NANOGLYCOGEN, A NEW FUNCTIONAL CARBOHYDRATE POLYMER

Master Thesis by

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12th May, 2017

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Abstract

Highly-branched glucose polymers, derived from starch have shown interesting potential industrial applications in areas such as food, medicine, cosmetics and pharmaceuticals. Glycogen, like starch, is also a natural glucose polymer that shows more favorable features, since it is readily soluble in cold water and more accessible by enzyme.

In the present study, glycogen was extracted from extremophilic red microalga *Galdieria Sulphuraria.* The material properties of this peculiar biopolymer were exploited with special emphasis in the rheology. Glycogen showed significantly decreased viscosity in solution compared to other highly-branched glucose polymers derived from starch and relative high surface activity, properties conferred by its shorter side chains and higher branch density.

Consequently, the extracted glycogen was substituted with octenyl succinic anhydride through a 24h reaction. Phytoglycogen octenyl succinate (PGOS) and waxy potato starch octenyl succinate (POS) were also prepared as reference. ^{1}H-NMR spectra were performed to facilitate the structure identification of the prepared polymers and for the determination of the degree of substitution.

Acknowledgments

At the beginning of this thesis, I would like to express my gratitude to all of the people who supported this project and made it an unforgettable experience for me.

First of all, I would like to express my sincere gratitude to Prof. Dr. Marc van der Maarel for trusting my capabilities and for giving me the opportunity to deepen my knowledge in the field of Aquatic Biotechnology and Bioproduct Engineering. Working on this field was something completely new for me and his systematic guidance, help and advice largely contributed to the fulfilment of this project.

I acknowledge my gratitude to my second assessor Prof. Dr. Francesco Picchioni for his valuable contribution to this project as well as his help and advice throughout the course of my thesis.

Special thanks to Marta Martinez-Garcia, Alle van Wijk and Anastasia Prima Kristijarti for helping me when I needed assistance, being always next to me to answer my questions, resolve my problems and for making my daily routine in the lab nice and happy. It was a pleasure working with them every single day.

I would also like to thank the ABBE, PPBBE and Product Technology groups for all the nice moments and experiences that we shared all of these months.

For an excellent secretarial assistance during my research I would like to express my sincerest thank to Inge Meijerink-Wever.

I would like to express my gratitude to my family for giving me the opportunity to attend this master in the University of Groningen and also for their encouragement, attention and continuous support.

Finally, I wish to thank all my friends both from Groningen and Greece, who gave me strength and support to continue this project.

This project is dedicated to them.

Abbreviations

CMC Critical Micelle Concentration

Cryo-TEM Crogenic Transmission Electron Microscopy

D_2O Deuterium Oxide

DMSO DiMethyl SulfOxide

DP Degree of Polymerization

FTIR Fourier Transform Infrared Spectroscopy

GOS Glycogen Octenyl Succinate

HBS Highly Branched Starch

HBS-AMG Highly Branched Starch treated with Amyloglucosidase

NMR Nuclear Magnetic Resonance Spectroscopy

PGOS Phytoglycogen Octenyl Succinate

POS Potato starch Octenyl Succinate

TCA TriCloroAcetic Acid

TFA TriFluoroAcetic Acid

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1. Introduction

Carbohydrates are the most abundant organic compounds in nature. They appear in animal and plant kingdoms and they play an important role in a large variety biological and biochemical processes, including, inter alia, energy storage, structural support and energy transport between the cells (Stick and Spencer Williams, 2010).

Carbohydrates are produced in bulk amounts. Furthermore, they are inexpensive and possess a high chemical as well as enantiomeric purity. Combined with the demand for environmentally friendly products and processes, sugars reveal to be competent raw materials. In the near future more and more products will be based on these natural sources. (Kennedy and Lloyd, 1992) Certain types of carbohydrates such as cellulose, sucrose, starch, alginate, carrageenan and agar are also biotechnologically relevant raw materials and together with their derivatives, are widely used in vast range of industrial applications (B.S. Albuquerque et al., 2016).

Agar, carrageenan and alginate are cell wall polysaccharides produced by various species of brown and red microalgae and are the only biotechnologically relevant carbohydrates that are not originate from terrestrial plants (Martinez-Garcia, 2017). Sucrose is the major energy transport carbohydrate in plants. Tones of sucrose are employed, nowadays,by the food industry as sweeteners. In recent years, sucrose has also gained attention as raw material for production of several chemicals thanks to the catalytic specificity of enzymes for the convention of sucrose into valuable products (Röper, 2002).

Cellulose and starch are by far the largest bulk of the annually renewable carbohydrate in terms of industrial relevance and their use as basic organic raw materials in industry (Lichtenthaler and Mondel, 1997). Cellulose The basic structural component of plant cell walls. Of great economic importance, cellulose is processed to produce papers and fibres and is chemically modified to yield substances used in the manufacture of such items as plastics, photographic films, and rayon (Klemm *et al.*, 2005). Starch is a carbohydrate extracted from agricultural raw materials and which is present in, literally, thousands of everyday food and non-food applications (Ellis *et al.*, 1998). The European Starch Industry, nowadays, produces 10,7 million tonnes of starch per year while EU consumption of starch and starch derivatives was 9,3 million tonnes in 2015 (www.starch.eu).

1.1. From starch to highly branched glycose polymers

Starch is the main form in which plants store carbon. It occurs as granules composed of two glucose polymers, amylose and amylopectin (Rolland-Sabaté *et al*., 20078. The morphology of a starch granule is determined by the amylopectin fraction which constitutes about 70–80% of normal starch (Gallant, Bouchet and Baldwin, 1997). Amylose has a linear structure and glucose units are linked α-(1-4) glycosidic bonds whereas amylopectin has a branched structure with a linear backbone of α-(1-4) linked glucans and side-chains attached though α-(1-6) glycosidic bonds (Manners, 1989) (Figure 1). A glycosidic bond or glycosidic linkage is a type of covalent bond that joins a carbohydrate molecule to another group, which may or may not be another carbohydrate. Starch is converted by hydrolysis or re-arrangement of these glycosidic linkages to produce novel type of molecules such as cyclodextrins (Biwer, Antranikian and Heinzle, 2002) and highly brached glucose polymers (Backer, 2017), (van der Maarel and Leemhuis, 2013).

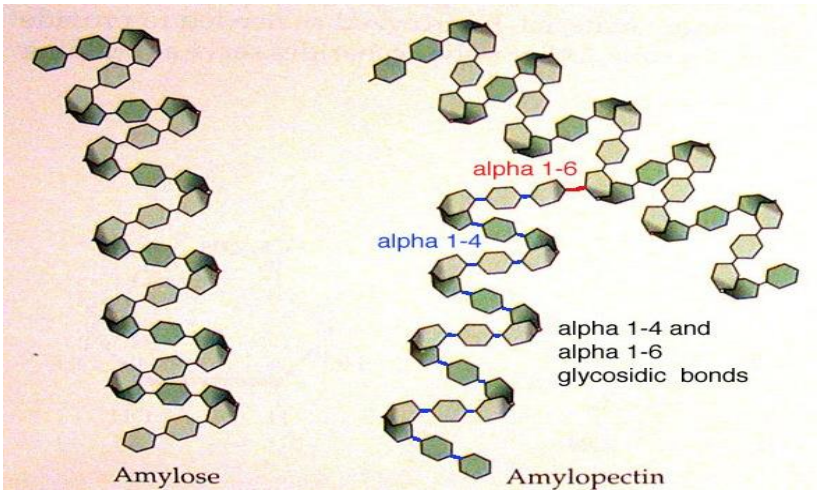


Figure 1: Schematic representation of amylose and amylopectin structure

1.1.1. Highly branched glucose polymers

As the name indicates, highly branched glucose polymers are starch derivatives in which the proportion of α-(1-4) branching linkages is considerably increased in comparison to starch. Highly branched glucose polymers exhibit advantageous properties compared to those of starch because of their high branching density. (Functional carbohydrates from the red microalga Galdieria sulphuraria, 2016) Highly branched glucoge polymers industrial applications have been increased dramatically the last few decades, especially food and pharmaceutical industry (B.S. Albuquerque *et al*., 2016). During this project, we investigated the properties and we

tried a chemical modification of two highly-branched glucose polymers, glycogen and pytoglycogen.

Glycogen

Glycogen is a highly branched glucose polymer that serves as the secondary long-term energy storage in animals, fungi, bacteria, yeasts, and archaea and it is analogous to the starch in plants (Ball *et al*., 2011). Glycogen structure is, in some ways, similar to amylopectin. However, glycogen has higher branching density than amylopectin (Fernandez, Rojas and Nilsson, 2011). The proportion of branching linkages in amylopectin is about 5% while for glycogen is between 8 and 13% depending on the glycogen source (Manners, 1991)

Figure 2: Schematic representation of glycogen structure

Glycogen is considered as a high molecular weight polymer (10^7-10^9 Da) (Manners, 1991). Regarding the physical form of the molecules, glycogen consists of two size populations specified as α- and β-particles (Sullivan *et al*., 2010). The β-particles constitute the primary structure of α-particles and have an average molecular mass of 10^7 g/mol. The α-particles are referred to be made up of as many as 50 β-particles subunits (Rolland-Sabaté *et al.*, 2008). However, the exact structure the type of bonds or interactions that keep α-particles together is still under investigation (Geddes, Harvey and Wills, 1977).

<u>Glycogen from Galdieria sulpuraria</u>

Glycogen can be obtained from human and animal tissue and bacteria according to various methods. In the present work, glycogen was extracted from *Galdieria sulphuraria*. *Galdieria sulphuraria* is a eukaryotic extremophilic red microalga. It is thermophile, as well as acidophile and inhabits highly acidic springs at temperatures higher than 40^{o}C and acidic environment (pH 2). *Galdieria sulphuraria* appear yellow or green in its natural environment (Moreira *et al.*, 1994) (Figure3).

Figure 3: Galdieria sulphuraria

Glycogen from *Galdieria sulphuraria* forms a crystalline granule and it has been reported to have a very remarkable chain lenght distribution with high amount of short chains and very low proportion of long chain. (Shimonaga *et al.*, 2007) (Shimonaga *et al.*, 2008) Similar chain length distribution profiles have been previously observed in polysacharides accumulated by a similar species, *Galdieria maxima*, (Stadnichuk *et al.*, 2007) while Sakurai *et al.* also indicated, at

their recently published work, the very short-chained structure of glycogen from *Galdieria sulphuraria* microalga (Sakurai *et al.*, 2016).

Martinez-Garcia *et al.*, in their recent published worked, showed that the glycogen from *Galdieria sulphuraria* displays a unique chain length distribution which differs significantly from that of other glycogens in its lack of chains of degree of polymerization (DP) larger than 10 (Martinez-Garcia, Stuart and van der Maarel, 2016). They also proved that the glycogen from *Galdieria sulphuraria* has a weight-average molecular weight (Mw) of 2.5×10^5 Da which is at least one order of magnitude smaller than that of other glycogens. Furthermore, they measured the particle size of *Galdieria sulphuraria* glycogen using cryogenic transmission electron microscopy (cryo-TEM) and they found that *Galdieria sulphuraria* glycogen particles were signigicantly smaller than other glycogens, which appeared as bigger β-particles and in some cases were arranged as α-rosettes of up to 100nm (Martinez-Garcia, Stuart and van der Maarel, 2016).

In the light of the above, one can claim that the glycogen from *Galdieria sulphuraria* constitutes a very peculiar type of nanoglycogen -taking into account its 'nano' particle size- with a unique chain length distribution that may have interesting properties regarding possible applications.

Phytoglycogen

Phytoglycogen is a water-soluble polysaccharide present in plants with a highly branched structure similar to the structure of glycogen and high molecular density in dispersion. (Wong *et al.*, 2003) The largest source of phytoglycogen is the kernel of the commercial sweet corn. In addition, it has been reported that, similar to nanoparticles from *Galdieria sulphuraria*, phytoglycogen particles range from 30 to 100 nm under transmission electron microscope (TEM). (Scheffler, Huang, *et al.*, 2010)

1.1.2. Applications

Medical applications

One of the most important medical applications of highly branched glucose polymers is for enteral and parenteral nutrition and in peritoneal dialysis cleaning fluids (Backer and Saniez, 2004). Peritoneal dialysis is a treatment for kidney failure. During the treatment, a hypertonic fluid is introduced in the abdomen through a tube that is placed in the peritoneal cavity and filters waste products from the blood. Glucose is safe, easily metabolized by the organism and constitutes a proper osmotic agent for this type of hypertonic solutions. Nevertheless, the effectiveness of glucose as osmotic agent is questionable in long periods of treatment, as it can easily cross the peritoneal membrane and get absorbed into the bloodstream. As a result the osmotic gradient in

the peritoneal cavity will decrease which is unfavorable for the treatment. Highly branched glucose polymers provide a good alternative to glucose as they do not easily get assimilated into the bloodstream and, despite their polymeric nature, can create osmotic pressure and induce water filtration through the peritoneum by a phenomenon known as colloid osmosis (Mistry and Gokal, 1993). Baxter Healthcare (USA) patented Extrameal, a mixture of glucose polymers derived from fractionation of hydrolyzed corn starch, with a proportion on α-(1-6) branching likages >10 % and molecular weight of $1.3\text{-}1.9 \times 10^4$ Da (Moberly et al., 2002). Ten years later, Deremaux *et al.* reported that high degree of branching of these polymers can improve their performance as osmotic agents and induce lower glucose release into the bloodstream (Deremaux *et al.*, 2013)

Another promising application of highly branched glucose polymers in medicine is related to drug delivery and tissue targeting. Dutcher and Graham presented, in 2010, polysaccharide nanoparticles of highly branched glucose homopolymers as drug delivery systems and fluorescent diagnostics (Dutcher and Graham, 2010). They claimed hydrophilicity, monodispersity and low solution viscosity of the carbohydrate nanoparticles to be properties of vital importance for these applications. In the same direction, Filippov et al reported in 2012 oyster glycogen nanoparticles modified with gadolinium and fluorescent labels that can be used as drug delivery nanocarriers (Filippov *et al.*, 2012).

Nutritional applications

A very promising food application of highly-branched glucose polymers is in sports drinks. A typical sports drink is a blend of carbohydrates, water and electrolytes. Carbohydrates delay depletion of muscle carbohydrate which is the main cause of fatigue while working out, by replenishing body energy reserves and counteract dehydration by allowing the fast fluid absorption from the stomach into intestine at the same time (Maughan, 1998). Due to high proportion of α-(1-6) linkages, highly branched glucose polymers are slowly degraded by digestive enzymes and this leads to a slower insulin response, as glucose appears in the bloodstream gradually (Taaki 1998) Furthethermore, it has been proven that highly-branched glucose polymers have negligible contribution of the osmotic value of the solution due to their high molecular weight (Takii 2015). The company ‘Clico’, in Japan, produces Cluster Dextrin, a highly branched clustered Dextrin generates from the cycliation reaction of a branching enzyme on corn amylopectin.

1.2. Chemical modification with OSA

Chemically modified starches are generally made by treating starch with agents that introduce chemical substituents via reaction with hydroxyl groups in the starch molecule (Niaounakis, 2015). This type of starches have physicochemical properties that differ significantly from the parent starch, thus widening their usefulness in many applications in food manufacturing and other industrial processes (Alcázar-Alay and Meireles, 2015). The first chemical modification of starch was patented by Caldwell and Wurzburg in 1953, although this patent did not specifically use OSA as substituent (Caldwell &Wurzburg,1953).

The starch derivative is prepared by a standard esterification reaction in which cyclic dicarboxylic acid anhydride and starch suspended in water and mixed under alkaline conditions (Figure 4) (Sweedman *et al.*, 2013) .When the hydrophilic starch reacts with hydrophilic OSA, the whole molecule acquires an amphiphilic character(Tizzotti *et al.*, 2011). Starch octenyl succinate derivatives are and in pharmaceutical and industrial areas, especially in food production, due to its good filming properties and excellent emulsion-stabilizing properties (Bao *et al*., 2003). .

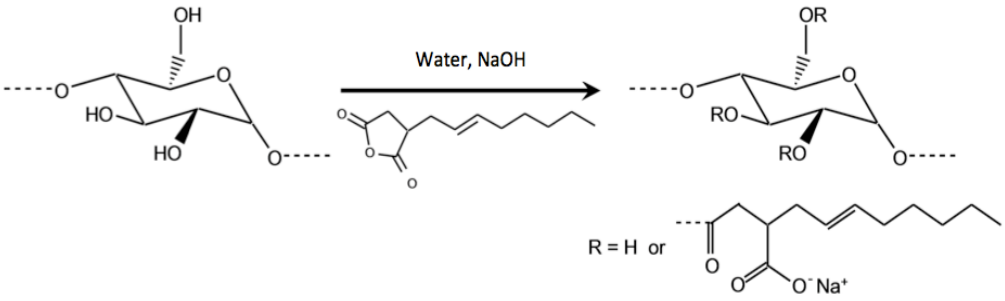


Figure 4: Reaction scheme of OSA- modified starches (Sweedman et al., 2013)

In recent years, Scheffler *et al*. successfully tried to modify phytoglycogen using OSA with intention to study in vitro digestibility and emulsification properties of phytoglycogen octenyl succinate (PGOS) amphiphilic nanoparticles and examine their potential to stabilize emulsions forming the so called 'Pickering emulsions'(Scheffler, Wang, *et al.*, 2010).

A Pickering emulsion is an emulsion that is stabilized by solid particles Pickering emulsions are emulsions of any type, in place of surfactants (Binks, 2002)(Figure 5) . The stabilization by solid particles brings about specific properties to Pickering emulsions while the high resistance to coalescence is a major benefit of the

stabilization by solid particles (Chevalier and Bolzinger, 2013) and OSA modified starch and phytoglycogen particles are already used in such emulsions.

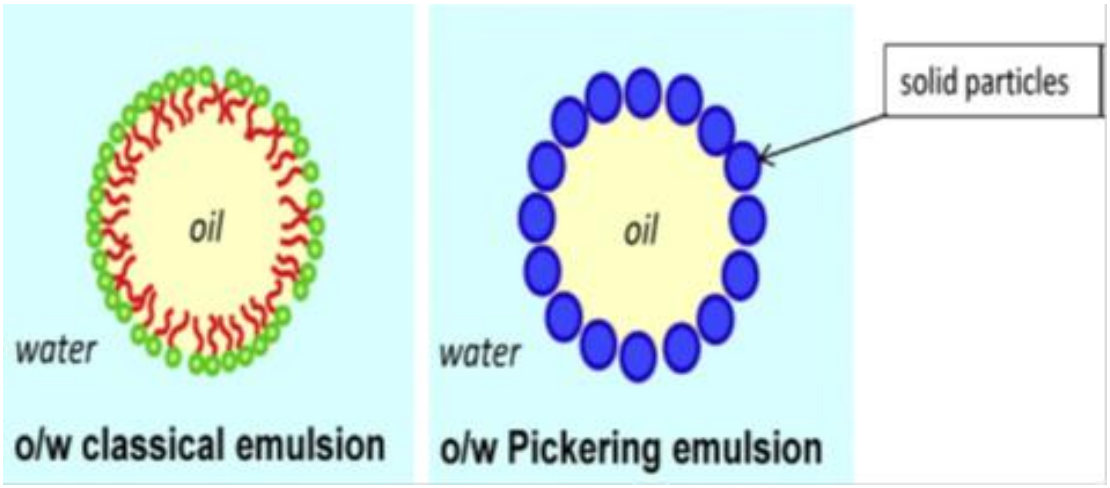


Figure 5: Sketch of a Pickering emulsion and a classical (surfactant-based) emulsion (Chevalier and Bolzinger, 2013)

Those significant similarities triggered our interest, so the second big part of this research project was the modification of our peculiar glycogen nanoparticles with OSA with purpose the investigation their surface activity and consequently, their emulsification properties.

1.3. Aim of this thesis

The structural properties of glycogen from *Galdieria Sulphuraria* have already been investigated before (Martinez-Garcia, Stuart and van der Maarel, 2016). Nevertheless, a lot is still unknown regarding then solution and rheological properties of this molecule as well as and also the influence that its structure can have on these properties. Therefore the ultimate scope of this project is to investigate the properties of this special carbohydrate in context of functionality and rheology.

The research project, which was carried out at the ABBE group (Aquatic Biotechnology in the Bioproduct Engineering, can be divided into two main parts. These include the extraction and the characterization of native nanoglycogen from *Galdieria Sulphuraria* red microalgae as well as the chemical modification of the extracted nanoglycogen with OSA and comparison with phytoglycogen and waxy potato starch.

In the first part of this project, *Galdieria Sulpuraria* red microalgae were cultivated and grown in vitro conditions. The native nanoglycogen was extracted by the harvested cells and solution properties such as viscosity, ζ-potential and surface tension were measure afterwards. During the second part, glycogen nanoparticles were substituted with OSA through a 24h reaction. In Chapter 2 all extraction and characterization methods that were used in the present investigation are described in detail. In order to obtain an insight into the functionality and the properties of *Galdieria Sulphuraria* nanoglycogen the results of the measurements are presented and discussed in Chapter 3. This thesis ends by reporting the major conclusions and some recommendations for future research in chapters 4 and 5 respectively.

2. Materials and methods

2.1 Materials

Galderia sulphuraria strain SAG 108.79 was supplied by the culture collection of the University of Gottingen (Sammlung von Algenkul-turen, Germany). Dried kernels of white sweet corn Sugar Pearl F1 were purchased from Vreeken's Zaden (Dordrecht, the Netherlands). Potato starch and Eliane C100 (waxy potato starch) were kindly provided by Avebe (Veendam, the Netherlands). 1-Octenyl succinate anhydride was purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. Galderia Sulphuraria cultivation

Galderia sulphuraria was grown until exponential phase in Allen medium supplemented with 1% glycerol at pH 2 in a rotating incubator at 40℃. The exact composition of Allen medium is referred in Appendix 1. Cell growth was monitored by measuring the optical density of the cells at 800 nm.

2.2.2. Glycogen extraction

Figure 6: *Galdieria sulphuraria* nanoglycogen

Glycogen was extracted by *Galderia sulphuraria* as described by Martinez-Garcia et al., 2016 (Martinez-Garcia, Stuart and van der Maarel, 2016). Cells were harvested by centrifugation at 5,000 × *g* for 5 minutes at room temperature and then washed twice. The wet cells were re-suspended in ultra-pure water, mixed with half volume of glass beads and disrupted by shaking in a Mixer Mill (MM400, Retsch) at frequency of 30 Hz for 8 minutes. The cell lysate was centrifuged at 20,000 × *g* at 4℃ for 10 minutes. The supernatant, free of unbroken cells and cell debris, was incubated in a water bath at 100 ℃ for 15 minutes in order to precipitate the proteins. The second supernatant was centrifuged at 20,000 × *g* at 4℃ for 20 minutes and then transferred to a new tube and 0.1 volumes of 50% trichloroacetic acid (TCA) were added for further precipitation of the residual proteins. The sample was incubated in the freezer for 10 minutes and then centrifuged at 20,000 × *g* for 20 minutes. The clear supernatant was mixed with 1 volume of 100% ethanol and was kept in the freezer (-40 ℃) overnight in order to precipitate glycogen. The precipitated glycogen was recovered by centrifugation at 10,000 × *g* for 10 minutes and afterwards it is freeze dried. The dry glycogen was re-suspended in water and then precipitated in 1 volume of 100% ethanol in the freezer

overnight. The precipitated glycogen was recovered by centrifugation at 10,000 × g for 10 minutes and it was finally freeze-dried.

2.2.3. Phytoglycogen extraction

Phytoglycogen was extracted from sweet corn kernels as described by Scheffler *et al.* (Scheffler *et al.*, 2010). Sweet corn kernels were ground into grits in a Perten laboratory mill and then mixed with 5 volumes of demineralized water. The mixture was homogenized using a Fisher Scientific (IKA) ultra turrax homogenizer and then it was centrifuged at 8,000 ×g for 20 min. The solid pellet was re-suspended with deionized water and further extracted by centrifuging t 8,000 ×g for 20 min twice whereas the supernatants at each batch were collected, combined, then passed through a 270-mesh sieve and precipitated in 1 volume of 100% ethanol in the freezer overnight. The precipitated phytoglycogen was recovered by centrifugation at 10,000 × g for 10 minutes and it was finally freeze-dried.

2.2.4. Characterization

Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR spectra were recorded using a Perkin-Elmer Spectrum 2000 spectrometer, with a diamond crystal for ATR. Glycogen and phytoglycogen samples were characterized through an ATR setup Greasby Specac-reflection. A region from 4000cm^{-1} to 600 cm^{-1} was used for scanning with a resolution of 4 cm^{-1} and a total of 16 scans. For the measurements, small amounts of the extracted glycogen and phytoglycogen were used in the form of powder.

Zeta potential

Zeta-potential analysis was carried out using a Brookhaven ZetaPALS zeta potential and particle size analyzer. The glycogen samples were suspended in aqueous solutions of 10 % w/v, 25 % w/v and 40 % w/v before the measurement. The laser angle was set at 90° and a total of 10 runs were performed for each sample (the reported value is the average).

Viscosity

Dilute aqueous solutions of glycogen (10 % w/v, 25 % w/v, and 40 % w/v) were prepared before the measurement. Viscometric measurements were performed on Haake Mars III (ThermoScientific) rheometer, by using a plate plate measuring geometry. The viscosity of the glycogen aqueous solutions was, first, measured as function of temperature by increasing the temperature from 4℃ to 50℃ at

continuous oscillatory shear of 5 1/s. Flow curves were also measured at 4℃, 20℃, 37℃ and 50℃ respectively, by increasing the shear stress by regular steps each time and waiting for equilibrium at each step. The shear rate (γ) was varied between 0.1 and 1000 s^{-1}.

Surface tension

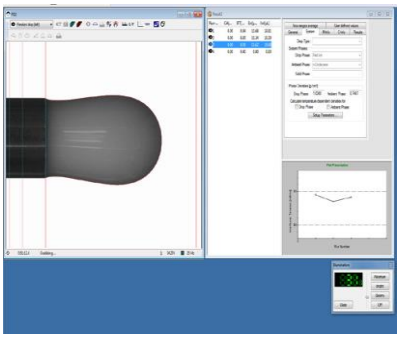


Figure 7 : Analysis of surface tension

Surface tension was measured on an OCA 15EC measuring device for professional contact angle measurements and drop shape analysis. The surface tension of the nanoglycogen solutions was determined using the pendant drop method. The main principle of this technique is to determine the change of the surface tension with time based on the shape of an axisymmetric hanging drop of a liquid. A needle with an outer diameter of 0.65 mm was attached to a plastic 1 ml syringe. The measurements performed in this study were achieved over a period of 180 min at room temperature (22°C). Different concentrations of glycogen solutions were obtained by dilution of a 10 % (w/v) solution that was prepared the same day of the measurement. The analysis of the results is computer controlled and based on the Young–Laplace equation. The critical micelle concentration (CMC) was obtained from the plot of the surface tension against the concentration by taking the line of best fit in two places and noting the concentration at the intersection.

2.2.5. Chemical modification using 1-Octenyl Succinic Anhydride

Chemical modification using 1-Octenyl Succinic Anhydride

To the aqueous solution of glycogen (20% w/w) and the suspensions of phytoglycogen, potato starch and Eliane C100 starch 20% (w/w) 250 μl of 1-octenyl succinic anhydride was added. The pH was adjusted to 7.5 by adding NaOH (% 3v/v). The reaction was conducted at room temperature (20 °C) for 24 h. The pH was maintained between 6.5 and 9.5 approximately by pumping NaOH (1% w/w) when it was necessary. The reaction was terminated after 24h by reducing the pH to 6.5 using HCl 1N. Three volumes of ethanol (99%) were added to the reaction mixture in order to precipitate the modified glycans and the mixture was kept in the freezer overnight. The precipitated materials were recovered by 3 cycles of centrifugation at 10,000 $\times g$ for 10 min and were finally freeze-dried.

Nuclear Magnetic Resonance Spectroscopy—^{1}H-NMR

Liquid nuclear magnetic resonance (NMR) is a useful tool for revealing the molecular structure of a material. A homogeneously dissolved sample is essential if well resolved NMR signals are to be obtained. D_2O has been generally used as a solvent for NMR-samples of starch and starch-related polysaccharides (Gidley and Bociek, 1985). In the present work 1mg/mL solutions of native glycogen and phytoglycogen were prepared in D_2O for analysis. For the OSA- treated glycogen and phytoglycogen samples DMSO-TFA was used as solvent, as due to their more hydrophobic nature, the polymers are not soluble in D_2O.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a a Bruker Avance NMR spectrometer operating at a Larmor frequency of 500.13 MHz for 1H, equipped with a TXI5z probe (Bruker Biospin). During the measurements, 16 scans per spectrum were collected in total, the relaxation delay was 10 and the pulse andle was set to 90°. (Čížová *et al.*, 2007)(Tizzotti *et al.*, 2011)

Degree of substitution

The degree of substitution was determined using the 1H-NMR Procedure for the Characterization of Native and Modified Food-Grade Starches by Tizzotti. (Tizzotti *et al.*, 2011)

3. Results and discussion

3.1. Native glycogen

3.1.1. Material characterization

FTIR

The infrared spectra were performed for the identification of the functional units of the biopolymers. In Figure 8, the spectra of the extracted nanoglycogen and phytoglycogen are illustrated. The broad band at around 3317 cm^{-1} is assigned to the stretching mode of the O-H bonds while the intense band 1651 cm^{-1} illustrates the first overtone of O-H bending vibration. The band at 2922 cm^{-1} is ascribed to C-H stretching while the band at 1145 cm^{-1} is attributed to C-O stretching. The two strong bands at 1076 cm^{-1} and 991 cm^{-1} are due to CH2-O-CH2 stretching vibrations. (Pal, Mal and Singh, 2006)

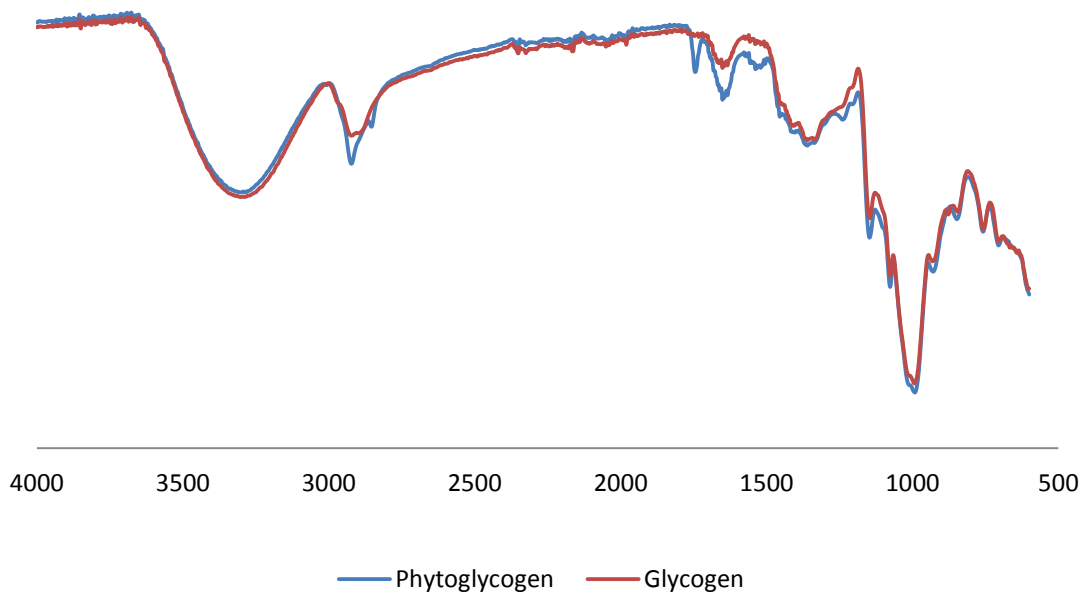


Figure 8: FTIR spectra of *Galdieria sulphuraria* nanoglycogen and phytoglycogen from sweet corn kernels

Surface tension

The surface tension of native nanoglycogen solutions was determined using the pendant drop method. In Figure 9 the change in dynamic surface tension is depicted as a function of native nanoglycogen concentration. Native nanoglycogen showed remarkable surface activity especially if we take into account the hydrophilic nature of the molecule.

By now, there are no liteture data related to surface tension of glycogen from *Galdieria sulphuraria* or other glycogen sources, so we tried to make a correlation of our data with surface tension measurements of starch that we found in literature. The minimum value of surface tension for native potato starch that has been reported in literature is around 60 mN/m (Prochaska *et al.*,2007), while for *Galdieria sulphuraria* glycogen is close to 50 mN/m (Figure 6). Nevertheless, hence there are many factors that affect the surface tension molecule such as the structure of the molecules and their molecular weight a further comparison between these two molecules in context of surface activity would not be considered significant.

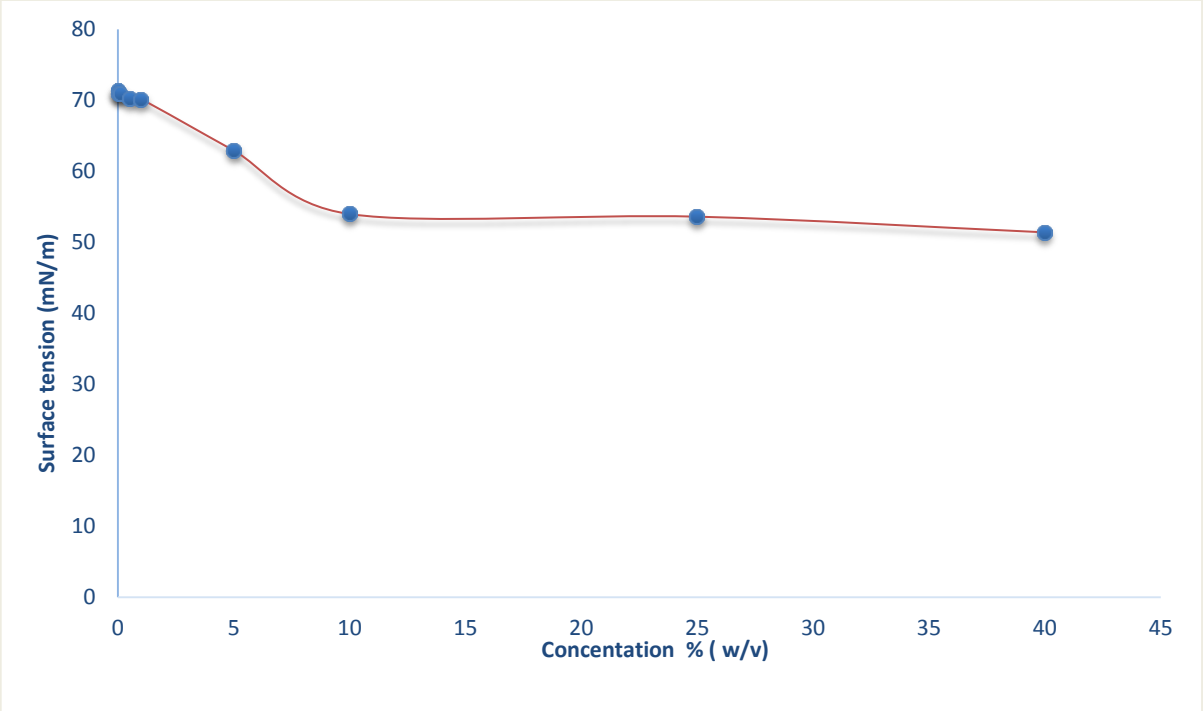


Figure 9: Surface tension as function of concentration for native *Galdieria suplpuraria* nanoglycogen

Consequently, the surface tension of OSA-treated nanoglycogen solutions was determined for comparison purposes. In Figure 7 the change in surface tesion of OSA-treated nanaglycogen is illustrated as function of concentration. To our surprise native glycogen and OSA-treated glycogen apprear to have exactly the same surface

tension with the native polymer at all different concentrations. The moleucular weight and the degree of substitution are two factors that significantly affect the surface tension. The degree of substitution of our OSA-treated nanoglycogen was calculated and the value was around 0.03-0.05. However, while this value is sufficient to claim the success of the OSA substitution (see Section 3.2), it might be quite small to cause any change at the surface activity of the molecule.

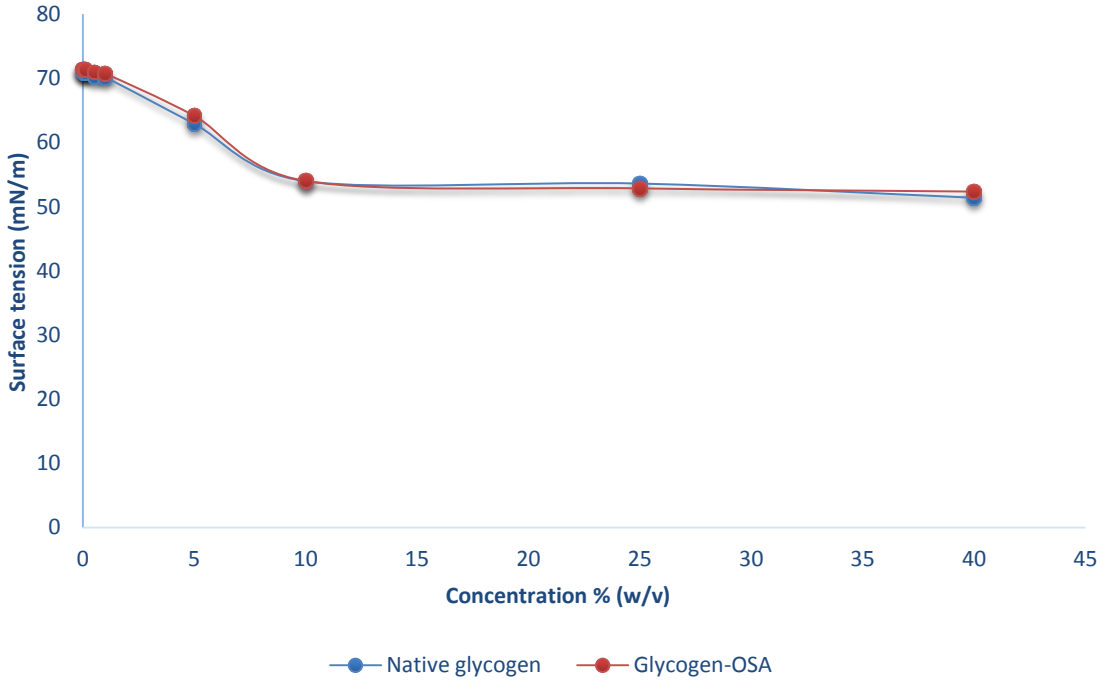


Figure 10: Surface tension as function of concentration for native Galdieria Suplpuraria nanoglycogen and OSA-modified nanoglycogen

In addition, in both Figures 9 and 10 one can notice that after a certain concetration value, the surface tension remain stagnant with respect to concentration. The concentration after which surface tension remains virtually constant with further increase in concentration is called “Critical Miscelle Concentration". In colloidal and surface chemistry, the critical micelle concentration (CMC) is defined as the concentration of surfactants above which micelles form and all additional surfactants added to the system go to micelles. The CMC that was obtained for Figure 10 for both native glycogen and OSA-treated glycogen was 10 g/L. According to liturature, a typical CMC for OSA-modified starches are of about 5 g/L. (Varona, Martín and Cocero, 2009) While the two values are at same range, the lower CMC value of starch can be attributed to its higher molecular weight compared to glycogen.

Zeta potential

The particles in a colloidal suspension or emulsion usually carry an electrical charge. The charge is more often negative than positive and it may arise in a number of ways. Sometimes the surface of the particles contains chemical groups that can ionize to produce a charged surface. Sometimes the surface itself preferentially adsorbs ions of one sign of charge in preference to charges of the opposite sign. In other cases there may be deliberately added chemical compounds that preferentially adsorb on the particle surface to generate the charge. (Hunter, 2001)

In the present work zeta potential was measured with purpose to examine the presence of charges that may affect the stability of the prepared nanoglycogen solutions. Three different concentrations (10, 25 and 40 % w/v) of native nanoglycogen aqueous solutions were measured using a Brookhaven ZetaPALS zeta potential analyzer. For all three solution concentrations, the charge was negative and the measured value was very close to zero (on average -1 to -2 mV) which is, in principle, the charge value of the –OH groups in the glycogen surface.

3.1.2. Rheology

Viscosity studies provide a reasonable evaluation of the bulk macroscopic solution behavior. The viscosity of the extracted nanoglycogen was measured in solution at three different concentrations (10, 25 and 40 % (w/v)), first as function of temperature and fixed shear rate and afterwards as function of shear rate at temperatures that we consider more relevant in the view of possible applications.

3.2.1. Viscosity as function of temperature

Figure 11 shows the effect of temperature on the viscosity, measured at fixed shear rate γ=5 1/s. As expected, the viscosity of all nanoglycogen solutions tends to decrease, and, more specifically, to be halved at higher temperatures. This inverse relationship can be attributed to the incidence of a freer molecule to molecule interaction at higher temperatures. Since viscosity is an indication of the resistance to flow, freer interaction between the molecules that is triggered by the increase of temperature, is expected to reduce the flow resistance of the polymer (Viswanath, 2010).

In particular, in Figure 11 we can observe that at concentration of 10% (w/v) and 25 % (w/v) the viscosity values of glycogen in solution are almost at the same order and close the viscosity value of tap water, while at a concentration of 40 % (w/v) there is a noticeable increase in viscosity. This can be attributed to the higher chance of chain

entanglement in more concentrated solutions than in less concentrated ones (Guo *et al.*, 2016).

The increase in viscosity is even more intense if we look at carbohydrate structures quite different from glycogen such as highly branched starch (HBS) (Table 1) (Martinez-Garcia, Krompa and van der Maarel, 2017). Longer polymer chains (DP>10) present in structure of HBS, but absent in glycogen, get entangled with each, slowing down the movement of the polymer molecules more drastically (Martinez-Garcia, Krompa and van der Maarel, 2017). In addition, it has been shown that for polysaccharides such as cellulose and highly-branced cyclodextins when solid concentration is high, the viscosity increases because of stonger hydrogen bonding with hydroxyl groups and the distortion in the velocity pattern of the liquid by hydrated molecules of solute (Togrul, 2003) (Szejtli and Davies, 2010) .

Another indication of the presence of strong hydrogen bonds that break at high temperatures is the fact that at solution concentration 40 % (w/v) we can observe that after 50°C viscosity tends to increase. This in turn implies the presence of hydrogen bonds at high solution concentrations that break at tempetures higher than 50°C. This in combination with the relative high surface activity of the molecule suggest some kind of unexpected hydrophobic interactions that might take place in solution. The released hydrophobic groups assosiate intermolecularly, thus intensifying the viscosity (Billon and Borisov, 2016).

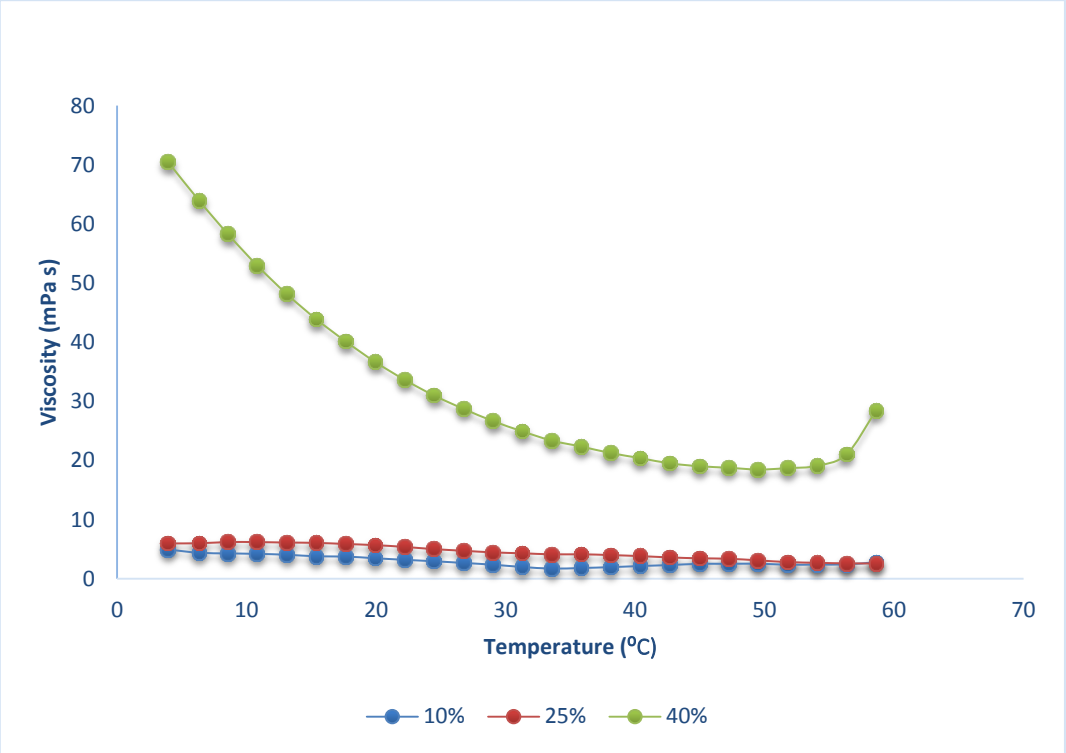


Figure 11 : Viscosity as function of temperature for nanoglycogen solution concentrations 10,25 and 40 % (w/v)

T (°C)	Viscosity (mPa-s)			
	Nanolycogen		HBS	
	25%	40%	25%	40%
4	22	52	59	246
20	13	27	40	209
38	8	15	24	109

Table 1: Viscosity of nanoglycogen and HBS solutions at concentrations of 25 and 40 % (w/v) and temperatures 4,20 and 38^{o}C.

3.2.2. Viscosity as function of shear rate

The vast majority of polysaccharide solutions are pseudoplastic. Their high molecular weight and concentration form an entangled network in solution impedes flow, thus their solutions may deviate substantially from Newtonian flow (Wang and Cui, 2005).

The following figures show typical shear flow curves of native glycogen aqueous solutions at concentrations of 10, 25 and 40 % (w/v) and temperatures 5°C , 20°C, 37°C and 50°C. From the diagrams (Figures 12,13 and 14), it is evident that the majority of the prepared solutions exhibit a clear Newtonian behavior and generally the extracted biopolymers don not show shear thinning behavior or degradation not even in very high shear rates (above 300/s). This can be attributed to the the fact that glycogen has shorter branches than many common carbohydrates creating a less entangled network, thus the interactions between the branches are not that intense.

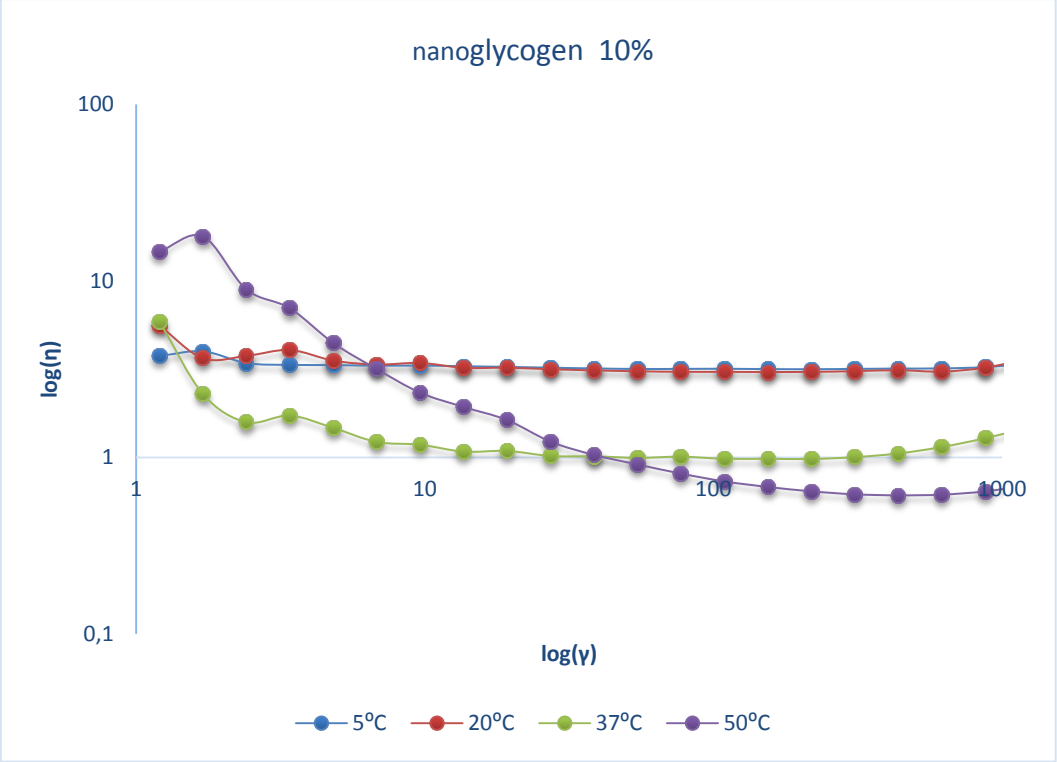


Figure 12: Viscosity as function of shear rate at 2,20,37 and 50 ° for nanoglycogen solution concentration 10 % (w/v) (in logarithmic scale)

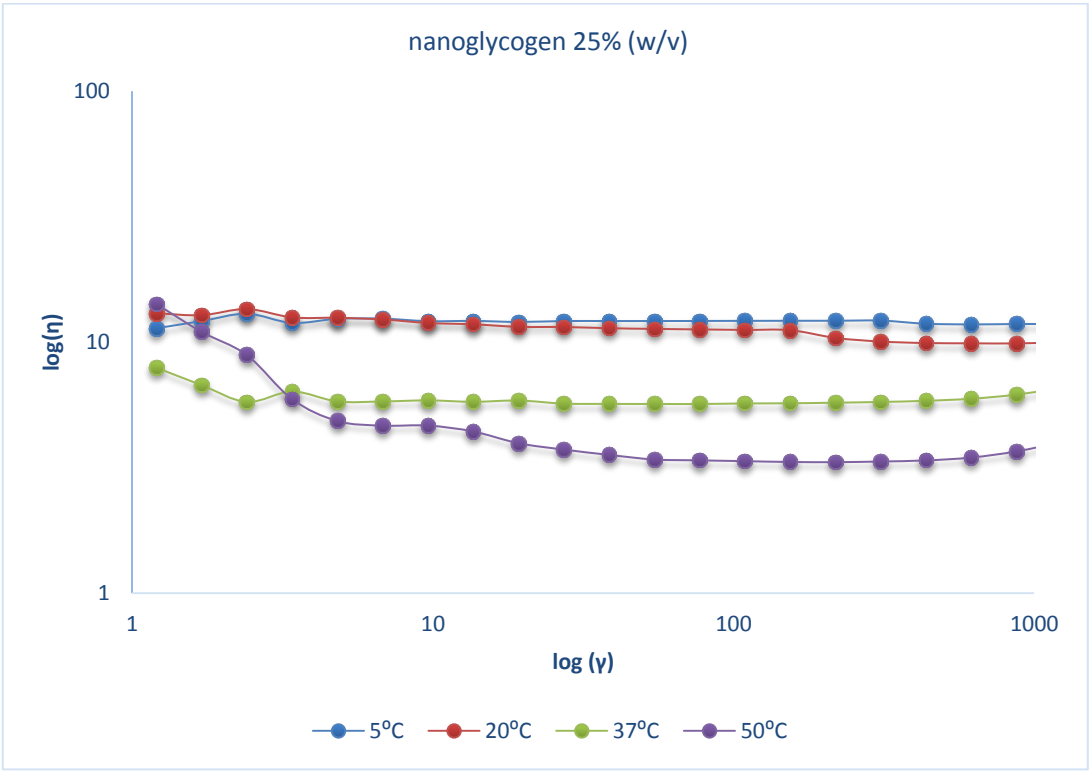


Figure 13: Viscosity as function of shear rate at 2,20, 37 and 50 °C for nanoglycogen solution concentration 10 % (w/v) (in logarithmic scale)

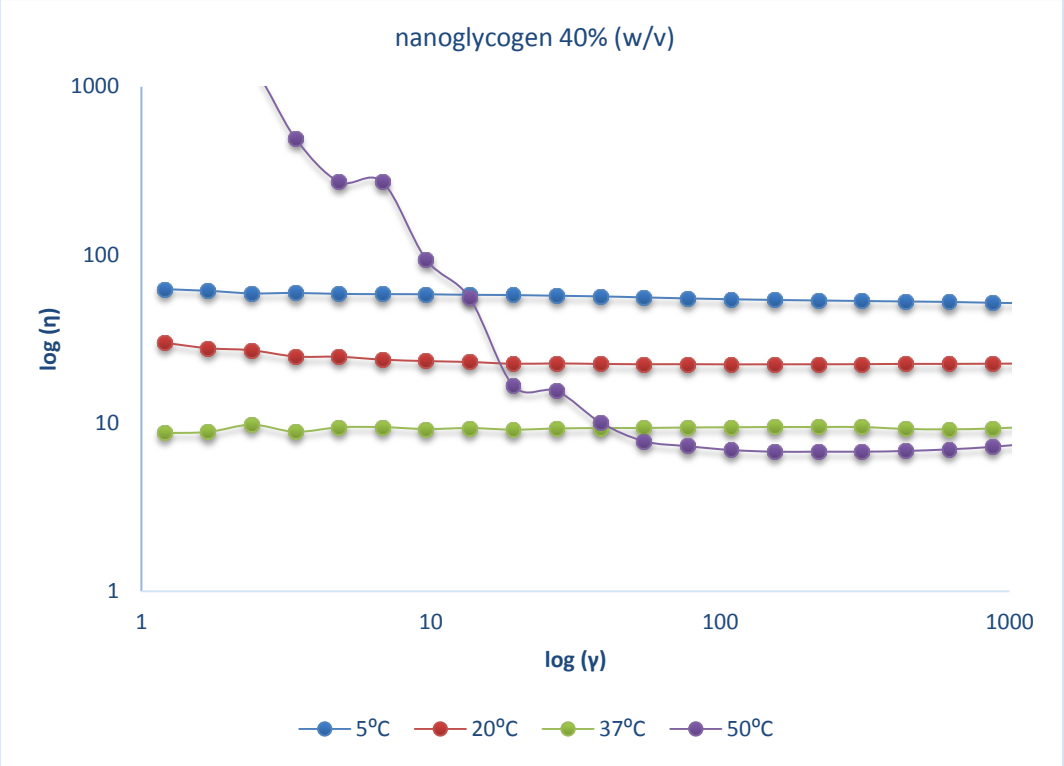


Figure 14 : Viscosity as function of shear rate at 2,20, 37 and 50 °C for nanoglycogen solution concentration 40 % (w/v) (in logarithmic scale)

What is very interesting to observe is that, again, at temperatures above 37°C, the viscosity values of the polymers are very high at low shear and after certain rate (close to 5/s) they level off. In Figures 15,16 and 17, it is evident that the highest the temperature the most intense this phenomenon is. In addition, one we can observe that this applies not only for high solution concentrations (close to 40% (v/v)), as it seems at Figure 11 ,but also for low concentrations close to 10 % (v/v). Again, this is a strong indication of the presence of hydrogen bonds that possibly break at high temperatures. This suggests some kind of hydrophobic interactions that might take place in solution. There are released hydrophobic groups that assosiate intermolecularly, thus intensifying the viscosity (Billon and Borisov, 2016). In addition, high shear rate seems to weaken these intermolecular assosiations of the hydrophobic groups and, thus, after a certain value of shear rate (close to 5/s) the viscosity levels off. After that, the viscosity values of all the polymer solutions are independent of the rate of shear, hence the behavior of the polymers in clearly Newtonian.

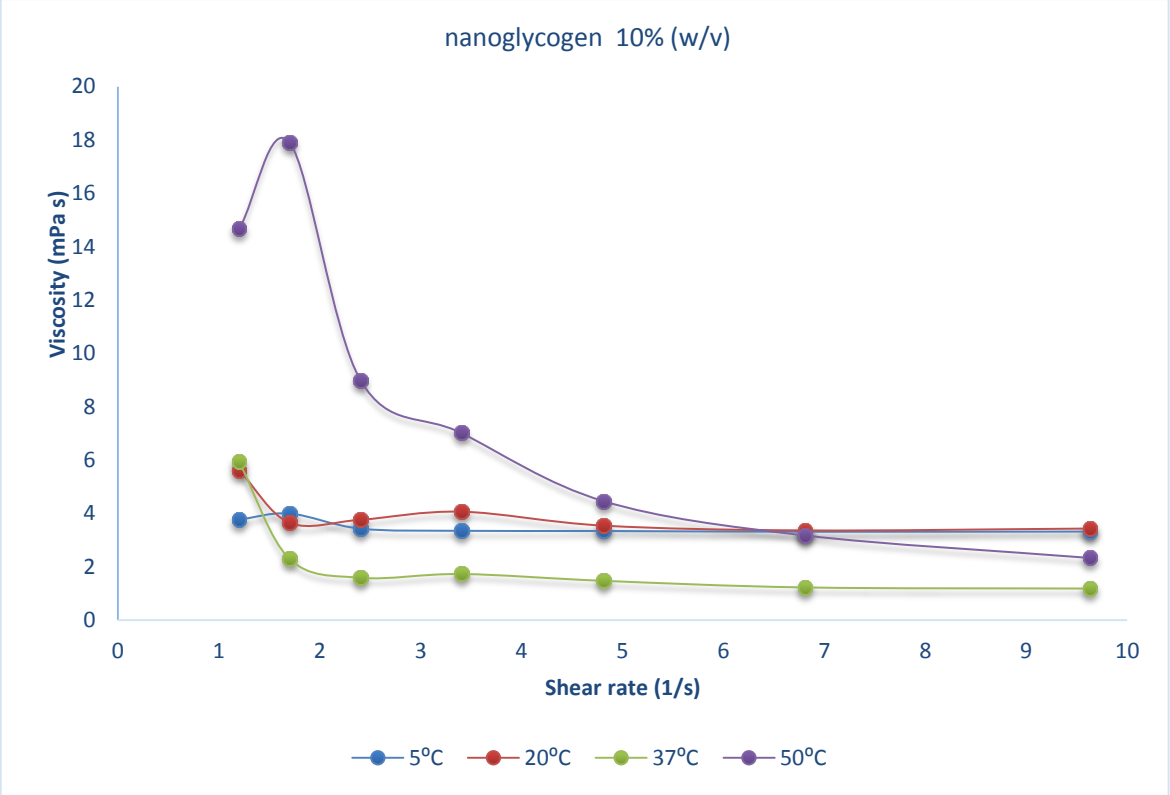


Figure 15: Viscosity as function of shear rate at 2,20, 37 and 50 °C at low shear rate for nanoglycogen solution concentration 10 % (w/v)

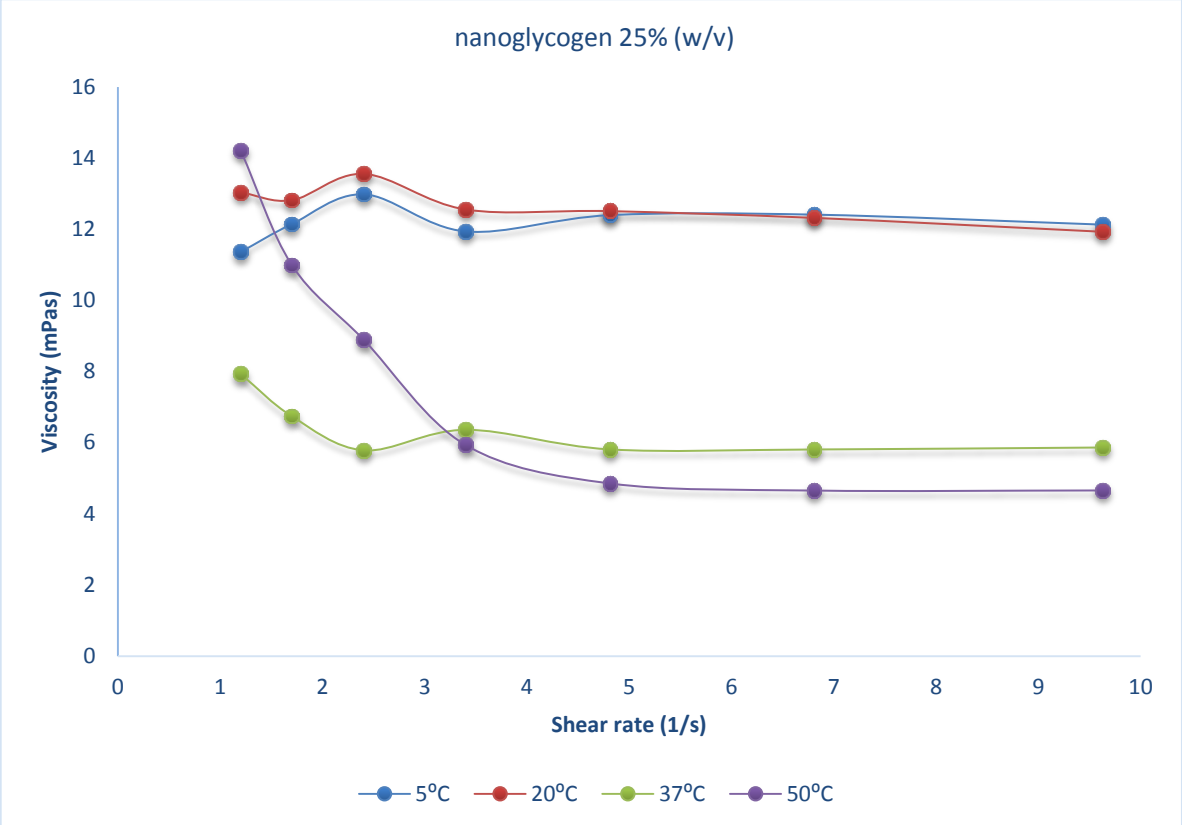


Figure 16 : Viscosity as function of shear rate at 2,20, 37 and 50 °C at low shear rate for nanoglycogen solution concentration 25 % (w/v)

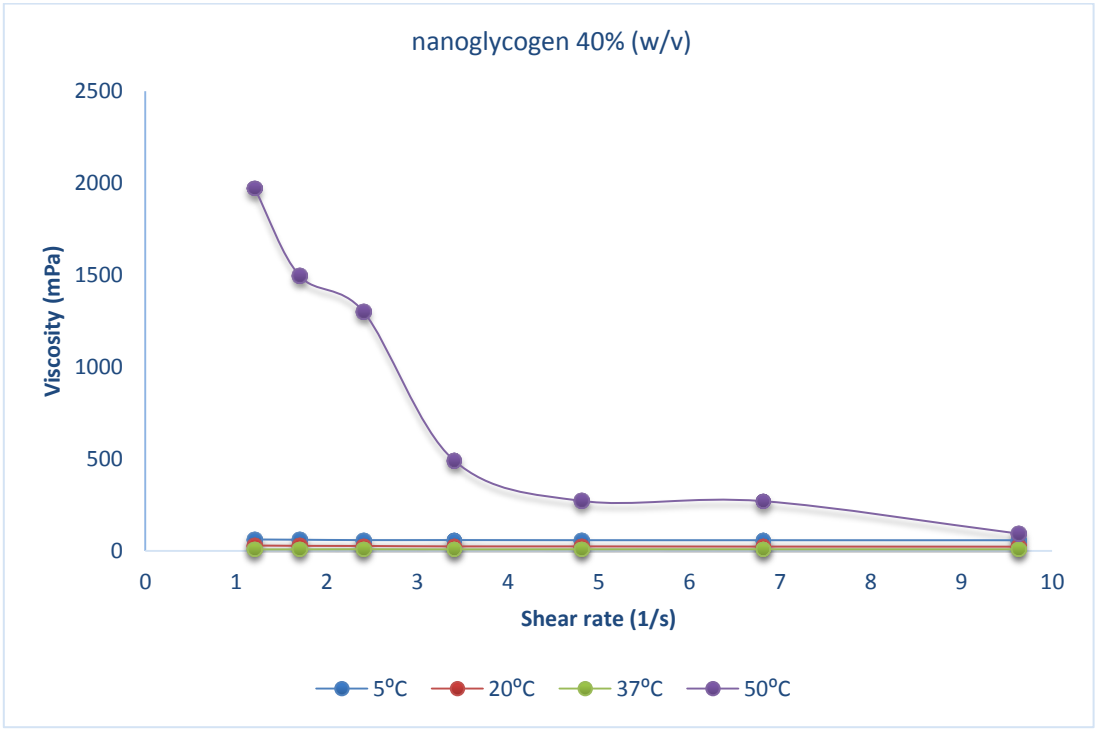


Figure 17 : Viscosity as function of shear rate at 2,20, 37 and 50 °C at low shear rate for nanoglycogen solution concentration 40 % (w/v)

3.2. OSA-treated material

3.2.1. Material Characterization

NMR

^{1}H-NMR spectra were mainly performed to facilitate the structure identification of prepared polymers and for the calculation of the degree of substitution. For analysis, the publication ^{1}H-NMR Procedure for the Characterization of Native and Modified Food-Grade Starches ' by Tizzoti et al was used as a reference (Tizzotti *et al.*, 2011). PGOS and GOS, because of their amphiphilic character, were not soluble in D_2O as their native counterparts, so DMSO-TFA was used as solvent. In Figure 18 the ^{1}H-NMR spectra of both GOS and PGOS after 9 hours of reaction are presented.

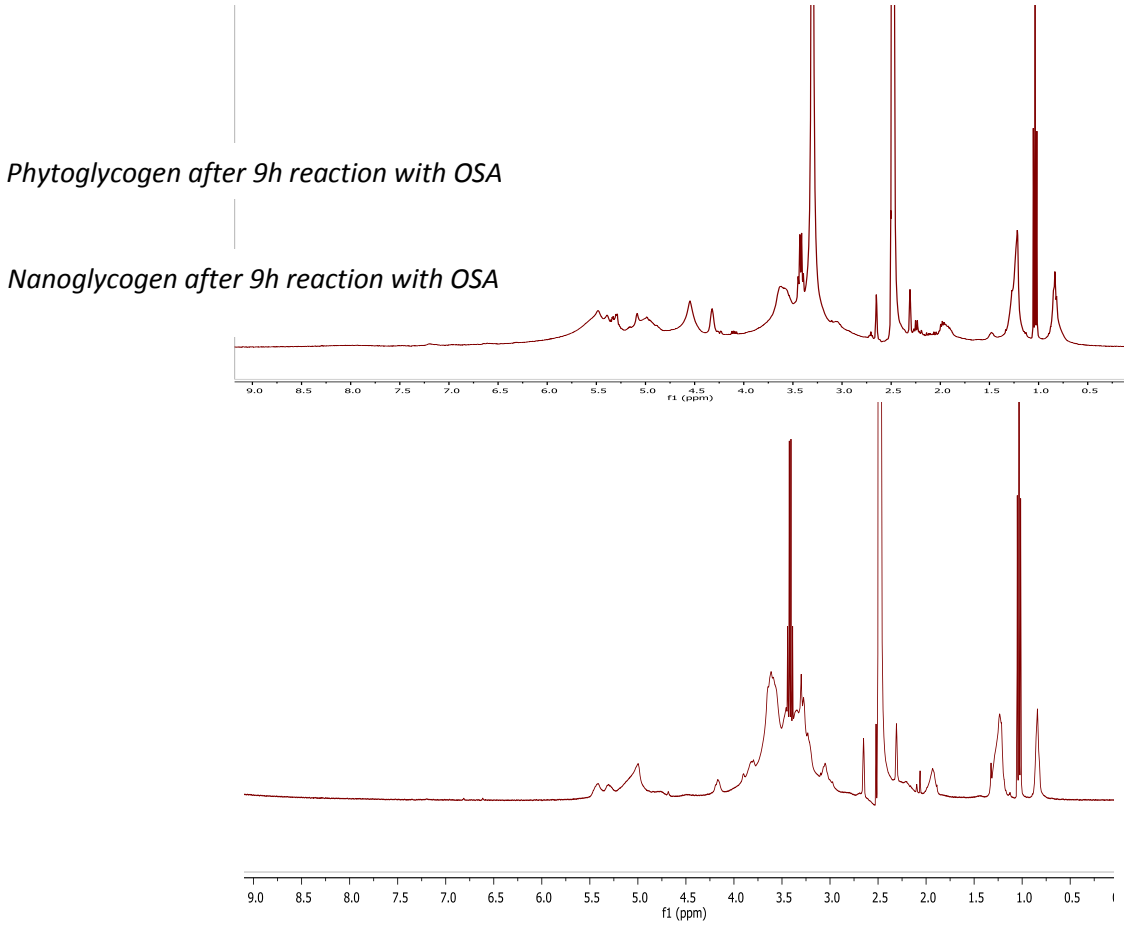


Figure 18: NMR spectra of OSA-modified *Galdieria sulphuraria* nanoglycogen and phytoglycogen from sweet corn kernels after 9 hours of reaction

Degree of substitution

The degree of substitution of the polymers was calculated based on 1H-NMR Procedure for the Characterization of Native and Modified Food-Grade Starches by Tizzotti. (Tizzotti *et al.*, 2011), by using the formula:

$$DS = \frac{I_{0.89}}{4(I_{\alpha-1.6} + I_{\alpha-1.4} + I_{r-e)}} \qquad \text{(Eq. 1)}$$

In the formula, $I_{0.89}$ is the ^{1}HNMR integral of the signal of the CH_3 group of OSA in d6-DMSO and $I_{r\text{-}e}$ corresponds to the reducing chain ends (Čížová *et al.*, 2007). $I_{\alpha\text{-}1,6}$ are based on the glycogen α-(1,4) linkages at 5,3-4,8 ppm and $I_{\alpha\text{-}1,4}$ based on are based on the glycogen α-(1,6) at 4,8-4,6 ppm (Martinez-García, 2017). (Figure 18)

The degree of substitution of glycogen after 24 hours of reaction was 0.05 and it is at same range of the structurally similar OSA-modified phytoglycogen and starches invested by Scheffler and Tizzoti respectively (Scheffler *et al.*, 2010; Tizzotti *et al.*, 2011).

4. Conclusions

During this research project, the extraction, characterization and the chemical modification with OSA of nanoglycogen from *Galdieria Sulphuraria* were successfully accomplished.

The nanoglycogen from *Galdieria Sulphuraria,* due to its peculiar highly branched structure, showed decreased solution viscosity, a clearly Newtonian behavior and no shear-thinning effects even for very high shear rates. However, all the prepared glycogen solutions demonstrated some thickening effects and the viscosity values increased dramatically at high temperatures and low shear rates. This fact in combination with the remarkably high surface activity of the the molecule may imply some unexpected hydrophobic interactions that may take place in solution.

In addition, it was proven that the G*aldieria Sulphuraria* nanonglycogen can be easily substituted with OSA, through a 24 hour reaction. The degree of substitution was found to be at the same range of the already widely used OSA-modified starches. This in combination with the relative high surface activity of the molecule can be very promising in context of the emulsification properties of the polymers and their potential use as emulsifiers in food industry.

Hopefully, the present investigation will help the further research on the field of carbohydrate functional biopolymers.

5. Recommendations

While a significant progress has been achieved, this research has the potential to continue and lead to great advances. Future work that is recommended includes the following paths:

- Better characterization of the molecule, using elemental analysis, in order to investigate in debth the nature of the molecule and explain the indications of hydrophobic interactions that are present in *Galdieria suplphuraria* glycogen aqueous solutions.

- Further investigation on the in vitro growth conditions of red microagla *Galdieria Sulphuraria* and on the extraction methods of nanoglycogen from *Galdieria Sulphuraria* cells for increasing the yield of the extracted nanoglycogen.
- A complete investigation on the emulsification properties of the OSA-modified nanoglycogen from *Galdieria Sulphuraria*.
- More research on the reaction mechanism of nanoglycogen from *Galdieria Sulphuraria* for optimizing the reaction conditions, increasing the possibility of a higher degree of substitution.

References

Alcázar-Alay, S. and Meireles, M. (2015). Physicochemical properties, modifications and applications of starches from different botanical sources. *Food Science and Technology (Campinas)*, 35(2), pp.215-236.

Backer (2017). *Soluble highly branched glucose polymers and their mode of production*. US 20040014961 A1.

Ball, S., Colleoni, C., Cenci, U., Raj, J. and Tirtiaux, C. (2011). The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. *Journal of Experimental Botany*, 62(6), pp.1775-1801.

Bao, J., Xing, J., Phillips, D. and Corke, H. (2003). Physical Properties of Octenyl Succinic Anhydride Modified Rice, Wheat, and Potato Starches. *Journal of Agricultural and Food Chemistry*, 51(8), pp.2283-2287.

Billon, L. and Borisov, O. (2016). *Macromolecular self-assembly*. Hoboken (N.J.): John Wiley & Sons, Inc.

Binks, B. P. (2002) 'Particles as surfactants - Similarities and differences', *Current Opinion in Colloid and Interface Science*, 7(1–2), pp. 21–41. doi: 10.1016/S1359-0294(02)00008-0.

Biwer, A., Antranikian, G. and Heinzle, E. (2002) 'Enzymatic production of cyclodextrins', *Applied Microbiology and Biotechnology*, 59(6), pp. 609–617. doi: 10.1007/s00253-002-1057-x.

B.S. Albuquerque, P., C.B.B. Coelho, L., A. Teixeira, J. and G. Carneiro-da-Cunha, M. (2016). Approaches in biotechnological applications of natural polymers. *AIMS Molecular Science*, 3(3), pp.386-425.

Chevalier, Y. and Bolzinger, M. A. (2013) 'Emulsions stabilized with solid nanoparticles: Pickering emulsions', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. Elsevier B.V., 439, pp. 23–34.

Čížová, A., Koschella, A., Heinze, T., Ebringerová, A. and Sroková, I. (2007) 'Octenylsuccinate derivatives of carboxymethyl starch - Synthesis and properties', *Starch/Staerke*, 59(10), pp. 482–492. Dutcher, J. R. and Graham, L. G.- (2010) 'Pubhcatlon Classl ? catlon as Wellpas modirigers if', (19). doi: 10.1016/S0065-2318(10)64004-8.

Deremaux, L., Petitjean, C. and Wills, D. (2013) *Soluble highly branched glucose polymers for enteral and parental nutricion and peritoneal dialysis*. US 8445462 B2.

Dutcher, A., Robert, J. (2010). *Polysacharide naoparticles*. US 20100272639.

Ellis, R. P., Cochrane, M. P., Dale, M. F. B., Duþus, C. M., Lynn, A., Morrison, I. M., Prentice, R. D. M., Swanston, J. S. and Tiller, S. a (1998) 'Starch Production and Industrial Use', *Journal of the Science of Food and Agriculture*, 77, pp. 289–311.

Fernandez, C., Rojas, C. C. and Nilsson, L. (2011) 'Size, structure and scaling relationships in glycogen from various sources investigated with asymmetrical flow field-flow fractionation and 1H NMR', *International Journal of Biological Macromolecules*. Elsevier B.V., 49(4), pp. 458–465. doi: 10.1016/j.ijbiomac.2011.05.016.

Filippov, S. K., Sedlacek, O., Bogomolova, A., Vetrik, M., Jirak, D., Kovar, J., Kucka, J., Bals, S., Turner, S., Stepanek, P. and Hruby, M. (2012) 'Glycogen as a Biodegradable Construction Nanomaterial for in vivo Use', *Macromolecular Bioscience*, 12(12), pp. 1731–1738.

Gallant, D. J., Bouchet, B. and Baldwin, P. M. (1997) 'Microscopy of starch: evidence of a new level of granule organization', *Carbohydrate Polymers*, 32(3–4), pp. 177–191.

García, M. (2017) 'Functional carbohydrates from the red microalga Galdieria sulphuraria Marta Martínez García'.

Geddes, R., Harvey, J. D. and Wills, P. R. (1977) 'Hydrodynamic Properties of 2-Mercaptoethanol-Modified Glycogen', 472, pp. 465–472.

Gidley, M. J. and Bociek, S. M. (1985) 'Molecular Organization in Starches: A Study', *Journal of Americal Chemical Society*, 107(10), pp. 7040–7044.

Guo, L., Hu, J., Zhang, J. and Du, X. (2016) 'The role of entanglement concentration on the hydrodynamic properties of potato and sweet potato starches', *International Journal of Biological Macromolecules*, 93(October), pp. 1–8.

Hunter, R. (2001). *Foundations of colloid science*. Oxford: Oxford University Press.

Kennedy, J. and Lloyd, L. (1992). Carbohydrates as Organic Raw Materials. *Carbohydrate Polymers*, 18(4), p.311.

Klemm, D., Heublein, B., Fink, H. and Bohn, A. (2005). Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *ChemInform*, 36(36).

Lichtenthaler, F. W. and Mondel, S. (1997) 'Perspectives in the use of low molecular weight carbohydrates as organic raw materials', *Pure and Applied Chemistry*, 69(9), pp. 1853–1866.

Manners, D. J. (1989) 'Recent developments in our understanding of amylopectin structure', *Carbohydrate Polymers*, 11(2), pp. 87–112. dManners, D. J. (1991) 'Recent developments in our understanding of glycogen structure', *Carbohydrate Polymers*, 16(1), pp. 37–82.

Martinez-Garcia, M., Kormpa, A. and van der Maarel, M. J. E. C. (2017) 'The glycogen of Galdieria sulphuraria as alternative to starch for the production of slowly digestible and resistant glucose polymers', *Carbohydrate Polymers*. Elsevier Ltd., 169, pp. 75–82.

Martinez-Garcia, M., Stuart, M. C. A. and van der Maarel, M. J. E. C. (2016) 'Characterization of the highly branched glycogen from the thermoacidophilic red microalga Galdieria sulphuraria and comparison with other glycogens', *International*

Journal of Biological Macromolecules. Elsevier B.V., 89, pp. 12–18.

Maughan, R. J. (1998) 'The sports drink as a functional food: formulations for successful performance.', *The Proceedings of the Nutrition Society*, 57(1), pp. 15–23. Mistry, C. D. and Gokal, R. (1993) 'Can ultrafiltration occur with a hypo-osmolar solution in peritoneal dialysis?: The role for "colloid" osmosis', *Clin Sci (Lond)*, 85(4), pp. 495–500.

Moberly, J. B., Mujais, S., Gehr, T., Hamburger, R., Sprague, S., Kucharski, A., Reynolds, R., Ogrinc, F., Martis, L. and Wolfson, M. (2002) 'Pharmacokinetics of icodextrin in peritoneal dialysis patients.', *Kidney international. Supplement*, 62(81), pp. S23–S33. doi: 10.1046/j.1523-1755.62.s81.5.x.

Moreira, D., López-Archilla, A.-I., Amils, R. and Marí-n, I. (1994) 'Characterization of two new thermoacidophilic microalgae: Genome organization and comparison with Galdieria sulphuraria', *FEMS Microbiology Letters*, 122(1–2), pp. 109–114.

Morris, E., Cutler, A., Ross-Murphy, S., Rees, D. and Price, J. (1981). Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions. *Carbohydrate Polymers*, 1(1), pp.5-21.

Niaounakis, M. (2015). *Biopolymers*. Oxford, U.K.: William Andrew.

Pal, S., Mal, D. and Singh, R. P. (2006) 'Synthesis, characterization and flocculation characteristics of cationic glycogen: A novel polymeric flocculant', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 289(1–3), pp. 193–199.

Prochaska, K., Kędziora, P., Le Thanh, J. and Lewandowicz, G. (2007). Surface activity of commercial food grade modified starches. *Colloids and Surfaces B: Biointerfaces*, 60(2), pp.187-194.

Rolland-Sabaté, A., Mendez-Montealvo, M., Colonna, P. and Planchot, V. (2008). Online Determination of Structural Properties and Observation of Deviations from Power Law Behavior. *Biomacromolecules*, 9(7), pp.1719-1730.

Röper, H. (2002). Renewable Raw Materials in Europe-Industrial Utilisation of Starch and Sugar [1]. *Starch - Stärke*, 54(3-4), pp.89-99.

Sakurai, T., Aoki, M., Ju, X., Ueda, T., Nakamura, Y., Fujiwara, S., Umemura, T., Tsuzuki, M. and Minoda, A. (2016) 'Profiling of lipid and glycogen accumulations under different growth conditions in the sulfothermophilic red alga Galdieria sulphuraria', *Bioresource Technology*. Elsevier Ltd, 200, pp. 861–866.

Scheffler, S. L., Huang, L., Bi, L. and Yao, Y. (2010) 'In vitro digestibility and emulsification properties of phytoglycogen octenyl succinate', *Journal of Agricultural and Food Chemistry*, 58(8), pp. 5140–5146.

Scheffler, S. L., Wang, X. U. E., Huang, L. E. I., Gonzalez, F. S. M. and Yao, Y. (2010) 'Phytoglycogen octenyl succinate, an amphiphilic carbohydrate nanoparticle, and ??-polylysine to improve lipid oxidative stability of emulsions', *Journal of Agricultural and Food Chemistry*, 58(1), pp. 660–667.

Shimonaga, T., Fujiwara, S., Kaneko, M., Izumo, A., Nihei, S., Francisco, P. B., Satoh, A., Fujita, N., Nakamura, Y. and Tsuzuki, M. (2007) 'Variation in storage α-polyglucans of red algae: Amylose and semi-amylopectin types in Porphyridium and glycogen type in Cyanidium', *Marine Biotechnology*, 9(2), pp. 192–202.

Shimonaga, T., Konishi, M., Oyama, Y., Fujiwara, S., Satoh, A., Fujita, N., Colleoni, C., Bul A., Putaux, J. L., Ball, S. G., Yokoyama, A., Hara, Y., Nakamura, Y. and Tsuzuki, M. (2008) 'Variation in storage glucans of the Porphyridiales (Rhodophyta)', *Plant and Cell Physiology*, 49(1), pp. 103–116.

Stadnichuk, I. N., Semenova, L. R., Smirnova, G. P. and Usov, a. I. (2007) 'A highly branched storage polyglucan in the thermoacidophilic red microalga Galdieria maxima cells', *Applied Biochemistry and Microbiology*, 43(1), pp. 78–83. doi: 10.1134/S0003683807010140.

Stick, R. and Spencer Williams (2010). *Carbohydrates: The Essential Molecules of Life*. Elsevier Science.

Sullivan, M., Vilaplana, F., Cave, R., Stapleton, D., Gray-Weale, A. and Gilbert, R. (2010). Nature of α and β Particles in Glycogen Using Molecular Size Distributions. *Biomacromolecules*, 11(4), pp.1094-1100.

Sweedman, M. C., Tizzotti, M. J., Schäfer, C. and Gilbert, R. G. (2013) 'Structure and physicochemical properties of octenyl succinic anhydride modified starches: A review', *Carbohydrate Polymers*. Elsevier Ltd., 92(1), pp. 905–920.

Szejtli, J. and Davies, J. (2010). *Cyclodextrin Technology*. Dordrecht: Springer Netherlands.

Takii H., Ishihara, K., Kometani, T., Okada, S. and Fushiki, T. (1999). Enhancement of Swimming Endurance in Mice by Highly Branched Cyclic Dextrin. *Bioscience, Biotechnology, and Biochemistry*, 63(12), pp.2045-2052.

Takii, H., Takii (Nagao), Y., Kometani, T., Nishimura, T., Nakae, T., Kuriki, T. and Fushiki, T. (2005). Fluids Containing a Highly Branched Cyclic Dextrin Influence the Gastric Emptying Rate. *International Journal of Sports Medicine*, 26(4), pp.314-319.

Tizzotti, M. J., Sweedman, M. C., Tang, D., Schaefer, C. and Gilbert, R. G. (2011) 'New 1 H NMR Procedure for the Characterization of Native and Modified Food-Grade Starches', *Journal of Agricultural and Food Chemistry*, 59(13), pp. 6913–6919.

Togrul, H. (2003). Flow properties of sugar beet pulp cellulose and intrinsic viscosity–molecular weight relationship. *Carbohydrate Polymers*, 54(1), pp.63-71.

Van der Maarel, M. and Leemhuis, H. (2013). Starch modification with microbial alpha-glucanotransferase enzymes. *Carbohydrate Polymers*, 93(1), pp.116-121.

Varona, S., Martín, Á. and Cocero, M. J. (2009) 'Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches', *Chemical Engineering and Processing: Process Intensification*, 48(6), pp. 1121–1128.

Viswanath, D. (2010). *Viscosity of liquids*. Dordrecht: Springer.

Wang, Q., & Cui, S. W. (2005) *Understanding the physical properties of food polysaccharides, 2005*.

Wong, K.-S., Kubo, A., Jane, J., Harada, K., Satoh, H. and Nakamura, Y. (2003) 'Structures and Properties of Amylopectin and Phytoglycogen in the Endosperm of sugary-1 Mutants of Rice', *Journal of Cereal Science*, 37(2), pp. 139–149.

Appendices

Appendix A: Allen Medium composition

Macro-elements				Trace elements			
$(NH_4)_2SO_4$	10 mM	$(NH_4)_2SO_4$	1.32 g/L	H_3BO_3	46 µM	H_3BO_3	2.8 mg/L
KH_2PO_4	2 mM	KH_2PO_4	272 mg/L	$MnCl_2$	9.1 µM	$MnCl_2*4H_2O$	1.8 mg/L
$MgSO_4$	1 mM	$MgSO_4*7H_2O$	246.5 mg/L	$ZnSO_4$	760 nM	$ZnSO_4*7H_2O$	0,218 mg/L
$CaCl_2$	0.5 mM	$CaCl_2*2H_2O$	73.5 mg/L	$CuSO_4$	310 nM	$CuSO_4$	0.05 mg/L
$FeCl_3$	71 µM	$FeCl_3$	11 mg/L	NH_4VO_3	200 nM	NH_4VO_3	0.023 mg/L
				$Na_2MoO_4*2H_2O$	100 nM	$Na_2MoO_4*2H_2O$	0.0242 mg/L

Appendix B: HBS-AMG production and some viscosity results

In order to have a wide picture of the rheological properties of our nanoglycogen at certain temperatures of our interest (fridge temperature, room temperature and temperature of the human body), we used enzyme-treated nanoglygen, HBS and enzyme-treated HBS as reference. The enzyme we used was amyloglucosidase and here is the protocol that we used to produce HBS-AMG and the results we got.

- *Production of HBS-AMG*

Highly Branched Starch (HBS) was dissolved by stirring at concentration of 10 g/L in sodium acetate buffer 200 mM pH 4 + 5 mM $CaCl_2$ + 0.02% NaN_3. The reaction volume was 6 L and it was divided in smaller volumes of 1L. The substrate solution was kept in a shaking incubator at 40°C overnight to allow the reaction volume to reach the temperature. Afterwards, 307 µL of of amyloglucosidase per liter of reaction were added and the reaction mixture is incubated while shaking (or stirring) at 40°C for 180 min. The enzyme was inactivated by adding NaOH at the reaction mixture until Ph 7.5.

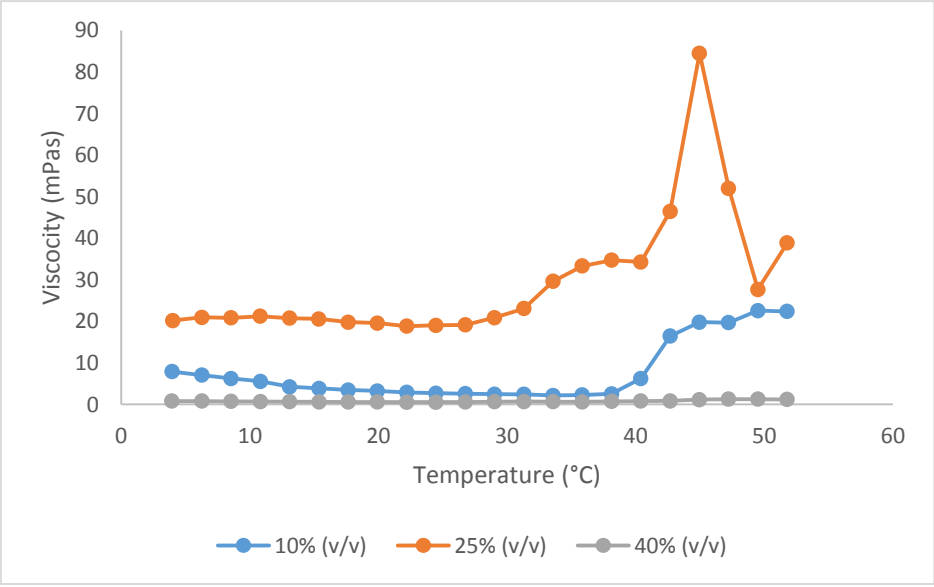
After enzyme inactivation, each reaction volume was evaporated until 500 mL left in each bottle, using the rotary evaporator at 60°C pressure of 72 mbar. Then, 1.5 volumes of ethanol 100% (v/v) were added to each concentrated reaction volumes and

after that they were kept precipitating at 4°C overnight. The precipitated polymer was recovered by centrifugation at 20000 g, 10-15 min, 4°C and then it was freeze-dried. The dried polymer was re-dissolved in water and transferred to a dialysis membrane and it dialyzed against ultra-pure water while stirring at 4°C overnight (*Dialysis eliminates possible salts from the buffer and glycerol from the enzyme solution.)* The dialyzed sample was transferred from the membrane to a bottle. After that, 1.5 vol. of ethanol 100% (v/v) were added and then the polymer precipitated at 4°C overnight. Finally, the precipitated polymer was recovered by centrifugation at 20000 g, 10-15 min, 4°C and freeze-dried.

- *Viscosity vs Temperature results*

T (°C)	Viscosity (mPa-s)							
	Glycogen		Glycogen-AMG		HBS		HBS-AMG	
	25%	40%	25%	40%	25%	40%	25%	40%
4	22	52	15	42	59	246	45	58
20	13	27	15	22	40	209	27	49
38	8	15	11	12	24	109	9	29

Appendix C: Viscosity vs Temperature for phytoglycogen solution concentrations 10.25 and 40 % (w/v)



Appendix D: Cloud Point and Pour Point Measurement

Cloud point and pour point were measured using TANAKA's MPC-102A/102L Pour/Cloud Point Testers. PP measurement is by "Air Pressure Method" (ASTM D6749), and CP measurement is by "Small Test Jar Method" (ASTM D7683). The glycogen samples were cooled up in a glass cuvette and inspected at intervals of 1°C until -10°C. The temperature at which the first cloud or haze appeared was automatically recorded as cloud point and the temperature at which the formation of the first wax crystal structure was detected was recorded as pour point.

For all nanoglycogen solution concentrations, the CP and PP values were close to 0^{o}C. These values are more likely to represent the freezing temperature of water and not the real CP and PP of the solutions. We would suggest that the measurement would be repeated by increasing the temperature above room temperature, so the possibility to find the CP and PP of the nanoglycogen solutions is higher.