Characterization of gellan-like polymers for new biotechnological applications

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“A person who never made a mistake never tried anything new”

Albert Einstein

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Resumo

O estudo dos mecanismos moleculares subjacentes à síntese de exopolissacáridos (EPS) bacterianos têm despertado grande interesse, sendo a bactéria não-patogénica *Sphingomona elodea* ATCC31461 um excelente exemplo. Esta bactéria produz gelano, um EPS tradicionalmente usado nas indústria alimentar e farmacêutica e mais recentemente, também em engenharia de tecidos. O desenvolvimento de suportes para a proliferação de condrócitos, requer que o polissacarídeo se mantenha liquído acima da temperatura corporal, mas que gelifique a 37ºC criando uma estrutura 3D. Isto desencadeou a nossa pesquisa de derivados do gelano que cumpram estas características. Para tal, criaram-se diversas mutações na proteína GelE de *S.elodea* e estudou-se o seu efeito nas propriedades do polímero. As mutações na folha-β existente em C-terminal e em Y198F levaram à obtenção de polímeros com baixas viscosidades, enquanto as mutações N99A e Y209F, conduziram a maiores viscosidades. O gelano nativo e os produzidos na presença das mutações N99A, C75K, V231E, e Y198F na proteina GelE foram purificados com o solvente orgânico clorofórmio. O gelano nativo foi o único que gelificou na presença de Ca^{2+}, mas estes géis revelaram-se instáveis no meio de cultura para condrócitos. Para encontrar novos polímeros bacterianos com potenciais aplicações estudaram-se isolados do género *Massilia*. Estes produziram um polímero extracelular com composição química desconhecida, mas com propriedades reológicas diferentes do gelano. No geral, confirmou-se a possibilidade de obter derivados do gelano através da modificação de proteínas que regulam o comprimento das cadeias de EPS. Dado que estes polímeros apresentam propriedades reológicas distintas, estudos adicionais deverão ser feitos para encontrar novas aplicações.

Palavras-Chave:

Goma de gelano; Gelificação; *Shingomonas elodea*; Autocinase GelE; Propriedades reológicas

Abstract

Studies on the mechanisms underlying the synthesis of bacterial exopolysaccharides (EPS) have raised great interest, being the non-pathogenic bacterium *Sphingomona elodea* ATCC31461 one of the best examples. This bacterium produces gellan gum, traditionally used in food and pharmaceutical industries and more recently also in 3D-scaffold material for tissue engineering. To prepare scaffolds for chondrocyte proliferation, a polysaccharide that remains liquid slightly above body temperature, but jellifies at 37ºC creating a 3D-structure is needed. Our aim was to search for gellan-like polymers fitting these requirements. To achieve that, mutations in the autokinase GelE of *S. elodea* were introduced and their effect on gellan properties determined. Mutations in the C-terminal β-strand and in Y198F led to lower viscosity polymers, while mutations N99A and Y209F led to EPS solution with higher viscosity. Native gellan and gellan-like polymers produced in the presence of GelE mutations N99A, C75K, V231E, and Y198F were further purified with organic solvents, being chloroform the best choice. Native gellan was the only one forming gels in the presence of Ca^{2+}, but these gels were unstable in standard chondrocytes growth medium. In an attempt to find new bacterial polymers with potential applications, soil isolates of the genus *Massilia* were studied. These produced an extracellular polymer of unknown chemical composition, but with very different rheology from gellan. Overall, this study confirms the possibility to obtain gellan-like polymers by modifying proteins involved in EPS chain-length regulation. Since these polymers display different rheological properties, additional characterization should be made to find new potential applications.

Key-words:

Gellan gum; Gelation; *Sphingomonas elodea*; GelE autokinase; Rheological properties

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Abbreviations

α-MEM	Minimum Essential Medium Eagle
BSA	Bovine Serum Albumin
DMEM	Dulbecco´s Modified Eagle Medium
EPS	Exopolysaccharide
Glc	Glucose
GlcA	Glucuronic acid
Glucose-6-P	Glucose-6-Phosphate
HEMA	2-Hydroxyethyl methacrylate)
IN	Inner Membrane
ON	Outer membrane
PEO	Poly (ethylene oxide)
PgmG	Phosphoglucomutase
PHB-free gellan	Polyhydroxybutyrate free gellan
PVA	Poly (vinyl alcohol)
Rha	Rhamnose
UDP-D-Glucose	Uridine diphosphate glucose
UgpG	UDP-glucose pyrophosphorylase
UgdG	UDP-glucose dehydrogenase

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1. Introduction

1.1 - Gellan gum

Bacterial exopolysaccharides (EPS) have diverse biological roles, as virulence factors in plant and animal pathogens, signaling molecules in bacteria–plant interaction and contributing to cell protection from environmental aggression. Some of these EPS are potential or accepted products of Biotechnology (Vartack et al, 1995). Among those is gellan, discovered in 1977 through a scrupulous screening of more than 30000 bacterial isolates in the search for new polysaccharides that could provide high solutions viscosity.

This commercial gelling agent, gellan, is an extracellular EPS produced by the non-pathogenic bacterium *Sphingomonas elodea* ATCC 31461, formerly known as *Pseudomonas elodea* or *Sphingomonas paucimobilis.* It is aerobic, rod-shaped with a single flagellum and forms round mucoid yellow pigmented colonies in a defined medium (Kang and Veeder, 1981). The mentioned heteropolysaccharide has an estimated molecular mass of 500 kDa (Fialho et al, 2008). This straight-chain is composed of a repeat-unit of four monosaccharide molecules, i.e., two D-glucose, one D-glucuronic acid and one L-rhamnose. The native form of gellan also presents two lateral carboxyl groups on its glucose residue immediately after production: one L-glycerate and one of O-acetate (1 mol and 0.5 mol, respectively, per repeat-unit) (Omoto et al, 1999; Mota, 2010) as shown in Figure 1.

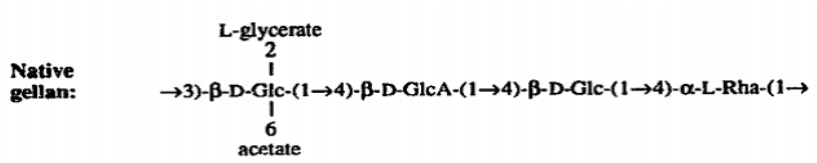

Figure 1 - Chemical structure of the repeat-unit of gellan gum (adapted from Jasson et al 1983).

Figure 2 shows the typical fermentation process for gellan production and its purification at laboratory scale.(Figure 2, A1). The viscosity of the culture medium increases during the exponential and stationary phases and reaches, at the end of the process, a very high value (Figure 2, A2). Gellan production is growth-associated with a maximum production of 12 g/l. This increase of viscosity creates a great problem in terms of separating gellan from cells and before precipitation, it is necessary the dilution of the culture broth with saline solution in order to try reduce the viscosity, followed by centrifugation to separate de cells. The supernatant is precipitated with cold isopropyl alcohol (Figure 2, A3), dried and resuspended in water, followed by dialysis and lyophilization (Figure 2, A4). Finally gellan can be resuspended in water to produce a gel (Figure 2 A5) (Fialho et al, 2008).

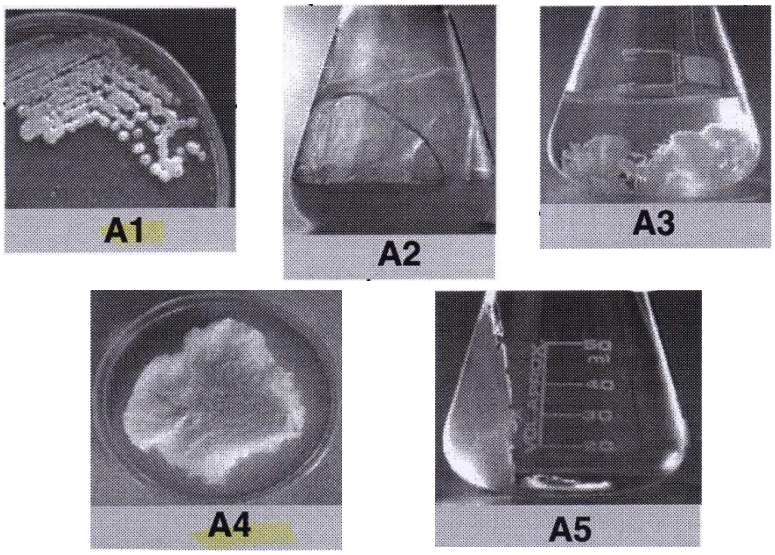

Figure 2 - Typical fermentation process for gellan production and its purification at laboratory scale (adapted from Fialho et al, 2008).

Gellan belong to a family of sugar polymers known by sphingans including welan, rhamsan, gellan, diutan, S-7, S-88, and S-198 (Fialho et al, 2008). The physical properties of sphingans vary considerably, depending on the nature and location of the side chains and in the presence of certain acyl groups. Relatively to the chemical structure of gellan, while acetyl esters are common on EPS, the glyceryl group is an uncommon substituent that has not been reported as a component of other polysaccharides (Fialho et al, 2008). The two acyl substituents control the gel-like properties of this polysaccharide, drastically affecting the rheology of the gels formed with various cations. Chemical deacylation of the native form results in a change from soft (weak), elastic thermoreversible gels to harder (firmer, strong) and more brittle gels (Sá-Correia and Fialho, 2002; Chandrasekran and Radha, 1995).

1.1.1 - Genes and enzymes involved in gellan biosynthesis

Many studies were made to identify and characterized the genes involved in the biosynthesis of gellan. Among these is the work of Harding et al (2004) which isolated a cluster of 18 genes homologous to the genes required for the synthesis of *sphingan* polysaccharide. This cluster of genes encode several proteins that are essential for cellular metabolism and formation of gellan tetrasaccharide unit (Figure 3).

Figure 3 - Physical organization and putative functions of the *gel* cluster of genes involved in gellan biosynthesis in *Sphingomonas elodea* ATCC 31461 (adapted from Moreira et al, 2004).

The gellan biosynthetic pathway is a multi-step process that can be divided into three sequential steps: intracellular synthesis of activated sugar-nucleotide precursors, assembly of the tetrasaccharide repeat-units linked to the inner membrane, and translocation of the repeat-units to the periplasmic space followed by their polymerization and export through the outer membrane (Fialho et al, 2008). The first step starts with the cytosolic formation of activated nucleotide-sugar precursors and three of seven enzymes required for the synthesis of these sugar nucleotides which are phosphoglucomutase (PgmG), UDP-glucose pyrophosphorylase (UgpG) and UDP-glucose dehydrogenase (UgdG), have been biochemically characterized (Fialho et al, 2008).

Figure 4 shows all the chemical reactions required for the synthesis of UDP-D-glucose, dTDP-L-Rhamnose and UDP-D-glucuronic acid. These nucleotide sugar precursors are the donors of monomers for the formation of the tetrasaccharide repeat-unit. While the *rmlABCD* genes involved in dTDP-L-rhamnose are located in the *gel cluster*, genes *pgmG*, *ugpG* and *ugdG* are somewhere in the *S. elodea* genome.

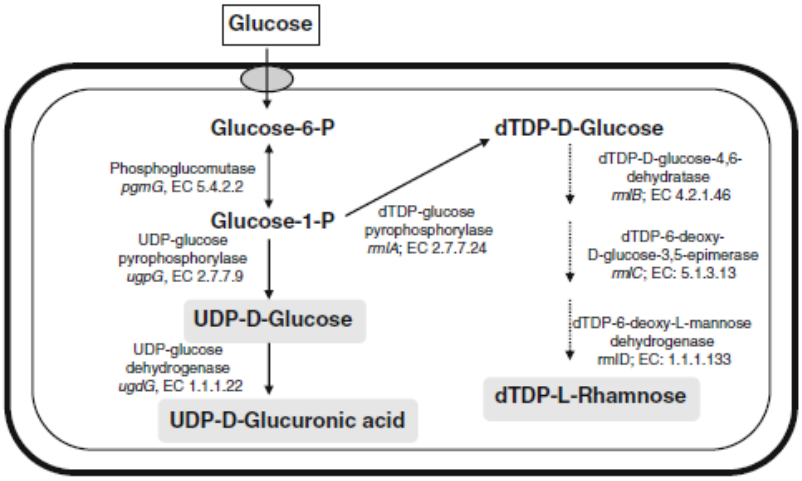

Figure 4 - Proposed pathway leading to the nucleotide-sugar precursors involved in gellan biosynthesis with glucose as the substrate (adapted from Fialho et al 2008, Sá-Correia et al, 2002)

The synthesis of sugar nucleotide precursors is followed by the formation of the repeat-unit by sequential transfer of the sugar donors to an activated lipid carrier by committed glycosyltransferases (Figure 5).

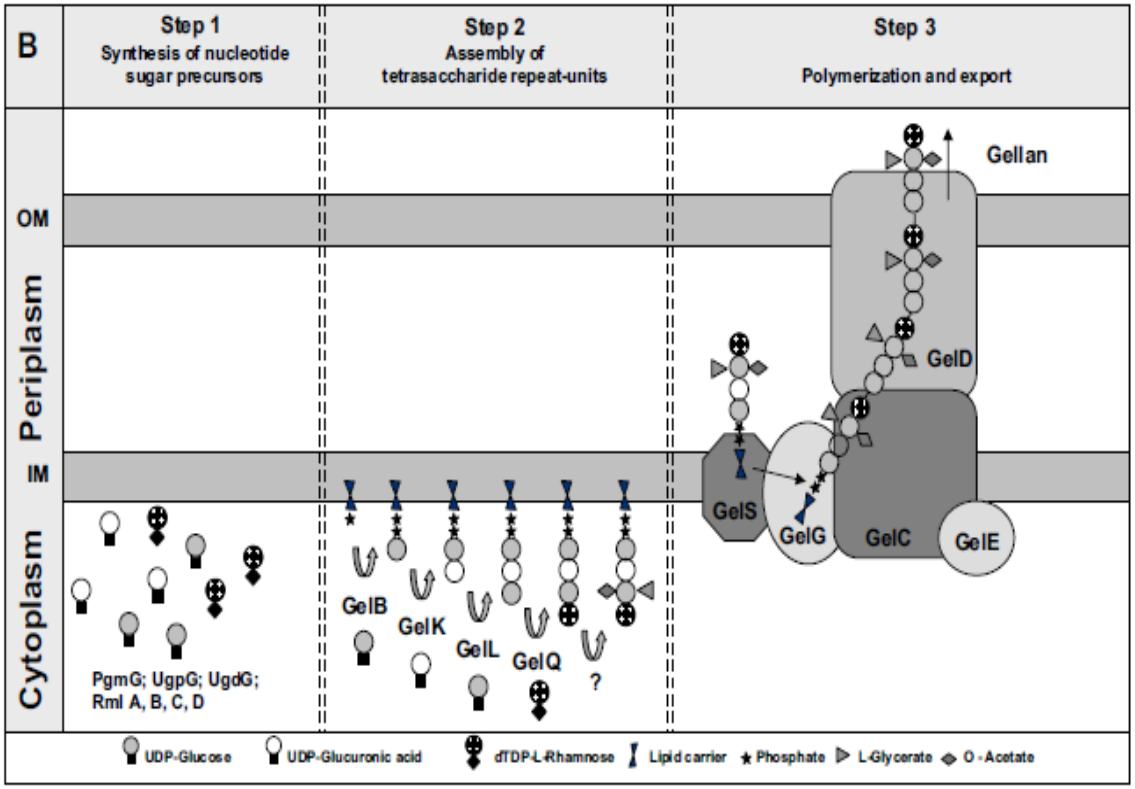

Figure 5 - A model for biosynthesis and assembly of gellan (adapted from Fialho et al, 2008).

The undecaprenyl pyrophosphate-linked tetrasaccharide repeat-unit is assembled at the interface between the cytoplasm and the inner membrane by sequential activity of GelB, GelK, GelL, and GelQ glycosyltransferases. These newly synthesized undecaprenyl pyrophosphate linked repeat-units are then translocated across the membrane in a process requiring GelS, and provide the substrate for GelG polymerization and also require GelC/GelE proteins, possibly by forming oligomers that connect polymerization to export and regulate polysaccharide chain length. Gellan chains are then exported by GelD, which may act as a channel (reviewed in Fialho et al, 2008).

Genes *gelC* and *gelE* encode two polypeptides homologous to the activator domain and to the kinase domain, respectively, of bacterial tyrosine kinase proteins (Moreira et al, 2004). As opposed to what would be expected, the pair GelC/GelE exhibit a genetic organization similar to Gram-positive bacteria, being composed of two independent polypeptides instead of a single polypeptide of gram-negative bacteria. Moreira et al (2004) studied further the characteristics of these two important genes, namely in gellan biosynthesis. These authors showed that the deletion of *gelC* or *gelE* genes from *S. elodea* resulted in a non-mucoid phenotype and the total absence of gellan from the culture supernatant. The absence of gellan biosynthesis in the *gelE* deletion mutant also suggests an important role for this protein, probably regulate *gelC* activity. In fact, GelE has ATP-binding activity, but the introduction of several point mutations in the Walker A or Walker B ATP-binding motifs did not prevent wild-type levels of gellan biosynthesis. Despite the several attempts, it was not possible to assess a tyrosine autokinase activity to GelE (Moreira et al, 2004). However, the tyrosine residue at position 198 was shown to be essential for the synthesis of high-molecular weight gellan as compared to the wild-type. Both the amount and the viscosity of the EPS produced were much lower. Nevertheless, the authors were not been able to clarify if the tyrosine residue at position 198 of GelE is important for structure/stability *in vivo* or to phosphorylation. It was also found, at computational level, that GelE structure has an amphipathic helix at the C-terminal region, which may indicate that is involved in association with the plasma membrane and thus possibly interacts with GelC (Moreira et al, 2004).

1.1.2 - Environmental and genetic engineering of gellan production

Although the production yields, composition, structure and properties of the gellan produced by *S. elodea* ATCC 31461 are genetically determined, it is possible to influence these factors by modifying culture conditions such as temperature (Martins and Sá-Correia, 1994), oxygen transfer and growth medium composition, in particular the carbon and the nitrogen source (Fialho and Martins, 1999). Gellan gum biosynthesis is temperature dependent, with a maximal production yield at 20 – 25°C, which is well below the optimal range for growth (30–35°C) and for maximal activities of gellan enzymes in the producing cells (30–35°C). In addition, the biopolymer synthesized at 20°C gives rise to solutions with maximal viscosity. Despite the uncertainties characteristics of biopolymers synthesized at various temperatures, it was proposed that a more rapid turnover of the lipid carrier, at temperatures causing higher specific growth rates, may lead to an earlier release of a polymer with a shorter chain length (Martins and Sá-Correia, 1994).

Comparison of gellan biosynthesis by *S. elodea* ATCC 31461 in a synthetic medium containing glucose or lactose (5–30 g/l) and in diluted sweet cheese whey (Fialho and Martins, 1999) indicates that alteration of the growth medium can markedly affect the polysaccharide yield, acyl substitution level, polymer rheological properties and susceptibility to degradation. In addition, it was shown that a number of complex organic and inorganic nitrogen sources support gellan production by *S. elodea* ATCC 31461, increasing production yield when a complex nitrogen source is present instead of ammonium sulfate (West and Strohfus, 1998). The role of fermentor hydrodynamics on gellan fermentation kinetics and the rheological properties of the EPS synthesized were also studied, increasing gellan production when oxygen transfer capacity is improved (Kang and Veeder, 1981).

1.1.3 - Physicochemical properties of gellan

Gelation of gellan solutions occurs abruptly upon heating and cooling of gellan gum solutions in the presence of cations. Such sol-gel transitions are considered as phase transition. The gelation of gellan gum is a function of polymer concentration, temperature, and presence of monovalent and divalent cations in solution (Yuguchi et al, 1993). At low temperature gellan forms an ordered helix of double strands, while at high temperature a single-stranded polysaccharide occurs, which significantly reduces the viscosity of the solution. The transition temperature is approximately 35°C, but can range from 30–50°C. Below transition temperature, a stiff structure is obtained (setting point), and results in gel formation (Bajaj et al, 2007). The gelation mechanism involves the formation of double helical junction zones followed by aggregation of the double helical segments to form a three-dimensional network by compilation with cations and hydrogen bonding with water (Moris et al, 2012). Addition of monovalent or divalent cations during cooling markedly increases the number of salt bridges at junction zone, thereby improving the gelling potential of gellan gum (Bajaj et al, 2007) as showed in Figure 6.

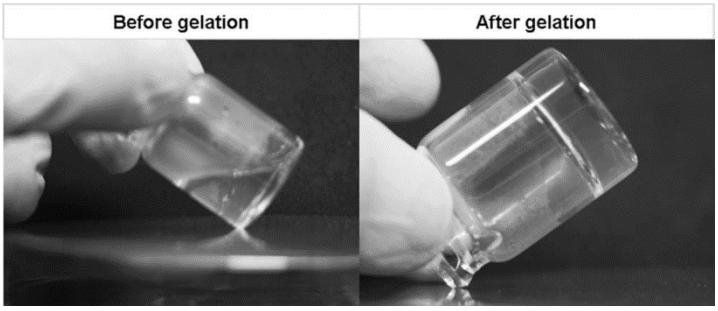

Figure 6 - Sol-gel transition occurring in a gellan gum solution containing $CaCl_2$ (adapted from Oliveira et al (a), 2010).

Acetyl content, type and concentration of ions, pH and presence of hydrophilic ingredients affect the gel strength. The study developed by Mao et al, (2000) proved that the most important factor that affect the gel strength is the acetyl content. Gellan gum with different acetyl content gives gels with different properties. Native gellan gum provides soft, elastic, thermoreversible gels, and is very weak because of bulky acetyl and glyceryl groups that prevent close association between gellan polymers packing of the cross-linked double helix. Deacetylated gellan gum forms firm, brittle and thermoreversible gel because of the absence of acetyl and glyceryl groups (Bajaj et al 2007, Mao et al, 2000). The different concentration of ions in addition to having an impact on gel strength, also has in brittleness. Gellan does not form gel in deionized water, but the addition of salts of calcium, potassium, sodium, and magnesium causes an increase in these two properties. Divalent cations such as calcium and magnesium are more effective in achieving this, even in gellan gels of very low concentration a high strength (Huang et al, 2004). Change in pH does not alter the setting point of the gel, but affects melting temperature in some cases. Gels prepared with very low levels of monovalent ions melt at around 70°C at neutral pH, but at pH=3.5 the melting temperature is slightly increased (Sanderson et al, 1984).

Addition of hydrophilic ingredients like sucrose tends to decrease the ion concentration required for optimal gellan gel strength. Tang et al, (2002) proved that gelling temperatures of gellan solutions generally increase with the addition of sucrose, whereas addition of fructose had no effect. Incorporation of fructose and sucrose markedly increased the gel clarity. Effect of sucrose on gel strength was dependent on cation concentration. At low cation concentrations, sucrose strengthened the gels; but at high cation concentrations, sucrose weakened them (Tang et al, 2002).

Gellan gum is stable at higher temperatures and maintains its strength at 90°C. The melting temperature can be below or above 100°C, depending on the conditions of gel formation. The most important factor responsible for the flexibility of the melting point is concentration of cations in the gels, because monovalent and divalent cations markedly increase the number of junction zones in gels and make them more resistant to temperature. Modification of the melting point can successfully replace other conventional thickeners/stabilizers, while used in much lower concentration (Bajaj et al, 2007; Giavasis et al, 2000).

Several studies were developed to find out what happened with textural properties of gellan gum when mixed with other food hydrocolloids. The solutions with sodium alginate dissolved in calcium chloride show a sharp increase in rigidity on cooling, and convert to permanent gels on storage at low temperature, but did not significantly change their elasticity, indicating that the gellan acts as strong 'filler' in an alginate matrix (Papageorgiou et al, 1994). Hardness, brittleness, cohesiveness and springiness were measured on mix gellan-gelatin gels and ion calcium concentration, in tests developed by Lau et al, 2000. The results suggested that there was a weak positive interaction between gellan and gelatin when no calcium was added. At higher concentrations, gellan formed a continuous network and gelatin the discontinuous phase. Hardness was dependent on the concentration of gellan gum in the mixture, whereas brittleness, springiness and cohesiveness were very sensitive to low levels of calcium but less sensitive to higher calcium, concentrations and gellan/gelatin ratio (Bajaj et al, 2007; Lau et al, 2000). Studies were also made in gellan-carrageenan and gellan-xanthan mixtures with aim to determine the contribution of both polysaccharides to the viscoelastic behavior of this mix. It was observed that strength of gellan alone was the highest, and gel strength of the two-component gels decreased as the proportion of gellan was reduced. Mixed gels having a gellan concentration equal to or lower than 50 % mass of the total concentration were less stiff and brittle, hence were more elastic (Rodríguez-Hernández and Tecante, 1999). The textural properties of gellan gum in the presence of chelatants was studied too. The effect of different concentrations of sodium citrate, sodium metaphosphate and EDTA on gellan gel setting temperature and rheological properties was performed by Camelin et al (1993). Results showed that at lower concentrations temperature setting decreased progressively for all chelatants. This effect was accompanied by a significant decrease of gel strength, and might be attributed to the binding of divalent cations required for chain association during gelation by chelatants.

1.2 - Gellan gum applications

Nowadays due to its rheological properties, gellan has been used in several application. US and EU approved this hydrogel as a gelling, stabilizing and suspending agent, either alone or in combination with other hydrocolloids in food and personal care applications (lotions, creams and toothpastes) (Fialho et al, 2008; Sá-Correia et al, 2002). It has also been employed as a gelling agent in plant biotechnology and in bacterial culture media in place of agar (Mota, 2010; Sutherland, 2002). Compared with other polysaccharides, gellan has many advantages such as an excellent thermal and acid stability, adjustable gel elasticity and rigidity, high transparency and good flavor release (Fialho et al, 2008).

Gellan is commercially available in three forms according on acyl content. Without this group, Gelrite®, with low quantity, Kelcogel® F and with high values Kelcogel® LT100 (Fialho et al, 2008). Gelrite® is used as a substitute of agar for the culture of thermophilic bacterial species an in plant tissue culture media (Fialho et al, 2008; Lin and Casida, 1984). This hydrogel also has potential in environmental applications such as in the biodegradation of gasoline and for transportation of gel-encapsulated bacteria for bioaugmentation of contaminated aquifers (Moslemy et al, 2003, 2004). Kelcogels are food-grade gellans used as gelling agents in foods and personal care applications such as those mentioned above, being mainly used as a stabilizer and suspending agent in a wide variety of applications in the food industry (Fialho et al, 2008, Sutherlan, 2001). Icings and glaze sauces, microwavable foods, dessert gels, and puddings are food products that incorporate gellan.

More recently it was also shown that gellan can be used in ocular, nasal, gastric and drug delivery applications directly related with the biomedical field including its use as a pharmaceutical excipient (Fialho et al, 2008; Rozier and Mazuel, 1989; Deasy and Quigley, 1991). Hydrocolloid beads based on gellan have been shown to be useful for slow drug release (Gal and Nussinovitch, 2007). Li et al, (2001) showed that gellan might be an ideal candidate in the development of protein delivery systems, when a gellan film for insulin delivery in diabetic was implanted.

Several gellan patents covering a wide range of applications are filed and published every year. Table 1 summarized some of the gellan applications.

Table 1 - Select list of worldwide-issued and published patents covering the use of gellan gum categorized into applications field (adapted from Fialho et al, 2008).

Application	Patent Title	Country/ Patent nº
Food  50/117	Gellan gum beverage and process for making a gelled beverage	US5,597,604
	Gelatin-free gummy confection using gellan gum and carrageenan	US6,586,032
	Calcium stable high acyl gellan gum for enhanced colloidal stability in beverages	CN101001538
	Food containing native gellan gum	JP2005253473
Medical/ Pharmaceutical  22/117	Gellan gum tablet film coating	US2004033261
	Gellan gum based oral controlled release dosage forms-a novel platform technology for gastric retention	US2006177497
	Liquid aqueous ophthalmic composition containing gellan gum	NZ217662
	Spray able wound care compositions comprising gellan gum	NZ523126
	Controlled release compositions comprising gellan gum gels	WO9922768
Production process  18/117	PHB-free gellan gum broth	US5300429
	Mutant strain of *Sphingomonas elodea* which produces non-acetylated gellan gum	US2003100078
	Genetically purified gellan gum	US2006003051
	Modified gellan gum and its production	JP11341955
Personal care  14/117	Cosmetic composition comprising gellan gum and carrageenan	GB2384705
	Oil-in-water emulsion comprising gellan and a particular surfactant and uses	WO0078442
	Composition of toothpaste having improved physical properties and stability, comprising gellan gum	KR20050023598
	Gellan seamless breakable capsule and process for manufacturing thereof	WO2006136198
Other Applications [a]  13/117	Process using gellan as a filtrate reducer for water-based drilling fluid	US5744428
	Paper coating composition comprising gellan gum/starch blend	US6290814
	Purification and use of gellan in electrophoresis gels	US2004168920
	Media and methods for promoting maturation of conifer somatic embryos	US20050003415

2 Use of hydrogels in tissue engineering

Since the pioneering work of Wichterle and Lim, (1960) on cross-linked HEMA (poly (2-hydroxyethyl methacrylate)) hydrogels, and because of their hydrophilic character and potential to be biocompatible, hydrogels have been of great interest to biomaterial scientists for many years. In the same decade it was demonstrated the successful application of calcium alginate microcapsules for cell encapsulation (Lin and Sum, 1980) and was tested the incorporation of natural polymers such as collagen and shark cartilage into hydrogels for use as artificial burn dressings (Yannas et al, 1989). Nowadays, hydrogels based on natural and synthetic polymers have a special interest for cell encapsulation, drug delivery, serve as adhesives or barriers between tissue and material surfaces, and tissue engineering as matrix for repairing and for regenerating a wide variety of tissue and organs due unique biocompatibility, flexible methods of synthesis, range of constituents, and desirable physical characteristics (reviewed in Hoffman, 2012). Every year millions of patients suffer the loss or failure of an organ or tissue as a result of accidents or diseases. Tissue or organ transplantation is a generally accepted therapy to treat these patients. However, this approach is extremely limited by a donor shortage. So the various developments made in the biomedical field are an exciting and revolutionary strategy to treat patients who need new organs or tissues. The main steps of this strategy are shown in Figure 7.

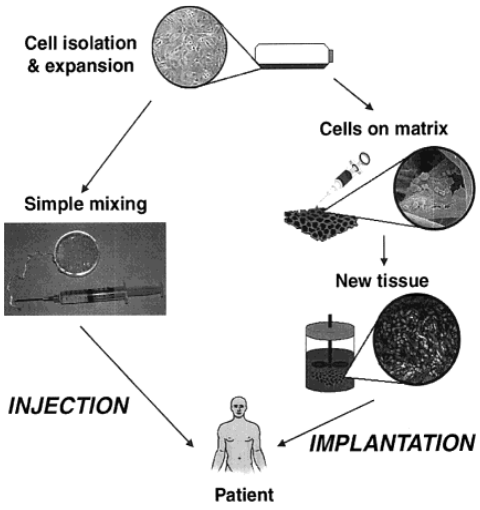

Figure 7 - Schematic illustration of typical tissue engineering approaches (adapted from Hoffman, 2012).

In this strategy, tissue-specific cells are isolated from a small tissue biopsy from the patient and harvested in vitro. The cells are subsequently incorporated into 3D polymer scaffolds that act as analogues to the natural extracellular matrices found in tissues. These scaffolds deliver the cells to the desired site in the patient body, provide a space for new tissue formation, and potentially control the structure and function of the engineered tissue (Lee and Mooney, 2001). Nowadays a variety of tissues are being engineered using this approach including fabricated artery, bladder, skin, cartilage, bone, ligament, and tendon.

The critical element of tissue engineering approach is the polymer scaffold. This polymer potentially mimics many roles of extracellular matrixes found in tissues like bringing cells together and control the tissue structure, regulate the function of the cells and allow the diffusion of nutrients, metabolites and growth factors and must be taken in account the biocompatibility of this material. This has to have the ability to exist within the body without damaging adjacent cells or lead to significant scarring or otherwise elicit a response that detracts from its desired functions. This may be especially problematic as the inflammatory response to a hydrogel can affect the immune response toward the transplanted cells and vice-versa. However, the use of these types of polymer scaffolds requires the surgeon to make incisions sufficiently large to enable placement of the polymer/cell constructions. An alternative approach to cell delivery for tissue engineering is the use of hydrogels that can be injected into the body. This approach enables the clinician to transplant the cell and polymer combination in a minimally invasive manner (Lee and Mooney, 2001).

Various types of polymers have been studied and utilized in tissue engineering field. Hydrogels are hydrophilic polymer networks which may absorb from 10-20% up to thousands of times their dry weight in water. This polymers may be chemically stable or they may degrade and eventually disintegrate and dissolve (reviewed Hoffman, 2012). Biological hydrogels have been formed from agarose, alginate, chitosan, hyaluronan, fibrin, and collagen, and many others. The most used in tissue engineering approach are the hydrogels from natural polymers. However, limitations of gels from natural polymers have motivated approaches to modify these polymers as well as to use various synthetic polymers. A wide range of synthetic polymers may potentially have suitable chemical and physical properties for these applications. Beside the biocompatibility, the controlled degradation of hydrogels is also critical in tissue engineering, whether the gels are originated from natural resources or are synthetically created. Degradation of hydrogels can be due to hydrolysis, the action of enzymes, and/or dissolution. The interactions of cells with hydrogels significantly affects their adhesion as well as migration and differentiation and this issue is also important take into account. The adhesion may be cell-type specific and is dependent on the interaction of specific cell receptors with ligands that are a component or adsorbed onto the materials. Inappropriate interactions could cause undesirable tissue formation (Lee and Mooney, 2001).

Table 2 – Examples of natural and synthetic hydrogels used in tissue engineering

Hydrogels from Natural Polymers	Hydrogels from Synthetic Polymers
Collagen and Gelatin	Poly(acrylic acid) and Its Derivatives
Hyaluronate	Poly(ethylene oxide) and Its Copolymers
Fibrin	Poly(vinyl alcohol)
Alginate	Polyphosphazene
Agarose	Polypeptides
Chitosan	

Table 2 shows the polymers used in tissue engineering in nowadays. All of this network structures have specific properties in common that are essential for their use in recent applications. The physical structure is characterized by junctions or tie points, which may be formed from strong chemical linkages (such as covalent and ionic bonds), permanent or temporary physical entanglements, micro-crystallite formation, and weak interactions (such as hydrogen bonds) (Slaughter, 2009). In terms of ionic charge, hydrogels can be neutral, cationic, anionic, or ampholytic as determined by pendant groups incorporated into the gel backbone. With regard of rubber elasticity, hydrogels under mechanical stress can exhibit a range of responses from rapid, elastic recovery following an applied stress or strain to a time-dependent recovery approaching viscous behavior. Effective solute transport is one of the most critical design parameters for these hydrogels. Mass transport parameters determine how nutrients, gasses, waste products, and bioactive agents, such as growth factors that stimulate natural tissue growth are exchanged within scaffolds or are delivered by the gel. Diffusion alone is regarded as the driving transport phenomenon.

Collagen is the most widely used tissue-derived natural polymer, and it is a main component of extracellular matrices of mammalian tissues including skin, bone, cartilage, tendon, and ligament. Physically formed collagen gels are thermally reversible and offer a limited range of mechanical properties. However, the weakness of the gels has been a problem, and a number of chemical modification methods have been investigated in order to improve the mechanical properties of gelatin gels (Slaughter, 2009).

Hyaluronate is one of the glycosaminoglycan components in natural extracellular matrices and plays a significant role in wound healing. This hydrogel has shown excellent potential for tissue engineering applications such as artificial skin, facial intradermal implants, wound healing and soft tissue augmentation. Hyaluronate requires thorough purification to remove impurities and endotoxins that may potentially transmit disease or act as an adjuvant in eliciting an immune response which is a disadvantage of this polymer. In addition, hyaluronate gels typically possess low mechanical properties that limited the applications of this hydrogel (Slaughter, 2009).

Fibrin has been used as a sealant and an adhesive in surgery as it plays an important role in natural wound healing. Fibrin gels can be produced from the patient's own blood and can be used as an autologous scaffold for tissue engineering. No toxic degradation or inflammatory reactions are expected from this natural component of the body. This gels might promote cell migration, proliferation, and matrix synthesis through the incorporation of platelet-derived growth factors. This polymer has been utilized to engineer tissues with skeletal muscle cells, smooth muscle cells and chondrocytes. However, fibrin gels are limited in mechanical properties which limits it is application (Slaughter, 2009).

Alginate is a well-known biomaterial obtained from brown algae and is widely used for drug delivery and in tissue engineering due to its biocompatibility, low toxicity, relatively low cost, and simple gelation with divalent cations such as Ca^{2+}, Mg^{2+}, Ba^{2+}, and Sr^{2+}. Alginate has found uses to date as an injectable cell delivery vehicle as well as wound dressing, dental impression, and immobilization matrix. Alginate gel beads have also been prepared and used for transplantation of chondrocytes, hepatocytes, and islets of Langerhans to treat diabetes. Despite its advantageous features, alginate itself may not be an ideal material because it degrades via a process involving loss of divalent ions into the surrounding medium, and subsequent dissolution. Another potential limitation in using alginate gels in tissue engineering is the lack of cellular interaction. This polymer cannot interact with mammalian cells due to hydrophilic character, so modified alginate polymers with specific binding were made. These modified alginate gels have been demonstrated to provide for the adhesion, proliferation, and expression of differentiated phenotype of skeletal muscle cells (Slaughter, 2009).

Agarose is another type of marine algal polysaccharide, but unlike alginate it forms thermally reversible gels. The physical structure of the gels can be mainly controlled by using a range of agarose concentrations, which results in various pore sizes. The large pores and low mechanical stiffness of the gels at low concentrations of agarose may enable the migration and proliferation of cells (Slaughter 2009).

Chitosan, due to its biocompatibility, low toxicity, structural similarity to natural glycosaminoglycans, and degradation by enzymes such as chitosanase and lysozyme appear in various tissue engineering applications. However, chitosan is easily soluble in the presence of acid, and generally insoluble in neutral conditions as well as in most organic solvents due to the existence of amino groups and the high crystallinity what compromised it is use in this applications of this field (Slaughter, 2009).

As regards synthetic polymer hydrogels one of the most studied is the hydrolytically stable cross-linked HEMA. The permeability and hydrophilicity of these gels are dependent on the crosslinking agents. Poly(HEMA) has been used for ophthalmic uses including contact lens, as well as in many drug delivery applications. These polymers are also being investigated as an injectable delivery vehicle for cartilage and pancreas engineering. This hydrogel has non-degradable cross-links which is a limitation (Slaughter, 2009).

Poly (ethylene oxide) (PEO) has been used for several medical applications due to its biocompatibility and low toxicity. These gels may be useful in tissue engineering as they can be easily formulated with protein drugs or cells at room temperature or lower, and subsequently delivered to the desired site in a minimally invasive manner (Slaughter, 2009).

Poly (vinyl alcohol) (PVA) is a hydrophobic and soluble hydrogel. In most of physiological situations PVA is not degradable and that is a limitation. PVA hydrogels have been utilized in tissue engineering for regeneration of artificial articular cartilage, hybrid-type artificial pancreas and bone-like apatite formation (Slaughter, 2009).

2.1 - Gellan gum in tissue engineering

Smith et al, (2007) reported the first evaluation of gellan gum as a material for tissue engineering applications. Using 1% gellan gum solution cross-linked with Minimum Essential Medium Eagle (α-MEM) it was also shown the self-supporting nature of such constructs and that these were sufficiently robust to permit handing with forceps (Mota, 2010). They also demonstrated cell viability with in these constructs and the rheological potential of gellan in tissue engineering. The variations on acyl form on the structure of this polymer lead directly with a variation of rheological properties that makes possible the use of gellan gum both as an injectable material and indirectly by culturing the cells *in vitro* and then implanting them. Another characteristic of gellan gum comes from the optical clarity of gel formed gels, which allows an in depth visualization of the encapsulated cells using conventional microscopy (Mota, 2010).

Figure 8 - Photograph shows the self-supporting nature of gellan gum cylinders (adapted from Smith et al, 2007).

Oliveira et al, (2010a) showed the versatility of gellan gum by processing it into different structures through temperature and pH dependence reactions (Figure 9). These researchers introduced gellan gum as a new biomaterial in cartilage tissue engineering and showed that gellan gum discs possess suitable material properties to be used in this field. This material has similar mechanical properties to the other hydrogel used in identical cartilage regenerative approaches. Gellan has an elastic nature, some damping capability and also presents the advantage of gelling at physiological conditions and being able to efficiently encapsulate human nasal chondrocytes with a homogeneous distribution. These hydrogels are noncytotoxic, maintain cell viability and allow them to encompass active cell division (Oliveira et al, 2010a).

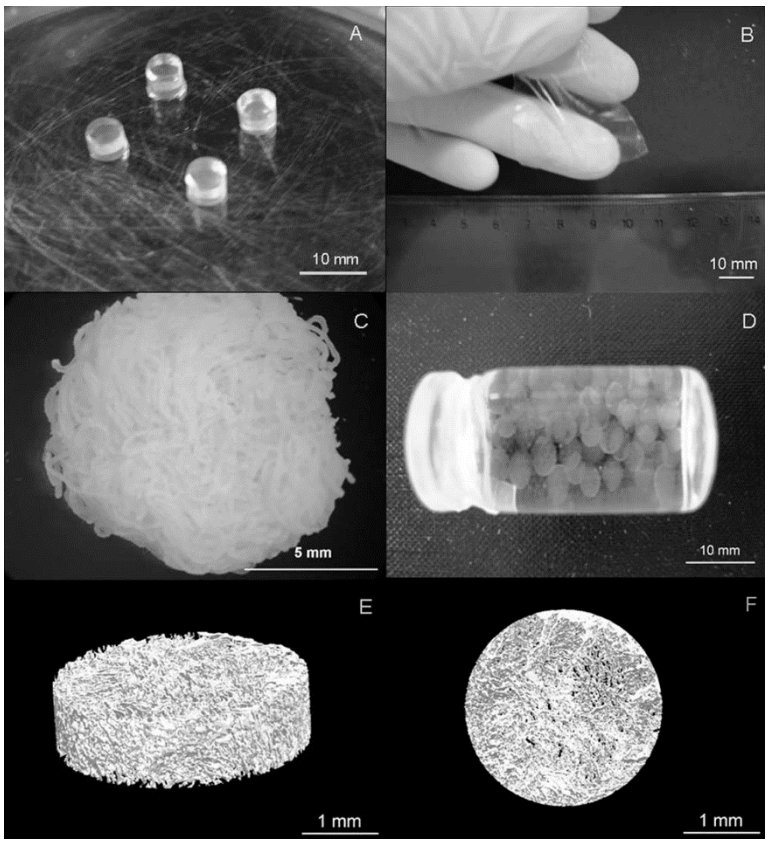

Figure 9 - The versatility of Gellan gum structures that can be formed using simple polymer processing technologies: (A) discs; (B) membranes; (C) fibers; (D) particles; (E) and (F) 3D lyophilized scaffolds (adapted from Oliveira et al, 2010a).

The injectable approach using gellan gum is a challenging one since its gelation temperature is too high. Normally the gelation temperature of commercially available and unmodified gellan is over 42ºC and must be decreased in order to suspend the mammalian cells at physiological temperature (~37.5ºC). Optimization of gelling point was performed by Gong et al, (2009) by adjusting the molecular weight of gellan gum. They assume that a molecular weight decrease would make assemble and aggregation of gellan molecular chains more difficult, lowering the gelation temperature. This way, they demonstrated a increasedr performance of these modified gellan gels in long-term *in vitro* in comparison with agarose (Oliveira et al, 2010).

In another work, Oliveira et al (2009b) continued their investigation on the performance of gellan gum hydrogels combined with human articular chondrocytes *in vivo*, by subcutaneously implanting them in nude mice. The results obtained were promising because revealed normal cell proliferation, as well as a decrease of collagen type I that is linked to the differentiation process that occurs in 2D culture and in collagen type X that is frequently expressed by hypertrophic chondrocytes that are associated with matrix mineralization. An increase on both collagen type II and aggrecans that are important components of the articular cartilage of extracellular membrane were also observed. They also proved that the integration of the gels within the host tissue does not trigger persistent inflammatory response (Oliveira et al, 2009b).

Again, Oliveira et al (b), (2010) tested gellan gum hydrogels coupled with autologous adipose stem cells to regenerate rabbit full-thickness articular cartilage defects, based on previous studies performed by Fan et al, (2010). These authors did pioneering studies of coupling gellan gum hydrogels with other types of cell sources, for example mesenchymal stem cells. Both of these studies suggest that gellan gum constructs combined with different types of cells have the potential to be applied in the treatment of articular cartilage defects.

3 Aims of this study

In the last years many studies have been developed to better understand the molecular mechanisms and regulatory processes underlying the synthesis of bacterial exopolysaccharides (EPS) as well as their potential applications. In the search solutions for biotechnological applications, gellan gum produced by *Sphingomonas elodea* ATCC 31461 emerges as a new promising extracellular polysaccharide due to its importance as commercial gelling agent. It is used in food and pharmaceutical industries and more recently in others fields, like biomedicine applications. The rheological proprieties of this hydrogel showed that it can be used in several applications, such as construction of scaffolds in tissue engineering due to its ability as a cellular support or its multi-functional capacity like synergy with other polysaccharides, which allows the production of wide range of textures.

So the principal aim of this work was to test mutant polymers derived from gellan and verify if their properties could improve those of the gellan gum and consequently explore their ability to work as scaffold, drug delivery or encapsulating gel. To do that, the several gellan-like polymers were produced, quantified and viscosity measurement of a polymer solution was determined. For the selected ones, purification protocols were established and several tests were performed to assess gelation properties.

During this work it was also tested the ability of strains from the *Massilia* genus and their characteristics. The unknown polymer obtained from this strain was subjected to various tests, namely rheological tests with aim of understand in order to know if it could be used in biotechnological applications.

4 Materials and Methods

4.1 - Strains and plasmids, media and culture conditions

Strains used in this study were the native strain *S. elodea* ATCC 31461 that is the major producer of gellan; *S. elodea ⊿gelE* mutant strain (SpLM21-4) with the recombinant plasmid containing the *gelE* gene (pHA010-3) and other mutant plasmids with Y189F, D179N, V231E, C75K, G74A, Y121F, Y235F, Y209F, YF1F4, C75A, pBBR1MCS, pLM51-1, YF1F3F4, V227D, I228D, N99A, D79A, L232E. Strains form the genus *Massilia* were designated RAG-1 and RAG-2.

S. elodea strains were grown in S medium (Moreira et al, 2004) supplemented with the appropriated antibiotic. Strains RAG-1 and RAG-2 were grown in Lennox broth (LB) with or without of 5 g/l glucose (LBG). All strains were incubated at 30ºC with 250 rpm if in liquid culture.

4.2 - Preparation of competent cells and electrotransformation

S. elodea ⊿gelE mutant was inoculated into S medium and grown overnight at 30ºC with stirring of 250 rpm. The pre-inoculum was added to 150 ml of S medium and grown until O.D. 600 nm of 1.2 was reached. After that, cultures were cooled on ice during 15 minutes and centrifuged during 15 minutes at 8000 rmp at 4°C. Supernatant was discarded and cell pellet was resuspend in 200 ml of deionized, cold and sterile H_2O. This process was repeat four times, followed by different glycerol 10% volumes, 40 ml, 8 ml and 4 ml, respectively. The final cellular suspension was aliquoted in 100 µl fractions and kept at -80°C. Prior to electrotransformation, plasmids were extracted using the *ZR Plasmid MiniPrep Kit* according to manufacture's instructions. After that, cells and plasmid DNA were mixed and an electrical shock was applied. The cells were incubated with 1 ml of LB medium during 3 hours at 30°C with stirring and plated on to solid S medium with chloramphenicol. Plates were then incubated during 4 days at 30°C.

4.3 - Gellan production and purification

The strains under study were inoculated in 250 ml of S medium and grown at 30 °C for 48 hours. After that time, the culture was diluted with NaCl to facilitate cell removal by centrifugation at 8000 rpm during 15 minutes at room temperature. The supernatant was precipitated with cold ethanol 96%, followed by successive washes with ethanol to remove cell debris. The polysaccharide was dried overnight and then weighted.

To further purify the polysaccharide it was necessary to test other approaches, such as treat the samples with chloroform, phenol and both. For that, 1g/l solutions of the different polymers were prepared in water. Then, to each 5 ml of sample were added 1.5 ml of ammonium acetate. Then, 5 ml of chloroform (or phenol) were added and the mixture centrifuged for 5 minutes at 13000 rpm. The aqueous phase containing the polysaccharide was recovered and stored at 4ºC.

4.4 - Viscosity measurements

The viscosity of each solution was determined at 30 °C using the viscometer Brookfield model DV-II with the spindle n. 40. Results are the means of at least three independent viscosity determinations.

4.5 - Methods for protein quantification

The quantification of proteins was determined by two different approaches: the method Bradford and the method of Lowry.

Lowry method: Preparation of diluted albumin (BSA) standards were performed as indicated in Table 3.

Table 3 - Preparation of diluted albumin (BSA) standards.

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	250 µL	750 µL of Stock	1500 µg/mL
B	625 µL	625 µL of Stock	1000 µg/mL
C	310 µL	310 µL of vial A dilution	750 µg/mL
D	625 µL	625 µL of vial B dilution	500 µg/mL
E	625 µL	625 µL of vial D dilution	250 µg/mL
F	625 µL	625 µL of vial E dilution	125 µg/mL
G	800 µL	200 µL of vial F dilution	25 µg/mL
H	800 µL	200 µL of vial G dilution	5 µg/mL
I	800 µL	200 µL of vial H dilution	1 µg/mL
J	1000 µL	0	0 µg/mL

40 µL of each standard on unknown sample was pipetted into microplate well and added 200 µL of Modified Lowry Reagent and mixed for 30 seconds. Microplate was then incubated at room temperature for 10 minutes, followed by the addition 20 µL of 1X Folin-Ciocalteu Reagent to each well. The microplate with the samples was agitated for 30 seconds and incubated at room temperature for 30 minutes. After that period, the absorbance at 750 nm was measured.

Bradford Method: Preparation of reagent Coomassie: It was added to 100 mg of Coomassie Brilliant Blue G-50 (Sigma), 50 ml of 95% ethanol,100 ml of phosphoric acid 85% (w/v) and distilled water to a final volume of 1L. The reactant was filtered before being stored in a dark bottle at 4ºC. In a test tube was mixed 2.5 ml of reagent and 50 µl of extract to be analyzed and stirred vigorously. Absorbance at 595 nm was read between 5-15 minutes after shaking the sample. To plot the calibration curve (Figure 10) aqueous solutions of BSA (Bovine Serum Albumin) were used.

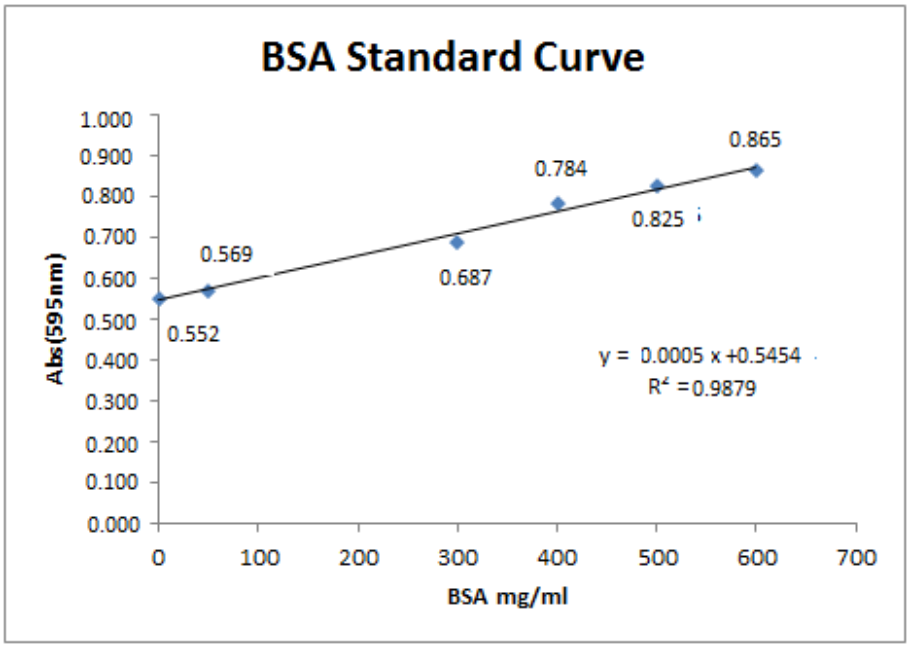

Figure 10 - Graphical representation of the calibration curve used for the determination of protein concentration by the Bradford method.

4.6 - Gelation of modified gellan gum samples

The selected samples of gellan-like polymers were mixed with sterile distillated water under constant stirring at room temperature. The solution was progressively heated at 90ºC until homogeneous dispersion and maintained at that temperature for 15-20 minutes. Different concentrations of several salts, such as NaCl, $CaCl_2$, and $BaCl_2{\cdot}2H_20$ were added to the solution and progressively decreased the temperature until 40ºC.

To test the gelation of the mutants in Dulbecco's Modified Eagle's medium (DMEM) sample were kept in an incubator (37ºC, 5% CO_2).

5 Results

5.1 - Production and rheological properties of gellan-like polysaccharides

Sphingomonas elodea produces gellan gum in high yield, giving rise to mucoid yellow colonies in plates containing S medium (Figure 11-A). Contrastingly, the deletion of the *gelE* gene encoding a putative tyrosine autokinase produces colonies that are slightly orange and dry (Figure 11-B).

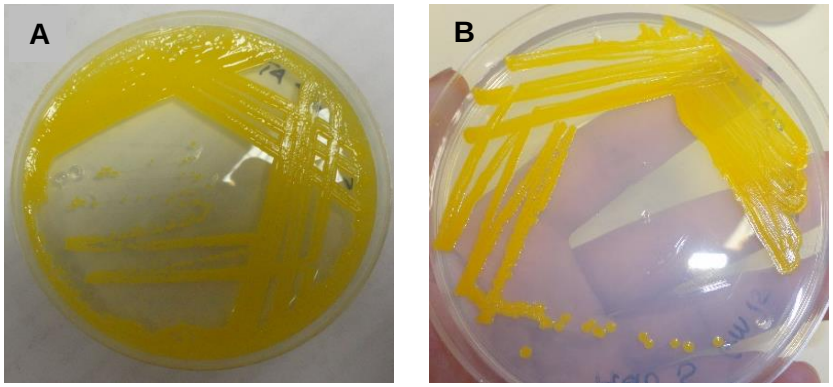

Figure 11 – Morphology of *S. elodea* ATCC (A) or Δ*gelE* mutant (B) grown for 3 days at 30ºC in solid S medium.

Analyses to GelE secondary structure evidenced several conserved regions, such as the ATP-binding pocket composed of the walker A, walker B and Mg^{2+}-binding region, an amphipathic helice, a C-terminal β-strand, and several tyrosine residues (Figure 12).

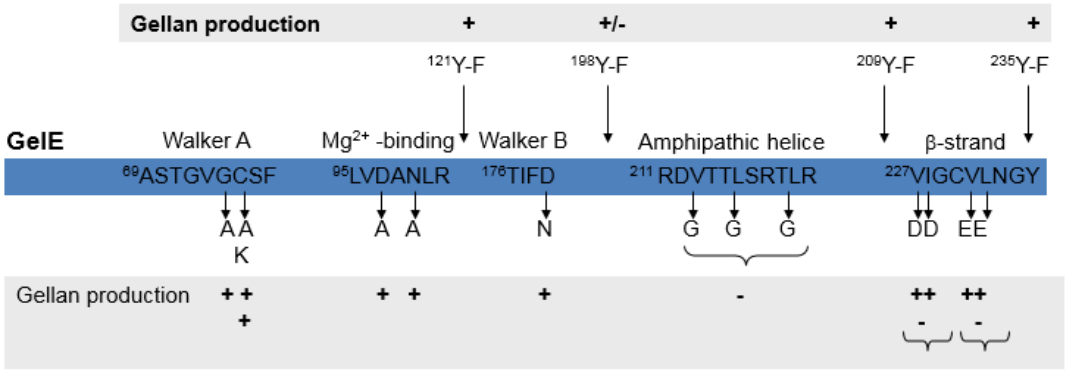

Figure 12 - Point mutations introduced into GelE coding sequencing and respective amino acid exchange. The effect of these mutations on gellan recovery from the growth culture is also indicated as + or -.

To evaluate the role of these regions in GelE activity regarding gellan production, several point mutations were previously introduced (Moreira et al, unpublished). The six mutations introduced in the ATP-binding pocket of GelE (G74A, C75A, C75K, D97A, N99A and D179N) still complement the gellan deficient phenotype of the Δ*gelE* mutant (Figure 12). Contrastingly, the three simultaneous mutations in the amphipathic helice did not restore gellan production. When single mutations were introduced in the C-terminal β-strand domain (V227D, I228D, V231E and L232E) gellan was still produced, but the simultaneous deletion of two amino acids (VI or VL) prevent gellan production. As GelE is a putative tyrosine autokinase, 4 tyrosine residues were mutated (Y121F, Y198F, Y209F and Y235F). All of this mutations restored gellan biosynthesis by the complemented Δ*gelE* mutant, although Y198F mutation gave rise to a lower amount of polymer and drastic changes in its rheology (Moreira et al, 2004).

To understand whether these mutations were giving rise to gellan-like polymers with different rheological properties, viscosity of the growth medium and of a polymer solution, and quantity of produced polymers were determined. For that, the Δ*gelE* mutant was transformed by electroporation with the empty vector (pBBR1MCS), with this vector carrying native *gelE* gene (pHA010-3), with pLM51-1 having the two last amino acids of GelE deleted, and plasmids carrying all the mutations of Figure 12 that still complement the EPS-producing phenotype. Three different colonies were chosen for each strain and grown in S liquid medium for 48 hours. After that period, growth medium viscosity was measured and data presented in Figure 13.

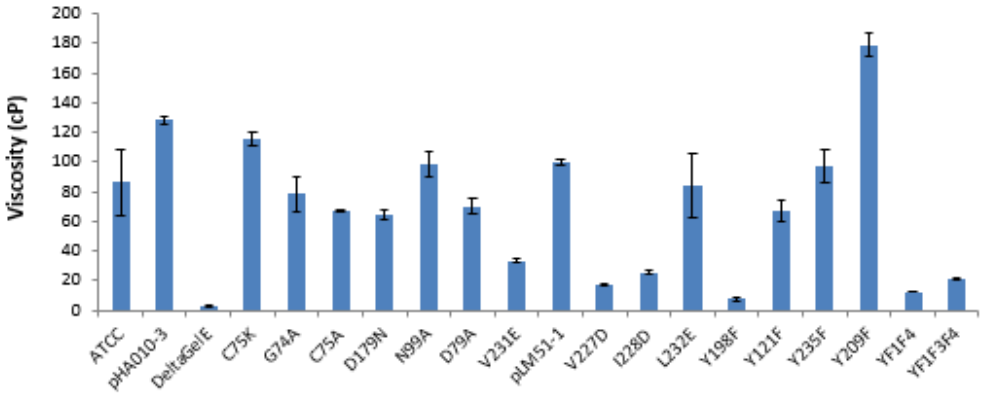

Figure 13 – Growth medium viscosity (shear rate 12 s^{-1}) of *S. elodea* ATCC 31461 and Δ*gelE* mutant complemented with the empty pBBR1MCS vector or with *gelE* carrying the indicated point mutations, after 48 hours of growth in S medium at 30ºC with 250 rpm agitation. Data are average of three biological replicates.

As expected, the growth medium of Δ*gelE* mutant carrying pBBR1MCS does not show significant viscosity, but when complemented with *gelE* gene (pHA010-3), viscosity is restored to a level similar to the *S. elodea* wild type strain. Regarding the mutations in the different domains of GelE, the ones with higher impact in viscosity reduction were in the C-terminal β-strand (V227D, I228D and V231E) and in all containing the Y198F mutation ((Y198F; Y121F/Y235F (YF1F4) Y121F/Y209F/Y235F (YF1F3F4)) (Figure 13). This analysis showed that although all GelE variants restore gellan production by the Δ*gelE* mutant, their effect on medium viscosity is variable, suggesting the present of polymers with different rheological properties.

To assess whether growth medium viscosity was comparable with the viscosity of the precipitated/dissolved polymer, the polysaccharide present in the culture medium of each strain was precipitated with ethanol, washed several times to remove cell debris and air dried. Then, 1 g/l solution of the gellan precipitates were prepared and viscosity was measured. Data here obtained confirmed previous results of Moreira et al (2004) since Δ*gelE* supernatant precipitate does not show viscosity and the viscosity of the complemented mutant (pHA010-3) precipitated gellan does not reach the value of the wild-type *S. elodea* ATCC 31461 (Figure 14-A). In comparison to the viscosity of the polymer of the native GelE complemented strain, ATP-binding site mutation C75A, D179N, N99A and D79A have a positive effect in polymer viscosity (Figure 14-A). Mutations in the β-strand C-terminal region had different effects depending on the mutation. V231E, V227D and pLM51-1 had a negative effect on gellan viscosity while I228D and L232E led a positive effect. Regarding the tyrosine residues, Y198F mutation had a negative effect, but Y209F mutation displayed a strong increase of gellan solution viscosity (Figure 14-A).

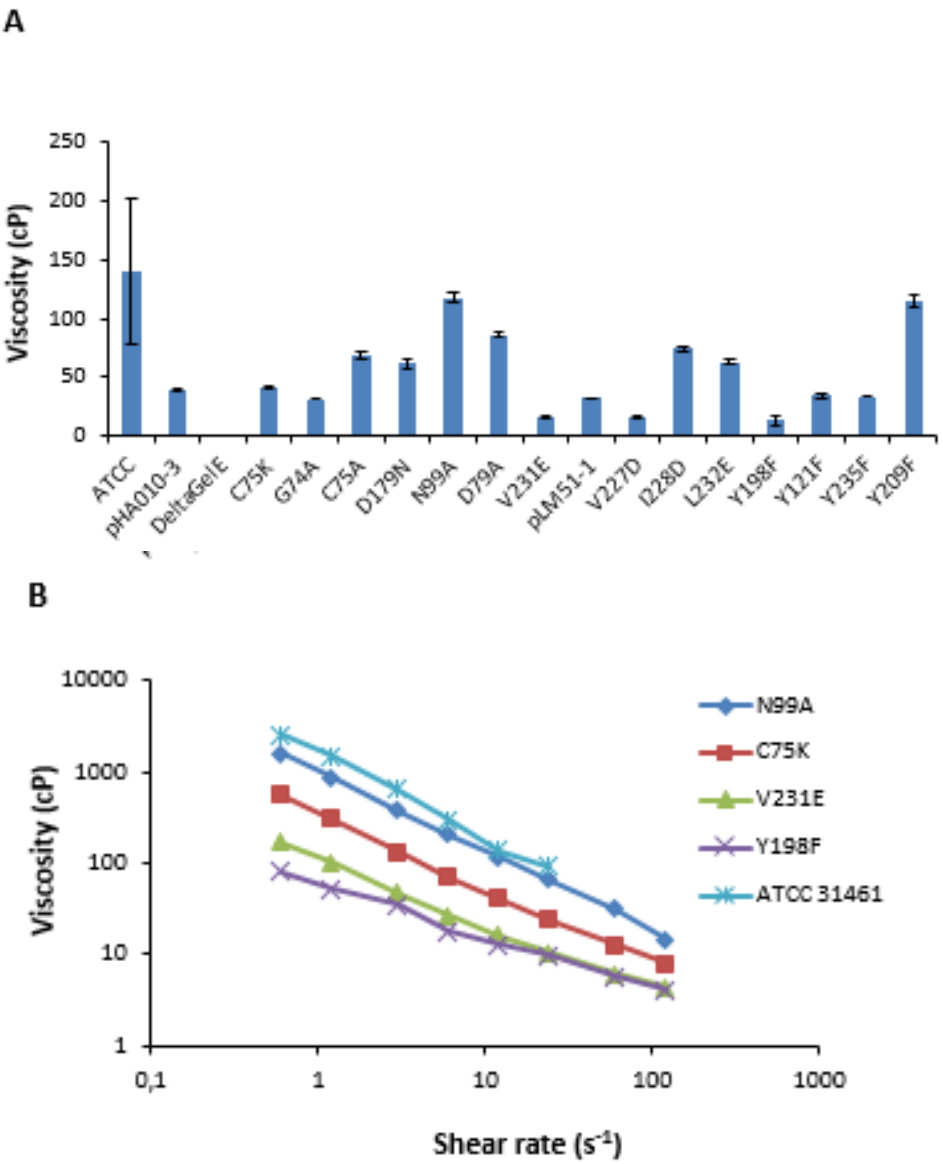

Figure 14 – Viscosity at 12 s^{-1} (A) or at different shear rates (B), of the aqueous solutions prepared with 1 g/l of the ethanol-precipitated polymers isolated from the supernatants of the indicated strains, grown for 48h at 30ºC in S medium. These bars (curves) are based on the median values of three viscosity measurements of polymer solutions obtained from three independent growth experiments.

The aim of the experiment shown in Figure 14-A was to identify strains producing polymers with different viscosities to assess properties such as gelation as it will be further described. Therefore, we were choosing for further analysis strain N99A producing a polymer with a viscosity comparable to the wild-type ATCC 31461, C75K with polymer display 30% of the wild type and V231E and Y198F for lower polymer viscosity. Figure 14-B shows the viscosity at different shear rates for the above mentioned strains, confirming the different rheologies.

Next step was to assess if the strains producing the chosen gellans were good producers by determining the dry weight of the ethanol-precipitated polymer. Data shown in Figure 15 demonstrate that V231E and C75K mutations give rise to 8.3 and 7.4 g/l, respectively, of polymer while Y198F produces approximately 4.4 g/l. The value for this last mutations confirms results previously published (Moreira et al, 2004). Due to unknown reason, no EPS was recovered from the strain with N99A mutation in this particular experiment and therefore no value can be shown.

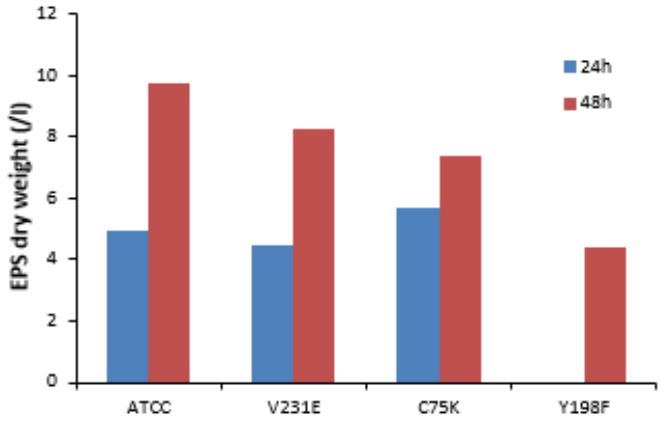

Figure 15 – Gellan production assessed by dry weight of the ethanol precipitated polysaccharide in the wild-type strain and when the mutant genes are expressed in the *S. elodea* Δ*gelE* mutant.

5.2 - Purification and gelling properties of the gellan-like polymers

For future applications of these polymers, they need to be as pure as possible. Therefore, the next aim was to remove contaminants like proteins that collapse with the EPS when ethanol is added. As a first approach, EPS sample were run in a SDS-PAGE gel, but due to EPS viscosity, proteins were not separated (data not shown). Then, purity of the EPS samples was followed by measuring the total protein content. For EPS sample deproteinization, samples were mixed with chloroform or phenol, centrifuged and the aqueous phase recovered. The protein content of the EPS solutions was determined by the modified Lowry method using the equation obtained in Figure 16.

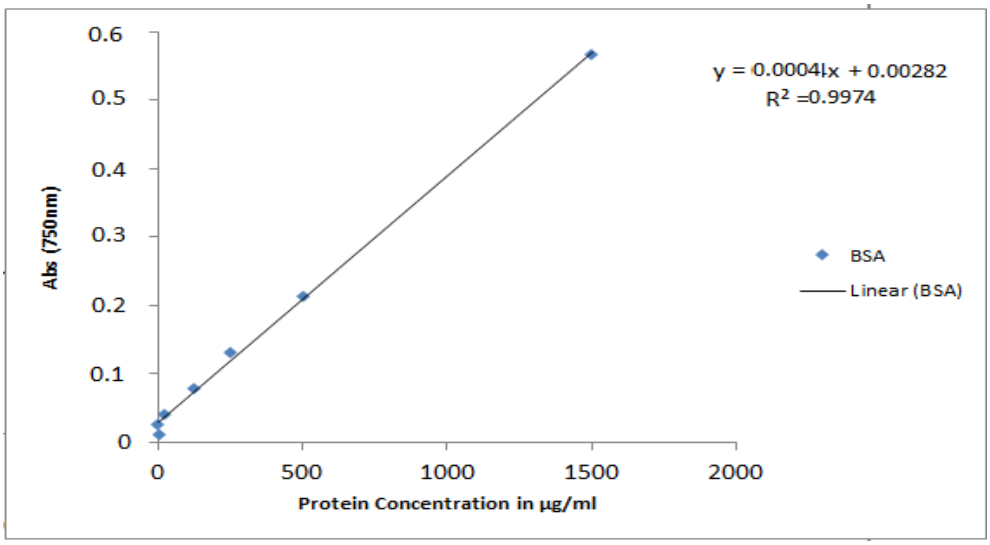

Figure 16 – Modified Lowry Protein Quantification Standard Curve prepared with serum bovine albumine (BSA).

Data obtained is shown in Table 4. Comparing with protein concentration of untreated EPS samples, deproteinization using chloroform was effective for most of the samples, except for the native gellan. Nevertheless, significant amount of proteins were still measured for Y198F and N99A EPS. Samples treated with phenol were interfering with the Lowry method and negative concentrations or huge concentration values were obtained (Table 4).

Table 4 – Protein concentration in µg/ml of EPS samples.

Strain	Without treatment	Chloroform	Phenol
ATCC 31461	617.4	717.1	*
Y198F	1577.4	666.3	*
N99A	1036.6	674.6	*
V231E	522.2	71.5	7118.7
C75K	858.9	64.7	5407

*-negative values were obtained.

Due to interference of phenol with the Lowry method it was used instead the Bradford method to estimate protein concentration. After using BSA as standard for the calibration curve (Figure 10), the protein concentration are shown in Table 5.

Table 5 – Concentration in µg/ml of the proteins present in EPS samples by using the Bradford method.

Strain	Without treatment	Chloroform	Phenol
ATCC 31461	468.2	19.2	211.2
Y198F	497.7	275.2	591.2
N99A	438.2	245.2	269.2
V231E	778.2	239.2	739.2
C75K	959.7	223.2	456.2

The Bradford method seemed to be more suitable to determine EPS-samples protein contamination, since consistent values were obtained. According to this data the best compound for deproteinization of EPS samples was chloroform since it was observed, at least, a reduction of 50% in the amount of contaminant proteins (Table 5).

After gellan-like polymers purification protocol was established, the following step was to determine the best conditions for gelation. It was known from previous published data that NaCl, $CaCl_2$ or sucrose could help in the gelation of gellan (Morris et al, 2012). But as sucrose could have detrimental effect or stem cell viability, only NaCl and $CaCl_2$ were tested. Using the native gellan produced by *S. elodea* ATCC 31461 and the Δ*gelE* mutant expressing mutant GelE (V231E) in presence of NaCl, none of the samples made a gel (data not shown). When concentrations of $CaCl_2$ ranging from 0 to 200 mM were added to the EPS solutions, it was observed the formation of a gellified solution for the native polymer, especially to the concentrations around 20 mM $CaCl_2$. An example of the type of gel formed is shown in Figure 17.

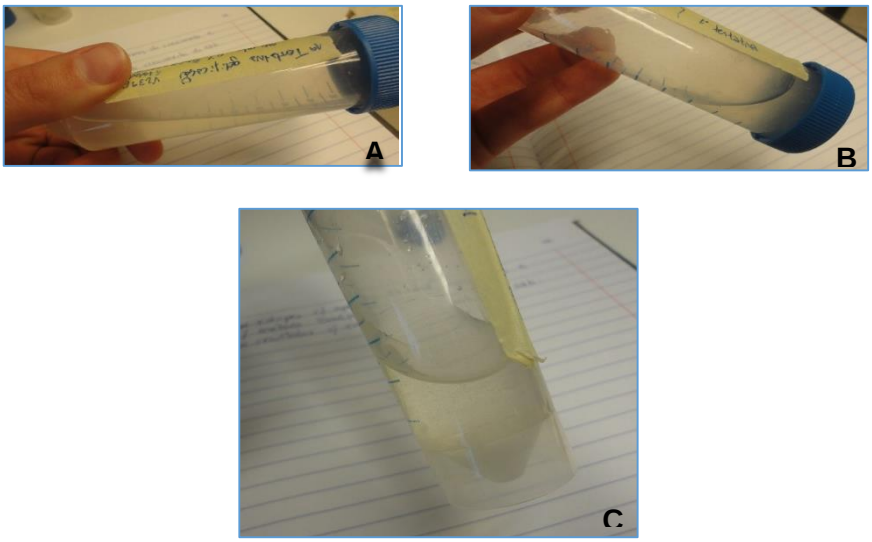

Figure 17 – Images showing attempts of gelation on V231E (A), C75K (B) and wild-type (C) polymers with $CaCl_2$.

To determine the stability of the formed gel, drops formed with a syringe were transferred to water, PBS buffer and a 20 and 200 mM $CaCl_2$ solution. The gellified droplets were dissolved in water, but remained as a gel in PBS and $CaCl_2$ solutions. As the main goal of using gellan-like polymers would be to immobilize stem cells for growth and differentiation, it was needed to test whether the gels produced in the presence of $CaCl_2$ were stable in DMEM medium, usually used to grow this type of cells. Therefore, a gellan solution of the native polymer was gellified with $CaCl_2$ (Figure 18-A) and then incubated overnight in DMEM with serum at 37ºC. As shown in Figure 18-B, the gel must have dissolved because after removing the medium no gel-like structure remained.

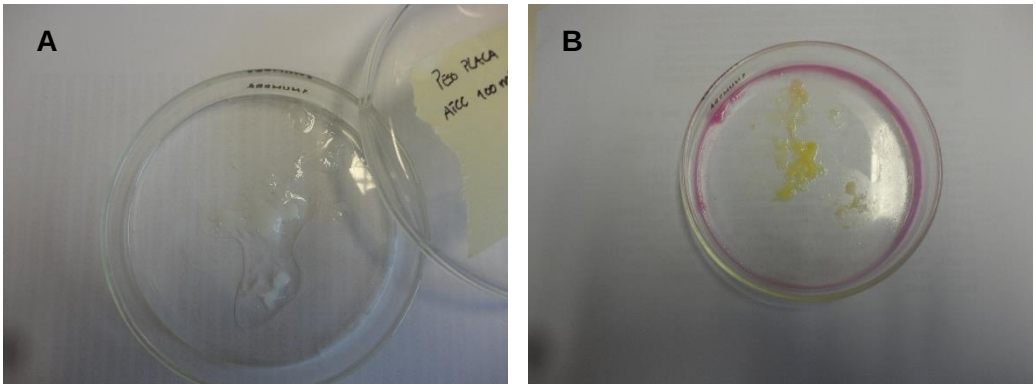

Figure 18 – Images showing the *S.elodea* ATCC 31461 native gellan after incubation with $CaCl_2$ and gelation (A) and after gel incubation in the presence of DMEM supplemented with serum overnight at 37ºC (B).

Due to this last result, another ion (Ba^{2+}) was tested for gelation. In spite of that, the gelation properties of gellan were not improved. This type of tests were also carried out with the gellan-like polymers V231E and C75K, but with these polymers no significant gelation was obtained (Figure 17).

5.3 - Assessment of polymer production in strains of the *Massilia* genus

With the aim of finding new carbohydrate polymers with potential biotechnological applications, we started the characterization of two soil isolates, which after several days grown on LB plates, produce purple and highly mucoid colonies. These two isolates, named RAG-1 and RAG-2, do not have a species attributed, but they may belong to the genus *Massilia* (António Veríssimo, personal communication).

The first experiment was to assess growth rate in two different media, LB and LB supplemented with glucose (LBG). Data shown in Figure 19-A indicate that the growth rate is similar for both strains in both media, but while RAG-1 maintained the same optical density in stationary phase, RAG-2 showed a slight decrease, suggestive of cell lysis. To quantify mucoidy strains were cultivated in liquid medium, from which several samples were taken, and ethanol precipitated. Precipitated dry-weight increases along the growth period with RAG-1-LB having a maximum at 72 hours, RAG-1-LBG at 96 hours, and RAG-2 at 120 hours in both media (Figure 19-B). To evaluate if this polymer increased the viscosity of the growth media over time, we measured growth medium viscosity at different shear rates. Figure 19-C shows the results for the shear rate 1.2 s^{-1}. Despite some fluctuations, the general trend is an increase in viscosity, especially for strain RAG-1. Possibly due to the cell lysis observed in stationary phase, RAG-2 growth media shows lower viscosity when compared to RAG-1. As a final experiment to assess the rheology of these polymers, the ethanol-precipitable material produced by the two strains in both media were dissolved in water (1 g/l solution) and viscosity was measured. Data represented in Figure 19-D shows that the most viscous polymer at 96 hours of growth is from RAG-1 grown in LB. Although the medium viscosity for RAG-1-LBG at 96 hours is similar to RAG-1-LB, it shows considerably lower viscosity, implying that the RAG-1-LB polymer has higher molecular mass.

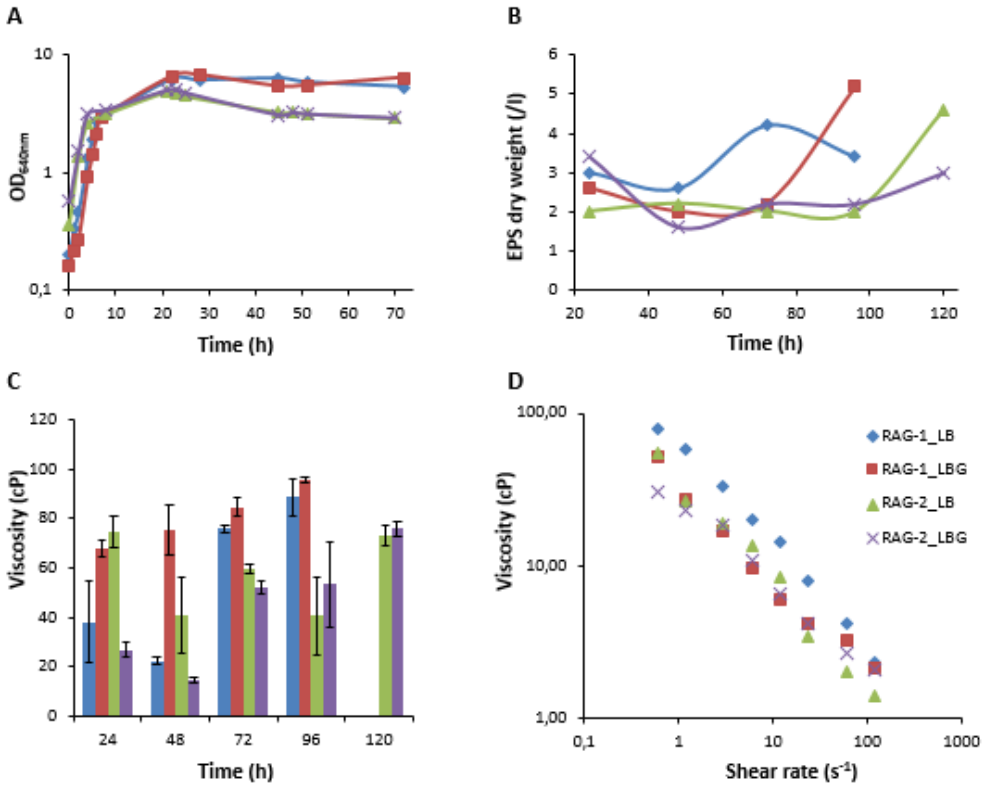

Figure 19 – (A) Comparison of growth curves of RAG-1 and RAG-2 grown at 30ºC in LB or LBG media; (B) exopolysaccharide production, assessed based on the ethanol-precipitated supernatant; (C) growth medium viscosity at 1.2 s^{-1}shear rate; (D) viscosity at different shear rates of the aqueous solutions prepared with the same concentration (1g/l) of the ethanol-precipitated material isolated from different cultures grown, for 96 hours, at 30ºC. These curves are based on the medium values from triplicate experiments. Strains legend for all the graphics is in (D).

6 Discussion

In this study it was evaluated whether different polymers produced by the Δ*gelE* deletion mutant of *S.elodea* complemented with mutated variants of *gelE* gene could have different rheological properties suitable for cell tissue engineering approaches. GelE is expected to interact with membrane protein GelC (named activator domain) and regulate gellan chain length. Previous studies have shown that deletion of *gelC* or *gelE* gene leads to gellan production abrogation, but when a mutated variant of GelE having a phenylalanine residue instead of a tyrosine at position 198, gellan is still produced although in lower amount and with lower molecular mass (Moreira et al, 2004). This suggests that mutations putatively affecting the structure/function of GelE may result in different interaction with GelC and possibly to polymers with different rheological properties. To investigate this hypothesis, mutations of GelE conserved motifs were introduced by site-directed mutagenesis (Moreira, unpublished). Here, we analyzed the effect of these GelE mutations in the viscosity of the gellan-like polymers produced. Regarding the viscosity of the growth medium after 48 hours of incubation, the mutations with higher impact in medium viscosity were in the C-terminal β-strand and Y198F which gave rise to lower viscosity and possibly to EPS with lower-molecular mass. Contrastingly, mutation affecting Y209F seems to indicate that an EPS with higher molecular mass is being produced. The C-terminal region of GelE seems to be determinant for gellan rheology, but the reason for this is unknown. An hypothesis is that this region is perhaps important for interaction with GelC, regulating then the activity of the polysaccharide polymerase enzyme GelG and consequently the size of gellan chains.

When viscosity of the gellan-like polymers 1g/l solutions were determined, there was a good correlation with the growth medium viscosity. Exceptions are the mutations that lack the two last amino acids of GelE (pLM51-1) and I228D, with opposite effects. As representative polymers for further studies we were choosing mutation giving rise to polymers with high viscosity (N99A), intermediate (C75K) and low (V231E, Y198F). In addition we had determined the amount of EPS production in the presence of these mutations. With the exception Y198F mutation, all the others mutations do not interfere significantly with the amount of EPS produced. This is an important feature regarding possible biotechnological applications.

For putative application in tissue engineering, a gellan-like polymer needs to have the right rheological properties for cell growth and differentiation, but also requires a high degree of purity, not to be toxic to eukaryotic cells. Due to gellan viscosity it is not always possible to remove cells by centrifugation and quite often cells are precipitated with the polymer by the addition of ethanol. These samples are highly contaminated with bacterial proteins that have to be removed. In this work we tested whether organic solvents like phenol and chloroform, known for deproteinization could be used.

Our data showed that chloroform was more effective in reducing the protein content of the EPS solutions, but still not all protein contaminants were removed. Phenol does not seem to be a good choice, because it is very difficult to remove from the EPS sample and is toxic to cells. Nevertheless, some extra work can be done for EPS sample deproteinization, such as incubation with a protease, followed by dialysis and lyophilization, or purification by chromatography.

Since an important goal was to test gelation of the different polymers, several conditions were tested. The aim was to obtain a polymer that is in the liquid state at temperatures higher than 37ºC, but at body temperature makes a gel able to be used as scaffold for eukaryotic cell growth. With that in view we tested the native gellan in the presence of divalent cations such as Ca^{2+}, being able to produce a gel. When this gel was tested for stability in solutions containing salts (PBS, $CaCl_2$) the gel was stable, but in the medium for stem cell growth, apparently the gel was dissolving. In addition, some of gellan-like polymers did not gelify under the tested conditions and other conditions have to be tested. One possibility is to crosslink these polymers with other compounds, to obtain a stable network of fibers with a tridimensional structure. Despite our results, this gellan-like polymers should not be left without consideration in further studies, because gellan is one of the few bacterial gums with gelling properties and one of the most promising tools for new biotechnological applications.

With the goal of searching for new bacterial exopolysaccharides with putative interest in the biotechnological field, we did the first preliminary studies with two soil isolates from the genus *Massilia* which comprises Gram-negative bacteria. RAG-1 and RAG-2 isolates produce a polymer which gives a mucoid morphotype to the purple colonies and that after cell-free supernatant precipitation gave a white material, not so dense as gellan. Viscosity measurements of the growth media or of a polymer solution showed very low values when compared to gellan, indicating that these polymers have different rheological properties. The nature of these polymers is unknown, and further studies including sugar content determination and structure determination should be conducted. From the two tested isolates, RAG-1 seems to be more promising since it has a highest production and viscosity when compared to RAG-2. Nevertheless, these are preliminary studies and new media, temperature of growth, pH should be tested. One important difference between *S. elodea* ATCC 31461 and RAG-1/ RAG-2 is that gellan production is stimulated by the high carbon/nitrogen ratio (S medium) while here we did not see significant differences between LB and LBG. A medium like the S medium was not suitable for growth of neither RAG-1 nor RAG-2 isolates.

Overall this work showed that mutations in proteins regulating polysaccharide chain length are good strategies to obtain polymers with different rheological properties. In addition, we started the characterization of other polymer producing microorganisms, hoping to find new interesting features for possible future applications.

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