this case that the glucose mole % of identified sugars decreased as mannose increased. In reality the weights of material glc, rha and man identified all increased as total sugar content values increased also.

Table 6.4: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.001% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS									TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	
22	5.8	4.6	0.1	nd	0.2	nd	nd	0.05	0.4	11.2
44	7.7	6.0	0.6	nd	0.2	nd	nd	0.04	0.3	14.9
75	8.6	6.7	1.4	nd	0.3	nd	nd	0.05	0.4	17.5
96	10.5	8.5	1.7	nd	0.3	nd	nd	0.1	0.4	21.4

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	51.4	44.8	1.4	nd	2.4	nd	nd
44	50.6	44.3	3.7	nd	1.4	nd	nd
75	48.4	41.8	8.0	nd	1.8	nd	nd
96	48.2	42.4	7.7	nd	1.6	nd	nd

nd = not detected

Table 6.5: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.0005% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS									TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	
22	4.4	3.2	0.1	nd	0.06	nd	nd	0.1	0.3	8.3
44	7.9	5.6	0.4	nd	0.2	nd	nd	0.03	0.4	14.5
75	8.4	6.5	1.0	nd	0.2	nd	nd	0.04	0.4	16.6
96	9.9	7.4	1.4	nd	0.2	nd	nd	0.1	0.4	19.4

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	54.8	42.9	1.7	nd	0.06	nd	nd
44	53.3	42.0	3.0	nd	1.7	nd	nd
75	49.8	42.4	6.2	nd	1.5	nd	nd
96	50.4	41.6	6.8	nd	1.0	nd	nd

nd = not detected

Table 6.6: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.0002% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS									TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	
22	3.3	2.4	0.2	nd	0.4	nd	nd	nd	0.3	6.6
44	9.3	6.9	0.5	0.03	0.2	nd	0.03	0.1	0.4	17.4
75	7.6	6.1	0.8	nd	0.2	nd	nd	0.1	0.4	15.1
96	9.5	9.0	1.2	nd	0.3	nd	nd	0.1	0.4	20.5

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	51.1	40.2	2.4	nd	6.3	nd	nd
44	52.2	43.2	2.6	0.2	1.6	nd	0.2
75	50.1	43.4	4.3	nd	2.4	nd	nd
96	45.6	47.1	5.7	nd	1.6	nd	nd

nd = not detected

Table 6.7: Table showing the neutral sugar composition of exopolysaccharide elaborated by *Sphingomonas paucimobilis* PA4 strain growing in Davis Mingioli medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.001% $MgSO_4$. Results are shown in two tables and are expressed either as composition of EPS as % (w/w) of total EPS or composition of EPS as mole % of identified sugars. Data shown are expressed as mean values of two duplicate analyses.

TIME (h)	COMPOSITION OF EPS AS % (w/w) OF TOTAL EPS										TOTAL SUGAR CONTENT (%)
	GLC	RHA	MAN	GAL	FUC	ARA	XYL	X_1	X_2	X_3	
22	4.5	3.1	0.07	0.04	0.1	0.01	0.2	0.2	1.0	0.1	9.3
44	6.2	4.9	0.6	0.03	0.1	0.02	0.02	0.2	0.9	0.05	13.0
75	6.9	5.4	1.2	nd	0.1	0.02	0.3	0.3	1.2	0.2	15.2
96	7.8	6.4	1.8	0.04	0.2	0.01	0.2	0.2	1.2	0.2	17.9

TIME (h)	COMPOSITION OF EPS AS MOLE % OF IDENTIFIED SUGARS						
	GLC	RHA	MAN	GAL	FUC	ARA	XYL
22	53.6	41.2	0.9	0.6	1.9	0.2	1.8
44	50.0	43.2	5.0	0.2	1.3	0.1	0.2
75	48.6	42.4	7.8	nd	0.9	0.2	0.2
96	45.8	41.4	10.3	0.2	1.1	0.8	0.4

nd = not detected

CHAPTER 7

DISCUSSION

7. DISUCUSSION

7.1 Chemical Composition Of Gellan Gum

7.1.1 Neutral sugar composition of gellan gum elaborated by *S. paucimobilis* wild type

The monosaccharide composition of gellan gum was determined by gas chromatography using Stones method for the preparation of alditol acetates. Samples of hydrolysate were removed, during the procedure to determine uronic acid content using the colorimetric assay of Blumenkrantz & Asboe Hansen (1973) Neutral sugar analysis revealed the presence of 39% glucose, 33% rhamnose and 28% glucuronic acid. In theory, the glc:rha:glcA ratio for gellan gum would be 2:1:1. However, in practice, this is normally closer to 1.5:1:1, when determined by GC, due to an underestimation of glucose attributed to the stability of the glucuronyl→ glucose linkage (O'Neill *et al.*, 1983). This stability is diminished on methylation. In this study, the glucose value was slightly lower and the rhamnose value slightly higher than expected. When the glcA value was excluded, the glc and rha values were 54% and 46% respectively, which was also less than the 60:40 ratio reported by Jansson *et al.* (1983).

It is very difficult to hydrolyse complex acidic polysaccharides completely into their monosaccharide constituents and the data obtained are dependent on the hydrolysis conditions, the derivatives used and the correction factors applied (Furneaux, 1983). A combination of these factors could account for the lower ratios in this study. Under the conditions used by Jansson *et al.* (1983), the sugar analyses of gellan gum gave D-glucose and L-rhamnose in the molar ratio 1:0.47 which was close to the actual values of 1:0.5.

The structure of gellan gum was elucidated simultaneously by O'Neill *et al.* (1983) and Jansson *et al.* (1983). Jansson *et al.* (1983) showed from NMR studies that of the sugars in the tetrasaccharide repeat unit, one was in the α-configuration and three were β-pyranosides. The rhamnose was suspected to be α linked. O'Neill *et al.* (1983) thought originally that the glcA was in the α-configuration also. When they later discovered that the Jansson group had found glcA to be of the β-configuration from their NMR studies, this

discrepancy was attributed to the insufficient reaction time with the Chromium Trioxide oxidation used in this study.
The configuration of the sugar residues was not determined, but by analogy with related polymers (Powell, 1979), glucose and glucuronic acid were assumed to be D, and rhamnose to be L (O'Neill *et al.*, 1983).

7.1.2 GC/MS methylation analysis of gellan gum

The Hakamori and sodium hydroxide methods were used for the preparation of permethylated alditol acetates (PMAAs) for the analysis of the neutral sugars in gellan gum by GC/MS. With gc/ms neutral sugar analysis, gellan gum would be expected to yield equimolar amounts of 1,3 glc, 1,4 glc and 1,4 rha. The results from the Hakamori procedure show close correlation to this with 30.4% 1,3 glc, 31.0% 1,4 glc and 32.6% 1,4 rha respectively. These values were obtained by combining the fragmented values obtained for each sugar.
With the NaOH method the rhamnose concentration was high at 38.1%. Glucose was present as 13.4% 1,3 glc and 44.7% 1,4 glucose. The sum of the two glucose values, 58.1% was close to the expected value of 66%. Instead of 1,4 glc and 1,3 glc being present in a 1:1 ratio the actual ratio was 3:1. This could be due to incomplete hydrolysis with the NaOH method of sugar derivitization. There was an absence of minor peaks with this method but hexitol hexaacetates were identified at 3.7%. Although the NaOH method was easier to carry out, the results were not as accurate as the Hakamori method.

7.1.3 GC/MS of carboxyreduced samples of gellan gum

The tetrahydrofuran method was used for the carboxyreduction of gellan gum and two methods of methylation analysis were compared. The Hakamori method yielded the expected ratios of monosaccharides in gellan gum with 24% 1,4 rhamnose, 24% 1,3 glucose, 26% 1,4 glucose and 25% 1,4,6 glc (corresponding to glcA). As with non-carboxyreduced samples this method produced extensive ion fragmentation. Many of these ions could be attributed to either 1,3 glucose or 1,4 glucose due to the breakdown products

released. For example, 1,2,3 glucose and 1,3,6 glucose both come from 1,3 glucose whereas 1,3,4 glucose and 1,2,3,4 glucose could come from either the 3 linked or the 4 linked glucose moiety. The results from the Hakamori method found 3 linked glucose at a lower concentration than 4 linked glucose. Due to this, the ion fragments of glucose were added to the lower concentration of 3 linked glucose where possible. Arabinose was detected at a concentration of 0.4% which was considered to be insignificant. The presence of 0.5% deoxy pentaacetate and 0.8% hexitol hexaacetate suggested only slight undermethylation.

The monosaccharide composition detected using the NaOH method were rather different. As in the non-carboxyreduced samples, rhamnose was underestimated (18.7%). The presence of only 18.2% 1,4,6 glucose was lower than the expected yield of glucuronic acid. The values for 1,3 and 1,4 glucose were close to the expected 25%. With the NaOH method, arabinose and mannose were identified at 5.5% and 2.9% respectively. Arabinose was almost always present as a contaminant in sugar analyses even in "purified" commercial preparations of polysaccharide. However, the concentration of arabinose in this sample was rather high to overlook as typical contamination. The presence of 8.3% hexitol hexaacetate was also much higher than the 0.8% detected with the Hakamori method.

With the NaOH method, for both carboxyreduced and non-carboxyreduced samples, under-methylation was greatest and sugar concentrations were often lower than expected. Other researchers routinely use the NaOH method with no adverse effect for other polysaccharides. The more time-consuming Hakamori method was thus preferred for analysis of gellan gum.

Following carboxyreduction, the 1,4 linked glucose concentration is expected to increase two-fold indicating the presence of glucuronic acid (O'Neill *et al.* 1983). The same increase was found in this study. The rha:glc ratio for gellan gum was reported to be 1:2.8 (Jansson *et al.*, 1983). In this study the rha:glc ratio increased to 1:3.1 following carboxyreduction which was very close to the expected value.

7.2 Growth and exopolysaccharide production in twenty mutant strains of *S. paucimobilis*

Twenty strains of *S. paucimobilis*, including the wild type strain, were grown in Davis Mingioli medium for 96 hours. The EPS yield, chemical composition and physical properties of the polysaccharide produced were compared.

7.2.1 Mutant strains of *S. paucimobilis*

Many of the strains studied during this project were kindly provided by Dr. I. Sá-Correia, Instituto Superior, Tecnico, Lisbon (Table 3.7). Six strains were selected for study which were found following EMS mutagenesis.

Several strains which were resistant to the antibiotic rifampicin were also studied. One of these strains R40, was found to produce greater quantities of gellan gum, than the wild type strain, especially in complex non-defined medium. Several colonial variants of *S. paucimobilis* were also studied in the course of this project. Lobas *et al.* (1992) recently described a variant of *S. paucimobilis* which produced greater quantities of gellan gum than the wild type strain. Colonial variants have also been described for *X. campestris* and *X. juglandis* which differed physiologically from the wild type stains. The EPS produced by these organisms was altered in yield, composition and physical properties (Cadmus *et al.*, 1976; Evans *et al.*, 1979).

Like many polysaccharide-producing microorganisms, spontaneous reversion to a non-mucoid phenotype was apparent with *S. paucimobilis*. The same problem was reported by Martins & Sá-Correia (1991) although reversion to the non mucoid phenotype by *S. paucimobilis* was less frequent than found with *P. aeruginosa* (Sá-Correia *et al.*, 1987). The instability of slime production in *P. aeruginosa* has been reported by many workers (Doggett, 1969; Mian *et al.*, 1978; Govan *et al.*, 1978). Spontaneous mutants of *P. fluorescens* were also reported following continuous cultivation of this organism (Pringle *et al.*, 1983).

It was noted that, following reversion to a mucoid phenotype, several strains of *S. paucimobilis* produced a fine precipitate material following acetone prccipitation of cell free culture supernatant. This

material differed considerably from gellan gum produced by the wild type strain. Whitfield *et al.* (1981) reported a very similar situation with crenated mutants of *X. campestris*. Material from the culture supernatants of these crenated mutants formed an almost granular precipitate which could only be removed following 1-2 hours settling.

7.2.2 Physiology of EPS production with *S. paucimobilis* mutant strains

It was apparent that the mutant strains studied were physiologically very different from each other. Maximum absorbance values were found to vary between 4.09-8.40. By comparison, the cell weights that were determined, ranged between 1.35-1.82 g l^{-1}. The greatest cell yields were found with, supposedly, EPS$^-$ strains of *S. paucimobilis* or, in this case, strains producing a fine precipitate of polymeric material. This may be because these cells do not produce high amounts of extracellular polysaccharide so carbon substrate is used instead for the production of cell material and/or intracellular storage polymer. Glucose utilisation was between 51-65% for the strains of *S. paucimobilis* studied.

EPS yields varied considerably with strain as did culture medium viscosities. EPS yield was determined by the colorimetric carbohydrate assay of Dubois *et al.* (1956). Some strains were found to have a very low EPS yield but the culture medium viscosity was quite high. This could mean that the EPS produced is of poorer quality i.e. of lower molecular weight. Evans *et al.* (1979) noted that certain cultures of *X. juglandis* had a poor culture viscosity compared with the amount of acetone precipitated material (APM) produced and related this to high protein content of the APM. Following industrial fermentation of gellan gum, about 50% of the material produced was found to be carbohydrate. The other 50% was attributed to insoluble material including protein (Kang & Veeder, 1982).

7.2.3 EPS yield from *S. paucimobilis* mutant strains

The yield of gellan gum was found to vary with mutant strain. In this study simple defined medium was used, namely Davis Mingioli medium although, very high yields of gellan gum were found when *S.*

paucimobilis strain R40 was grown in complex, non-defined medium (Martins & Sá-Correia, 1991). When EPS yield was determined by the colorimetric, phenol-sulphuric acid assay, the values were approximately 12 times less than the amount of polymer found by acetone precipitation. *S. paucimobilis* strains R40, WT and MJ216, were the only strains found to have EPS yields greater than 1 g l^{-1} when measured by the carbohydrate assay. The carbohydrate assay did not seem to show any relationship to the culture medium viscosity. For example, strains HM2 and B4 had very low values (both 0.19 g EPS l^{-1}) for the carbohydrate assay but their culture medium viscosity values were found to be quite different at 11.20 and 3.84 mPa s respectively. *S. paucimobilis* strain M118 had a low value for the carbohydrate assay (0.18 g EPS l^{-1}) but had quite a high viscosity (17.0 mPa s). The fine precipitate producing strains all had low viscosity values but the carbohydrate values, as measured by the phenol/sulphuric acid assay, were found to range between 0.12-1.49 g EPS l^{-1}. When these values were compared to the EPS yields measured by acetone precipitation of cell free culture broth, WT had the greatest EPS yield at 8 g l^{-1} (compared with only 1.4 g EPS l^{-1} determined by the phenol/sulphuric acid assay). Strains HM2 and B4 had yields of 3.9 g l^{-1} and 2.5 g l^{-1} EPS respectively whilst, strain M118 had an EPS yield of 5.85 g l^{-1} by acetone precipitation which was 32 times more than the value found by the Dubois assay. There was a strong correlation between culture medium viscosity and amount of acetone precipitated material (APM). Samples with a low yield of APM were generally found to also have a low culture medium viscosity.

The amount of EPS (as APM) produced in small (250 ml) flasks was compared to that produced in large flasks (2 l). Cultures of *S. paucimobilis* were grown in DM medium in 100 ml amounts in small 250 ml flasks and in 800 ml amounts in 2 l flasks over a 96 hour period. The results were quite surprising in that approximately ten times less EPS was produced from growth in the larger flasks. This could be an oxygen-limitation effect. Many polysaccharide-producing organisms require optimum aeration for the production of EPS (Duguid & Wilkinson, 1953).

The production of gellan gum from *S. paucimobilis* was found to almost cease with oxygen-limiting conditions (Lobas *et al.*, 1992).

7.2.4 Chemical composition of EPS produced by mutant strains of *S. paucimobilis*

The polysaccharide from *S. paucimobilis* analysed during the course of these experiments, either had a gellan type composition or produced fine precipitate material. There were 5 sub-classes of these 2 main compositional types found:-

(1) Gellan like composition - without mannose
(2) Gellan like composition - with mannose
(3) Fine precipitate - predominantly glucose composition
(4) Fine precipitate - predominantly mannose composition
(5) Fine precipitate - containing galactose

The gc analyses for these mutant strains unfortunately do not show how much of the original EPS sample was accounted for by neutral sugar analysis. It is evident from Table 3.10 that half of the polymers analysed contained 1.9 - 12.7 % mannose.
Seven of the strains studied produced fine precipitate material which was generally of one major sugar type, often glucose or mannose. With strain MJ216, three batches of polysaccharide were prepared, in duplicate, at different times. Two of these batches of EPS were composed mostly of glucose, but also contained rhamnose. This was called MJ216A (Table 3.10). On the other occasion, the major sugars found were rha and glc but gal and man were also present in smaller amounts. This batch was called MJ216B.
Whitfield *et al.* (1981) reported two mutant types different from the wild type *X. campestris*. Non-mucoid mutants produced trace amounts of EPS which was similar in composition to wild type EPS. Crenated mutants produced EPS of an unusual composition which contained sugars normally found in LPS. Different batches of EPS which were produced under identical conditions, were found to have different polysaccharide composition, similar to *S. paucimobilis* strain MJ216 in this study.

On another occasion, two different polymers originated from the same culture flask following acetone precipitation of *S. paucimobilis* strain MJ200. One polymer fell to the bottom of the flask as fine precipitate material and was termed, MJ200 PPT. The other polysaccharide wrapped itself around the glass rod used to stir the acetone-polysaccharide mixture and was termed MJ200 ROD. The EPS composition of these 2 polymers were very different. MJ200 ROD had a similar composition to gellan gum, as shown in Table 3.10. MJ200 PPT, was found to contain mostly glucose. The production of two polymers from one batch of growth medium is not too surprising since many of the non-mucoid (fine precipitate producing) strains were spontaneous mutants of *S. paucimobilis* wild type strain.

The acetyl content of the mutant strains of EPS ranged between 1.4 - 7.7 %, with an average value of 4.7%. This was a little less than the 6% acetyl content normally reported for gellan gum (O'Neill *et al.*, 1983; Jansson *et al.*, 1983).

The monosaccharide compositions of the mutant strains were also analysed by HPLC and GC/MS. HPLC analysis was qualitative only, and was used to give some indication of the sugar ratios present in the sample. GC/MS data have been included to compare with the other sugar analysis data. For many of the EPS samples studied, there was a large amount of under-methylation with GC/MS which introduces error into the results. For most of the strains, the most abundant sugar found with GC was also found with HPLC and GC/MS also.

7.2.5 Gelation of gellan gum

Polysaccharide gels like gellan are composed of 99% water and yet are solid, rigid structures (Attwool, 1987). A wide range of gel types are available for gellan depending on the quantity of gum, the type and quantity of cations and the extent of acylation. Deacylation markedly affects the mechanical properties of gels (Moorehouse, 1981).

The divalent cations Ca^{2+} and Mg^{2+}, were found to produce gels at levels 1/25 of those required for gelation by the monovalent ions Na^{+} and K^{+} in a study carried out to investigate the gelation of gellan

gum by Sanderson & Clark (1983). Gellan gelation was studied in test tubes containing either $CaCl_2$ or $MgSO_4$ with varying concentrations of gellan gum. Calcium ions produced gels at slightly lower levels than magnesium ions.

Less calcium ions than monovalent cations are needed for gelation in gellan gum since divalent ions form cross-links between the double helices. The association was much stronger with Ca^{2+} double helix interactions than, for example, K^+ (Chandrasekaran *et al.*, 1988a).

In the present study, gellan gum gelation was studied with native and deacylated gellan gum with either $CaSO_4$ or $MgSO_4$ present in the solutions. At 0.4% gellan concentration, deacylated gellan gum formed a very rigid gel whereas native gum only produced a viscous solution. No difference in gelation was noticed between Ca^{2+} and Mg^{2+} containing solutions in the assay used. This technique may not be sufficiently sensitive to detect small changes in gel quality between cations.

Although gelation would not be expected in deionised water, in tap water, if the natural water hardness was sufficiently high, the ionic concentration may be enough to cause gelation (Sanderson & Clark, 1983).

The L-glycerate in gellan gum described by Kuo *et al.* (1986) is probably the major difference between the native and alkali-treated (deacylated) polymer preventing gelation rather than the acetic ester (Kuo *et al.*, 1986; Chandrasekaran & Thailambal, 1990).

The presence of one acetyl group every second repeat unit was sufficient to hinder gelation in the EPS of *K. aerogenes* K54 while the similar structure XM6, which had no acetyl, could gel (Atkins *et al.*, 1987).

In this study, the polymer entitled MJ200 ROD which had a chemical composition similar to the wild type gellan could gel; MJ200 PPT was unable to form gels.

7.2.6 Rheology studies with EPS from mutant strains of *S. paucimobilis*

The characteristic of gel strength and texture is vitally important to the industrial utilisation of polysaccharides. Both producers and users require objective data relating firmness of the gel to polymer

concentration and, where appropriate, ion concentration too. In general, rheological testing involves the application of some force to the sample under investigation followed by its response. If a cube of material on a bench was squashed by a force applied perpendicular to the bench, this is called compression. The force applied per unit cross-sectional area is termed "stress" while the fractional change in height is the strain (Attwool, 1987).
The two simple type of response to the applied force are:-

i) perfect elastic gels and,
ii) perfect Newtonian liquids.

Most gels exhibit both solid-like and liquid-like behaviour and are conventionally termed viscoelastic. The elastic and viscous responses may be conventionally resolved by mechanical spectroscopy where an oscillatory shearing motion is applied. Under these conditions an elastic solid possesses a stress that is exactly in-phase with the imposed strain. In contrast, in the viscous case, the stress is maximised at the midpoint of the cycle when the net deformation is greatest. In this instance, the stress and strain are exactly out of phase (Morris, 1984). In practice, the viscoelastic gel contains both of these components which may be detected by resolving the resultant stress into its in-phase and out-of-phase components. The ratio of in-phase stress to applied strain is the shear storage modulus G'. By analogy, the corresponding ratio for out-of-phase stress is the shear loss modulus, G". The overall response of the sample may be characterised by the complex modulus G^*

$$G^* = G' + G''$$

Also a dynamic viscosity η^* may be defined:

$$\eta^* = G^*/\omega$$

where ω is the frequency of oscillation. Normally a measurement of the storage modulus will suffice to characterise the properties of the gel (Attwool, 1987).

Rheological measurements were undertaken for eight of the EPS samples produced by mutant strains of *S. paucimobilis*. Strain WA formed a very strong gel, closely followed by NM, M118 and Rif 40. Samples Rif 60 and WI formed very unstable gels and E3 and B4 remained in a liquid state. There did not appear to be any relationship between physiological behaviour or monosaccharide composition and the ability to form strong gels, in the samples studied. It may be that the EPS formed by these organisms differed in solubility or acylation. Examples are presented of WA, which set to form a strong rigid gel (figure 3.11). This sample also had a very high culture medium viscosity and contained some mannose.
Sample B4, remained in a liquid-like state and was not a true gel (figure 3.12). This sample had a low culture viscosity prior to acetone precipitation. The polysaccharide had the same chemical composition as gellan gum.
Acetyl assays were not carried out for every sample and glycerate values are not known. As already discussed, glycerate is known to occupy a bulky position in the gellan helix (Chandrasekaran & Thailambal, 1990). Strain differences in terms of acylation extent may be more interesting and more relevant to study than repeat unit structure. Another factor not considered in this study is molecular weight. It would be interesting to determine if the differences between the fine precipitable material and gellan structure relate also to molecular weight.

7.3 Physiological responses to nutrient limitation

Many microorganisms must grow in nutrient limited conditions. Most organisms express only that part of their genome that enables them to become structurally and functionally adjusted to a certain set of conditions, like a nutrient-limited environment (Harder, 1983). Macromolecular components of bacterial surfaces, for example, LPS, protein and exopolymers, vary in quantity and composition with varying growth conditions (Jarman *et al.*, 1978; Ellwood & Tempest, 1972). Changes in nutrient conditions in aquatic habitats will affect the attachment of individual bacterial species which influences the population structure of developing biofilms (McEldowney & Fletcher, 1986). Limitation of the primary nutrients, C, N, P, S, K and Mg

have a greater effect on cells than growth-limiting by less essential nutrients (Harder, 1983).

In this study, the effect of reduced levels of carbon, nitrogen and magnesium on the growth and extracellular polysaccharide production in *Sphingomonas paucimobilis* was investigated. Most studies on nutrient limitation in microorganisms have been concerned with nitrogen-limiting conditions. Many of the changes in physiology found in the course of this work with reduced carbon and magnesium concentrations were also found with reduced nitrogen levels.

Some differences were noted in chemical composition of EPS produced by the strains studied. The effect of limiting nutrients on the chemical composition and physical properties of EPS will be discussed in section 7.3.6 of this chapter.

7.3.1 The effect of nitrogen limitation on growth and EPS production in microorganisms

Polysaccharide production in microogranisms has been found to be enhanced when growth medium contains a high carbon:nitrogen ratio. Organisms showing this effect include *A. aerogenes* (Duguid & Wilkinson, 1953), *C. violaceum* (Corpe, 1964) and *P. pullulans* (Catley, 1971). The production of xanthan gum by *X. campestris* was also greatest with nitrogen limitation (Souw & Demain, 1979) as was the production of a galactoglucan by *Pseudomonas* species 11264 reported by Williams & Wimpenny (1977).

For many species conversions of 70-80% utilised carbon into EPS was commonly found (Sutherland, 1979), although this conversion efficiency may include cellular debris and 50-60% conversion of carbon into EPS may be a more realistic estimate.

Not all polymer producing organisms benefit from growth in nitrogen deficient medium. Cellulose production in *A. xylinum* was found to be lower in growth medium with reduced levels of nitrogen (Dudman, 1960).

7.3.2 Growth and EPS production of *S. paucimobilis* strains WT, SB10, PA4 and MJ8 in Davis Mingioli medium

Growth and EPS production were compared for four strains of *S. paucimobilis* growing in Davis Mingioli medium with 2% glucose, 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4$ (referred to as the 'control' condition) which represented the greatest nutrient concentrations studied.

Specific growth rate was lowest in the non-mucoid strain MJ8 at 0.067 h^{-1}, although the values for the other 3 strains of *S. paucimobilis* were quite similar. Maximum cell weight found during the 96 hour growth period were also recorded for strain MJ8 (Table 4.3).

In a recent study by de Souza and Sutherland (1994), greater levels of the storage polymer, PHB, were found in an EPS^- mutant of *Alcaligenes* sp. 31961, compared with the polymer producing strain. This organism produces a polymer with the same backbone structure as gellan gum. Similar cell growth was found in both EPS^- and EPS^+ strains although a greater amount of PHB was accumulated in the non-mucoid strain. Exopolysaccharide production and glycogen accumulation in *Enterobacter aerogenes* were also studied in both EPS^+ and EPS^- strains. In both species, the levels of storage polymer were greatest in EPS^- mutants suggesting that carbon substrate was converted into EPS at the expense of storage polymers in the mucoid organisms.

Accumulation of PHB may be the reason for the greater cell yield in *S. paucimobilis* strain MJ8 grown under these conditions of carbon excess.

The yield of EPS and cells for the 3 mucoid strains of *S. paucimobilis* were very different. The greatest cell and EPS yields were measured for *S. paucimobilis* strain PA4. The cell and EPS yields for strains SB10 and WT were much lower than the values found from strain PA4.

Kang *et al.* (1982) reported a 50% conversion of carbon substrate into gellan gum for *P. elodea*. In a recent study by Lobas *et al.* (1992), a high producing polysaccharide variant of *A. elodea* was studied. Variant E2 of *S. paucimobilis* was found to produce approximately 8 kg/m^3 of gellan from an original glucose

concentration of 30kg/m^3 whilst *A. elodea* wild type strain, produced only about 2kg/m^3 polymer from the same initial glucose concentration.

The type of growth medium used was also a factor in the production of gellan gum by Martin and Sá-Correia (1991). Four times as much gellan was produced in medium B (which contained casamino acids and yeast extract) compared with medium A (peptone/yeast extract medium). This represents conversion efficiencies of 20% and 5% for *P. elodea* grown in medium B and medium A respectively.

Very different conversion efficiencies of carbon substrate into EPS have been found by these other authors. Earlier in the course of this work greater EPS yields were found for the wild type strain than the values outlined here perhaps due to strain deterioration.

The EPS/cell values were lower for *S. paucimobilis* strains SB10 and WT compared with strain PA4.

Conversion of glucose into EPS was most efficient in strain PA4 (Table 4.4) with 15% EPS formed from glucose utilised.

During the production of microbial polysaccharides oxygen limitation becomes a real problem in culture fluid of high viscosity. Oxygen transfer may be reduced which results in decreased EPS yield (Margaritas, 1978). The poor yield of gellan gum found with *S. paucimobilis* WT and SB10 may be due to poor oxygen transfer in these cultures, although this would not account for the higher EPS yield found for strain PA4.

For many organisms, excess nitrogen in the growth medium, has an inhibitory effect on EPS production (Souw & Demain, 1979). Under such conditions growth is normally stimulated even although polymer yield is reduced. EPS and cell yield were lower for *S. paucimobilis* strains SB10 and WT than those found for strain PA4.

Cell growth entered stationary phase at about 40 hours. Glucose consumption continued until the end of the 96 hour growth period.

A similar situation was found with polymer production in *Zoogloea ramigera* where cell growth finished after 20 hours and glucose consumption continued beyond this (Norberg & Enfors, 1982).

With strains WT and SB10, EPS yield reached a maximum value then declined during the 96 hour growth period. Due to the high

culture medium viscosity, it is impossible to tell whether viscosity also declined with the decrease in EPS yield.

Gellanase enzymes have been isolated from other bacteria which break down gellan gum and some other members of the gellan family of polymers (Kennedy & Sutherland, 1994; Mikolajczak *et al.*, 1994). A variant of *Auromonas elodea*, termed variant E1, was isolated by Lobas *et al.* (1992) which appeared to sink into solid medium containing gelrite as the gelling agent. This strain would not produce polysaccharide in submerged cultivation. This variant did not grow on gelrite as the sole carbon source. When variant E1 was cultivated in shake flasks in the presence of gelrite, some reduction in solution viscosity was noted. This strain may produce a gellanase enzyme which breaks down the polysaccharide gellan, although the reason variant E1 sinks into the gelrite medium is not known.

A comprehensive study of EPS and storage polymer (PHB) production has recently been carried out on *Alcaligenes* sp. 31961 which produced a polymer similar to gellan gum (de Souza and Sutherland, 1994). This organism was grown in culture broth with differing C:N ratios. In medium with 0.5% glucose and 0.05% $(NH_4)_2SO_4$, the glucose substrate was consumed and EPS and PHB were produced. A slight decline in EPS was also noted with these growth conditions, although the organism is not known to produce polysaccharases.

During the course of these experiments, the starting pH of the Davis Mingioli medium was 7.00. There was not a great change in the pH of the culture medium with limiting nutrient conditions. With this 'control' composition of Davis Mingioli medium, when all nutrients were at the maximum concentration studied, the greatest decline in pH of the culture broth was noted. For the mucoid strains of *S. paucimobilis* the culture medium pH was 5.74-5.98 at the end of the 96 hour growth period for strains PA4, WT and SB10 respectively. The pH of the culture fluid decreased to 6.20 for the non-mucoid strain MJ8. This pH change might reflect the acidic nature of the polysaccharide produced. The pH optimum for EPS production varied with species but was close to neutrality for most organisms (Wilkinson, 1958).

7.3.3 The effect of reduced levels of nitrogen on the growth and EPS production of *S. paucimobilis* strains WT, SB10, PA4 and MJ8

With all the strains of *S. paucimobilis* investigated, maximum cell yield was found to decrease with decreasing amounts of nitrogen. However, cell yield was not proportional to the initial nitrogen concentration. Norberg & Enfors (1982) found that the C:N ratio was very important in the production of exopolysaccharide in *Z. ramigera*. The final amount of EPS was negatively affected if the C:N ratio was less than 38 with a fixed glucose concentration of 25 g l^{-1}. Instead, the culture responded by producing more cells. In the study with *Z. ramigera*, the yield coefficient $Y_{X/N}$ was constant irrespective of the C/N ratio. This is reportedly a common characteristic of polysaccharide production by yeast (Catley, 1971) and bacteria (Duguid & Wilkinson, 1953).

For the *S. paucimobilis* strains studied, EPS yield decreased with decreasing nitrogen content. However, EPS produced per cell (g g^{-1}) increased with decreasing nitrogen content of the culture broth.

Cell yields were found to decline drastically after maximum values were reached in the first 25-40 hours of growth. Such a decrease in cell yield was also noted for *Alcaligenes* sp. 31961. This decrease in cell yield was attributed to the utilisation of storage polymer following carbon substrate exhaustion when grown under reduced carbon levels (de Souza & Sutherland, 1994). In this study, glucose consumption by *S. paucimobilis* strain PA4 ceased between 25-40 hours, even although glucose was still present in the growth media. Following this, cell yield decreased, which may suggest utilisation of the storage polymer, PHB, which may account for up to 80% cell dry weight in some bacterial species, for example, *Alcaligenes eutrophus* (Dawes, 1986).

With *S. paucimobilis* strain PA4, a decrease in EPS yield was found at all reduced nitrogen concentrations. There was no accompanying decrease in viscosity. Interestingly, no such decline in EPS yield was found with strain WT, at any nitrogen concentration.

7.3.4 The effect of reduced levels of carbon on the growth and EPS production in S. *paucimobilis* strains WT, SB10, PA4 and MJ8

In *Erwinia amylovora* the quantity and composition of the EPS produced *in vitro* were affected by the nature and concentration of the sugar (or sugar alcohol) supplied and the nature of the nitrogen source (Bennett & Billing, 1980).

Some microorganisms are unable to produce exopolymer under carbon limiting conditions. Galactoglucan polysaccharide described by Williams & Wimpenny (1978) was not formed with carbon limitation. Also, *Klebsiella aerogenes* did not produce EPS with glucose limitation (Neijssel & Tempest, 1975). For both of these organisms, nitrogen limitation was the favoured growth limitation for the production of EPS.

The production of xanthan gum by *X. campestris* was found to be greatest in ammonium-deficient medium and least in carbon-deficient medium (Davidson, 1978; Tait *et al.*, 1986). In *S. paucimobilis*, gellan gum was produced with carbon limiting conditions, but the yield of polysaccharide produced was lower than found with nitrogen limitation.

EPS/cell values (g g^{-1}) did not change very much when the culture broth contained less glucose. The conversion of glucose into EPS was more efficient at 0.2% glucose than at 2% glucose. *S. paucimobilis* strain PA4 seemed to produce 0.43 g l^{-1} EPS from only 0.2 g l^{-1} glucose initially. This was not the only occasion that greater concentrations of EPS were formed than initial carbon substrate available. It could be that the initial glucose concentration was greater than 0.2 g l^{-1} or that lyophilised EPS was not completely dried when it was weighed.

Cell yields for the four strains of *S. paucimobilis* studied, decreased towards the end of the 96 hour growth period. The decline in cell yield was very dramatic with strain PA4. EPS yield decreased for *S. paucimobilis* strains SB10 and PA4. For strain PA4, there was also some loss of viscosity accompanying this decrease in EPS yield. *S. paucimobilis* strain WT used the available glucose much slower than the other two mucoid strains, and, in this strain the decline in cell yield was less marked and there was no significant decrease in EPS

yield. With carbon substrate limitation, many organisms show increased synthesis of enzymes or enzyme systems involved in the initial metabolism of the limiting nutrient. Whereas, in some organisms the response may be synthesis of a high affinity enzyme system that could involve a different metabolic sequence for the metabolism of the substrate. Such a response allows the organism to sustain a higher rate of metabolism of the growth limiting nutrient (Harder, 1983).

Cornish *et al.* (1988a & b) described 3 major periplasmic proteins in *A. radiobacter* studied for a potential role as glucose binding proteins. Two of these proteins, GBP1 and GBP2, bound glucose with high affinity. During glucose limited growth both GBP1 and GBP2 were hyper-produced accounting for about 27% of total cell protein. The 2 binding proteins had different functions for different strains. When cells were switched to NH_4 - limited cultures, there was a rapid decline in glucose uptake. However, GBP1 had greater uptake capacity that GBP2. These cells produced succinoglucan possibly as a means of disposing of excess carbon (Neijssel & Tempest, 1975).

7.3.5 The effect of reduced levels of magnesium on the growth and exopolysaccharide production of *S. paucimobilis* strains WT, SB10, PA4 and MJ8

Magnesium is an essential nutrient for living cells and their basic requirement for it does not seem to alter much for widely different organisms (Aiking *et al.*, 1977). Under a variety of growth limiting conditions, including Mg limitation, the cellular Mg content increased with increasing growth rate in parallel with the cells ribosomes content. This led Tempest (1969) to the conclusion that most of the cell bound magnesium was associated with the ribosomes.

Magnesium is also present in bacterial cell walls and its abundance in the walls varies greatly with growth conditions (Tempest & Strange, 1966). Specific carrier systems for Mg have been reported (Konings *et al.*, 1981). However, nothing is known about a possible modulation of the activity of properties of these systems under conditions of Mg limitation.

Magnesium limited *B. subtilis* cells excrete compounds that bind Mg thereby improving their ability to compete with other organisms for growth limiting amounts of Mg (Meers & Tempest, 1968). Similarly, cell walls of this organism under Mg-limitation showed an increased Mg binding efficiency over Mg sufficient cells (Meers & Tempest, 1970).

When *S. paucimobilis* was grown at a range of $MgSO_4$ concentrations specific growth rate was between 0.09-0.11 h^{-1}. This was in contrast to the situation found with reduced nitrogen levels in the culture broth. In this case, specific growth rate was found to decrease with decreasing nitrogen concentration. Although both nitrogen and magnesium are essential nutrients for the cell, the amount of magnesium required for growth is far less than the amount of nitrogen necessary.

Culture viscosity generally decreased with decreasing concentration of $MgSO_4$ in the culture medium. However, culture medium viscosity readings were low at 0.005% and 0.0012% $MgSO_4$. It could be that the wrong concentration of $MgSO_4$ was present in these flasks or perhaps the polymer was broken down in some way. This experiment would have to be repeated before any firm conclusions could be made. Certain bacteria produce polysaccharases which break down the polysaccharide they produce. Examples are *Azotobacter* sp. which produce alginate lyases (or alginases) that can degrade alginate (Kennedy *et al.*, 1992). As already mentioned, there is no firm evidence to suggest that *S. paucimobilis* can produce gellanase enzymes capable of degrading gellan gum.

Growth and exopolysaccharide production in *S. paucimobilis* strains WT, SB10, PA4 and MJ8 were studied in DM medium containing reduced levels of $MgSO_4$.

Less variation was found with $MgSO_4$ limitation than found with nitrogen or glucose limitation.

Cell yields were greatest with *S. paucimobilis* strain MJ8 which was non mucoid.

For strain PA4, EPS yield did not vary greatly with $MgSO_4$ concentration. Yields of 2.03-2.36 g l^{-1} EPS were found even although fifty times more $MgSO_4$ was present at 0.01% concentration than found at the lowest Mg concentration studied.

With the wild type and strain SB10, 2.5-3.6 g EPS l^{-1} was produced with reduced Mg conditions. Maximum EPS yields were produced when *S. paucimobilis* was grown with reduced levels of $MgSO_4$.
Maximum culture viscosities were all greater than 20 mPa s for the mucoid strains of *S. paucimobilis*.
At 0.0005% $MgSO_4$, 3.57 g l^{-1} EPS was produced, yet the culture broth viscosity was only 27.6 mPa s. With culture medium containing 0.01% $MgSO_4$ only 0.75 g l^{-1} EPS was produced, yet the culture broth viscosity was too viscous to measure, after 48 hours growth. It may be that the EPS formed with reduced levels of $MgSO_4$ is of poorer quality e.g. lower molecular weight. Or, the high viscosity readings at 0.01% $MgSO_4$ concentration may be due to gellan aggregation or gelation in the presence of high concentrations of Mg++.
Gellan gum gels, when deacylated, in the presence of Mg++ or Ca++. The hardness of the gel produced, may be changed using different gum concentrations, ion concentration or type of cation (Sanderson & Clark, 1983).
From all of the conditions studied, it was apparent that gellan gum was produced by *S. paucimobilis* in a partly growth linked process. This was found for gellan gum production from *S. paucimobilis* variant E2 studied by Lobas *et al.* (1992). In this case productivity decreased at the end of the growth phase and continued to decrease in the stationary phase.
EPS synthesis was found to be growth associated for *Acetobacter* (Dudman, 1960), *A. vinelandii*, (Deavin *et al.*, 1977), and *P. aeruginosa* (Mian *et al.*, 1978). In contrast, EPS synthesis commenced during late stationary phase, with most EPS being produced after growth had ceased, for another strain of *P. aeruginosa* (Piggott *et al.*, 1982), *E. aerogenes* (Duguid & Wilkinson, 1953) and in *Pseudomonas* sp. 11264 (Williams & Wimpenny, 1977). A mutant of *A. vinelandii* produced alginate throughout both exponential and stationary phases (Horan *et al.*, 1981). It was suggested by Tait *et al.*, (1986) that these differences may be related to the different functions of the polysaccharides.

7.3.6 The effect of nutrient limitation on the chemical composition of EPS produced by *S. paucimobilis*

Samples of EPS were analysed using gas chromatography to determine the neutral sugar composition and any changes in monosaccharide constituents with time and strain of *S. paucimobilis*. In this text, each sample will not be discussed individually, rather general findings from all reduced nutrient conditions will be discussed.

Three findings were thought to be significant:-

① Different amounts of EPS were accounted for following hydrolysis.
② Glc:Rha ratios were often different from expected.
③ Mannose was often detected in EPS analyses.

The amount of EPS accounted for following hydrolysis of a known sample of polysaccharide varied with strain and nutrient limitation. With the control composition of Davis Mingioli medium, 32% of the starting amount of polymer was accounted for following sugar analysis, with *S. paucimobilis* strain PA4. Only about 10% of the polysaccharide samples analysed was accounted for with both *S. paucimobilis* strains PA4 and SB10 with the control DM composition. Following industrial production of gellan gum about 50% of polymer produced was found to be composed of insoluble material which was found to contain protein (Kang & Veeder, 1982). This may explain some of the unidentified material not measured by neutral sugar analyses. Another factor which could be important is the hydrolysis procedure. In theory, optimal hydrolysis conditions should be determined for EPS from each strain, each nutrient condition and different time points. As already mentioned, it is very difficult to hydrolyse complex, acidic polysaccharides completely into their monosaccharide constituents. The data obtained will depend greatly on the hydrolysis conditions used (Furneaux, 1983).

Under nitrogen-limiting conditions the amount of EPS accounted for following hydrolysis was found to decrease with decreasing $(NH_4)_2SO_4$ concentration for all strains. The total sugar content (i.e. % of total EPS analysed) was found to increase with time for all

strains. The amount of EPS identified was greater at the 96 hour time point than at any time point in the earlier part of the time course.

This difference in percentage of polymer identified with time strain and nutrient limitation may be an effect of the extent of acylation of the polysaccharide produced. Tait *et al.* (1986) found that acylation in xanthan gum was low in the exponential phase of growth, maximal in EPS isolated as the growth rate fell and usually lower after this time.

There are several reports in the literature of EPS being produced with different composition with varying growth conditions. This was found for *Pseudomonas atlantica* (Uhlinger & White, 1983) and for *Alcaligenes faecalis* (Hisamatsu *et al.*, 1978). The choice of limiting nutrient was found to affect not only the composition of cellular polymers but also the properties (Evans *et al.*, 1979).

Davidson *et al.* (1977) suggested that the presence of acetate esters would be expected to influence the hydrophilic nature of alginate produced by *A. vinelandii.* If this is also true for gellan gum, this would surely affect the solubility of the polysaccharide and also determine the success of hydrolysis.

The glc:rha ratio for gellan gum should be 1.5:1.0 (O'Neill *et al.*, 1983; Jansson *et al.*, 1983). In this study the glc:rha ratio was closer to 1:1 and often the amount of rhamnose found following hydrolysis was greater than the amount of glucose. This may relate to the poor solubility of the polysaccharide, the extent of acylation or sub-optimal hydrolysis conditions used.

Different ratios of glc:rha with strain, time and growth conditions were apparent. Also, the greatest glc:rha ratio was found for some strains at very early time points, about 4-6 hours.

In many of the gc traces, certain unidentified components were routinely found. These components may have been derived from the EPS sample, so were calculated in the amount of material accounted for. These components were called X_1-X_3. They were not taken into account for mole % calculations as no correction factors or molecular components were known. The main reason these components were highlighted, was that sometimes mannose was detected in the EPS samples. Although the mole % of mannose

reached approximately 11% in some instances, in other cases, the amount of mannose found was less than the unknown components. To put the mannose detected into perspective, it is important that all the components identified, or found, with gc, are shown. The greatest mole % mannose was found with Mg limitation (0.0005% $MgSO_4$) of *S. paucimobilis* strain SB10, where 10.3% of identified sugars was mannose.

It may be that under these conditions *S. paucimobilis* produces 2 polysaccharides or polysaccharide of altered composition. There has been some evidence, during the course of this study, that both of these suggestions could happen.

Lawson & Symes (1977) found that xanthan gum produced from complex nitrogen limited cultures contained two polysaccharides. One polymer had a composition similar to xanthan, whilst the other was composed of glucose and rhamnose. The ratio of the 2 polymers varied with culture conditions.

Evans *et al.* (1979) reported that EPS synthesised by *X. juglandis* in continuous culture (0.03 h^{-1} dilution rate) was composed of longer, unbranched molecules than that formed at higher dilution rate. This may be attributed to an increased proportion of a second EPS synthesised by this strain, containing glc and rha rather than a modified form of xanthan (Tait *et al.*, 1986).

From the results discussed here, it is apparent that the structure of gellan gum does show differences in composition (and probably also physical properties) with strain and nutrient limitation. During this work, no attempt was made to study the acylation of gellan gum in connection with nutrient limitation. As the commercial properties of gellan gum depend so much on its extent of acylation, this would be an interesting continuation of this work. Gellan contains in addition to the tetrasaccharide repeat structure, acetyl groups and L-glycerate. Acetyl may be determined by colorimetric assay (Hestrin, 1949) or HPLC (Cheetham and Punruckvong, 1985). In a study by Kuo *et al.* (1986), L-glycerate was identified by selective solvolysis of the rhamnosyl linkages with anhydrous liquid hydrogen fluoride at -40°C and a combination of nuclear magnetic resonance (NMR) spectroscopy, fast atom bombardment mass spectrometry (fab-ms) and gas liquid chromatography (glc). These procedures would not

be suitable for routine analysis due to the complexity of the techniques and the specialist equipment required. If a simpler method for L-glycerate determination could be found this would make analysis easier.

7.4 The Future

Monteiro *et al.* (1992), suggest that the cloning and identification of genes essential for the synthesis of gellan gum would allow the development of polysaccharides with distinct chemical and physical properties by the manipulation of the biosynthetic pathway. The work of Vanderslice *et al.* (1987) has shown that this is a real possibility and that by genetic engineering a family of polymers may be produced in the same manner as xanthan.

The modification of growth medium allows polymers of varying structure and function to be produced. Modification at the molecular level may be the way forward for future researchers.

CHAPTER 8

REFERENCES

8. REFERENCES

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