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Development of a new microcarrier culture based in matrix of natural polymer

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(2º ciclo de estudos)

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Dedicatory

To my mother

"Focused, hard work is the real key to success. Keep your eyes on the goal, and just keep taking the next step towards completing it. If you aren't sure which way to do something, do it both ways and see which works better."
- John Carmack

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Resumo

A goma de gelano é um polissacárido de baixo custo, produzido por fermentação microbiana do microorganismo Sphingomonas *paucimobilis*. Este polímero é vulgarmente utilizado na indústria alimentar e farmacêutica. A goma de gelano é solúvel em água, e, quando aquecida e misturada com catiões mono- ou divalentes, forma géis mediante a diminuição suave da temperatura. A goma de gelano pode ser encontrada em duas formas, uma com elevado conteúdo em grupos acetilados, designada por goma de gelano de alta acetilação e outra forma adicional com baixo conteúdo em grupos acetilados, denominada por de goma de gelano de baixa acetilação. Cada forma de goma de gelano apresenta diferentes características nos géis que resultam destes produtos. A goma de gelano de alta acetilação permite a formação de géis fluídos, já a goma de gelano de baixa acetilação forma géis rígidos e transparentes.

A cultura de células de adesão em suspensão por microtrasportadores é um método que se tem tornado cada vez mais importante, não só porque permite o crescimento e desenvolvimento de um grande número de células mas também por possibilitar a formação de microambientes celulares sustentáveis. Devido a destas características, esta técnica tem vindo a ser utilizada não só pela indústria farmacêutica para a produção em larga escala de alguns fármacos como também para testar e avaliar fármacos numa fase inicial do seu desenvolvimento através de modelos 3D. No entanto os microtransportadores disponíveis no mercado apresentam algumas desvantagens tais como a opacidade, a baixa densidade dos ou a baixa porosidade dos microtransportadores. Também o elevado diâmetro dos microtransportadores macroporosos os torna desvantajosos para algumas funções como a visualização das células aderidas. Mas a principal desvantagem prende-se com o custo de processamento elevado.

Associando as principais características do polímero goma de gelano e a necessidade de desenvolver novos microtransportadores surge o objectivo principal deste trabalho, que consiste no desenvolvimento de um novo transportador celular através da conjugação das duas formas de acetilação da goma de gelano. De modo a concretizar este objectivo o trabalho foi dividido em dois grandes tópicos, sendo eles, a produção e recuperação de goma de gelano de alta acetilação e o desenvolvimento e caracterização de um novo microtransportador com base na goma de gelano.

Para a biossíntese de goma de gelano foram testados 3 métodos, com protocolos e meios distintos. Os resultados obtidos indicaram que o meio que permite uma maior produção de goma de gelano apresenta baixo nível de azoto e uma solução de sais composta por 10 g/L de Na_2HPO_4, 3 g/L de KH_2PO_4, 1 g/L de K_2SO_4, 1 g/L de NaCL, 0.2 g/L de $MgSO_4.7H_2O$, 0.01 g/L de

$CaCl_2$, 0.001 g/L de $FeSO_4.7H_20$. Para além disso, foi também constatado que para uma melhor adaptação e crescimento da bactéria durante a fermentação, o processo de transição da pré-fermentação para a fermentação deverá ser acoplado um passo de remoção da goma de gelano e interferentes majores do meio, tais como proteínas.

Relativamente à recuperação da goma de gelano na forma de alta acetilação foram testados três patamares para a obtenção do polímero alvo com elevado grau de pureza, nomeadamente a remoção do conteúdo bacteriano, a remoção de interferentes e a precipitação do polímero. Deste modo para a remoção do conteúdo bacteriano foi comprovado que o tratamento do caldo de fermentação através de dois passos de centrifugação melhora a qualidade da goma de gelano precipitada. Na remoção dos principais interferentes foram avaliados dois métodos, um por precipitação com sulfato de amónio e outro por diálise. Nenhuma das opções testadas demonstrou resultados satisfatórios em termos de remoção dos contaminantes presentes em maior percentagem. Finalmente, para a precipitação da goma de gelano, foi testado um passo de filtração por membranas que apresenta bons resultados a nível da pureza do polímero recuperado (espectros de FTIR muito semelhantes entre a amostra recuperada e a amostra comercial de goma de gelano de lata acetilação). No entanto apresenta algumas desvantagens tais como a baixa quantidade de polímero recuperado que o tornam num método pouco viável. Nesta fase analisou-se ainda a capacidade de vários solventes orgânicos, tais como o isopropanol, o etanol, a acetona e o acetonitrilo para precipitar a goma de gelano de alta acetilação. Os resultados mais satisfatórios foram obtidos utilizando a precipitação da goma de gelano de alta acetilação com acetonitrilo.

Na segunda fase do projecto usou-se a conjugação da goma de gelano de alta e baixa acetilação para desenvolver um novo microtransportador para cultura celular. A conjugação destas duas formas prende-se ao facto de tentar diminuir a densidade de cargas negativas da goma de gelano de baixa acetilação usando a goma de gelano de alta acetilação. Para além disso foi também pretendido analisar o efeito das duas formas na capacidade de inchaço e no grau de porosidade dos microtransportador finais. Foi então desenvolvido um desenho experimental de forma a obtermos as conjugações possíveis entre as concentrações da goma de gelano de alta (0%, 1%, 1.5%, 2%, 2.5%, 3%) e baixa acetilação (0%, 0.16%, 0.33%, 0.5%). Para a construção da estrutura do microtransportador foi utilizada a técnica de emulsão água em óleo. Os microtransportadores desenvolvidos foram avaliados quanto à sua capacidade de inchaço e grau de porosidade, de forma a ser desenvolvidos dois modelos iniciais para a previsão destas características. Para a avaliação da capacidade de inchaço foi medido o diâmetro dos microtransportadores na forma liofilizada e na forma hidratada. Através da variação de diâmetro foi obtido a capacidade de inchaço dos microtrasportadores. Para a avaliação da porosidade dos microtransportadores foi usado microporosimetria de mercúrio e foi avaliado a área total de poros de cada microtransportador. Também foi avaliada a

superfície dos microtransportadores recorrendo à técnica de microscopia electrónica de varrimento.

Posteriormente e de forma a avaliar a performance dos microtransportadores desenvolvidos foram testados dois microtransportadores com diferentes graus de porosidade (1.672 m^2/g e 0.209m^2/g) de modo a avaliar a sua sustentabilidade em termos de adesão e crescimento celular da linha COS-7 na sua superfície. Os resultados obtidos indicam que a porosidade dos microtransportadores pode influenciar uma maior adesão celular. Adicionalmente foi ainda avaliada a densidade de cargas. Deste modo foi utilizada albumina de soro bovino carregada positivamente e negativamente (dependendo do pH da solução) e testou-se o poder de ligação de cada forma da goma de gelano à superfície do microtransportador. Conclui-se que o microtransportador adsorve compostos positivos e por isso deve apresentar maioritariamente cargas negativas à superfície.

Através deste trabalho inicial, foram obtidos resultados que demostram a potencialidade destes novos microtransportadores em diversas áreas, tais como a entrega de fármacos controlada por variações de pH e como possível base para suportes de crescimento celular em suspensão.

Palavras-chave

Acetilação; Goma de gelano; Microtransportadores; *Spingomonas paucimobilis* ATCC31461.

Abstract

Gellan gum is a low cost polysaccharide manufactured by microbial fermentation of the *Sphingomonas paucimobilis* microorganism. This polymer is commonly used in food and pharmaceutical industry. It can be dissolved in water, and when heated and mixed with mono or divalent cations, forms a gel upon lowering the temperature under mild conditions. Gellan gum can be found in two forms, one with high acetylate content, called high acyl gellan gum and other form with low acetylate content, called low acyl gellan gum. Each form of gellan gum presents different gel characteristics.

Microcarrier cell culture seems to be a promising method for the growth of large amounts of cells. This technique has been used not only for the production of pharmaceuticals but also in biomedical engineering and in drug screening using 3D cell culture. However microcarriers available on the market have some disadvantages such as opacity, low density or low porosity. Also the large diameter of the macroporous microcarriers makes them disadvantageous for some functions such as viewing adhered cells. But the main disadvantage lies in the high cost of processing.

Bringing together these two topics, the aim of this work was create a new microcarrier made by the conjugation of the two forms of gellan gum. To achieve this aim, the work was divided in two main topics, the production and recovery of high acyl gellan gum and the development and characterization of the microcarrier structure.

For the production and recovery of high acyl gellan gum, different procedures were tested and results revealed that medium with low nitrogen source and salt solution containing 10 g/L of Na_2HPO_4, 3 g/L of KH_2PO_4, 1 g/L of K_2SO_4, 1 g/L of NaCL, 0.2 g/L of $MgSO_4.7H_2O$, 0.01 g/L of $CaCl_2$, 0.001 g/L of $FeSO_4.7H_2O$ is more suitable for the growth of bacteria. The procedure of two centrifugation steps showed the best results for the removal of bacteria content. In addition, acetonitrile was the organic solvent that showed the best results for the precipitation of high acyl gellan gum compared to the other organic solvent tested (isopropanol, ethanol and acetone).

In the second phase of the project was used the combination of gellan gum of high and low acetylation to develop a new microcarrier for cell culture. The combination of these two forms is related to the fact of trying to decrease the density of negative charges of low acyl gellan gum by using gellan gum high acetylation. Furthermore it was also desired to analyse the effect of the two forms in the swelling capacity and the degree of porosity of the final microcarrier. For this aim was then developed an experimental design in order to get the possible combinations between the concentrations of gellan gum high (0%, 1%, 1.5%, 2%, 2.5%,

3%) and low acetylation (0%, 0,16% , 12:33%, 0.5%). The water-in-oil emulsion technique was applied to the construction of microcarrier structures. The microcarriers developed were then evaluated for porosity degree by mercury microporosimetry and for swelling ability by the difference between the hydrated and lyophilized form of microcarrier. Through the results obtained, two initial models for the prediction of swelling behaviour and porosity degree were developed. Scanning electron microscopy was also performed to visualize the topographic surface of the microcarrier.

Microcarriers with high (1.672 m^2/g) and low level of porosity (0.209m^2/g) were chosen for a cell culture assay and demonstrated similar behaviour regarding cell adhesion. The ability to adsorb compounds with positive charge was proved with a bovine serum albumin adsorption study by the surface of the microcarrier.

In overall, these results suggest that the gellan microcarriers show advantageous characteristics that can be used for microcarrier cell culture. However more studies are needed in order to improve the microcarrier characteristics.

Keywords

Acetylation; Gellan gum; microcarrier; *Spingomonas paucimobilis* ATCC31461

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µg	Microgram
µL	Microliter
µm	Micrometre
^{1}H NMR	Proton Nuclear Magnetic Resonance
Abs	Absorbance
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CaCl2	Calcium chloride
cm^{-1}	número de onda
CO_2	Carbon dioxide
$CoCl_2 \cdot 6H_2O$	Cobalt(II) chloride hexahydrate
$CuCl_2$	Copper(II) Chloride
D-	Dextrorotatory
DMEM	Dublecco's modified Eagle's medium
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
$FeSO_4 \cdot 7H_2O$	Iron(II) Sulfate Heptahydrate
FTIR	Fourier-transform infrared
g	Gram
h	Hour
H_2SO_4	Sulfuric acid
H_3BO_3	Boric Acid
K_2SO_4	Potassium sulphate
KH_2PO_4	Potassium dihydrogen phosphate
KV	Quilovolt
L	Litter
L-	Laevorotatory
M	Molar
m/V	Mass/volume
mg	Milligram
$MgSO_4$	Magnesium sulphate
min	Minute
mL	Millilitre
mM	Milimolar
mm	Millimetre
$MnCl_2 \cdot 4H_2O$	Manganese(II) Chloride Tetrahydrate
N.A.	No Available
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ºC	Degree Celsius
OD	Optical Density
PBS	Phosphate buffered saline
PLGA	Poly(lactic-co-glycolic acid)

PLLA	Poly(L)lactic acid
Ppm	parts per million
Psia	Pound force per square inch
Rpm	Rotation per minute
SEM	Scanning Electron Microscopy
TSP	Sodim 3-trimethylsilypropanol
US $	American Dollars
USA	United State of America
UV	Ultraviolet
$ZnCl_2$	Zinc Chloride

Chapter 1 - Introduction

1.1. Exopolysaccharides: an overview

In recent decades, research in the field of biomaterials has been a subject of great interest at academic and industrial level. Many are the new biomaterials recently added to the market, with innovative properties for diverse applications (Langer and Tirrell, 2004). Different compounds are used for the creation of these new biomaterials, both from natural and synthetic source (Khan and Ahmad, 2013).

However, some of these compounds are not well accepted by the organism, due to toxic or inflammatory effects. To overcome these drawbacks, several techniques are used to modify the material in order to be considered a suitable biomaterial for biomedical applications (Shin *et al.*, 2003). Some of these techniques are not the most ecological or economically viable. In an attempt to reduce production costs and environmental problems, research centers and biotech companies have been increasing the research for biodegradable materials, such as polysaccharides, in order to reduce the disturbances in the environment and the raw material costs (Gross and Kalra, 2002).

The use of polysaccharides in the matrix for the development of new biomaterials has been a field of exponential growth. Polysaccharides have come to be used in biomedical field for new drug delivery system applications (Liechty *et al.*, 2010), for cell entrapment systems (Woerly *et al.*, 1996) or even for tissue engineering application, with the aim to improve cell growth especially for replacement of cartilage(Puppi *et al.*, 2010) (Vert, 2007).

Polysaccharides have numerous applications in biomedical field due to the wide variety of polysaccharides available and also due to the different properties they exhibit, like controllable biological activity, biodegradability and the ability to form hydrogels with controllable physical properties (d'Ayala et al., 2008).

Typically, the polysaccharides can be found in several live organisms, like plants, animals, algae, bacteria and fungi. But, the most interesting polysaccharides for academic or industrial application are extracellular polysaccharides produced by bacteria, because they allow an easy production, recovery and purification of polysaccharides from the medium in which they are formed, not requiring cellular lysis techniques that make the process more difficult, time consuming and also more costly (Freitas *et al.*, 2011).

The microbial extracellular polysaccharides also called exopolysaccharides are water-soluble polymers and they can be produced by several microorganisms during the fermentation process.

Exopolysaccharides have attracted the attention of the world as good and economic excipients for drugs, because of their unique physicochemical properties. They have multiaplicability in food and in the pharmaceutical industry as emulsifiers, stabilizers, gelling agents, lubricants and thickening agents. Besides these applications the exopolysaccharides began to emerge in the biomedical field due to their biodegradability, biocompatibility and bioadhesion abilities, chemical and mechanical resistance, swelling behavior and the ability to form reversible hydrogels. Gradually the exopolysaccharides have become promising biomaterials and economically competitive (Manjanna *et al.*, 2009), as can be seen in table 1.

Table 1 - Overview of the most relevant physicochemical and functional properties, main application areas and market value of the principal available exopolysaccharides. (Modified from Freitas *et al.*, 2011). N.A.- Not available

Exopolysaccharides	Main properties	Main applications	Market value (US$)
Xanthan	Hydrocolloid - High viscosity yield at low shear rates even at low concentrations; - Stability over wide temperature	Foods Petroleum industry Pharmaceuticals Cosmetics and personal care products	235 millions
Gellan	Hydrocolloid -Stability over wide pH range Gelling capacity Thermoreversible gels	Foods Pet food Pharmaceuticals Research: agar substitute and gel electrophoresis	15 millions
Alginate	Hydrocolloid Gelling capacity Film-forming	Food hydrocolloid - Surgical dressings - Wound management -Controlled drug release	N.A.
Cellulose	High crystallinity Insolubility in most solvents High tensile strength Moldability	Foods (indigestible fiber) -Wound healing -Tissue engineered blood vessels -Audio speaker	N.A.
Dextran	Non-ionic Good stability Newtonian fluid behavior	Foods Pharmaceutical industry: Blood volume expander Chromatographic media	N.A.
Hyaluronan	Biological activity Highly hydrophilic Biocompatible	Medicine Solid culture media	1 billion
Levan	Low viscosity High water solubility Biological activity: Anti-tumor activity Anti-inflammatory	Food (prebiotic) Feed Medicines Cosmetics Industry	N.A.
Curdlan	Gel-forming ability Water insolubility Edible and non-toxic	Foods Pharmaceutical industry Heavy metal removal	N.A.

Currently, several exopolysaccharides are available for research and application as biomaterials. One of the most studied and used is bacterial cellulose, produced by *Acetobacter xylinum,* and it has been used to form surface pellicle through aggregates of microfibrils. The Xanthan gum produced by *Xanthomonas campestris* is another example of famous exopolysaccharide, which has different physical properties like emulsion and foam stabilization, inhibitor of crystal formation, shear thinning and viscosity control and also suspending agent. Dextran produced by *Leuconostoc sp*. is perhaps the exopolysaccharide most used as a matrix of biomaterials due to its characteristics of biocompatibility, biodegradability and bioadhesion. The levan produced by *Halomonas sp*. present satisfactory properties, like good biodegradability, water solubility, and a low intrinsic viscosity. For last, gellan gum produced by *Sphingomonas paucimobilis* can form elastic and thermoreversible gels, and can also form strong and brittle gels due to the extensive intermolecular association (Chaabouni *et al*., 2014).

1.2. Gellan gum exopolysaccharide

Gellan gum is a bacterial exopolysaccharide with a high molecular weight (2 x10^6 Daltons), produced aerobically with high yields by the non-pathogenic strain *Sphingomonas paucimobilis* ATCC 31461. This bacterium was isolated after an extensive screening program done by the Kelco Company, from the surface of a plant of the Elodea genus (Kang *et al*., 1982). The *Sphingomonas paucimobilis* ATCC 31461 is a gram negative, rod shaped, chemoheterotrophic, and forms mucoid and yellow pigmented colonies in a defined medium, as can be observe in figure 1.

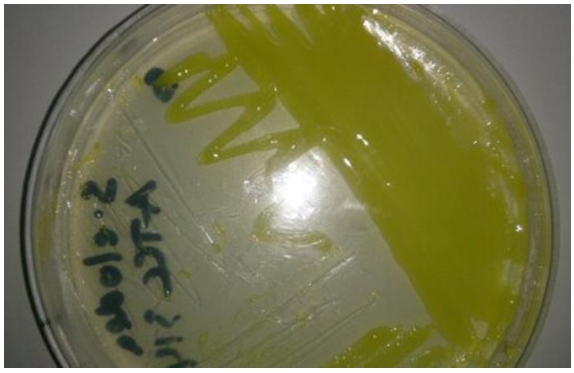

Figure 1 - Example of *Sphingomonas paucimobilis ATCC31461* culture plate.

1.2.1. Molecular structure

Gellan gum is a linear anionic exopolysaccharide with a tetrasaccharide repeat unit, composed by two molecules of D-glucose, one of D-glucuronic acid and one of L-rhamnose, as shown in figure 2. In its native form, two acyl substituents (*O*-acetate and L-glycerate) are linked to the same glucose residue (Jansson *et al*., 1983).

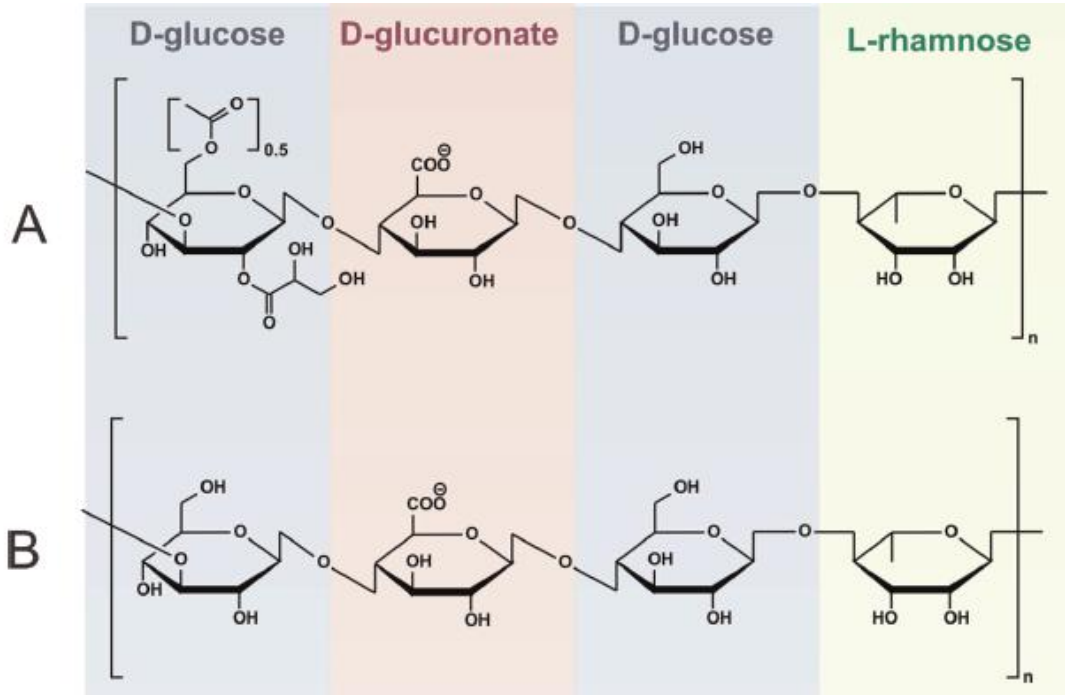

Figure 2 - Structure of high acyl gellan gum (A) and low acyl gellan gum (B)(Osmalek *et al.*, 2014).

In the solid state, gellan gum shows an ordered structure like a coaxial double helix (Chandrasekaran *et al.*, 1988), as can be seen in figure 3.

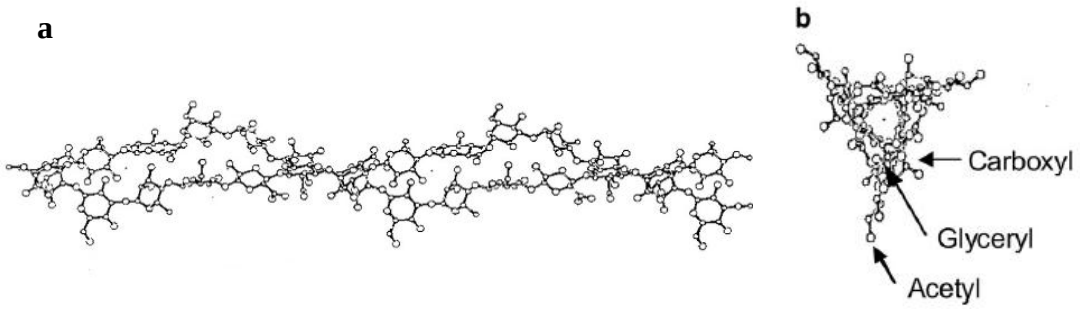

Figure 3 - Double helix structure of low acyl gellan gum, viewed perpendicular to the helix axis (a) and along helix axis (b) (Morris *et al.*, 2012)

Depending on the content of the acyl group, gellan gum can be divided in two groups, one group with high acyl content also called native gellan gum with commercial name KELCOGEL CG HA, and a second group with low acyl group also called deacetylated gellan gum with commercial name KELCOGEL CG LA. To convert native gellan gum into deacetylated gellan gum, the fermentation broth undergoes an alkaline treatment that removes the acyl content of the polymer (Kang and Veeder, 1982).

Gellan gum was first approved for food in Japan in 1988, in the Unite State of America in 1992, then in the European Union and is now allowed as a food additive in many other countries worldwide.

1.2.2. Production, recovery and purification

The gellan gum biosynthetic pathway can be divided into three sequential steps, intracellular synthesis of sugar-activated precursor, 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 trough the outer membrane (Sá-Correia *et al.*, 2002). Therefore and considering these data, it is possible to manipulate the gellan gum production yield and its chemical composition, structure and molecular mass by modifying environmental and nutritional requirements in order to promote bacteria growth and increase mass procuctivity of gellan gum (Prajapati *et al.*, 2013).

The main medium described in literature for the production of gellan gum consists of high levels of the carbon source, low levels of nitrogen source and inorganic salts. Also complex medium ingredients like molasses, tryptone, casamino acid, disodium hydrogen orthophosphate or manganese chloride, can be supplied to enhance the cell growth and production (Banik *et al.*, 2007). Comparison of gellan gum biosynthesis in defined medium containing diluted cheese whey and glucose or lactose, as the carbon source, revealed that the alteration of carbon source affects the gellan yield, acyl content and polymer rheological properties (Fialho *et al.*, 1999). In addiction the nature and concentration of nitrogen source also affects the gellan yield (West and Strohfus, 1998). The temperature and level of dissolved oxygen are also crucial parameters for optimal production of gellan gum. It was observed that gellan gum is biosynthesized at low temperature (20-25ºC) with higher molecular mass compared with those formed at an higher temperature range (30-35ºC) (Martins and Sa-Correia, 1994). Higher dissolved oxygen tension levels improved the viscosity and molecular weight of the polymer with change in acetate and glycerate content of the polymer (Banik and Santhiagu, 2006).

All the steps for production and recovery of gellan gum can be seen in a schematic model present in figure 4. Specifically, for the recovery of gellan gum it is used isopropanol, due to the insolubility of the polymer in typical organic solvents. Studies have been made to achieve the best volume of organic solvent ratio to improve the recovery of gellan gum (Kanari et al., 2002).

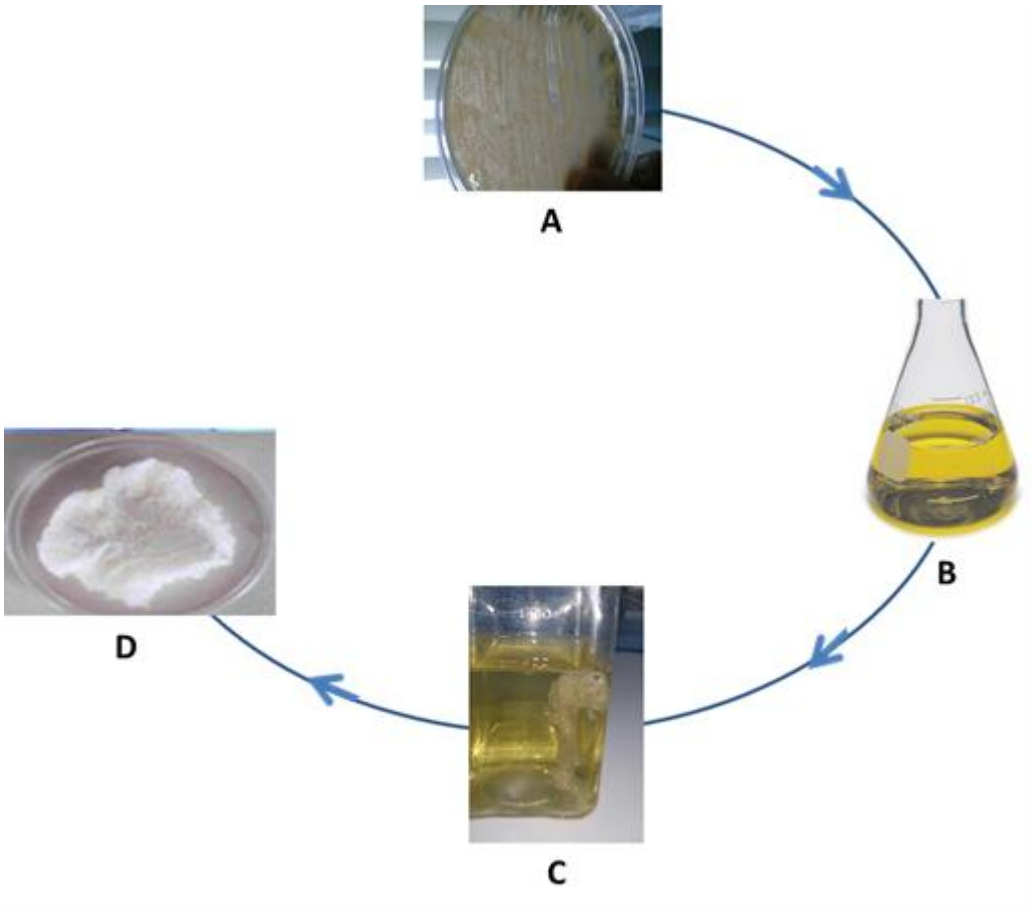

Figure 4 Typical fermentation processes for gellan gum production and its purification at laboratory scale. The mocoid solid culture of *Sphingomonas paucimobilis* ATCC 31461(A) Culture medium for the fermentation (B) Precipitation of gellan gum with isopropanol (C) Gellan gum lyophilized (D).

1.2.3. Gelling properties

One of the most interesting properties of gellan gum is the capacity to form thermoreversible gels. The gelation of gellan gum depends on several parameters like polymer concentration, acetyl content, temperature and the presence of monovalent and divalent cations in the solution (Morrisa *et al.*, 2013).

The first step in gelation of gellan is the conversion of the polymer from the disordered coil state to the double helix form. However, as it can be seen in figure 5, conformational ordering does not, in itself, give a cohesive network. Formation of true gels requires association of double helices into stable aggregates. Aggregation is inhibited by electrostatic repulsion between helices. So, cations have an enormous impact on gel strength and transparency mainly in low acyl gellan solutions, because the strength of gel is attributed to the binding of divalent cations required for helices association during gelatinization. The group I cations decrease repulsion by binding to the helices in specific coordination sites around the carboxylate groups of the polymer. It was verified that the strength of binding increased with the increasing of ionic size, like the extent of aggregation and effectiveness

for promoting gel formation. In addition, aggregation and consequent gelation with group II cations occurs by direct site binding of the divalent ions between gellan double helices. In fact, salt excess can weaken gellan networks by promoting excessive aggregation, leading to the collapse of the gel structure and ultimately the precipitation of the polymer. A reduction in pH also promotes aggregation and gelation by decreasing the negative charge on the polymer, and thus, decreasing the electrostatic repulsion between helices (Morrisa *et al*, 2013).

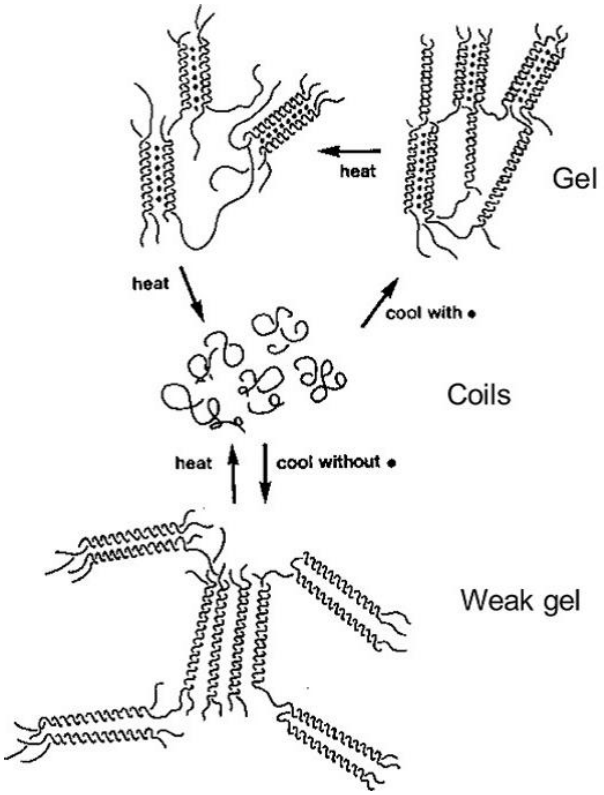

Figure 5 Model for gellan gum gelation (Morrisa *et al.*, 2013).

Gellan gum has a great ability to form gels, being only necessary a minimal concentration of polymer. A comparison of the require concentration between gels with high acyl gellan and deacetylated gellan indicate that a higher the acyl content lead to a lower polymer concentration necessary to form gel. However, the acetyl content is one of the most important factors affecting the gel strength. Gellan gum with high acyl content forms soft, elastic, termoreversibles and milky color gels (Lorenzo *et al.*, 2013). The formed gel is very weak because of bulky acetyl and glyceryl groups, which prevent close associations between gellan chains in bulk helix formation and hinder the compact packing of cross linked double helix. Indeed, gellan gum with low acyl content provides firm brittle, termoreversible and transparent gels because of the absence of acetyl and glyceryl groups (Mao *et al.*, 2000).

The hydration temperature of gellan gum is also a crucial factor to form gels. At low temperature, gellan gum forms an ordered helix of double strands, while at high temperature a single stranded polysaccharide occurs, which significantly reduces the viscosity of the

solution. Low and high acyl gellan requires a temperature value between 75ºC to 80ºC to achieve complete hydration (Huang *et al.*, 2004).

Gellan gum solutions easily form gels under cooling environments, so in the absence of specific cations, low acyl gellan gum forms gels at around 25ºC where high acyl sets around 65ºC. In contrast, if cations are added to gellan gum solutions the setting temperature increases (Flores-Huicochea *et al.*, 2013).

1.2.4. Overview of main applications

Due to its good and unique characteristics, gellan gum is a bacterial exopolysaccharides with big commercial potential for food, pharmaceuticals or biotechnological applications (Banik *et al.*, 2000). As mentioned before, gellan gum is a food additive approved by the main regulatory agency, and present applications as stabilizer, thickening agent, structuring and versatile gelling agent in a wide variety of ailments and can produce gel textures in food products ranging from hard and brittle to fluid texture.

The applications in food industry were the first focus for this polymer, but after 1991 the gellan gum also began to be used in cosmetic applications (Bara and Mellul, 1997), pharmaceutical (Sanzgiri *et al.*, 1993) and biotechnological industry (Groboillot *et al.*, 1994). In 1991, toxicological evaluation of gellan gum was performed, showing that gellan gum was poorly absorbed, and did not cause any death in rats. Studies in drug delivery also showed that there were no adverse effects that could be attributed to chronic exposure to gellan gum (Anderson *et al.*, 1988). In general, toxicological studies show that gellan gum is nontoxic polymer under physiological conditions.

In cosmetic applications, gellan gum can provide effective stabilization and suspension of different formulations, being ideal to products requiring a pseudo plastic rheology. At this moment, gellan gum is being used in lotions and creams, make up, face mask, hair care products, tooth paste and air freshener gels (Laurent and Pasquet , 2013).

Gellan gum has been used in pharmaceutical industry to produce easy-to-swallow solid dosage forms, such as gels and coated tablets, and also to modify the rate of release of active compounds from capsules. The specific gelling properties in different media led to the development of controlled release formulation based on gellan, several solid, semi-solid and liquid formulations have been studied for oral administration (Kubo *et al.*, 2003).

In biotechnology industry, gellan gum has been used as an alternative to agar for microbiological media (Shungu *et al.*, 1983), and for plant tissue culture (Garin *et al.*, 2000). Gels of gellan gum are also used as solid matrix for separation of DNA fragments onto

electrophoresis (Cole, 1999). Also, gellan gum has recently been applied in the development of a new matrix for ionic exchange chromatographic approaches (Gonçalves *et al.*, 2014).

Recently, gellan gum has taken the first steps in biomedical research, being proposed for cartilage tissue engineering application. In this field, gellan gum can function as a minimally invasive injectable system, gelling inside the body in situ under physiological conditions and efficiently adapting to the defect site (Oliveira *et al.*, 2009).

A specific study already demonstrated the benefices of using gellan gum gels as biomaterial matrix for spinal cord repair. The gellan gum modified with fibronectin, derived synthetic peptide, was used to improve cell survival after transplantation, as provide an ideal environment under physiological condition (Silva *et al.*, 2012).

Moreover, gellan based materials are now investigated in the field of gene delivery. Briefly, gellan gum was blended to branched polyethylenimine, for partial neutralization of its positive charge excess of polyethylenimine, with the aim to form nanocomposites. Subsequently, the nanocomposites formed were tested for transfection techniques and it was found that they had better efficiency when transfecting primary cells of mouse skin, when compared to transfection only with branched polyethylenimine or lipofectamine. Nanocomposites were also able to protect the plasmid deoxyribonucleic acid from nucleases and serum proteins present in the blood (Goyal *et al.*, 2011).

A new composite film made of microbial gellan gum was firstly used as an immobilization matrix to entrap proteins in order to study its bioelectrochemical properties. The results demonstrated that the hemoglobin molecule in the film kept its native structure and showed its good electrochemical behaviour. Therefore, as a novel substrate this kind of composite film offers an efficient strategy and a new promising platform for further study on the direct electrochemistry of redox proteins and the development of the third-generation of electrochemical biosensors (Wen *et al.*, 2008).

1.3. Microcarrier systems

Cell culture techniques have become vital to study animal cell structure, function and differentiation, and for the production of many important biological materials such as vaccines, enzymes, hormones, antibodies, interferons, and nucleic acids.

The cell culture can be classified as anchorage dependent or anchorage independent cells, depending upon the kind of cells in culture. For instance, if cells require attachment for growth, they are designated anchorage dependent cells. The adherent cells are usually derived from tissues of organs, such as kidney, where they are immobile and embedded in connective tissue. On the other hand, if cells do not require attachment for growth or do not attach to the surface of the culture vessels, are designated anchorage independent cells/suspension cells. All suspension cultures are derived from cells of the blood system because these cells are also suspended in plasma *in vitvo*.

Anchorage dependent cells grow in monolayer cultures, a continuous layer of cells, and usually one cell in thickness, which grows at the bottom of the T-flask. Although it is a technique widely used, it has many disadvantages, such as no homogeneity in the medium, so the growth is limited by surface area which may limit product yields. Besides, there is high risk of contamination related to the number of handling steps required to produce a given quantity of cells or products.

A few techniques appear to reduce the disadvantages of monolayer culture, by increasing the production of cell adhesion and its products, as for example, techniques that try to mimesis the suspension culture (Vertrees *et al.*, 2008). Microcarrier culture introduces new possibilities and, for the first time, it is possible high yield culture of anchorage dependent cells. In microcarrier culture, cells grow as a monolayer on the surface of small spheres or as multilayers in the pores of macroporous structures that are usually suspended in culture medium by gentle stirring.

The idea of culturing anchorage dependent animal cells on small spheres, microcarriers, kept in suspension by stirring (figure 6), was introduced by Van Weasel in 1907 to mass produce viral vaccines and biological cell products using mammalian cells (van Wezel, 1967). As can be seen in figure 6, in the initial steps, the cells need to grow in a monolayer culture. After 4 days, the cells can be transferred to rolling flasks and posteriorly into a bioreactor. This scale up increased the cell amount and the production yield. Since the introduction of the first microcarrier, a wide range of commercially available microcarriers have been successfully used for the production of a variety of biological products, at analytical and industrial scale (Martin *et al.*, 2011). The optimal microcarrier for anchorage dependent cell culture still has not been found. An indicator of this fact is the amount of new microcarriers available in market (table 2).

Figure 6 - Simplify design of microcarrier cell culture process.

Table 2 - Overview of microcarrier available in the market and their principal materials and characteristics. + characteristic present, - characteristic not present (Malda and Frondoza, 2006)

Name	*Size*	*Porous*	*Material*
			Dextran
Cytodex 1	147-248	-	Dextran matrix with substituted N,N-dimetylaminoethyl groups
Cytodex 2	135-200	-	Dextran matrix with a surface layer of N,N,N-trmethyl-2-hydroxyaminopropyl groups
Hillex	150-210	-	Dextran matrix with treated surface
			Plastic
Plastic coated	150-210	-	Plastic coated with denatured collagen
PlasticPlus coated	150-210	-	Plastic coated with denatured collagen promoting fast attachment
Biosilon	160-300	-	Polystyrene
Cytoline 2	400-2500	+	Polyethylene and silica
			Glass
Glass coated	150-210	-	Plastic with glass coating
			Cellulose
Cytopore 1	200-280	+	Cellulose
			Gelatin
Cultispher G and S	130-380	+	Cross linked porcine gelatin
			Collagen
Cytodex 3	141-211	-	Dextran matrix with thin layer of denatured pig skin derived
Cellagen	100-400	+	Highly cross-linked bovine collagen type I

1.3.1. General characteristics

Several characteristics have to be analyzed in the development of new microcarriers or in the choice of one type of microcarrier available in the market, such as roughness, rigidity, porosity, superficial charge density, toxicity, transparency and density (Zhou *et al.*, 2013). There are two parameters that control the principal characteristics of microcarriers, the material used in the matrix of microcarriers and the technique used to create them.

Over the last years, many articles describe techniques to develop microcarriers, each one presenting advantages and disadvantages. Thermally induced phase separation technique (Ahmadi *et al.*, 2011) is a technique commonly used. The procedure requires the use of a solvent with a low melting point, in order to be easy to sublime. The phase separation is induced through the addition of a small quantity of water and a polymer-rich and a polymer-poor phase is formed. Following the cooling below the solvent melting point and vacuum-drying to sublime the solvent, a porous scaffold is obtained.

With the technology development, a number of new methods and equipment are available to prepare microcarriers, like ultrasonic atomization method. This procedure can produce microcarriers in different sizes by changing the nozzle diameter, vibration frequency and the flow rate of the polymer.

Electrospray system uses a liquid delivery system and a needle with high electric potential to prepare the microcarriers. Changing the potential difference of inner and outer needles and the flow rates of the polymer solution, different shapes and sizes of microcarriers can be obtained (Hu *et al.*, 2013).

Emulsion technique is also a common method used to produce spheres and microcarriers. Emulsion is one of the methods more studied and it has been used because of the easy controlling of process parameters. Besides, emulsion techniques do not require expensive and complex instrument. This process is divided into two major parts, emulsification and removal steps. The first step, the emulsion or multiple emulsions, consists in a mixture of two or more liquids that are normally immiscible. The water-in-oil single emulsion method is the most used in the microcarrier preparation due to its simplicity. Briefly, the polymer is dissolved in water to form a polymeric solution, then is dropped into an oil solution and mixed in order to obtain an emulsion. Before the two phases are able to separate, the mixture is transferred into a solution that promotes the stabilization and reinforces the polymer solution to form solid structures. Then, the second step is oil removal phase, performed by centrifugation or suction (figure 7). After, the formed structures are washed and freeze-dried (Brun-Graeppi *et al.*, 2011).

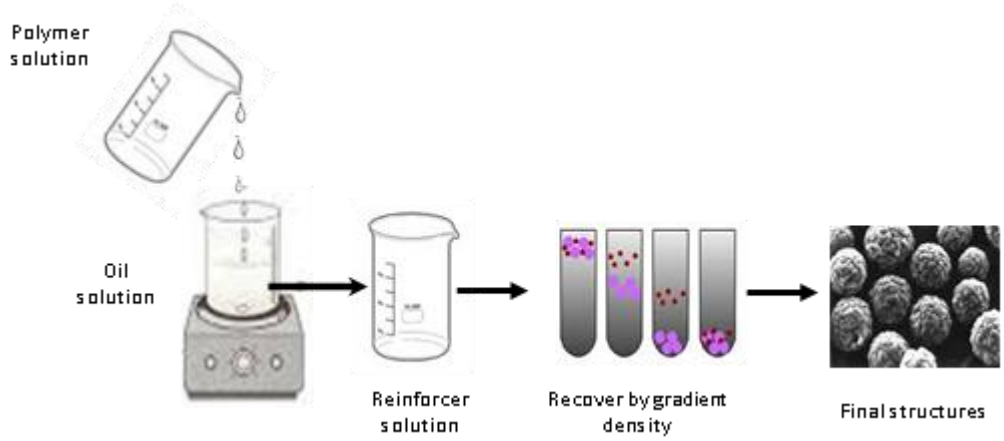

Figure 7 - Simplify design of water in oil proceed

In water-in-oil emulsion procedure, the characteristics of the final structure can be adjusted by changing factors in the water in oil process, such as the ratio between emulsifier and polymer, the type of emulsifier or polymer used, the type of stirring, and the speed, temperature and time of stirring. Also the solution used to stabilize and reinforce the microspheres is very important in the emulsion process (Brun-Graeppi *et al.*, 2011).

As aforementioned, the material used for the microcarrier development is a crucial factor. When selecting a material for microspheres fabrication, the chemical composition, physical and mechanical properties of material, mechanism and rate of biodegradation, toxicological profile, and regulatory history, have to be considered (Hu *et al.*, 2013).

Several materials listed in table 2 can be used in the development of microcarriers, such as dextran, cellulose, plastic, polyethylene and silica, suffer specific modifications in order to improve the microcarrier final characteristics (Zhou *et al.*, 2013). Dextran is a widely used polymer for tissue engineering applications since has good capacity for cell adhesion due to the non-ionic stability (Boo *et al.*, 2011). However, microcarriers based in dextran, until the present date, do not show a macroporous structure. This can be a drawback since this type of microcarriers does not allow good protection of cells and neither the formation of ideal microenvironments for anchorage of cells (Malda and Frondoza, 2006).

Recently, new materials have come to be used in the development of new microcarriers, for example the poly(lactic-co-glycolic acid) PLGA (Chun *et al.*, 2006) and poly(l)lactic acid PLLA (Tan *et al.*, 2010). There are two promising materials for the microcarrier development, because of safety and FDA approval for human application and the low market cost. Also some exopolysaccharides appear as new microcarriers, being the deacetylated gellan gum the most used (Wang *et al.*, 2008) due to the stability of gels. However, they present the drawback to be anionic polymers that form structures with negatively charge, decreasing cell adhesion. So many alterations to the polymer have been researched to improve cell adhesion,

for instance gelatin (Wang *et al.*, 2008), or by peptide modification (Silva *et al.*, 2012) or even by surface modification (Coutinho *et al.*, 2010).

1.3.2. Benefits and drawbacks

Microcarriers provide the ideal surface for growing anchorage dependent cells, increasing the number of viable cells in the culture when compared to standard monolayer culture vessels (Nor *et al.*, 2010). The most suitable application involves the production of large quantities of cells, viruses and cell products. Additionally, studies based on differentiation, cell proliferation, and uptake studies of labelled compounds and screening of drugs has been made based in microcarrier cultures (Justice *et al.*, 2009).

The principal benefit of using microcarriers is the increased production capacity when compared with traditional cell cultures. Since microcarriers have a very large surface area, they can enhance cell growth without having to resort to bulky equipment and tedious methodology. For an equal quantity of cells produced or cell products, the microcarrier culture, need much less space than other types of monolayer culture (Chu and Robinson, 2001). Once they need less space, also smaller volumes and fewer culture vessels are required. Besides, allows excellent control of culture parameters like pH and gas tension, leading to enhanced cell growth.

In cell culture, the risk of contamination is related to the number of handling steps, opening and closing culture vessels is required to perform the necessary procedures to allow the cell growth. In microcarrier culture, the number of handling steps is reduced, and so the contamination risk is also reduced when producing a large quantity of cells.

The harvest stream downstream process becomes simpler especially for larger scale production. Also, the monitoring and sampling microcarrier of culture is simpler than with any other techniques used to produce large numbers of anchorage dependent cells (van Wezel, 1967).

The use of macroporous microcarriers provides further advantages additionally to those already described. The macroporous microcarriers protect cell from bumps against the stirrer, especially at large scale production (Tharmalingam *et al.*, 2011). Macroporus structure allows the development of a microenvironment, where cells can exchange their own autocrine growth hormones, allowing the use of protein free medium, and thus, a considerable reduction of cost in cell culture can be possible (Kato *et al.*, 2003). If cells create their own microenvironment, better oxygenation is made and they are able to tolerate more chemical stress promote by lactate and ammonia levels.

On the other hand, some microcarriers have to be washed and stabilized first, for the successful of the final application. In scale up process, the harvesting of cells from microcarriers, especially from macroporous microcarriers, is more complex and harder than monolayer culture expansion. Also due to the higher cell density, cell enumerations are more difficult. Additionally, due to the higher density of cells, it is extremely difficult to efficiently infect all cells simultaneously in transfection techniques using microcarriers. Large microcarriers with small pores may restrict the diffusion of nutrients to some cells. Succinctly these are the main drawbacks of microcarrier cell culture systems (Warnock and Al-Rubeai, 2010). To overcome these problems, the development of new microcarriers is needed, with new characteristics and built using new materials and different technologies.

1.4. Aims of the work

The main goal of the present work is to biosynthesize, for the first time, a microcarrier from high acyl and low acyl gellan gum. In order to achieve this goal, the project was divided in two main topics.

The first topic consists in the production and recovery of gellan gum polymer in an acetylated form. For that purpose, it was evaluated the best fermentation procedure to produce gellan gum polymer. Thereafter, a recovery method that achieves high amounts of high acetylated form of gellan gum with satisfactory purity degree was also tested.

The second topic of the work is achieving the best conjugation of high acyl and low acyl form of gellan gum polymer to develop a new microcarrier. This step was performed by using the commercial gellan gum in high acyl and low acyl forms. Firstly, an experimental design to achieve the possible combinations of high acyl gellan gum with low acyl gellan gum was created. The water-in-oil technique was the procedure chosen to develop the microcarrier structure. After all the assays accomplished, the microcarriers were evaluated for swelling behavior and porosity degree. Finally, the microcarrier chosen was tested in cell culture.

Chapter 2 – Materials and methods

This section is divided into two broad subsections. Section 2.1 describes methods and materials applied in production, recovery and purification stage of biotechnological high acyl gellan gum. Section 2.2. describes the development and evaluation of the new microcarrier constructed with gellan gum.

2.1. Biotechnological gellan gum

This topic is related to materials and methods used in this work with the aim to produce and recover gellan gum in acetylated form. It was divided in three subsections. Subsection 2.1.1 is related with the production of gellan gum by fermentation of *Sphingomonas paucimobilis* ATCC 31461. Subsection 2.1.2 is dedicated to the methods used for the recovery of gellan gum. The characterization procedures, executed to evaluate the type and purity of gellan gum, are described in subsection 2.1.3.

2.1.1. Biosynthesis

With the aim to find the best method to produce gellan gum, three different procedures were evaluated. The next three topics briefly describe the procedures done for each fermentation, which have already been described in the literature.

2.1.1.1. Procedure 1- YPG medium with complex mineral solution

This strategy was previously described by Kanari and coworkers (2002). Briefly, the growth of *Sphingomonas paucimobilis* ATCC 31461 in a chemically defined YPG medium is promoted. This medium contains 3 g/L yeast extract, 5 g/L peptone, 3 g/L NaCl, 20 g/L glucose, and 20 g/L agar, and was supplemented with the addition of 1% of mineral solution. The mineral solution was composed by, 1.8 g/L of $MnCl_2 \bullet 4H_2O$, 2.4 g/L of $FeSO_4 \bullet 7H_2O$, 0.283 g/L of H_3BO_3, 0.0027 g/L of $CuCl_2$, 0.0021 g/L of $ZnCl_2$ and 0.007_4 g/L of $CoCl_2 \bullet 6H_2O$.

Firstly, the bacterium was grown in solid agar and was incubated for 48 h at 30 ºC. Then, to promote the adaption of the *Sphingomonas paucimobilis* to the liquid medium, a loop of *Sphingomonas paucimobilis* ATCC 31461 was transferred to a 250 mL Erlenmeyer with 100 mL of fermentation medium. The culture was maintained for 24 h in an orbital agitation at 30 ºC under 200 rpm. Then, 10% of the 24 h old-fermentation was transferred into 500 mL flasks containing 90 mL of fermentation medium in order to initiate the growth of the bacteria for gellan gum production. The fermentation was maintained in an orbital shake at 30 ºC under 200 rpm for 48 h.

2.1.1.2. Procedure 2 –YPG medium with simpler mineral solution

This strategy was previously described by Wang and coworkers (2006). Briefly, the strain was firstly grown in solid culture, incubated for 48 h at 30 ºC with YPG medium containing 3 g/L yeast extract, 5 g/L peptone, 3 g/L NaCl, 30 g/L glucose and 20g/L agar. After 48 h, one loop of bacteria was collected and transferred to 250 mL Erlenmeyer flasks containing 50 mL of the production medium. The production medium is composed by 1 g/L Na_2HPO_4, 10 g/L K_2SO_4, 3g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract, 5 g/L peptone, 3 g/L NaCl, and 30 g/L glucose. Pre fermentation was placed in orbital agitation under 200 rpm at 30 ºC for 24 h to promote bacterium adaptation to liquid medium. The next step was the transfer of 10% of the volume into 500 mL flasks containing 90 mL of the production medium. Finnaly, the Erlenmeyer flasks were incubated at 30 ºC, under 200 rpm for 48 h. This fermentation was made to promote the bacterium growth and gellan gum production.

2.1.1.3. Procedure 3 –S-medium with simpler mineral solution

The fermentation procedure was described by Fialho and coworkers (1999). Briefly, the *Sphingomonas Paucimobilis* ATCC 31461 was maintained in agar containing S-medium, which contained 10 g/L of Na_2HPO_4, 3 g/L of KH_2PO_4, 1 g/L of K_2SO_4, 1 g/L of NaCL, 0.2 g/L of $MgSO_4.7H_2O$, 0.01 g/L of $CaCl_2$, 0.001 g/L of $FeSO_4.7H_20$, 1 g/L of Casamino Acids, 1 g/L of yeast extract, 20 g/L of glucose, and 20 g/L of agar. The solid culture was maintained for 48 h at 30 ºC in an incubator. Posteriorly, a loop of bacterium from the solid culture was transferred for a culture with 100 mL S-medium and placed in 250 mL shake flasks overnight. This culture was incubated in orbital agitation at 30 ºC, under 250 rpm, for the adaptation of the bacterium to the liquid medium. After, the culture was centrifuged at 10 000 g and the pellets were resuspended in fresh S-medium in order to obtain an optical densities (OD) of 0.2±0.01. *Sphingomonas paucimobilis* was then grown in 500 mL shake flasks with 250 mL of S-medium and incubated at 30 ºC under 250 rpm for 48 h.

2.1.1.4. Monitoring growth techniques

To evaluate the growth of the bacteria and the production of gellan gum, aliquots of 5 mL (under intervals of 6 h) were collected, under sterile conditions. The aliquots were then centrifuged and the pellet and supernatant were stored.

To access the cell growth, the aforementioned stored pellet was resuspended in a solution of NaCl 0.8% (m/V) and OD was measured using a spectrophotometer at 640 nm wavelength (Fialho *et al.*, 1999).

For the evaluation of gellan gum production, 5 mL of acetone were added to the obtained supernatant. The solution obtained was maintained for 24 h with agitation to improve gellan

gum precipitation. The pellet formed was dried in an incubator set up to 50 ºC for 8 h and the dry pellet was then weighted in analytical balance in order to asses gellan gum mass.

2.1.2. Clarification and recovery

At the end of the fermentation procedure, the gellan gum should be recovered, in a high purity form. Therefore several steps for the clarification and recuperation of gellan gum were tested.

First, to the remove of bacteria content were analyzed alkaline treatment and centrifugation steps. To the remove of impurities were analyzed the ammonium sulfate precipitation and dialyze. After the removal of bacterial content and imputities, suitable methods for the precipitation of high acyl gellan gum were evaluated. The methods tested were the organic solvent precipitation method and filtration procedure. To improve the purity of high acyl gellan gum, ammonium sulfate precipitation was also tested.

2.1.2.1. Removal of bacterial content from the fermentation broth

The first method is the most described in literature and uses a step of alkyine treatment. Briefly, after the fermentation procedure, the fermentation broth was first heated in boiling water for 15 min to decrease the viscosity and then was cooled at room temperature and the pH was adjusted for 10.0 by adding 2 M NaOH. Once again, the broth was heated in boiling water for 10 min, cooled at room temperature and pH was next adjusted for 7.0 by adding 2 M H_2SO_4 (Kang and Veeder, 1982). Finally, the solution was twice centrifuged at 10 000 g for 45 min and a precipitation of the supernatant with 1:1 isopropanol was performed in order to recover the gellan gum. The pellet was stored for further analysis.

The second method used for the removal of bacterial content from the fermentation broth do not uses the alkyl treatment. Briefly, fermentation broth was immediately transferred to centrifuge tubes and twice centrifuged at 10 000 g for 45 min, in order to remove all bacterial content from the broth fermentation. Then 1:1 isopropanol was added to the supernatant in order to promote the precipitation of the polymer. The pellet formed was stored for further evaluation.

2.1.2.2. Removal of impurities from the free-bacteria supernatant

After the treatment used to remove the bacterial content of fermentation broth, the elimination of some interferences was necessary, in order to increase the gellan gum purity. With this purpose, ammonium sulfate method and dialysis technique were tested.

Briefly, the supernatant without bacterial content was treated with ammonium sulfate (1.5, 2, and 2.5 M of ammonium sulfate). To achieve the desired concentration, the respective

ammonium sulfate amount was directly added to the supernatant volume recovered from the broth centrifugation. Then, each sample was maintained in agitation for 30 min at 4 ºC, and after was centrifuged at 10 000 g for 30 min. In spite of, the pellet and the supernatant were stored and treated separately. However, both samples were treated with isopropanol in the proportion 1:1 and maintained in agitation at 4 ºC. After 24 h, the obtained pellets were stored for further analysis to evaluate the purity of the sample.

The dialysis technique was performed using dialysis tubing with a molecular mass cut off of 14 000 daltons, as described in the literature for purification of low acyl gellan gum (Jin H et al., 2003). The bacterium-free supernatant was loaded into dialysis tubing. The dialysis tubing was placed into an external chamber with deionized water (10x of the volume of bacterium-free supernatant used). The dialysis was performed for 3 days with five changes of deionized water. Content of the dialysis tubing was then withdrawn and isopropanol in the proportion 1:1 was added to promote the precipitation of the polymer.

2.1.2.3. Gellan gum precipitation

After the removal of bacterial content from the fermentation broth was also necessary to evaluate the best method for the precipitation of the polymer from the bacteria-free supernatant. At this stage the gellan gum precipitation was tested two techniques; organic solvent precipitation method and filtration procedure.

The precipitation of low acyl gellan gum using organic solvents is a very common procedure. In literature is described how different amount and types of organic solvent can influence the precipitation of low acyl gellan gum (Kanari *et al.*, 2002). In this work, three organic solvents, acetonitrile, acetone, isopropanol and ethanol, were evaluated for the precipitation of high acetylated form of gellan gum. The technique is very easily performed, at the bacteria-free supernatant recovered from centrifugation of fermentation broth was added one of each organic solvent tested in the proportion of 1:1. After addiction of each organic solvent into each sample, the mixture was maintained in agitation at 4ºC to improve precipitation of the polymer. After 24h the pellet formed was stored for further analysis to evaluate the purity of the sample.

For the filtration procedure was used bacteria-free supernatant recovered after centrifugation of the fermentation broth. This supernatant was filtered using a 2.2 and 4 µm cut-off cellulose filter with help of a vacuum pump. Both the permeated and the refined were stored for future analysis, to evaluate the purity of the samples.

2.1.2.4. Analysis method for gellan gum produced and recovered

All the recovered gellan gum samples were analyzed by Fourier-transform infrared (FTIR) spectroscopy and some samples with nuclear magnetic resonance (NMR) in order to compare with the commercial high acyl (Kelcogel LT100, CP Kelco, San Diego US) and low acyl gellan gum (Kelcogel AFT, CP Kelco, San Diego US).

The FTIR analysis of gellan gum was made in a FTIR spectrophotometer (Nicolet iS10) (Thermo Scientific, Waltham, USA). The FTIR spectrum were obtained after 164 scans with a spectral range between 500 and 4000 cm^{-1}, at a resolution of 4 cm^{-1}. For FTIR analysis, all of the samples were lyophilized and the resulting powders were placed in contact with a diamond window and spectra were collected. Data was process in the OMNIC Spectra software (Thermo Scientific).

Proton ^{1}H NMR spectroscopy was performed with a model Brüker Advance III 400 MHz spectrometer (Brüker Scientific Inc, USA) at different temperature (25 ºC to 60 ºC). Each sample was diluted in D2O (0.01% and 0.1% and 1%) and heated at 95 ºC. Then, the heated solution was transferred to 5 mm outside diameter tubes, also heated. In the tubes was also added 40 uL of 10 mM sodium 3-trimethylsilylpropanol (TSP). Data was processed by using the TOPSPIN 3.1 software (Brüker Scientific Inc.).

2.2. Gellan gum microcarrier

In this section, the presented work was performed using two forms of commercial gellan gum gifted by CP Kelco Company, San Diego, United States. The commercial high acyl gellan gum and the commercial low acyl gellan gum were used to ensure the desire purity of the polymer for the target application.

In this section, the methods used for the development of gellan microcarrier, the techniques used for characterization, and initial assessment of the adhesion between the cells and the microcarriers are described.

2.2.1. Gellan microcarrier development

This subsection is divided into two parts. Firstly the methods used to perform all the combinations between high and low acyl gellan gum used to develop the microcarrier are described. The second part is related to the techniques used to evaluate the characteristics of microcarriers formed.

2.2.1.1. Experimental design

For the development of a suitable gellan gum the possible combinations between the different amounts of high and low acyl gellan, an experimental design was created. The concentrations of low acyl gellan gum tested were 0%, 1%, 1.5%, 2%, 2.5%, 3%. For the high acyl gellan gum was tested 0%, 0.16%, 0.33%, 0.5%. After the conjugation of these concentrations, an experimental design with twenty eight assays was performed, each one corresponds to a unique microcarrier. In table 3 can be seen all the combinations obtained from the experimental design.

Table 3 - Assays obtained from the experimental design constructed by the conjugation of the different amount of low and high acyl gellan gum.

Microcarrier number	Concentration of high acyl form (%m/V)	Concentration of low acyl form (%m/V)	Final concentration
1	0	0	0
2	0	0.5	0.5
3	0	1	1
4	0	1.5	1.5
5	0	2	2
6	0	2.5	2.5
7	0	3	3
8	0.16	0	0.16
9	0.16	0.5	0.66
10	0.16	1	1.16
11	0.16	1.5	1.66
12	0.16	2	2.16
13	0.16	2.5	2.66
14	0.16	3	3.16
15	0.33	0	0.33
16	0.33	0.5	0.83
17	0.33	1	1.33
18	0.33	1.5	1.83
19	0.33	2	2.33
20	0.33	2.5	2.83
21	0.33	3	3.33
22	0.5	0	0.5
23	0.5	0.5	1
24	0.5	1	1.5
25	0.5	1.5	2
26	0.5	2	2.5
27	0.5	2.5	3
28	0.5	3	3.5

2.2.1.2. Microcarrier built: water-in-oil method

For the cronstructionconstruction of gellan microcarriers, single water-in-oil emulsion technique was used. Briefly, the high acyl and low acyl gellan gum were dissolved in water at 90 ºC. After full dispersion, the solution was quickly dropped into preheated 90 ºC vegetal oil, provided by a local distributor. The mixture was maintained in agitation at 990 rpm for 10 min. Then the emulsion was quickly transferred to 300 mL of 2% $CaCl_2$ solution and was maintained in agitation for 15 min, to reinforce the microcarrier structure (Wang *et al.*, 2008). To recover of the microcarriers produced, one centrifugation at 2 500 g for 15 min was performed and the pellet (microcarriers) were washed several times with PBS 1X. The amount of microcarriers produced was then lyophilized overnight, and stored for future analysis.

2.2.2. Characterization

To complete the aforementioned experimental design, two characteristics to evaluate the microcarriers built were defined. The characteristics defined were the swelling capacity and the porosity degree. In this section are described the methods used for each evaluation.

2.2.2.1. Swelling capacity

This procedure was done for the microcarriers in lyophilized form and in hydrated form. To achieve the hydrated form of the microcarriers, the microcarriers were mixed with PBS 1X, for 24 h in 37 ºC incubator. The microcarriers were chosen randomly and placed over microscope slides for visualization under inverted optic microscopic Axio Observer, Carl Zeiss). To determinate the diameters of each microcarrier, the AxioVision 4.8 software (Carl Zeiss MicroImaging GmbH, 2010) were used. After all diameters measured, the swelling capacity was calculated using the next correlation:

$$\text{Swelling capacity} = (\text{Hydrated diameter} - \text{Lyophilized diameter})$$

2.2.2.2. Porosity assessment

The porosity was studied by mercury porosimetry. It is a well-known technique that gives access to the size distribution of pores in solids, by applying various levels of pressure to a sample immersed in mercury. The pressure required to intrude mercury into the sample pores is inversely proportional to the size of the pores (Román *et al.*, 2010). By this technique, it is possible to obtain the total pore volume, the percentage of interstitial porosity, pore volume distribution by pore size and total pore area. In this study, a mercury micro porosimetry 9500 PoreSizer V1.07 (Micrometics, USA) was used. All the microcarriers were analyzed in a lyophilized state.

Specifically, the lyophilized microcarriers were loaded into a penetrometer and sealed. Then the penetrometer was placed at low pressure port, where the microcarriers are evacuated to remove air and moisture. The penetrometer was then backfilled with mercury. Excess of mercury is drained back, and only a small amount remains in the penetrometer. As pressure on the filled penetrometer increases, mercury intrudes into the microcarrier pores, beginning with those pores of largest diameter. The pressure range applied in this task was between 0.10 psia to 45 000 psia. The data collected allow to assess the porosity degree by the total pore area measure.

2.2.2.3. Scanning electron microscopy

Also scanning electron microscopy (SEM) analysis was performed in two microcarriers to visualize the microcarrier morphology. For that purpose, the lyophilized microcarriers were placed on a metallic support with a thin adhesive tape and then coated with gold under vacuum using an Emitech K550 sputter coater. The surface was scanned and photomicrographs were obtained with a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope with an acceleration voltage of 20 kV, at several magnifications degrees.

2.2.3. Microcarrier culture trials

After the evaluation of all microcarriers developed, some microcarriers were chosen to further cell culture assay. With the aim to verify if gellan gum microcarriers are suitable for microcarrier cell culture and if the total pore area of each microcarrier affects the adhesion and proliferation of cells, two microcarriers for cell culture were chosen, one with high level of porosity and the other with low level of porosity.

This section was divided in two parts; the first one describes the procedure used in cell culture with microcarriers. The second one depicts the methods to monitor the microcarrier culture.

2.2.3.1. Cell seeding

In this work, COS-7 cell line, derived from the kidney of the African Green Monkey, *Cercopithecus aethiops*, was used. Firstly, COS-7 (passage 7 to 10) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 1.5 g/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin, and they were maintained at 37 ºC in a humidified atmosphere of 5% CO_2. Cells were culture until a confluence of 90% was reached.

For microcarrier culture, gellan microcarriers were firstly sterilized with UV light for 30 min and fully hydrated with PBS 1X solution. Before 24 h until use the microcarrier were stabilized

in DMEM with the aim to reduce the shock with cells. The cell seeding procedure was conducted in 6- and 24-wells culture plates. The bottom of 6 well tissue culture plates were covered with gellan microcarriers and 250 000 cell/mL, freshly trypsinized, and were incubated for 24 h. After that, the unattached cells were removed by aspiration of the medium, the gellan microcarriers laden with cells were transferred to 96-weell and incubated with fresh medium. This procedure was repeated every day for 3days. The cultures were monitored by visualization of the plate every day in inverted optic microscope (Axio Observer, Carl Zeiss).

2.2.3.2. Cell visualization

For a better visualization of cells in microcarrier before cell seeding on microcarriers, cells were stained with neutral red (Repetto *et al.*, 2008).

All the solutions used were prepared immediately before their use to improve stability and avoid precipitation of the compound or medium proteins. All the solutions were sterilized through a 0.22 µm filter to ensure aseptic conditions.

Firstly, a neutral red stock solution (4 mg/mL) was prepared by dissolving neutral red dye in PBS 1X. This solution was dilute 1:100 in DMEM to prepare neutral red medium.

In cell cultures with confluence of 65%, the DMEM medium was removed and neutral red medium was added. After 24 h, the COS-7 cells incorporated the neutral red and retained the staining over several days.

2.2.4. Microcarrier surface evaluation

To evaluate the surface charge of the microcarriers used in cell culture trials, an assay with (Bovine serum albumin) BSA, at different pH values, was performed. The microcarriers have positive or negative charged by the capacity they have to capture the positive or negative form of BSA.

This section is divided in two topics, one with the description of the protocol used to adsorption and release of BSA and the other one describes the method used for quantification of the BSA released.

2.2.4.1. Adsorption and release of BSA from microcarriers

Three different solutions of BSA in sodium acetate buffer (0,2M) were prepared with different pH values taking into account the isoelectric point of BSA (4.7). Therefore, it was prepared a BSA solution (2 mg/mL) at pH 3.7, 4.7 and 5.7. At these three pH, BSA can be charge positively or negatively to promote or not more surface adsorption. Therefore, pH lowers

than 4.7 should favor the positive charge of the BSA and pH higher than 4.7 should promote the negative charge of this protein.

For the adsorption assay, the microcarriers were placed in 48 well plates and stabilized in sodium acetate buffer with the work pH. After 4 h, the buffer was removed and the solution of BSA in sodium acetate buffer was added. The microcarriers were maintained at room temperature. The solutions were replaced every hour.

After 4 h of BSA adsorption, the BSA solution in sodium acetate buffer was completely removed. To start the assay for releasing BSA the microcarriers with working pH of 3.7 was only added sodium acetate buffer at pH of 3.7, 4.7 and 5.7. The same procedure was made for the microcarriers at working pH of 4.7 and 5.7. The release of BSA from microcarriers was evaluated for 4 h with change of the sodium acetate buffer solution every hour, and the removed solution was stored at 4 ºC for analysis.

2.2.4.2. Quantification of BSA released

With the aim to measure the BSA released by microcarriers, the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA) was performed with the samples obtained during adsorption and release trials. Briefly, BCA assay is a method for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+} by a protein in an alkaline medium (the biuret reaction) with a highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}), by using a unique reagent containing bicinchoninic acid. The purple-stained reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. The water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/mL).

For this assay, standard samples of BSA were prepared to build a calibration curve and to quantify the unknown samples stored from the BSA release assays.

Briefly, 25 µL of each standard or unknown sample were pipetted into a 96 -microplate well, and then 200 µL of working reagent was added and mixed. Thereafter, the microplate was covered and incubated for 30 min at 37 ºC. The microplate was cooled to room temperature and the absorbance was read at 570 nm, by using a microplate reader. The calibration curve obtained can be observed in figure 8.

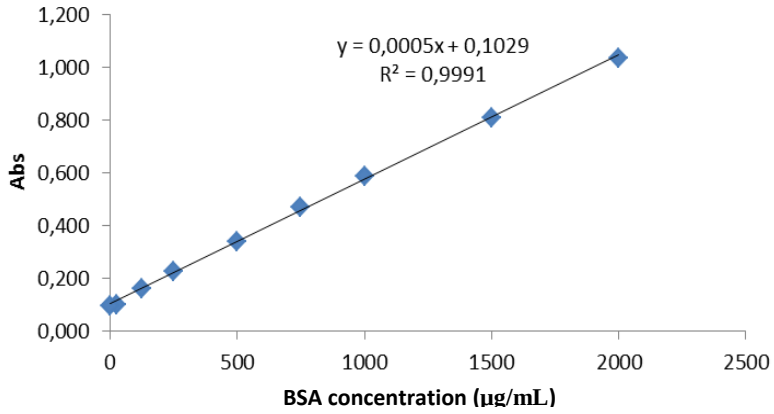

Figure 8 - Calibration curve of bovine serum albumin (20 µg/mL to 2000 µg/mL)

Chapter 3 – Results and discussion

In order to improve the production of large quantities of cells, viruses and cell products, the development of new microcarriers, that allow the growing of anchorage dependent cell and the increase of the number of viable cell in culture compared to standard monolayer culture vessels, are increasingly required (Justice *et al.*, 2009).

Recently, low acyl gellan gum has been proposed to create new structures for cell growth, especially for tissue engineering applications (Ferris *et al.*, 2013). Gellan gum presents good characteristics, like gel strength and transparency, biocompatibility and biodegradability for biomedical application as a sustainable matrix for new microcarrier structure. However, in the case of low acyl gellan gum, no cell adhesion to the surface of the formed structures is obtained, due to the negative charge of this polymer.

In this way the main goal of this thesis is to develop a new microcarrier structure that allows a better cell attachment. Therefore, it was proposed the formulation of a matrix based in the conjugation of low acyl gellan gum with high acyl gellan gum. By choosing these two forms of gellan gum, it was intended to combine the best properties of each form. The low acyl gellan gum forms gels with great strength and transparency but with high negative charge density. The high acyl gellan gum forms fluid gels but, also due to the acyl groups, it allows the formation of structures with lower charge density (Funami *et al.*, 2008).

Therefore, the work was divided in two phases. The first phase had the objective to produce and recover high acyl gellan gum. The second phase uses the high acyl gellan gum in combination with low acyl gellan gum to develop a new microcarrier for cell culture. In this way, the discussion of the work was also divided in two sections, the production and recovery of high acyl gellan gum in section 3.1 and the developing and characterization of a new microcarrier in section 3.2.

3.1. Biotechnological gellan gum

One of the objectives of this thesis was to produce, recover and purify the biotechnological gellan gum with high acyl content. For that purpose, the best fermentation conditions for maximal production of gellan gum was tested, as well as the best method for the recovery of gellan gum in high acyl form was evaluated. So, this chapter was divided into two main topics, one for the production of gellan gum and other for the recovery process.

3.1.1. Biosynthesis

In this section, it will be analyzed the best procedure for the production of high acyl gellan gum by fermentation of *Sphingomonas paucimobilis* ATCC 31461.

Several manuscripts describe modifications of culture condition such as temperature and dissolved oxygen tension. Also growth medium compositions, such as the carbon source or cation concentration, vary from article to article. In order to find the best medium composition, it was evaluated three different mediums already described in literature (Kanari *et al.*, 2002) (Wang *et al.*, 2006)(Fialho *et al.*, 1999). The three procedures were chosen because of their medium differences and because of the high production yield of gellan gum, which were claimed in the respective articles. Kanari and colleagues claimed in their article a production of 8.12 g/L of gellan gum (Kanari B *et al.*, 2002) and this procedure corresponds to the procedure 1. The group of Wang claimed a higher production of 14.75 g/L of gellan gum (Wang *et al.*, 2006), and this procedure correspond to the procedure 2 in this work. In the procedure described by Fialho and colleagues, it was claimed a production of 14 g/L of gellan gum (Fialho *et al.*, 1999), and this procedure corresponds to the procedure 3.

Figure 9 represents the fermentation progress of the three procedures. As it can be seen, the OD measure in the first 6 h was higher for procedure 3. This can indicate that bacteria adapt better to the liquid medium while the fermentation broth was centrifuged to remove the polymer. Only bacteria were transferred to the final fermentation medium, improving the bacterial growth. However, this final step increases the probability of contamination, which leads to a greater caution in the transfer step.

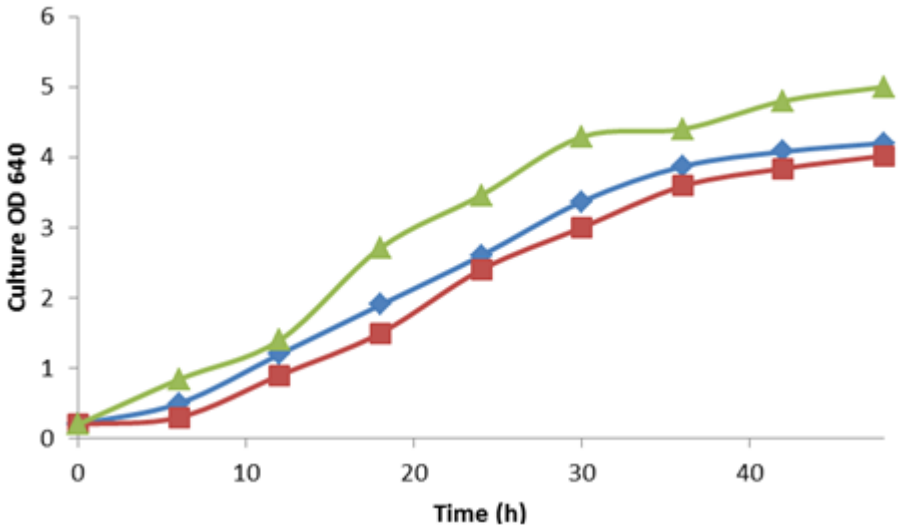

Figure 9 - Microorganism growth for 48 h in relation with the OD measure at 640 nm. Procedure 1 – YPG medium with complex mineral solution (♦), procedure 2 – YPG medium with simpler mineral solution (■) procedure 3 – S medium with simpler mineral solution (▲).

In the procedures 1 and 2, the bacterial growth is very similar; nonetheless the gellan gum obtained is slightly different (figure 10). Procedure 1 allows a higher initial production of gellan gum than procedure 2.

In terms of the total gellan gum produced, procedure 3 achieved the best yield (11.1 g/L) compared to procedure 1 and 2 with yields of 8.9 g/L and 7 g/L respectively. The fact that the procedure 3 has the best yield is in concordance with what it is described in literature

(Fialho *et al.*, 1999). Enzymes used in gellan gum biosynthesis by *Sphingomonas Paucimobilis* ATCC 31461 require low levels of nitrogen source and specific salts that may act as cofactors of these enzymes (Sá-Correia I *et al.*, 2002).

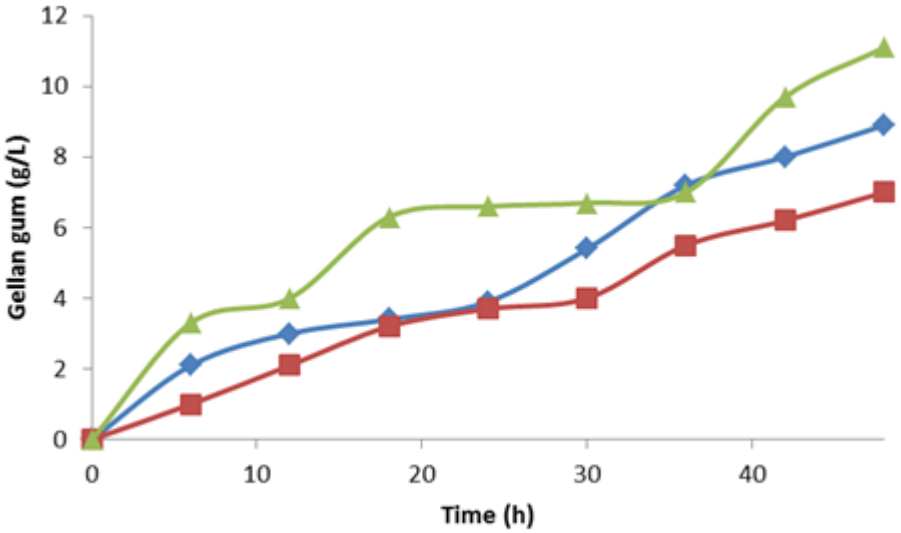

Figure 10 - Gellan gum production (g/L) for 48 h, Procedure 1 – YPG medium with complex mineral solution (♦), procedure 2 – YPG medium with simpler mineral solution (■) procedure 3 – S medium with simpler mineral solution (▲).

When the results obtained in this work are compared to the results described in literature, mentioned above, it can be seen that all results are slightly lower. This fact may be due to the values of gellan gum production in the articles were obtained by bioreactor production. In this work the values for gellan gum production were obtained only by small scale fermentation.

Since the procedure 3 – S medium with simpler mineral solution, show the highest gellan gum concentration, this strategy was then used in the remaining work for producing gellan gum.

3.1.2. Recovery

After these preliminary studies for identification of the best procedure to obtain high yield of gellan gum, the next step to be developed is the gellan gum recovery. With this objective, it was first evaluated the best method for the removal of bacterial content from the fermentation broth. After that, it was analyzed the best procedure to eliminate some interferents present in bacteria-free supernatant to increase the purity of gellan gum recovered. The last step was to evaluate the best method to recover gellan gum directly from free bacteria supernatant. Each of these steps is described in the next subsections.

With the aim of evaluating the samples obtained during the gellan recovery step, all FTIR samples were compared to FTIR commercial samples. In figure 11, it can be seen the FTIR

spectrum of the two forms of gellan gum, commercial high acyl (figure 11, blue line) and low acyl gellan gum (figure 11, red line).

The low acyl gellan gum has five characteristic wavelengths, the broad band centered at 3311 cm^{-1} (figure 11 peak a) due to the O-H stretching vibration, while the peak at 2896 cm^{-1} (figure 11 peak b) is related to the C-H stretching. The peaks at 1608 cm^{-1} and 1379 cm^{-1} (figure 11 peaks d and e) are typical for asymmetric and symmetric COO- stretching mod. Peaks extending between 1000 to 1100 cm^{-1} are assigned to the C-O stretching vibrations due to the carbohydrate back bone.

The high acyl form of gellan gum has the same wavelength peaks of interest and two more additional peaks due to acyl substituents, the C=O of O-acetyl ester bounds that is characterized by a band between 1723 and 1717 cm^{-1}(figure 11 peak c) supplemented by other peak between 1290-1230 cm^{-1}(figure 11 peak f).

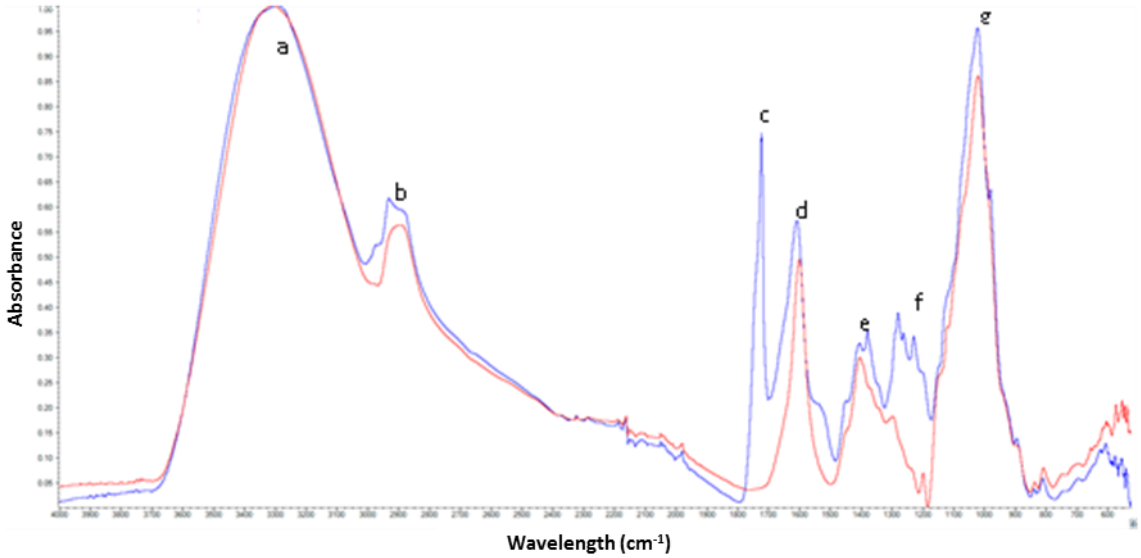

Figure 11 - FTIR spectrum of commercial high acyl gellan gum (blue line), and low acyl gellan gum (red line) with characteristic peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1379 cm^{-1}; f-1290 to 1230 cm^{-1}; g-1000 to1100 cm^{-1}

3.1.2.1. Removal of bacterial content from the fermentation broth

At these stage was used alkaline treatment to disrupt the cells and decrease the viscosity of the broth by changing the high acyl gellan gum into low acyl form. This viscosity reduction helps the removal of bacteria.

As described in material and methods, after the alkaline treatment, a centrifugation step was performed and the resultant supernatant was treated with isopropanol solution (1:1) for the precipitation of gellan gum. The pellet was lyophilized and the FTIR spectrum obtained is present in figure 12.

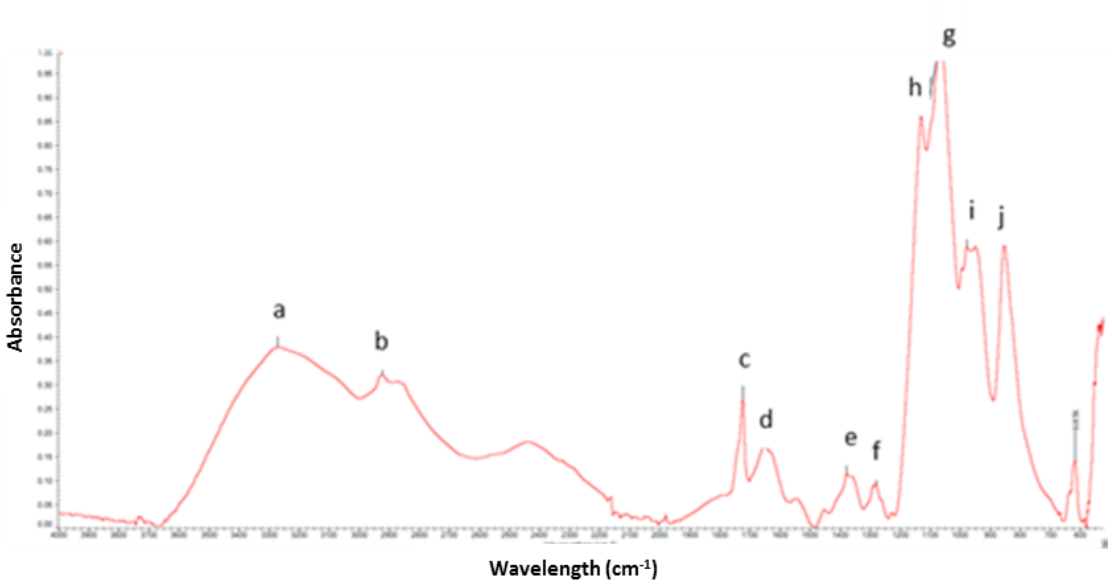

Figure 12 - FTIR spectrum of sample collected by using alkaline treatment with characteristic peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1378cm^{-1}; f-1280 cm^{-1}; g-1000 to1100 cm^{-1};h-1134 cm^{-1};i-970 cm^{-1}; j-864 cm^{-1}

In the analysis of the obtained FTIR spectrum, there are two main observations to be noticed by comparing this spectrum with the commercial samples of figure 11. The first observation is that the spectrum has an increase peak at C-O band (1000-1100 cm^{-1}, figure 12 peak g) differing with the O-H band (3311 cm^{-1}, figure 12 peak a) that is verified in commercial samples. Also some major peaks are not visualized in commercial sample spectrum (Figure 12 peaks h, i and j). One reason for that difference may be the impurities present in the gellan gum recovered from bacterial extract. Other observation that can be made is the presence of characteristic acyl substituent peaks (1724 cm^{-1}, 1378 cm^{-1} and 1280 cm^{-1}, figure 12 peaks c e and f respectively). These peaks were not supposed to appear once the alkaline treatment was made to remove the acyl substituents, so it can be conclude that the acyl treatment was not fully effective.

Since one of the goals of this project is to recover high acyl gellan gum, it is concluded that the alkaline method is not completely suitable for it. The alkyline treatment do not fully converts the high acyl gellan gum into low acyl gellan gum, as described in literature (Kang and Veeder, 1982). Thus, a new procedure to remove the bacterial content from fermentation broth without needing to convert high acyl gellan gum into the low acyl form was applied.

In this new procedure, the fermentation broth was quickly centrifuged at 10 000 g, for 45 min to remove the cells. The supernatant was recovered and precipitated with isopropanol 1:1 and the pellet was lyophilized and analyzed by FTIR. In figure 13 it is presented the spectrum of the sample resultant of this procedure, which exhibits the characteristic acyl substituent peaks (figure 13 peaks c and f). This result is in accordance with what it is described in the literature, since bacteria produce the gellan gum in high acyl form. On the other hand, it can

also be seen the emergence of new peaks (1132 cm^{-1}, 1100 cm^{-1}, 980 cm^{-1}, 853 cm^{-1}), probably due to major contaminates (cells and proteins) arrested in the high viscosity extract.

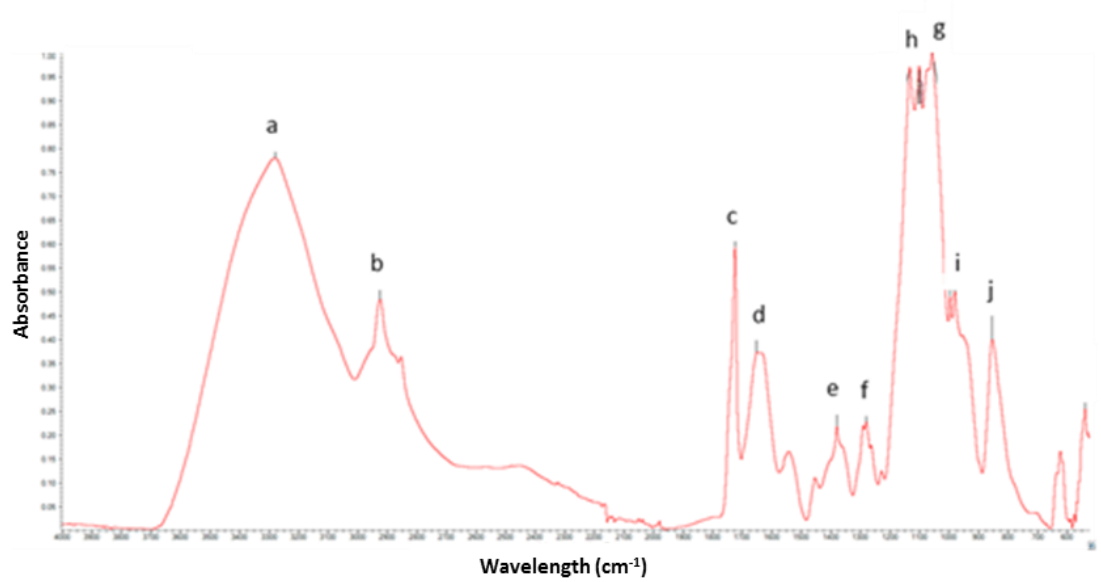

Figure 13 - FTIR spectrum of sample collected by using one centrifugation step and their characteristic peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1378cm^{-1}; f-1280 cm^{-1}; g-1000 to1100 cm^{-1};h-1132 cm^{-1};i-996 to990 cm^{-1}; j-865 cm^{-1}

As it can be observed, the relationship between the C-O band (figure 13 peak g) and the O-H band (figure 13 peak a) is better than procedure previous described (alkaline treatment), but when compared with the commercial high acyl gellan gum sample the ratio is not equal. If the two procedures were compared for the characteristic peaks of high acyl form (figure 13 peaks c and f), the spectrum obtained with centrifugation is more similar to the commercial sample. This can be an indicator that the chains of the produced gellan gum have acetyl substitutes in the correct proportion.

With the aim of remove the possible contaminants, there were evaluated two methodologies, one using dilution of the fermentation broth with equal volume of water and the other by adding another centrifugation step at 10 000g for 45 min of the supernatant recovered from the first centrifugation made in the previous procedure. After this second centrifugation/dilution, the supernatant was treated with isopropanol 1:1 and the pellet was recovered, lyophilized and the FTIR spectrum was obtained (figure 14).

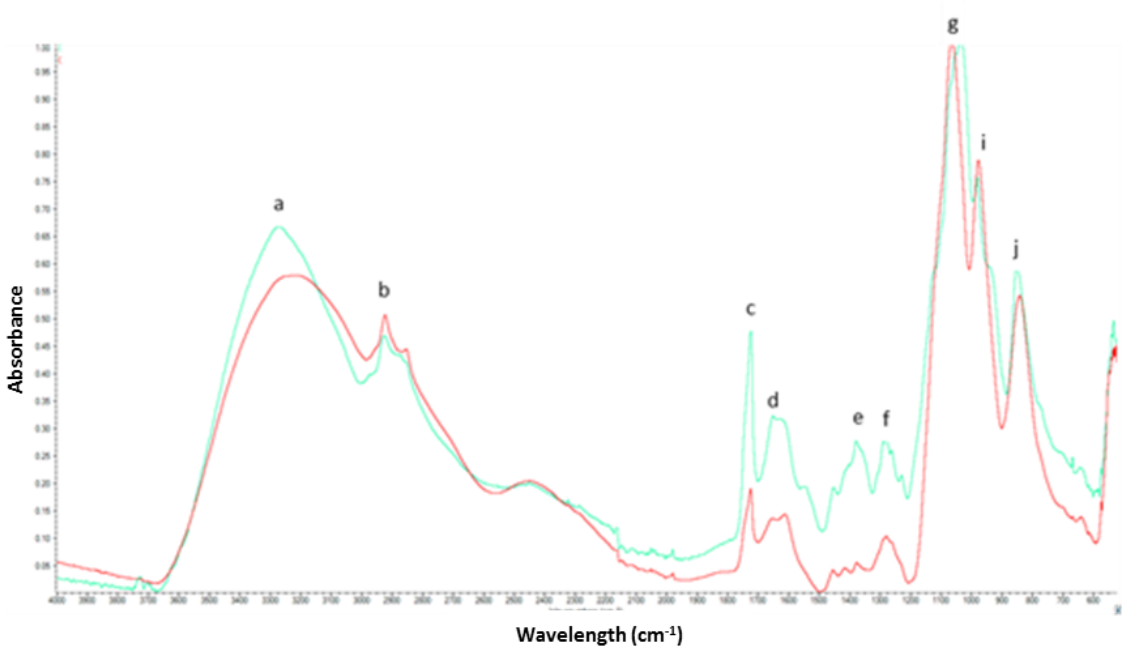

Figure 14 - FTIR spectrum of sample collected by using dilution methodologies (red line) and using two steps of centrifugation (blue line) and their characteristic peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1378cm^{-1}; f-1280 cm^{-1}; g-1000 to1100 cm^{-1};i-980cm^{-1}; j-814 cm^{-1}

As it can be seen in figure 14, some of the non-characteristic peaks present in figure 13 were removed which indicates a greater purity of the polymer. Both methodologies achieve higher polymer purity, probably due to the decrease in the viscosity of the broth caused by the dilution or by the second centrifugation that is used for a better removal of impurities. However, the application of dilution methodology increased not only the volume for treatment but also the volume of organic solvent required for the recovery of the same polymer mass. Furthermore, both spectrum report non characteristic peaks of gellan gum (980cm^{-1} and 814.78cm^{-1}) but with less intensity than in figure 13.

The relationship between the absorbance of each peak is not similar to what is observed in commercial samples, specially the ratio between the O-H band and the C-O band of the diluted sample, which may indicate the presence of interferents that increase the C-O bonds. However, the relation between the sample spectrum peaks recovered after two centrifugation steps (figure 14 blue line) were more similar to the commercial sample(figure 11 blue line). In conclusion, the two steps of the centrifugation method are more suitable to remove bacterial content from fermentation broth.

3.1.2.2. Removal of impurity content of the free-bacteria supernatant

Since the bacteria-free supernatant recovered from the two centrifugation steps contained a high level of impurities, it was necessary to add a new step for the removal of the remaining interferents.

It was known from the literature that the use of ammonium sulfate can be used for the precipitation of proteins. So, several concentrations of ammonium sulfate (1.5, 2, and 2.5 M ammonium sulfate) were tested in order to precipitate possible proteins present in the free-bacteria supernatant.

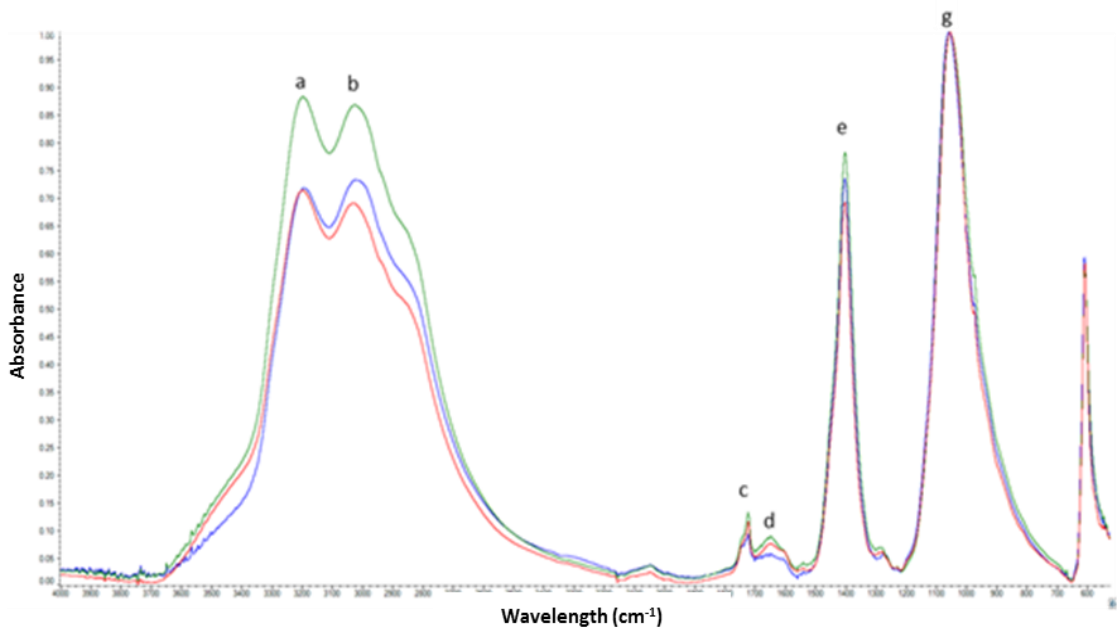

Figure 15 - FTIR spectrum of sample collected by using different concentration of ammonium sulfate, 1,5M-red line, 2M-blue line, 2.5M-green line and their characteristic peaks. a-3311 cm^{-1}; b-3090 cm^{-1};c-1719 cm-; d-1656 cm^{-1}; e-1410cm^{-1}; g-1000 to1100 cm^{-1}

In this strategy it was expectable that only the proteins would precipitate. However it was found that the ammonium sulfate concentration between 1.5 M and 2.5 M also promote the precepitation of gellan gum. This may be due to the aggregation of the chain polymer when the negative charges of the polymer are in contact with the ammonium cation. The formed pellet was lyophilized and was resuspended in 20 mL of acetone and maintained in 4 ºC overnight. Then it was recovered and washed several times with isopropanol, and lyophilized again. As we can see in figure 15 the ions attached to the gellan gum chain do not allow the identification of gellan gum characteristic peaks, so it can be considered that the method failed to remove interferents present in free-bacteria supernatant.

The process of removing the interferents from the free-bacteria supernatant was also evaluated by using a dialysis method. The dialysis was performed during 3 days with five changes of the deionized water. The final solution in the dialysis tubing was precipitated with isopropanol but no precipitate was formed, probably due to the absence of the recovered polymer. This may be owing to the length of the polymer chain produced during the fermentation, which can be smaller than the ones formed in low acyl gellan gum described in literature (Jin *et al.*, 2003). If the chains produced are smaller, the molecular weight of the gellan gum produced is also smaller, meaning that the polymer can easily pass through the dialysis tubing with a molecular mass cut-off of 14 000 daltons.

3.1.2.3. Gellan gum precipitation

Since no procedure was found suitable for the complete removal of impurity content on the free-bacteria supernatant, two additional methods for the precipitation of gellan gum from free-bacteria supernatant were evaluated.

The first evaluated method does not use an organic solvent for the precipitation of high acyl gellan gum. Free-bacteria supernatant was submitted to filtration step with filters that have a diameter pore of 0.22 µm and 0.4 µm. Due to the viscosity of the solution, it was impossible the pass the supernatant trough the 0.22 µm filter, which did not happened in the filtration with 0.4 µm filter. The refined pellet formed in the top of the filter was recovered, as well as the filtered solution. The permeated solution was precipitated with isopropanol and the pellet formed was lyophilized and analyzed by FTIR. The refined solution was only lyophilized. The FTIR spectrums are represented in figure 16.

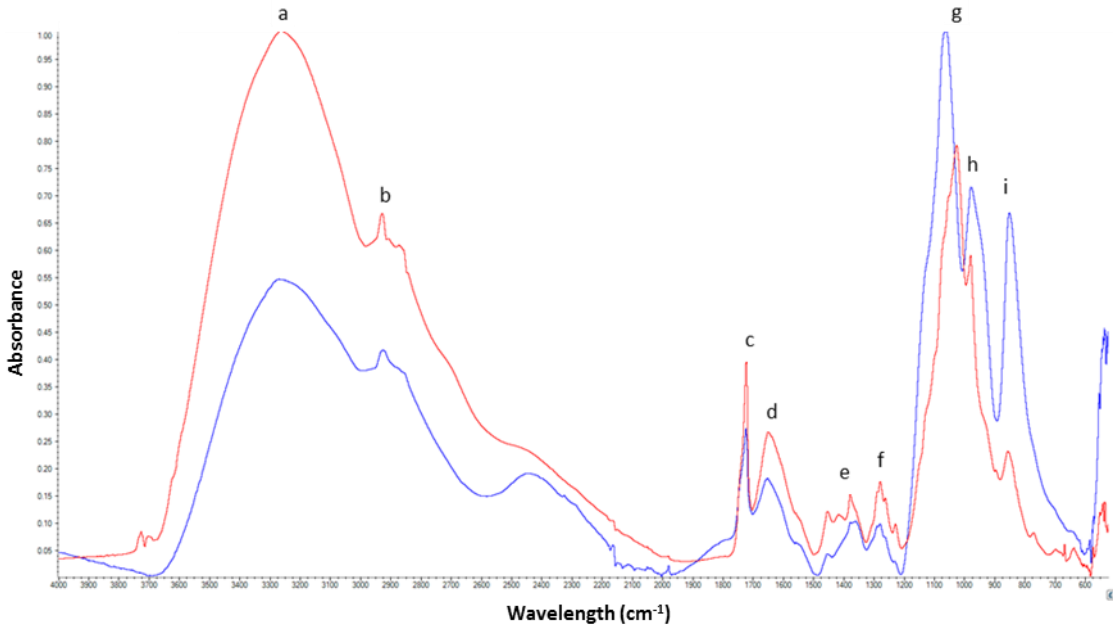

Figure 16 - FTIR spectrum of sample collected from refined solution (red line) and permeated solution (blue line) and their characteristics peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1367cm^{-1}; f-1292 cm^{-1}; g-1000 to 1100 cm^{-1};h-970 cm^{-1}; i-864 cm^{-1}

On one hand, the refined solution has the ratio and the characteristic peaks (figure 16 red line) very similar with the commercial high acyl gellan gum spectrum (figure 11 blue line), which may indicate an increase in sample purity.

In the other hand this process was very slow due to the viscosity of the free-bacteria supernatant. By the analysis of the permeated sample spectrum (figure 16 blue line), it was verified that it also had the characteristic peaks of high acyl gellan gum. So the procedure is not very efficient since it can promote a loss in high acyl gellan gum content during the filtration stage.

Since the gellan gum yields are better using precipitation with organic solvents, such as isopropanol, an evaluation of what is the best suitable organic solvent for the precipitation of high acyl gellan gum from free-bacteria supernatant was performed.

To the ability of isopropanol, ethanol, acetone and acetonitrile to precipitate the polymer, decreasing the interfering peaks, was analyzed. After the removal of bacterial content from broth fermentation, the organic solvent was added to the free-bacterial supernatants. The mixtures were maintained at 4 ºC with agitation for 24 h. The pellets were captured and lyophilized for FTIR analysis.

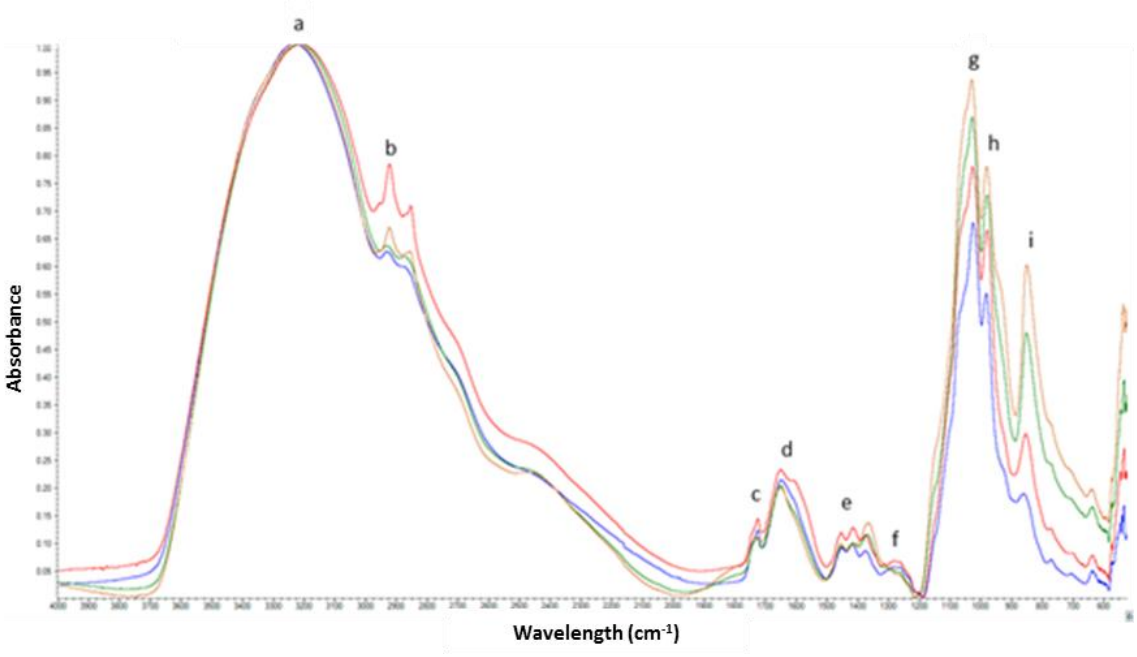

Figure 17 - FTIR spectrums of samples collected after precipitation with different organic solvent acetonitrile-blue line, ethanol-green line, isopropanol-orange line and acetone-red line, and their characteristics peaks. a-3311 cm^{-1}; b-2896 cm^{-1};c-1723 cm-; d-1608 cm^{-1}; e-1367cm^{-1}; f-1292 cm^{-1}; g-1000 to1100 cm^{-1};h-970 cm^{-1}; i-864 cm^{-1}

The resulting spectrums from these assays can be seen in the figure 17. Indeed, it is possible to observe that the relation between the peaks of O-H (figure 17 peak a) and C-O (figure 17 peak g) with the peak of acyl content C=O (figure 17 peak c) are very different than what was seen in the figure 11 (peaks a, g and c, respectively).

This behavior may be due to the non formation of acetylated substitutes in the chain of the polymer that decrease the presence of C=O (figure 17 peak c) bonds, characteristic from the deacetylated gellan gum, or it may be due to the presence of interferents that increase the peaks of C-O band (figure 17 peak g) and O-H band (figure 17 peak a).

In addiction it can be observed the presence of the peaks 970 cm^{-1} (figure 17 peak h) and 864cm-1 (figure 17 peak i) may be influenced by the nature of organic solvent applied. The

acetonitrile is the organic solvent that best removes this peak and for that reason it was chosen as the best organic solvent for recovery of high acyl gellan gum.

Succinctly all the characterization of the recovered samples was done based only in FTIR spectrum. Since the results are only based in one method, it is hard to confirm the gellan gan purity degree. As described in materials and methods, there was an attempt to make ^{1}H NMR, but due to the gel strength formed with the commercial and experimental samples, the results were not possible to be analyzed (figure 18).

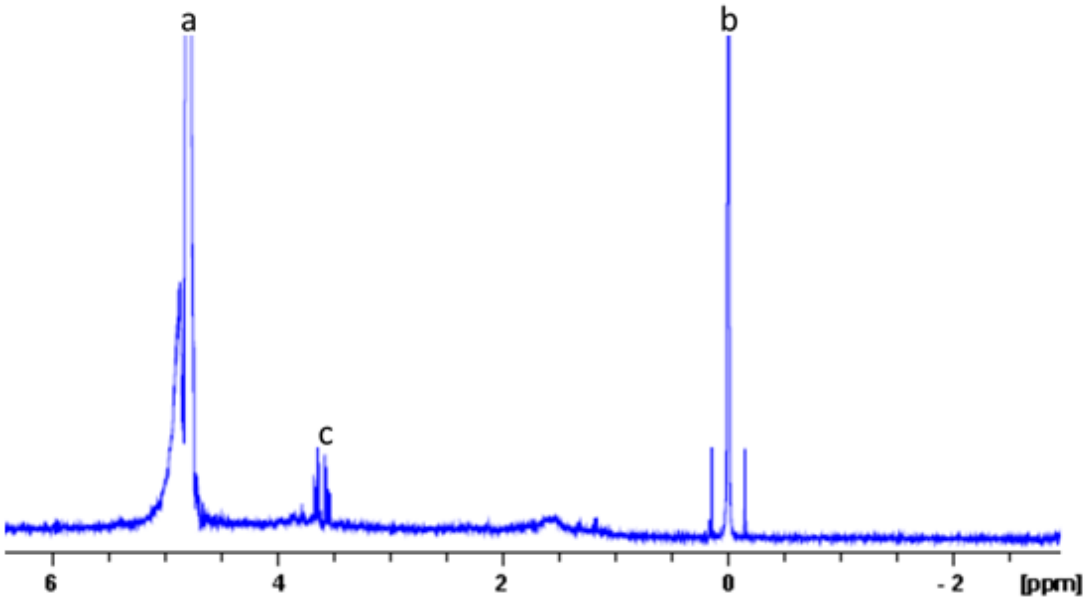

Figure 18 - Representative ^{1}H NMR spectrum of high acyl gellan gum commercial sample (0.1%), and characteristic peaks, a- D_2O peak; b- TSP peak; c- peaks with low resolution.

Also was tried to heat the solutions in the equipment at 60 ºC (maximum temperature allowed) but the gel strength continued to be high not allowing the correct collection of spectrum. It was also tried to decrease the concentration of the polymer used (0.01%, 0.1% and 1%) with the aim to decrease the gel strength of each sample but with a lower concentration no signal was detected.

In literature gellan ^{1}H NMR spectrum is described to be made with 1% (m/V) at 90 ºC but this temperature can not be achieved in the equipment used (Fialho *et al.*, 1999).

3.2. Gellan gum microcarrier

Since it was not possible to recover the high acyl gellan gum from the fermentation broth with a high purity level, the development of the new microcarrier was performed using commercial samples of high acyl and low acyl gellan gum. By using the commercial sample, a high level of purity of the polymer used in the development of the microcarriers was assured with the additional advantage of being more reliable for the replication of the assays.

In this section, it is described in a chronological order the development and characterization of the new gellan gum microcarrier.

3.2.1. Microcarrier development

To achieve a microcarrier with the best characteristics possible, an experimental design that uses the combination of the high acyl with low acyl gellan gum concentration range was proposed. Therefore, this experimental design has two input parameters, the concentration of high acyl gellan gum [0%, 0.16%, 0.33% and 0.5% (m/V)] and the concentrations of low acyl gellan gum [0, 0.5, 1%, 1.5%, 2%, 2.5% and 3% (m/V)]. These concentrations were chosen regarding to what has been described in the literature for gel formation with the high acyl and low acyl gellan gum. All the combinations are represented in table 3 (materials and methods).

The output proposed to evaluate the best microcarrier was the swelling capability and the porosity degree.

The technique chosen for the development of microcarrier structures was the water-in-oil emulsion technique. This technique was chosen, since, it is very easy to perform and the parameters that affect the formation of the structures by this technique have already been extensively studied (Rao and Geckeler, 2011). The only parameter that changed from test to test was the average concentration of each form of gellan gum. All the others parameters, like the ratio between polymer and oil solution, the stirring speed of oil solution and ion solution and the time and temperature of each step were maintained constant in every assay, so they would not influence the results.

During the procedure to develop all the 28 microcarriers, it was noted that when the concentration of low acyl gum is too low, it is not possible to form a final structure (microcarriers number 1, 2, 8, 9 and 15). These results are expectable, since the gellan gum with low acetylation is described to form gels with highest strength.

When the concentration is too high, the solution is so viscous that it is impossible to form structures with round shape, such as it was the case of microcarriers number 27 and 28.

All the other assays run according to the protocol. In figure 19 two examples of microcarriers developed can be seen, one in hydrated state and other in lyophilized state. The image was obtained using an inverted microscopic with lens of 10x magnification.

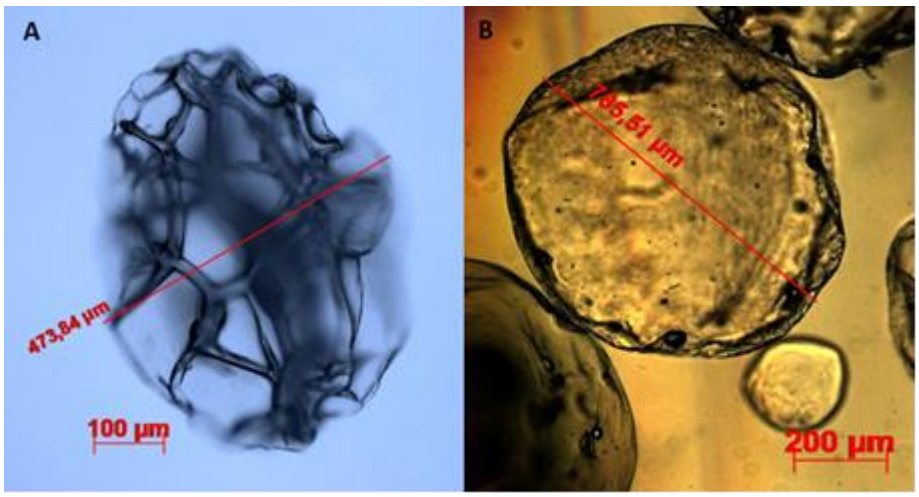

Figure 19 - Represent microcarriers in hydrated state (B) and lyophilized state (A)

3.2.2. Characterization

To assess the swelling capacity of each microcarrier, it was measured the diameter of 10 randomly chosen microcarriers from each assay performed. The diameters of the microcarriers were measured in lyophilized and hydrated state. Through the difference between the lyophilized and hydrated state, it was obtained the swelling capability for each microcarrier developed. These results are shown in table 4.

Table 4 - Results obtained after analyses of each microcarrier developed

Microcarrier number	Diameter of lyophilized microcarrier	Average deviation	Diameter of hydrated microcarrier	Average deviation	Swelling capability (μm)	Total pore area (m^2/g)
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	499.384	89.6852	692.208	79.2432	192.824	0.737
4	456.076	78.4334	644.151	61.9969	188.075	0.255
5	537.056	79.9384	804.889	109.1935	267.833	0.444
6	483.963	63.5273	708.045	87.7045	224.082	0.481
7	598.548	54.8588	925.12	148.913	326.572	0.671
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	340.576	52.3206	606.738	123.4762	266.162	1.152
11	314.918	45.9808	546.837	42.8109	231.919	1.672
12	425.943	57.6663	665.773	91.0053	239.83	0.26
13	625.416	82.2326	1073.7	138.983	448.284	1.121
14	892.465	329.5905	1211.138	233.0868	318.673	0.954
15	0	0	0	0	0	0
16	334.723	53.0753	773.525	120.5135	438.802	0.255
17	383.541	68.7051	570.74	53.056	187.199	0.209
18	1261.439	371.8959	2083.617	274.7847	822.178	1.536
19	348.291	66.5831	764.301	79.0839	416.01	0.231
20	508.176	79.4944	848.4	147.388	340.224	0.567
21	1005.451	109.6243	2037.195	179.1185	1031.744	1.06
22	0	0	0	0	0	0
23	454.365	85.1655	857.636	232.9764	403.271	0
24	471.668	56.0586	673.277	86.1737	201.609	0.355
25	531.058	92.8252	827.119	180.9329	296.061	0.546
26	752.121	115.4733	922.59	133.293	170.469	0.349
27	0	0	0	0	0	0
28	0	0	0	0	0	0

It is important to analyze the diameter measured of each microcarrier. As it can be seen in the figure 20, the size of the microcarriers increases with the final concentration of the polymer used for the formation of the microcarriers. Also the variation of measured diameters (average deviation) increases with the final concentration of gellan gum used. This may be due to the increased viscosity of the solution of gellan gum used in the formation of the microcarriers. It has been found in previous studies (Lorenzo *et al.*, 2013) that the viscosity of the solution increases with the increase of the concentration of high acetylation gellan gum.

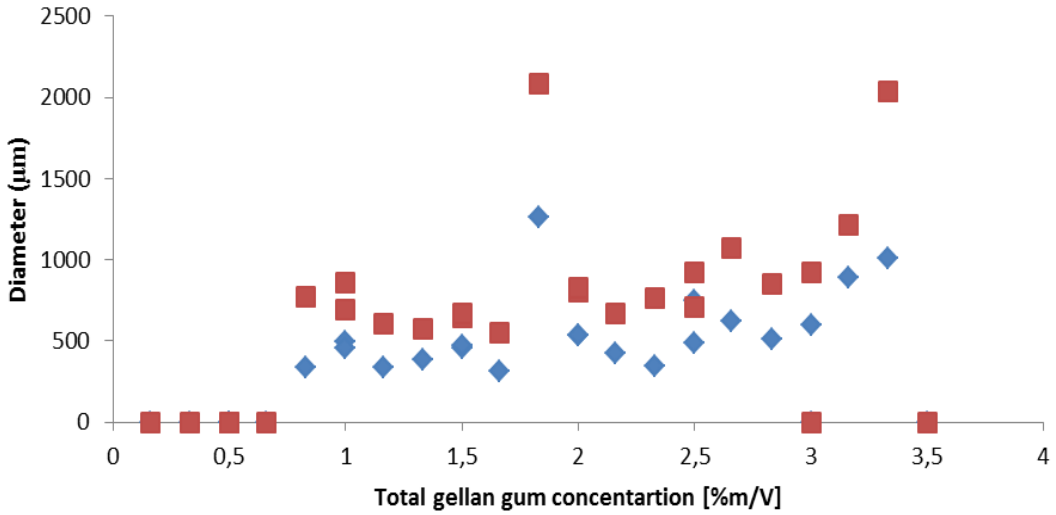

Figure 20 - Relationship between the diameter of each microcarrier in lyophilized sate (♦) and in hydrated sate (■)with the total amount of gellan gum used in their preparation.

Relatively to the swelling ability of the microcarrier, a model to predict the swelling capability through a neuronal network was developed. This evaluation was made in MATLAB software from MathWorks, USA. For the development of the model for the prediction of the swelling behavior, it was used as input the concentration of high acyl and low acyl gellan gum and as output the swelling capability verified for each microcarrier. The neuronal network was built with 5 neurons and was trained with 18 assays, validated with 3 assays and tested with 7 assays. In figure 21 the final model for the prediction of swelling ability of the microcarriers made with conjugation of high acyl and low acyl gellan gum can be seen. The correlation factor between the model and the assays made is only 0.6. This low correlation may be due to the few assays made that do not allow a better validation and test of the model. In other hand, the variation in swelling behavior is described in literature as being mainly influenced by the characteristics of the solution, where the structure is placed, and not by the characteristics of the matrix structure (De Silva *et al.*, 2013).

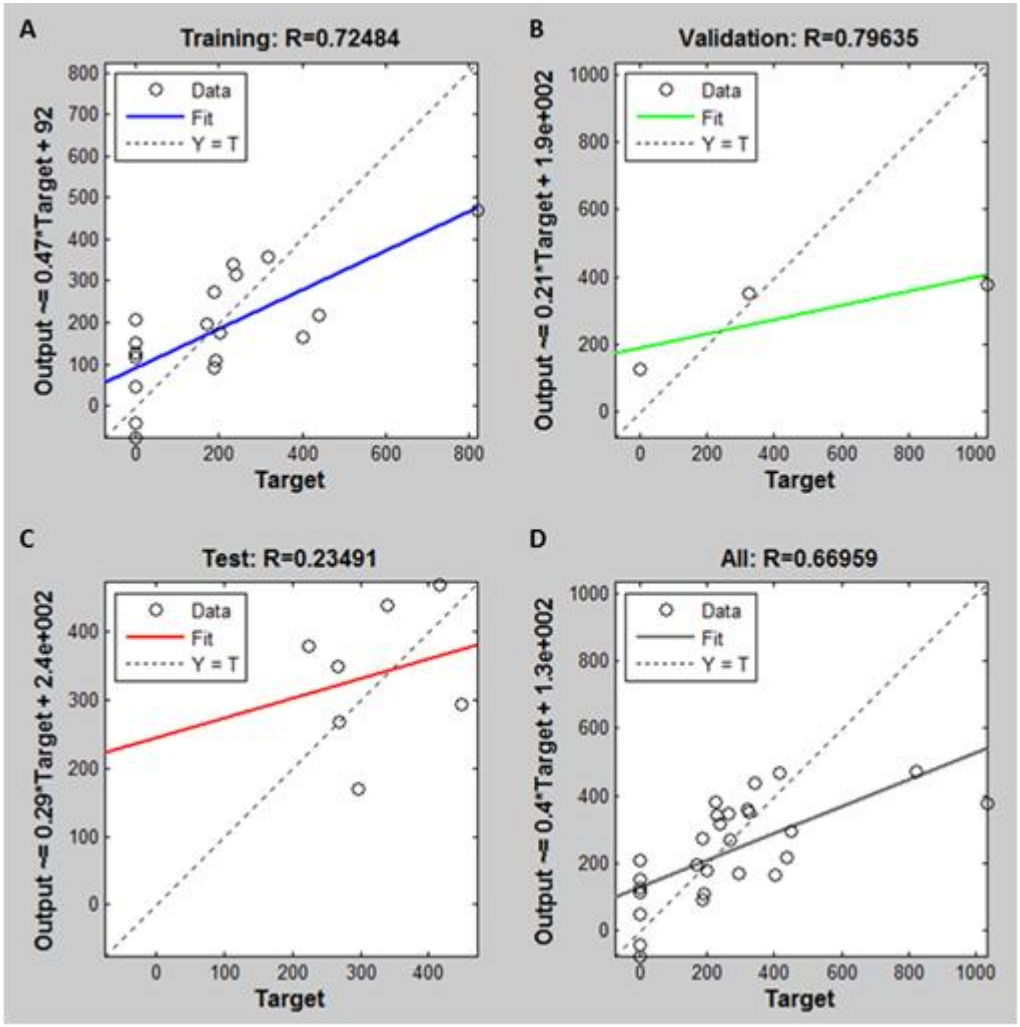

Figure 21 - Model obtained for the prediction of swelling ability of the microcarrier developed with different concentration of high acyl and low acyl gellan gum (D), the training model (A), the validation model (B) and the test model (C).

The porosimetry degree of the microcarriers was also evaluated with a mercury porosimetry. From the data obtained from this assay was possible to calculate the total pore area occupied by the pores in the microcarrier. Figure 22 is an example of what it is observed in an assay of mercury porosimetry. The peaks represent the amount of mercury intrusion in free spaces of microcarriers.

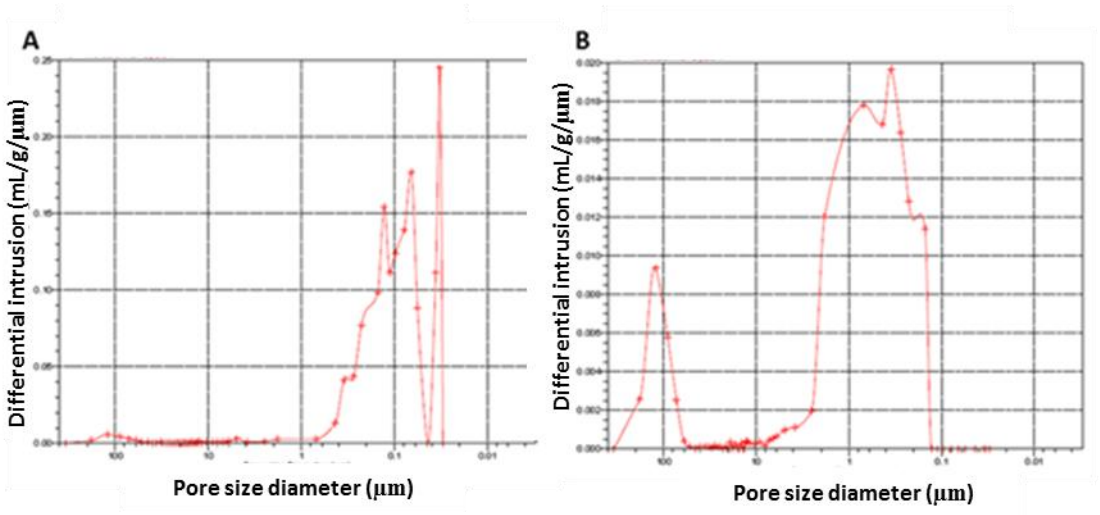

Figure 22 - Two examples of the graphics obtained by mercury porosimetry of differential intrusion of mercury and the relationship with pore size for the microcarrier number 11(A) and 17(B).

Through the peaks of figure 22 it is possible describe that the microcarrier 17 has a much more amount of small pores (<0.1 µm), than the microcarrier 11(>0.1 µm). For the determination of total pore area, it was calculated the area above each peak that determines the amount of volume occupied by mercury inside the microcarrier, which is equal to the free space in a normal microcarrier.

After analyzing every mercury porosimetry graphic, it was tried to develop an initial model to the prediction of the porosity degree trough neuronal network. This evaluation was made in MATLAB software from MathWorks, USA. For the development of the model for the prediction of the porosity degree, it was used as input the concentration of high acyl and low acyl gellan gum and as output the total pore area evaluated for each microcarrier. As described in material and methods, the total pore area was obtained by mercury porosimetry assay. The neuronal network was built with 5 neurons and was trained with 18 assays, validated with 3 assays and tested with 7 assays. In figure 23 the final model (figure 23 A) for the prediction of porosity degree of the microcarriers made with conjugation of high acyl and low acyl gellan gum can be seen. The relation is not very high (R=0.73), this low value is due to the few number of assays done, however the correlation factor is better comparing with model for swelling capacity.

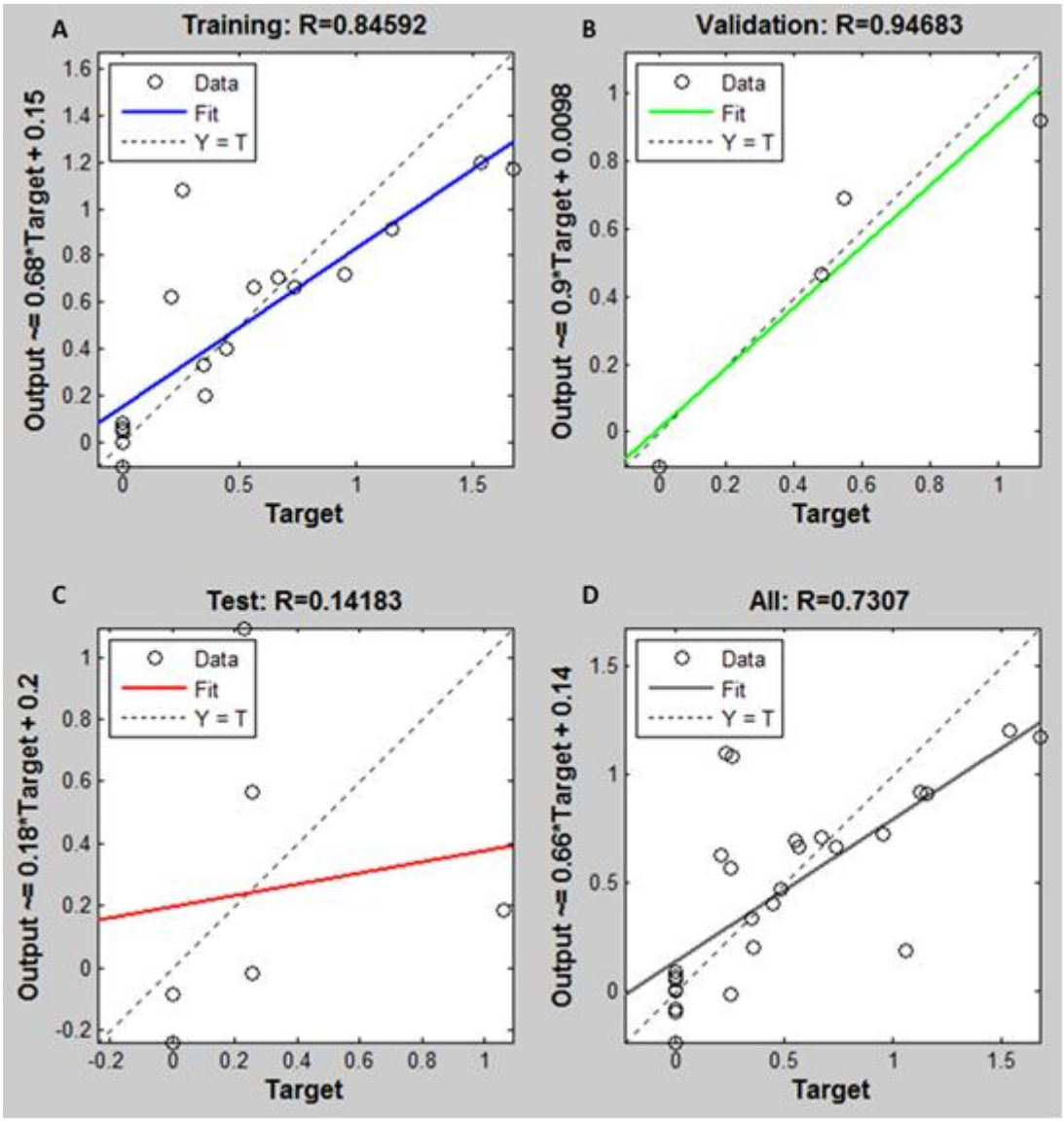

Figure 23 - Model obtained for the prediction of porosity degree of the microcarrier developed with different concentration of high acyl and low acyl gellan gum (D), the training model (A), the validation model (B) and the test model (C).

After the characterization of the swelling ability and the porosity degree, some microcarriers in lyophilized state were analyzed by SEM. This technology allows accessing the surface topography of microcarriers. There was no need to perform the SEM analyses to all the microcarriers, because they have a very similar surface topography. As it can be seen in figure 24, the microcarrier shows a very uniform structure for all the surface of microcarrier with some big cavity structures, which seem like a honeycomb or an alveolar structure. The cavity surface is not regular, it has some small cavities that do not drill the surface of the cavity from one side to the other. These small incisions make the surface rough. This fact may be due to the higher or lower porosity degree, since the microcarrier represented in figure 24 B (total pore area = 0.671 m^2/g) has a higher total pore area than the microcarrier represented on the figure 24 A (total pore area= 0.209 m^2/g).

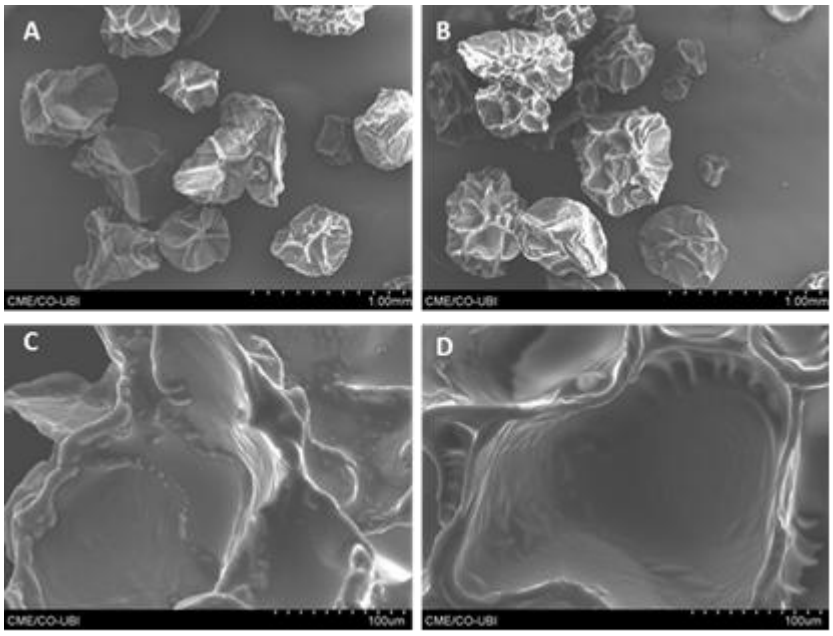

Figure 24 – Representative pictures of two different lyophilized microcarriers. Microcarrier number 17 with magnification of 47X (A) and with magnification of 400X (C). Microcarrier number 7 with magnification of 47X (B) and with magnification of 200X (D)

3.2.3. Microcarrier culture trials

For the microcarrier culture assays, two microcarriers regarding only their porosity degree were chosen. The parameter is described as a possible influence factor in adhesion and growth of anchorage dependent cells (Melchels *et al.*, 2011).

According to the results presented in table 4, the microcarriers that have the highest pore area and the microcarriers that have the lowest pore area were chosen. It was also chosen a third microcarrier that was prepared only with low acyl gellan gum, because it has better transparency and was chosen to compare the cell adhesion in the microcarrier only developed with negatively charged polymer. Thus, the microcarriers number eleven and seventeenth were chosen. The microcarrier number eleven has the highest total pore area of all microcarriers (1.672m^2/g), being considered the microcarrier with the best porosity degree. It was prepared with 0.16% of high acyl gellan gum and 1.5% of low acyl gellan gum. The microcarrier seventeen has the lower porosity degree (total pore area of 0.209m^2/g), and it was prepared with 0.33% of high acyl gellan gum and 1% of low acyl gellan gum. All this information can be confirmed in table 3 and 4.

The cell culture assay was performed with these microcarriers in order to evaluate the best gellan microcarrier for cell growth. For that purpose, a widely used cell line, COS-7, was employed. This cell line allows a very rapid and general evaluation of the gellan microcarrier in cell culture.

In the first assay performed with cells and microcarriers, it was difficult to visualize the adhesion and expansion of cells, so a method for staining the cells was evaluated (described in section materials and methods). As described in the literature, the best dye for live cell culture was neutral red, since the aim of this assay was to evaluate the ability of viable cells to incorporate and bind the dye in lysosomes. The uptake of neutral red depends on the cells capacity to maintain the pH gradients, through the production of adenosine triphosphate. At physiological pH, the dye presents a net charge close to zero, enabling its penetration through the cell membranes. However, inside the lysosomes a proton gradient maintains the pH lower when compared with the cytoplasm pH, thus the dye becomes charged and is retained inside the lysosomes.

The next assay was performed with recently trypsinized and stained COS-7. The cells were transferred to 6-wells plate with the bottom covered with the microcarriers number 17 and 11, separately. After 24 h, the medium was fully aspirated to remove unattached cells, some microcarriers were recovered and placed in the bottom of a 24-well plate with fresh medium, then placed in the incubator. 24 h later, the cells anchored to the microcarriers were observed under optic microscope. As shown in the figure 25 A and B, the microcarriers have attached cells on their surface but it appears that the microcarrier 17 has lower adhesion. Also it can be observed that control cells have a better expansion than the cells in the microcarrier (figure C). Since the agglomeration of red color in cells attached to microcarrier is more close to the nucleus cell than in the control cells.

To improve cell expansion, the medium was removed once again, and the microcarriers were incubated for more 24 h with fresh medium. Figure 25 D, E and F shows the results visualized with optic microscope. As can be seen, the cells continued viable and anchorage in the microcarrier after 48 h. It also seems that there was proliferation of cells because there was a increase in number of cells in the transporter. However it remains a small difference in the distribution of the dye in the cell, between the control cells and the cells of the microcarrier. Even when observing with a higher magnification this difference remains to be noticed.

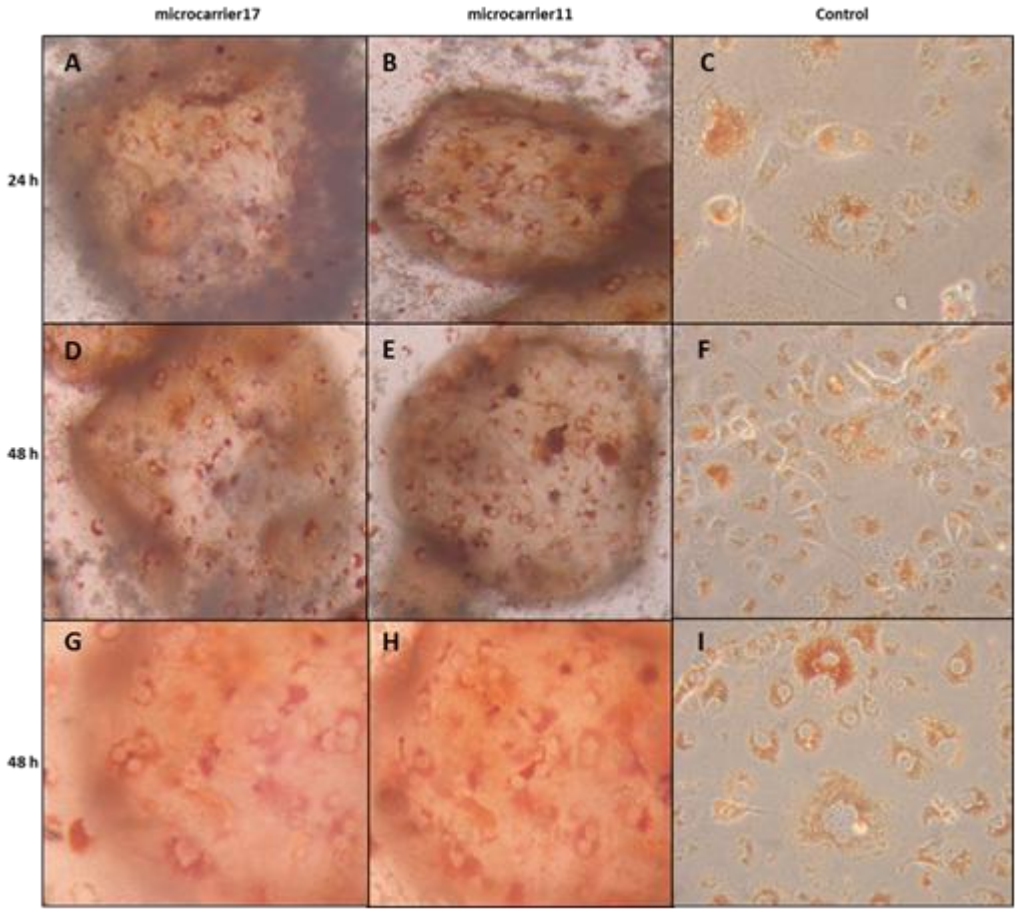

Figure 25 - Represents COS-7 culture in microcarriers. (A) Microcarrier 17 after 24 h culture with magnification of 10Xnumber 17 after 24 h (B) Microcarrier 11 after 24 h culture with magnification of 10X (C) Control culture after 24 h culture with magnification of 20X (D) Microcarrier 17 after 48 h culture with magnification of 10X (E) Microcarrier 11 after 48 h culture with magnification of 10X (F) Control culture after 48 h culture with magnification of 10X number (G) Microcarrier 17after 48 h culture with magnification of 20X (H) Microcarrier 17 after 48 h culture with magnification of 20X (I) Control culture after 48 h culture with magnification of 20X.

As an overview, the results of the study of cell adhesion were similar for both carriers used. However, the presence of pores on the surface of microcarriers may be facilitated cell attachment. The porosity can also be an important factor in a long duration culture of cells in microcarriers, because it facilitates the diffusion of nutrients and gases for suitable functions.

3.2.4. Surface evaluation

As referred above, some initial differences were observed between the adhesion process in the microcarrier 17 and 11. To assess if the microcarrier surface charge influences this process, it was studied the charge of the microcarrier. This study was performed by using BSA in their different charges, taking into account the isoelectric point of the protein.

To modify the charge of the BSA, three different pH solutions were prepared. One with pH of 3.7 where BSA is negatively charged other with pH of 4.7 where BSA is uncharged and a third

solution with pH of 5.7 where BSA is positively charged. These solutions were placed in contact with microcarriers 11 and 17 for 4 h with a change of solution every hour. After that the solution of BSA was completely removed.

To assess the amount of BSA adsorbed per microcarrier, the microacrrier in working pH of 3.7 was transferred to new solution only with acetate buffer at pHs of 3.7, 4.7 and 5.7. This way the charge of BSA was forced to modify and BSA can disconnect from the surface of the microcarrier. For the microcarriers at working pH of 4.7 and 5.7 the same procedures were made. The release of BSA from the microcarrier was evaluated for 4 h with change of the sodium acetate buffer solution every hour. All the aliquots recovered were analyzed by BCA method to quantify the amount of BSA release from each microcarrier (figure 26).

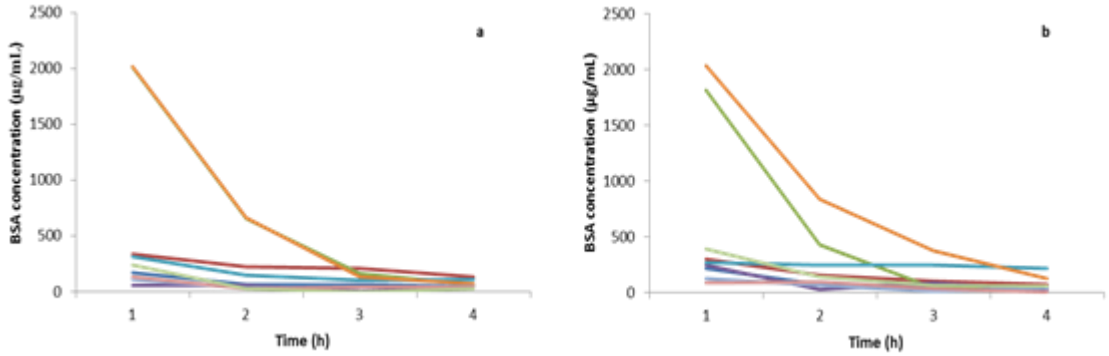

Figure 26 - Graphical representation of the BSA release from the microcarrier number 17(a) and 11(b). The different curve in each graphic represents the transition of pH solutions, 3.7 to 3.7(-), 3.7 to 4.7(-), 3.7 to 5.7(-),4.7 to 3.7(-), 4.7 to 4.7(-),4.7 to 5.7(-),5.7 to 3.7(-),5.7 to 4.7(-) and 5.7 to 5.7(-).

It is expected that if the microcarrier has a negative charge that it adsorbed at a pH of 3.7 and released when the microcarrier is changed to pH 5.7. If the microcarrier is positively charge the microcarrier would adsorb at pH of 5.7 and release when the microcarrier is transferred to pH of 3.7.

With the analysis of the figure 26, it can be concluded that our microcarrier has negative superficial charge. At pH of 3.7 the BSA has positive charge, so it can form ionic bonds with negatives surfaces, when it was placed in a solution with pH of 5.7 the BSA turns to negative charge and the ionic bond are broken so we can find BSA in the solution recovered.

If the capacity for adsorption between microcarriers was compared it can be said that both microcarriers have equal capacity to adsorb positive compounds. So the superficial charge of the two microcarriers is the same.

Also by the analysis of figure 26, it can be visualized that all the microcarriers have the capacity to absorb compounds independently of the charge since basal amounts of BSA are detected in all assays.

Chapter 4 – Conclusion and future perspectives

With the increase of the world population, the production of medicines at lower costs becomes an urgency. One of the techniques used by pharmaceutical companies to produce large quantities of drugs or virus is the production in bioreactors. However, when it is necessary to increase the production of these compounds, also the production costs increase. This is caused by the necessity to buy larger bioreactors or to increase the number of bioreactors available in the company to thereby increase the production.

In 1907, Van Weasel introduced a new method that allows the production of a larger quantity of drugs or viruses without having to invest in new bioreactors. This method is called microcarrier culture and has recently been used not only for the production of pharmaceuticals but also for the 3D growth of cells for drug screening.

Several microcarrier structures were introduced in the market in the last 10 years. All the microcarriers have their advantages and their drawbacks. The present work wanted to develop a structure for a new microcarrier that has the main characteristics of the existent microcarriers but with a lower cost.

To reduce the final cost of the microcarrier, gellan gum was chosen because this exopolysaccharide is very cheap. This polymer has two forms, one with low acetylation content and the other with a high acetylation content. The low acyl gellan gum forms transparent and strong gels due to the high negative charge density. On the other hand, high acyl gellan gum has low negative charge density so it forms more fluid gels.

In this work, the development of a new microcarrier through the combination of the two forms of gellan gum was proposed. To achieve this aim, the work was divided in two parts, one for the biosynthesis (production and recovery) of high acyl gellan gum and the second part for the development and characterization of the microcarrier produced.

In the first part, it was attempted the production and recovery of gellan gum in high acyl form, and it was concluded that the best fermentation procedure to the production of gellan gum is the procedure 3, which uses low content of nitrogen source and a specific salt solution. It was also concluded that the removal of supernatant in transition between pre fermentation and fermentation allows a better adaption and growth of bacteria which enables a higher production of gellan gum.

For the recovery of gellan gum it was concluded that the two step centrifugation is the best method for the removal of the bacterial content compared to dilution or the one step centrifugation method. In addition, it can be concluded that the filtration procedure is the method that allows the recovery of high acyl gellan gum with the highest purity degree. However, the filtration procedure is a very slow method and there is loss of high acyl gellan gum during the procedure, and thus it is not the best procedure for gellan gum recovery.

For the recovery of high acyl gellan gum it was also evaluated the best organic solvent to use in the precipitation and it was concluded that acetonitrile is the best solvent when compared with ethanol, isopropanol and acetone.

However it was not possible to recover the high acyl gellan gum with a high degree of purity comparatively with commercial samples. For future work on the recovery of gellan gum would be interesting to test the dialysis with a membrane cut off lower than 14 000 daltons and chromatographic methods. It would also be interesting to analyze the size of the chains of the produced gellan gum in fermentation and its degree of acetylation, using for example mass spectroscopy technique.

In the second part of the work, 28 microcarriers were proposed by experimental design tool and were developed by the combination of high acyl gellan gum and low acyl gellan gum. These microcarriers were evaluated for swelling ability and porosity degree. With the results obtained for these evaluations, it was possible to develop an initial model for the prediction of porosity degree according to the content of high and low acyl gellan gum. The model developed has a low correlation factor but it can be improved in future work with the performance of new assays. The same work can be done to improve the microcarrier swelling ability model. The analysis of the topographic surface of microcarriers by SEM was also done and it was concluded that the roughness of the microcarriers surface will be dependent of the porosity degree.

For the cell culture assays, two microcarriers with the follow characteristics were chosen: Microcarrier number 11 was made with with 0.16% of high acyl gellan gum, and 1.5% of low acyl gellan gum and has a total pore area of 1.672m^2/g and a swelling ability of 231.919 µm. Microcarrier number 17 was made with with 0.33% of high acyl gellan gum and 1% of low acyl gellan gum and has total pore are of 0.209m^2/g and a swelling ability of 187.199 µm.

It was observed that both microcarriers have a similar behavior in cell culture. So it is concluded that presence of pores on the surface of microcarriers may be facilitated cell attachment. It can also be an important fact for cell development in the microcarrier. Further work has to be made for this type of evaluation. It would also be interesting to evaluate the formation of microenvironments, and the importance of pores in the process.

In the process of evaluating the surface charge density, the adsorption of BSA by the surface of microcarrier number 11 and 17 was tested. The results show that both of them have a negative surface and may, for this reason, not verify a better cell adhesion. In future work seems important to analyze the influence of high acyl gellan gum in the decrease of negative surface charge density to better understand its importance in the development of microcarriers. Also seems interesting evaluate the possibilities for surface functionalization of these microcarrier to improve cell adhesion.

Overall, the combination of the high acyl and low acyl gellan gum seems to be a promising solution for the creation of a matrix for new microcarriers with macroporous structure. This combination allows the creation of transparent, rigid and rough structures with high ability to adsorb positive compounds. However more studies have to be made to determine the effect of the two types of gellan in the physic and chemical characteristics of the final structure.

Since this is an initial work many aspects can be improved and several are the possible applications for the new microcarriers developed like controllable drug release systems, cell entrapment system or protein entrapment system.

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