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Modulation of enzymatic activity – case study for lytic polysaccharide monooxygenases

Master's thesis in

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Preface

This project was initiated in 21st of February 2017 and finished in 31st of July 2017 and it took place through Erasmus Programme of the summer semester of 2017 between the National Technical University of Athnes (NTUA) as home university and Chalmers University of Technology. The project was carried out under the supervision of Doctor Ausra Peciulyte, Chalmers University of Technology. Doctor Katja S. Johansen, a visiting researcher at Chalmers University of Technology was a co-supervisor and Professor Lisbeth Olsson, Chalmers University of Technology was the examiner. Examiner at the NTUA was Professor Evangelos Topakas. The project was funded by the Swedish Energy Agency (Energimyndigheten) under the grant № 41259-1. Enzymes were provided by Novozymes A/S. Chalmers Student number is 19940226C151 and NTUA student number is 05112702.

Abstract

Lignocellulosic biomass is one of the most promising alternative resources for production of biofuels and added valued products. Conversion of lignocellulosic biomass into fermentable sugars is a very important step in lignocellulosic ethanol production. Saccharification of biomass using enzymes, called cellulases, is an attractive process but still, there is a great deal of scope for research to improve the enzymatic conversion efficiency. The efficiency of the saccharification is based on advances in the enzymatic cocktail ingredients, of which one of the most significant is the recent inclusion of Lytic polysaccharide monooxygenases (LPMOs). LPMOs are of increased biotechnological and scientific interest due to their implication in lignocellulosic biomass decomposition as they are the first enzymes that act to the crystalline cellulose making the substrate easier accessible to the other enzymes for the complete decomposition. Through the years, after the discovery of LPMOs, information about them that they have been published are about their classification, their structure, the identification of cooper as the metal in their active site, their mechanism, and what activates them. In this project, the effect of a plant extract that comes for a persimmon fruit to fungal LPMOs and to beta-glycosidase was investigated. The overall aim was the search of a modulator, which can be either an inhibitor or an activator, with the plant extract as a source for modulators. For this purpose, saccharification experiments were done using cellulose as substrate and LPMO and beta-glucosidase as enzymes. Also, fractionation of the plant extract took place using liquid-liquid extraction. With the saccharification experiments the effect of the plant extract and the fractions of it were tested on these two enzymes. Last the effect on tannins on these two enzymes was tested using dilutions of tannic acid in the saccharification experiments. It is concluded that the plant extract and the tannic acid has an inhibitory effect to the LPMOs and beta-glucosidase, with higher effect to LPMOs.

Key words: *Cellulose, cellulases, LPMOs, PASC, plant extract, liquid-liquid extraction, tannic acid*

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List of Abbreviations

AA	Auxiliary activity
BG	β-glucosidase
CBH	cellobiohydrolase
CBM	Carbohydrate binding module
CD	Catalytic Domain
CDH	cellobiose dehydrogenases
FC	Folin-Ciocalteu
HPLC	High-performance liquid chromatography
LLE	Liquid-liquid extraction
LPMO	Lytic Polysaccharide Monooxygenases
PASC	Phosphoric Acid Swollen Cellulose
PE	Plant Extract
PVPP	Polyvinyl PolyPyrrolidone
PVP	Polyvinylpyrrolidone
TaAA9A	Fungal LPMOs that used in the project

1. Introduction

Nowadays the population produces about 1.3 billion tons of solid waste per year and this could be increased up to 2.2 billion per year by 2025 (1). It is therefore understandable that the world must change the production and consumption model that the global economy is being using. After the Industrial Revolution, the global economy developed the Linear Economy – the 'take-make-use-dispose' model of consumption and now we can see that this model is not sustainable. Over the last decade the Circular Economy, an alternative model for production and consumption, has drawn attention worldwide which is a model based on continuous growth and proper resource management (2). The Circular Economy is the model that instead of the 'take-make-use-dispose' model aims to keep products, components, and materials at their highest utility and value at all times, distinguishing between technical and biological cycles (3).

Industrial biotechnology is one of the most promising new approaches to pollution prevention, resource conservation, and cost reduction. So, Biotechnology is a field that can offer to the Circular Economy major help. Specifically, Industrial biotechnology creates smart, sustainable products and processes based on renewable raw materials, enabling carbon to be re-cycled at the end of the product's life. One example of the industrial biotechnology aims, is to provide a sustainable and economical feasible process for conversion of biomass to biofuels for energy or bioproducts such as biochemicals with high added value using enzymes. Those processes which improved the conversion of biomass into biofuel are being developed and the market for enzymes for biofuels production is expected to become larger. The global market for enzymes was estimated at about $4.5 billion in 2012 and it is expected to grow at almost $8 billion in 2020. Europe is the world leader in key industrial biotechnologies such as enzyme technologies, with Novozymes A/S from Denmark holding a market share of 47% worldwide (4). There are more than 3000 known enzymes, of which about 150 are used commercially (5). As a term, enzymes are biologically produced proteins that catalyze chemical processes and speed up chemical reactions (up to millions of times faster), with many applications in food industry, chemicals, biofuels etc (6).

Lignocellulosic biomass, such as plants, is one of the most promising renewable resources that has the potential for meeting a significant portion of the demand for energy (7). Oil has been overused all these years as the main resource for energy and transportation so the need for an alternative and more environmentally friendly resource for fuel is becoming necessary. There is when industrial biotechnology comes and creates a fully developed and sustainable process for bioconversion of plant biomass to biofuels and added value products using enzymes and microorganisms or what is defined as biorefinery (8; 9). Biorefinery as an idea is analogous to an oil refinery except that the raw material is biomass. In general terms, the production of biofuels such as bioethanol from lignocellulosic biomass involves a degradation of the polymeric compounds, primarily cellulose and hemicellulose, to sugars, which are then fermented by microorganisms to ethanol. This process can be performed in a number of different ways (10).

One important step in this whole bioconversion, is called enzymatic hydrolysis and it is the conversion of the lignocellulosic biomass, after being firstly pretreated, to sugars. Utility cost of this process is low compared to acid or alkaline hydrolysis because enzymes work in mild conditions (pH 4,8-5 and temperature 45-50^{o}C) and it is more environmental friendly as a process (11). However, the cost of the enzymes is high and their reduction in cost is very important for their commercial use in biorefineries (12). For the conversion different enzymes, are acting with synergistically in order to complete the decomposition of the cellulose and hemicellulose, the two components of lignocellulosic biomass that can be hydrolyze to sugars. Two categories of enzymes are called cellulases and hemicellulases and each one category has different enzymes that they act differently to complete this process (13). As their name implies cellulases are the category of enzymes that can catalyze the hydrolysis process of cellulose while hemicellulases the category of enzymes that they can catalyze the hydrolysis process of hemicellulose. This project is going to be focused on cellulases, which their synergistic action is known almost 60 years ago as a system of two groups of enzymes, one enzyme known as ‘’C1’’ and one mixture of enzymes known as ‘’Cx’’ (14). C1 was the enzyme that acting first on the cellulose and the Cx were the enzymes, which hydrolyze the cellulose to sugars. Nowadays, the actual picture for the cellulases is different and the knowledge for them increases through the years.

The last decade, a new class of enzymes has been discovered that are called Lytic Polysaccharide MonoOxygenases (LPMOs) and they are the unknown ‘’C1’’ enzyme from the early 50s. LPMOs have significant economic benefits to the hydrolysis process, due to the fact that they can boost the activity of the other enzymes, because they provide access to parts of cellulose that are not easy to be hydrolyze from them. After this discovery, a lot of research has been conducted about finding LPMOs structure and classification. It has been found that LPMOs are oxidative enzymes, which means that use a different mechanism than the rest of cellulases and in order to act they require molecular oxygen and electron donation (15; 16; 17). However, there are still things that they are unclear about LPMO so research and new information about them are always useful in the academic community.

1.1. Aim of the project

This project is focused on the enzymatic hydrolysis process of cellulose and more specifically on fungal LPMO enzymes. The overall aim of the project is to look for a modulator for those LPMO enzymes which can be either an activator or an inhibitor for their activity. For this purpose, a plant extract that comes from astringent persimmon fruit has been examined on the saccharification of cellulose and used as a source to search for a modulator. This plant extract is a persimmon juice which is called Kakishibu and throughout the thesis from now on it will be called PE.

The project aims to answer the following research questions:

a) How the saccharification of cellulose by LPMO is affected by addition of PE?
b) Can the PE be fractionated using Liquid-liquid extraction and create modulators, either inhibitors or activators for LPMO?
c) In case presence of tannins have an inhibitory effect on LPMOs and BG, is there any different effect on them?

2. Theoretical part

2.1. Lignocellulosic biomass

Generally, biomass is any material derived from living organisms, such as plants. Biomass has potential to be a major resource in the future, because is the only renewable organic resource and is also one of the most abundant resources on Earth (8). Lignocellulosic biomass, which is the term that describes the biomass that comes from plants, can be used as a resource not only for biofuels but also for chemical compounds with high added value.

Lignocellulosic biomass contains three main components: cellulose, hemicellulose and lignin. These components are present in biomass at varying amounts, depending on the plant species. Cellulose, the main structural component of plant cell walls, is a long chain of glucose molecules, linked to one another primarily by glycosidic bonds. Cellulose was the substrate for the enzymes in this project and it will be described more detailed in the section 2.2.1. Hemicellulose, the second most abundant constituent of lignocellulosic biomass, is not a chemically well-defined compound but rather a family of polysaccharides, composed of different 5- and 6-carbon monosaccharide units, that link cellulose fibres and cross-links with lignin, creating a complex network of bonds that provide structural strength. Hemicellulose also contains acids, such as acetic acid, but generally its composition is depending on the source of the plant biomass. Finally, lignin, a three-dimensional complex phenolic polymer, can be considered as the cellular glue providing the plant tissue and the cellulose fibres with compressive strength and the cell wall with stiffness (18; 19; 20). Those three components together create structures called microfibrils, which their aggregates are forming a matrix known as macrofibrils (Figure 1). Macrofibrils exists in the cell walls, they can be visible using emission electron microscopy or scanning electron microscopy and their size is varying amongst cell wall types (21).

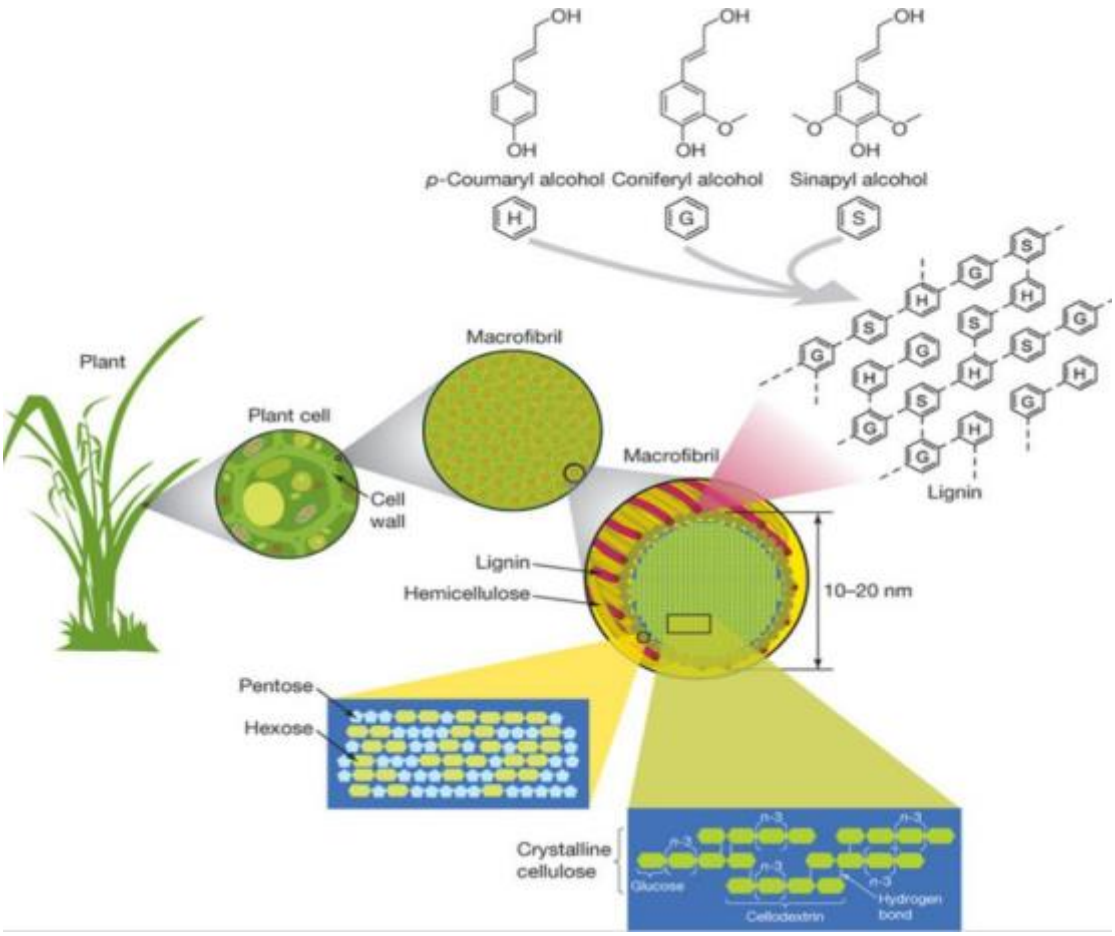


Figure 1. *The structure of lignocellulose* (22)

2.1.1. Cellulose

Cellulose is one of the most abundant organic biopolymers on Earth, representing around 1,5 x 10^{12} tons of the total annual biomass production (23) and it is the main component in lignocellulose. In plant cells cellulose has the form of fibres and between the cellulose fibres and the lignin there is the adhesive layer comprising hemicelluloses. Cellulose microfibrils are formed from glucose molecules linked together with glycosidic bonds. Chemically, cellulose it is a linear biopolymer composed of β-1,4-linked D-glucose chains (Figure 2).

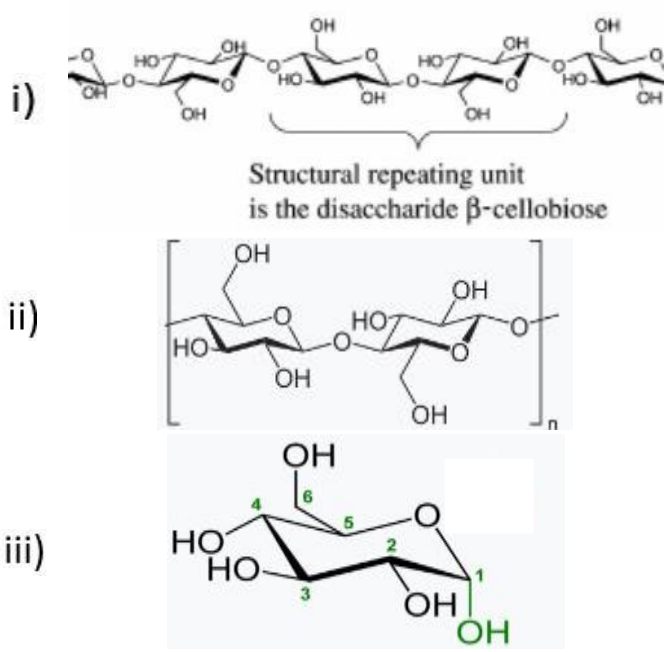


Figure 2. *Glucose molecules forming the cellulose linear chain i), repeating unit of cellulose, called cellobiose formed of two β (1-4) linked D-gucose monomers ii),* (24) *D-Glucose monomer with its with 6 carbon atoms iii)*

Cellulose recalcitrant structure is inherently resistant to hydrolysis making cellulose very stable biopolymer. It has been calculated that the uncatalyzed half-life of cellulose is approximately 5 million years (25). Although cellulose has a simple chemical structure, the spatial organization of these polymer chains makes the structure of cellulose very complex. Generally, the structure of cellulose can be determined on the macro-, micro- and nano-scale. Regions in the micro scale, within the microfibrils with high order are termed crystalline and regions with less order are termed amorphous. One of the suggested molecular architecture of crystalline and amorphous cellulose is that cellulose is semi-crystalline, with regions of high crystallinity (ordered), approximately 200 glucose monomers length separated by amorphous regions (disordered) (26; 27) (Figure 3).

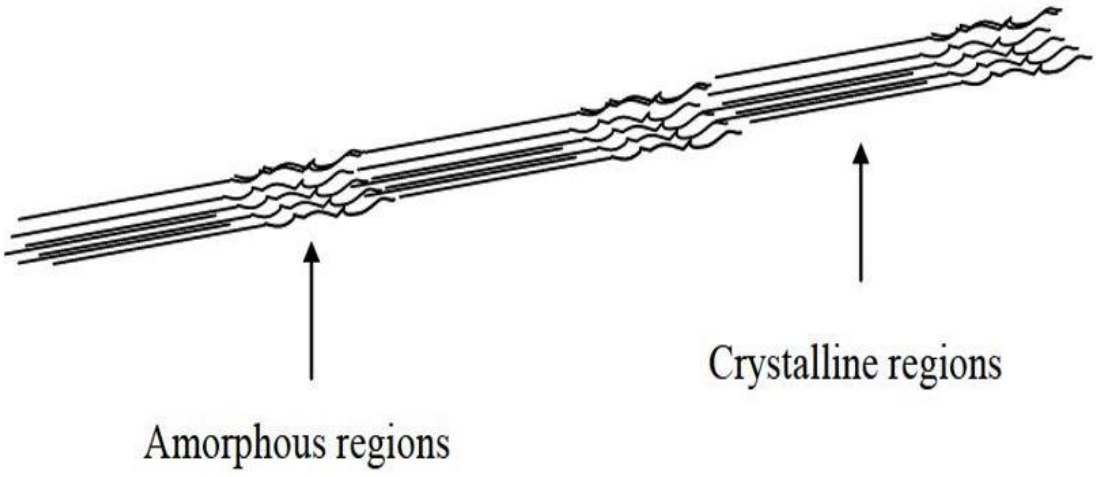


Figure 3. *Crystalline and amorphous regions are being repeated in horizontal dimension*

Cellulose degree of polymerization and degree of crystallinity are varying with the origin and the treatment of the raw material (28; 29). Native cellulose has approximately a degree of polymerization from 300-1700 such as wood pulp and from 800-10000 for cotton (23). Degree of crystallinity can be measured with different techniques usually is measured with X-ray diffraction method (XRD) and Nuclear Magnetic Resonance (29; 30). For cellulose, there are many model substrates such as Avicel, cotton, Wahtman No.1 filter paper, Phosphoric acid swollen cellulose (PASC), Valonia cellulose, Sigmacel, Solka Floc, (31; 32). In this project, the substrate that has been used is PASC which has been produced from Avicel. Avicel is a white, dry powder with particle size from 20-180 μm and it is derived by acid hydrolysis of wood or cotton and it is considered to be a crystalline substrate. Unlike Avicel, PASC is considered to be a representative of amorphous cellulose (33) and it is a viscous, white-cloudy liquid. Below the table summarizes some physical properties of those two model substrates.

Table 1*: Summary of some physical properties of cellulosic model substrates Avicel and PASC* (34)

Substrate	**Degree of polymerization**	**MW (g/mol)**	**Crystallinity index**	**Specific surface area (m^2/g)**
PASC	96	15,570	0.00-0.04	240
Avicel	150	24,320	0.5-0.6	20

PASC has become one of the most common cellulose substrates for cellulase activity assays due to the fact that is an easy substrate to be hydrolyzed by the enzymes (35). That happens because of the large disordered surface area that can provide for them. PASC can be produced from Avicel after phosphoric acid treatment as it is described in the section 3.2. As the Table 1 shows, PASC has lower degree of polymerization from Avicel showing that the treatment with phosphoric acid decreased the degree of polymerization of the substrate by more than one third. It is also known that properties of PASC are very dependent on the method was used to produce it such as acid concentration, incubation and mixing time, source of cellulose (35; 32). Furthermore, these results showed that phosphoric acid treatment resulted in generation of more reducing ends in PASC so more attackable sites for the enzymes that they act there (34).

2.2. Bioethanol from biomass

Biofuel production from biomass involves collection of biomass, physicochemical pretreatment, deconstruction of cell wall polymers into component sugars via enzymatic process and conversion of the sugars to biofuels (via fermentation) for example bioethanol (8; 18). Generally, as a definition, biofuels are the fuels for which the resource is biomass and not petrochemical resources and their chemical structure is not different with the fuels that can come from oil. Based on the source of biomass, biofuels are classified broadly into two major categories, first and second-generation biofuels. First can be derived from different sources such as sugarcane or starch. Sugars present in this biomass are fermented to produce bioethanol, an alcohol fuel which can be used directly in a fuel cell to produce electricity or serve as an additive to gasoline. Second-generation biofuels, on the other hand, utilize non-food-based biomass sources such as agriculture waste. This biomass mostly consists of lignocellulosic material, which is not edible and is a low-value waste for many industries (36; 37). The derivation of second-generation biofuels from non-edible woody plant biomass through biorefinery processes is therefore viewed as crucial (38; 39). The process from the lignocellulosic biomass to bioethanol is quite complex with many different individual steps and also there are different variations of the overall process (Figure 4) (40). After the pretreatment, the deconstruction of biomass can be thermochemical (41) and

biochemical (42). Biochemical approach relies on enzymes. There has therefore been much interest in understanding enzymatic degradation of cellulose (43) and similar polysaccharides, such as chitin (44). Those enzymes known as cellulases or cellulolytic enzymes have received considerable attention in the biotechnology area for their activity (43).

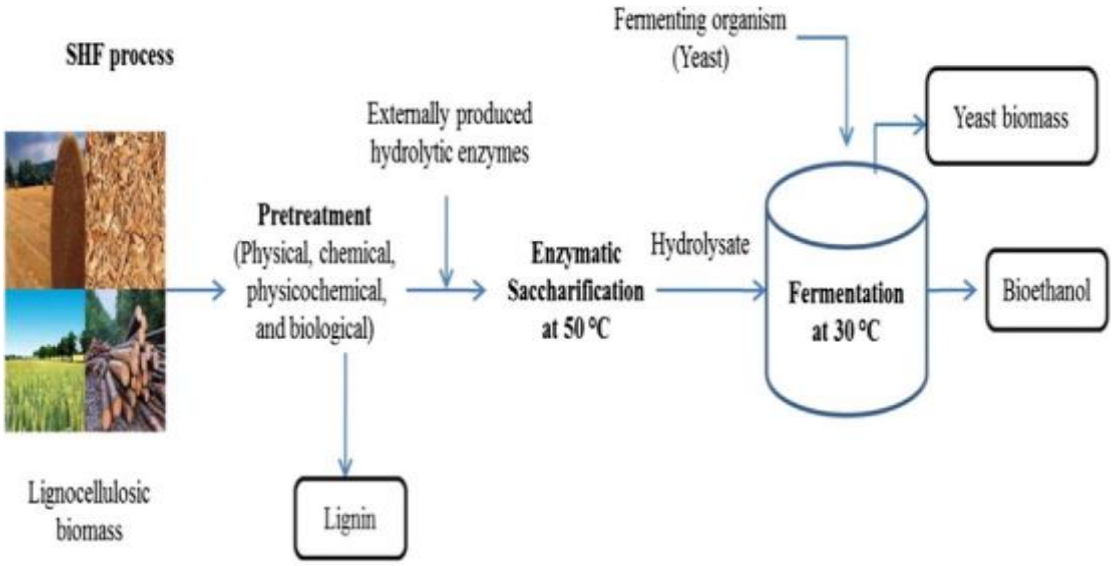


Figure 4. *Process flow for the production of bioethanol from lignocellulosic biomass using separate hydrolysis and fermentation (SHF)* (40).

2.3. Cellulolytic enzymes

Enzymes are produced by the microorganisms and they can produce a multiplicity of enzymes. For examples microorganisms that are efficient degraders polysaccharides in plant cell walls, such as *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) (a soft-root fungus) can produce a variety of enzymes which take different roles during degradation of the cellulose (45). These enzymes are called cellulases or cellulolytic enzymes. To degrade cellulose and hemicellulose cellulases and hemicellulases are required. In this project, the discussion will be focused on the cellulases since the substrate that was consists only of cellulose. However, it should be mention that enzyme systems that can degrade cellulose can also degrade (not fully) hemicellulose and hemicellulases show also activity on cellulose (46).

Enzyme system is very complex consisting from a few to even 100 enzymes (47) and the system consists of several enzyme classes based on their role during the hydrolysis process (31). In addition, most of the enzymes are complex molecules as individual modular proteins making the characterization of them not an easy work. Specifically, cellulases are composed of discrete units, referred to as either domains or modules. Commonly, cellulases consist of one catalytic domain (CD) and one carbohydrate binding module (CBM) usually linked together with a linker peptide (30-44 amino acids) (46) (Fig 5). The main role of CBM is helping the binding of the catalytic domain on the polymer of cellulose, although CBMs might also participate in the initial disruption of cellulose fibers (48).

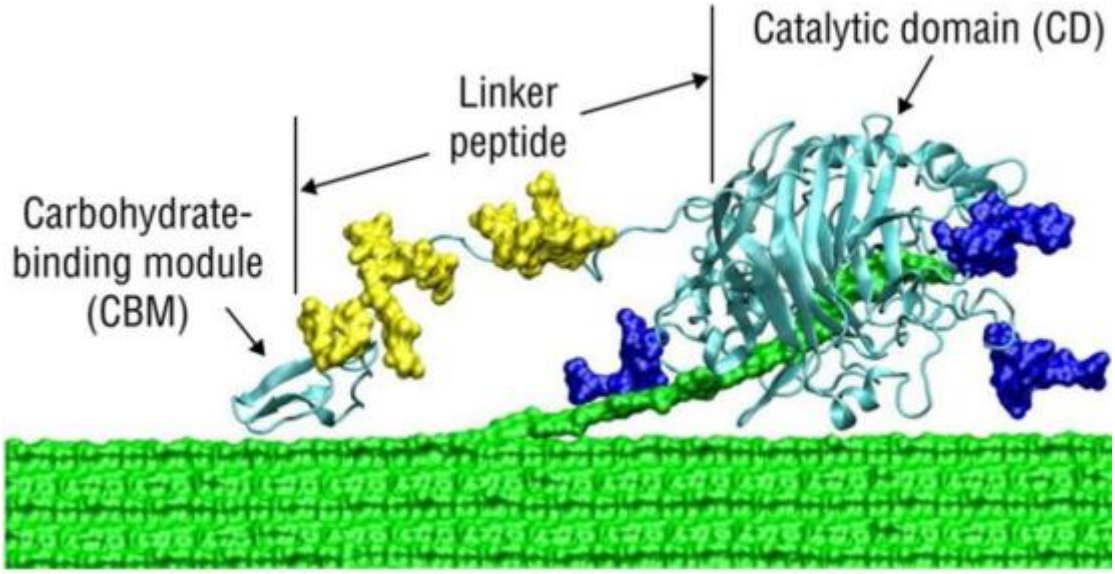


Figure 5. *Hydrolysis of cellulose by modular exo-cellulase (cellobiohydrolase, CBH). The figure shows the enzyme carbohydrate binding module (left); its linker peptide (middle) with attached polysaccharides (yellow) and the catalytic domain (right) linked to polysaccharides. Green is the cellulose chain and in dark blue is the disaccharide cellobiose which is a product released during enzymatic hydrolysis of cellulose* (49)

The enzymatic cellulase system exhibit higher collective activity than the sum of the activities of individual cellulase enzymes, a phenomenon described as synergism. Four forms of synergism for the degradation of cellulose to sugars have been reported (46):

(i) endo-exo synergy between endoglucanases and exoglucanases
(ii) exo-exo synergybetween exoglucanases processing from the reducing andnon-reducing ends of cellulose chains
(iii) synergy between exoglucanases and beta-glucosidases (BGs) that remove cellobiose (andcellodextrins) as end products of the first two enzymes
(iv) Intramolecular synergy between catalytic domains and CBMs

As it is being mentioned before, the enzymatic system consists of different enzymes more specifically β-1,4-endoglucanases, exoglucanases (including D-cellodextranases and CBH) and BGs. Their role in the hydrolysis process will be explained in section 2.4. For the proper archiving of glycoside hydrolases there is the carbohydrate-active enzyme database (CAZy, www.cazy.org). CAZy is a classification system which is based on amino acid sequence similarities, protein folds and enzymatic mechanisms.

This classification has been integrated and updated in the CAZy database since 1998. CAZy currently incorporates more than 300 sequence families subdivided into the following classes: glycoside hydrolases, glycosyl transferases, polysaccharide lyases, carbohydrate esterases, and auxiliary activities (AA) (50).

Generally, cellulase achieve the decomposition of cellulose by using a hydrolytic mechanism that involves conserved carboxylic acid residues within either channel- or cleft-type substrate loading sites (51). However, there are other enzymes that can cleave cellulose chains by using an oxidative mechanism at their planar active site and they are called Lytic Polysaccharide Monooxygenases enzymes (LPMO).

2.3.1. Lytic Polysaccharide Monooxygenases enzymes (LPMO)

As it been mentioned, different types of enzymes are required for the decomposition of cellulose to glucose and each one of them is doing different work in the hydrolysis process but they all act synergistically in order to achieve this decomposition. The enzymes thought to act first on the cellulose, making the substrate accessible for hydrolysis by other cellulases, remained unknown for 50 years (52). Now it is acknowledged that these enzymes are the metalloenzymes with copper as metal at their active site and they called LPMO. As it was mentioned in the introduction almost 60 years ago, two categories of enzymes were described by the pioneer work of Reese et al (1950), one unknown enzyme called ‘’C1’’ and one group of enzymes called ‘’Cx’’ and that unknown enzyme (‘’C1’’-enzyme) it was shown that has a major role in the decomposition of cellulose (14). Later Eriksson et al (1974) published that there are not only hydrolytic enzymes that involved in the decomposition of cellulose but also oxidative enzymes (53). In the late 2000s, it was reported that fungal LPMOs, which then were known as glycosyl hydrolase family 61 (GH61), have the ability to boost biomass degradation (54)**.** In 2010, it was discovered that LPMOs have an oxidative mode of action (55). A year later Quinlan et al (2011) (56) were the first ones to determine that the metal in their active site is copper and specifically the active site can be best described as a type II copper site. Now LPMOs are classified into four Auxiliary Activity (AA) enzyme families (AA9, AA10, AA11 and AA13) of the CAZy database (57; 58). This project is focusing on AA9 LPMOs, which are exclusively found in fungi and act preferentially in cellulose (59).

Specifically, LPMOs are enzymes that catalyze oxidative cleavage of glycosidic bonds after being activated by an electron donor that they can find in their environment. During the cleavage LPMOs oxidize C1, C4 or both of carbon atoms of glucose. LPMOs, besides cellulose, can also depolymerize other polysaccharides such as chitin (15), starch (60) and hemicellulose (61). It is therefore understandable that LPMOs offer great promise for further improvements in biomass deconstruction processes. LPMOs are found widespread in fungal (62) and bacterial organisms (15), as well as in viruses (63). Quinlan et al (2011) using electron paramagnetic resonance spectroscopy LPMO was shown to contain a single type 2 copper center, with a t-shaped coordination sphere termed the histidine brace, where two histidines provide three nitrogen ligands, two from N-His and one from the terminal amine (64) (Figure 6). There is also tyrosine involved together with those two histidines in the coordination of copper (65). In fungal LPMOs N-histidine is methylated, which is makes them special amongst the LPMOs and it is unknown why they have this difference (56).

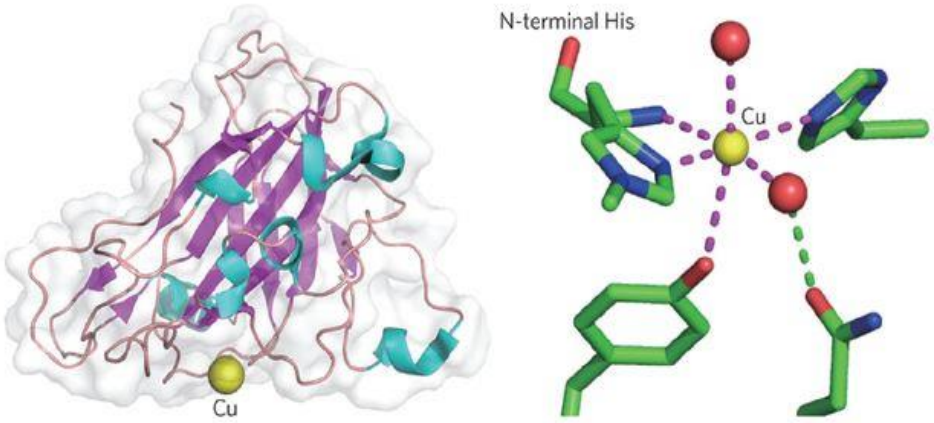


Figure 6. *Illustrations of the surface structure (left) and the copper active site (right) of the AA9 LPMO. The flat surface binds crystalline cellulose, and the metal center catalyzes oxidative cleavage of glycosidic bond* (66)

In order for LPMO to complete their catalytic cycle they need an extracellular electron donation from either proteins or plant-derived molecules plus they require oxygen molecules that is necessary for their activity (15). One major advantage that LPMO can offer is the reduction of the enzyme loading in the hydrolysis process (52). Less enzyme loading has financially benefits for the whole process but also environmental benefits due to the fact that the enzyme production has one of the highest contributions to the

Global Warming Potential and to the Photochemical Ozone Creation Potential including other processes such as fermentation, pretreatment and cultivation and harvesting of straw (67). However, LPMOs have a disadvantage which is that they release reactive oxygen species after the redox reaction. Reactive oxygen species are chemically reactive molecules containing oxygen, for example hydrogen peroxide and superoxide radical (68; 69) and they are harmful for the cell and for the enzymes and for LPMO itself.

2.3.2. Activation of LPMO

As it been mentioned, LPMOs need molecular oxygen and an external electron donor for their proper function and for completion of their catalytic cycle. However, the types of electron donors and the mechanism of electron transfer are still not completely clear (64). First, diphenols derived from plants showed that they can initiate LPMO activity on cellulose (70). Then Kracher et al. (71) using biochemical methods and genome data has characterize and compare different electron sources for LPMOs and both proteins and plant-derived molecules have been found to serve as electron donors (Figure 7).

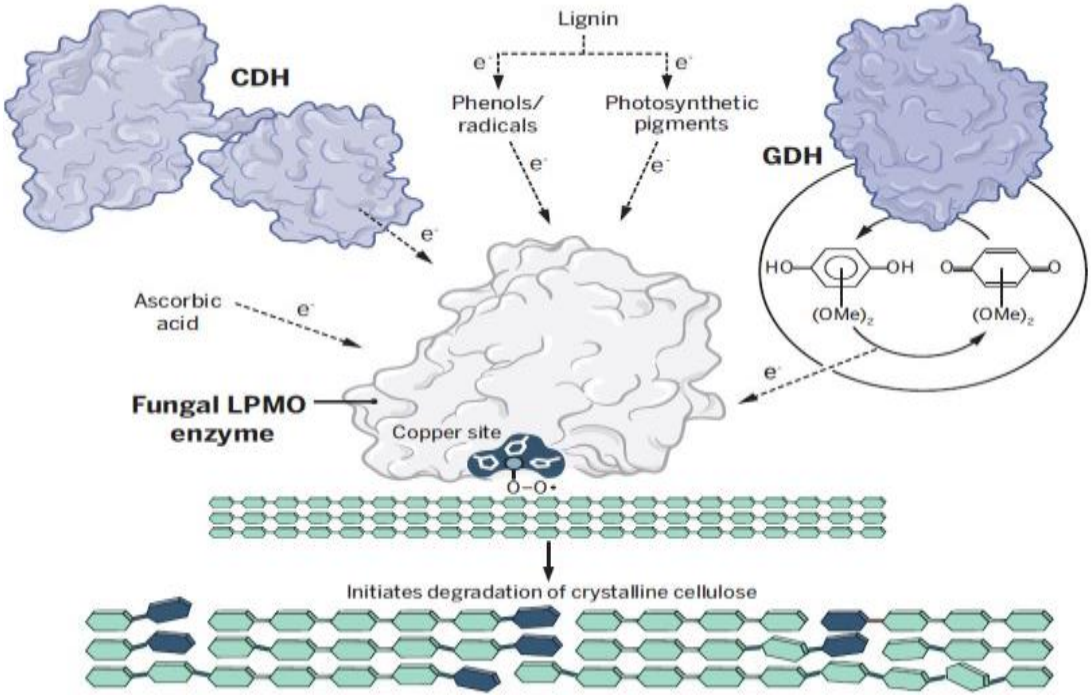


Figure 7. *Scheme of several natural electron sources that have been identified as activators to LPMOs, including enzymes (cellobiose dehydrogenases and glucose dehydrogenase), phenolic compounds and light-activated photosynthetic pigments* (16).

One protein electron donor for LPMOs is cellobiose dehydrogenases (CDHs) which has been reported that can act as an activator to LPMOs (72; 73). CDH are enzymes from the family oxidoreductases and they oxidize oligosaccharides and disaccharides, for example lactose and cellobiose using electron acceptors (74). About CDH-LPMO system it has been suggested that CDH transfer electron to LPMO from its catalytic dehydrogenase domain via its mobile cytochrome domain (75). Kracher et al. using stopped-flow spectroscopy showed that different combinations of LPMOs and CDHs can have high rates of electron transfers. They suggested that preferences of LPMOs for specific CDHs, which could be figured out by the different rates of electron transfers, can be due to variation in surface complementarity at the protein/protein interface. Another enzymatic source of electron to LPMOs are the enzymes glucose dehydrogenase, which belongs in the same family with CDH but they act differently as electron donors to LPMOs than CDH. That is because they do not act as direct electron donors but plant-derived or fungal diphenols and/or quinones serve as redox mediators between them and LPMO creating an efficient electron transfer system (71).

Non-enzymatic source for electrons to LPMOs can be natural reductants like phenolic compounds such as ascorbic acid, gallic acid, phenols from lignin (76; 56). In literature, ascorbic acid has often been used as electron donor in experiments but in our experiments gallic acid was used as an electron donor because it is more stable than the ascorbic acid. Another interesting source for electron are photosynthetic pigments, once activated by light. Specifically, Canella et al (2016) (64) they investigated the effect of chlorophyll pigments, once activated by light, to LPMOs and they report that the catalytic activity of LPMOs can be driven by light and it can reach in some cases 100 faster than with standard reductants such as extracted lignin.

2.4. Saccharification of Cellulose

The efficient enzymatic hydrolysis or degradation of plant biomass is one of the major industrial challenges of the 21st century. In order to obtain the constituent sugars from lignocellulosic biomass, the cellulosic and hemicellulosic portions of biomass should be separated from the lignin and depolymerized (77). When it comes to cellulose portion two important difficulties exist. Firstly, it is known that the structure of cellulose is resistant to hydrolysis attack and second is that the cellulose chains forming extensive intrachain hydrogen bonding. These two problems amplify each other, making cellulose degradation quite challenging. Furthermore, it is rational that multiple enzymatic activities are required in cellulose hydrolysis leading to soluble sugars that can then be metabolized by fermenting microorganisms. Briefly, Endoglucanases randomly cut internal sites on amorphous cellulose surfaces, generating and release new chain ends. CBH has two types, I and II and despite the basic functional similarity, their action appears to be little different (78). CBH I is suggested to start its action from the reducing end of the cellulose chain, while the CBH II prefers the opposite direction. Generally, CBHs act in a processive manner on the reducing or nonreducing ends of cellulose and liberate cellobiose as major products. β-Glucosidases hydrolyze soluble cyclodextrins and cellobiose to D-glucose and thus relieve the system from end product inhibition (79).

The term hydrolysis of cellulose is similar to the saccharification of cellulose but when it comes for LPMOs the term hydrolysis is not the proper one to describe their activity. That is because hydrolysis does not represent the action of LPMOs as they use an oxidative mechanism which is different from the classical hydrolytic mechanism that the cellulases use. LPMOs have emerged as key enzymes for the degradation of cellulose as they are the first enzymes that react with the crystalline cellulose, making the substrate easier accessible for the rest of cellulases boosting their activity. This boosting effect is from the creation of new chain breaks in crystalline regions of the substrate by LPMO activity (80). This chain breaks are creating when LPMOs use their single active-site copper ion to activate molecular oxygen and hydroxylate the polysaccharide backbone (73). This oxygen activation depends on external electron donors that LPMO can found in their environment. So, practically LPMOs provide a solution to the problem of accessing the active site of cellulases to crystalline regions of cellulose. In LPMOs, the active site is located on a planar surface of approximately

1200 Å to facilitate the metal dependent oxidative cleavage of the glycan chains, which help cellulase components to access cellulose fibers by producing nicks in between the chain (66). Therefore, the action of the LPMO together with EGs can help cellulase components such as CBHs to access cellulose fibers by producing nicks in between the chain (Figure 8).

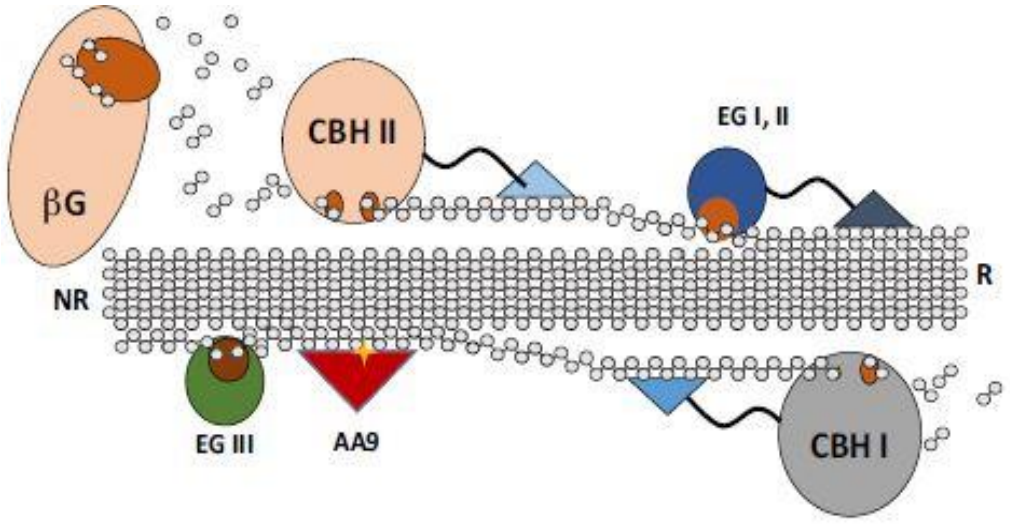


Figure 8*. Illustration of the enzymatic degradation of cellulose including AA9 LPMOs, endoglucanases (EG I, II and III), cellobiohydrolases (CBH I and II) and β-glucosidases. At the left side is the non-reducing end (NR) and at the right side is the reducing end (R) of the cellulose chain. The blue triangles that are connected with some of the cellulases are their cellulose-binding modules* (52).

First information about the mechanism of fungal LPMOs are from Harris et al (2010) that they showed the importance of the metal in the active site and that their mechanism is unlikely to be a classic hydrolytic one (81). Then more information about the mechanism of LPMO that can degrade chitin, which back their family was called CBM33 and nowadays AA10, is from Vaaje-Kolstad et al (2010), where they used isotopically labelled dioxygen to confirm overall monooxygenase activity on chitin (15). The chain cleavage of chitin was observed at the C1 carbon atom and it was revealed through the addition of a chitinase to release detectable products. Another isotope labelling study (82), used AA9 enzymes on PASC, show that LPMO-catalysed hydrogen atom abstraction at either C1 and/or C4 carbon atom positions followed by cleavage of the glycosidic C–O bond.

2.5. Factors that affect Cellulase's activity

Enzymatic hydrolysis is a complex process that can be affected by many parameters. We can divide the factors that affect the enzymatic degradation in two categories: the enzyme related factors and the substrate related factors. The first category contains many different factors such as:

- Enzyme system composition and concentration
- Adsorption
- End-product inhibition
- Reaction conditions of hydrolysis

Each one has different effect on the process but is difficult to quantify the influence individually. About the enzyme system the origin of the enzymes plays role, given the fact that enzymes can be produced from different sources such as bacteria, fungi and virus. The composition of the enzymatic mixture is also important due to the fact the right loading analogies of the different enzymes have major effect at the extent of the synergism of the system. About loading and concentration there is a general rule that the conversion of the substrate increases with higher dosage and concentration, however the increase conversion is not proportional with the increase of enzyme dosage (83). Adsorption on the enzymes on the substrate surface plays an important role in the efficiency of the decomposition of cellulose to glucose and as a general rule ''the better the adsorption the better the catalysis'' (84). There is also unproductive adsorption and this is when enzymes are not recovered and it is being reported that cellulases can have unproductive binding to lignin surface (85) or even to cellulose surface (86; 87). End product inhibition is considered an important enzyme factor related with the decrease of the conversion at the end of the process. Cellulases can be inhibited by cellobiose, also by glucose but to a lesser extent, which are the products of the hydrolysis. (88; 89). Continuous and fed batch processes are often adopted to cope with the inhibitory effect of such compounds (90). For example, in batch processes an additional quantity of BGs is added during the reaction in order the BGs to covert the inhibitory cellobiose to the less inhibitory glucose. Last the conditions of the process are very important for the hydrolysis and they should be related with the conditions that the enzymes are working more effective. Typical industrial hydrolysis process has temperature at 50^{o}C, pH

around 5 (11). The adsorption activities beyond 60^{o} C decreased, possibly because of the loss of enzyme configuration leading to denaturation of enzyme activity.

The second category is also important for the efficient action of the enzymes and it is about substrate related factors. Composition of cellulose, hemicellulose and lignin is very important for the final yield and the initial rate of hydrolysis, for example removal of lignin can increase dramatically the hydrolysis rate (91). When the substrate consists of cellulose, parameters such as concentration, crystallinity, surface area, degree of polymerization are affecting the final yield and the initial rate of cellulases. About concentration at low substrate levels, an increase of substrate concentration leads to an increase of the yield and the reaction rate. However, when high substrate concentration exists and depending on the ratio of the total substrate to total enzyme, there can be substrate inhibition, which substantially lowers the rate of hydrolysis (87; 92). Structural features of substrate are important and generally and they are related for example the degree of polymerization is related with the degree of crystallinity. The surface area is a very important characteristic of the substrate and declares the accessibility of the substrate from the enzymes. The higher the surface area means more adsorption sites for the enzymes to act (93). Therefore, substrate pretreatment methods often include cutting, that is, reduction in size, of the lignocellulosic material to increase the surface area.

2.6. Liquid-liquid Extraction

The Liquid-liquid extraction (LLE) is method that is used as a separation process for substances in liquid samples. In LLE, there are two immiscible liquids forming two phases with the denser phase on the bottom. One phase is an aqueous phase and the other phase is an organic solvent and solutes from the aqueous phase are extracted to the solvent phase. The solute is initially present in one of the two phases; after the extraction, it is present in both phases. Extraction efficiency which is the percentage of solute moving from one phase to the other, can be determined by the equilibrium constant for the solute's partitioning between the phases and any other reactions involving the solute. A solute's partitioning between two phases is described by a partition coefficient, K_D. The K_D is defined by the ratio of the concentration of solute

at the organic phase to the concentration of the solute to the aqueous phase. The larger value of K_D means that the extraction of solute into the organic phase is favorable. The partition coefficient is an equilibrium constant and has a fixed value for the solute's partitioning between the phases (94; 95; 96).

Number of extractions is an important factor for the efficiency of the method and instead of using one big quantity of solvent for one extraction, it can be used several extractions using smaller portions of solvent each time. Interaction between the two phases is also important for the extraction. Examples of reactions affecting extraction efficiency include acid–base reactions and complexation reactions (97). The solvents that are used in this method are based on their polarity and the immiscibility with the liquid sample. For example, ethers and ketones are among the most employed solvents for removing phenolics from water, whereas ethyl acetate and diethyl ether have been used for extracting low molecular weight phenolics from oak wood (98). Another case is that the different solvents can extract different concentration of compounds. For example, pentane, hexane, methylcyclohexane and iso-octane were used to extract different concentrations of trihalomethanes from water (99). LLE can be used as a method for the recovery of aromatic substances (100; 101) and also it can be used for the isolation of phenolic compounds in juices or fruit/plant extracts (102; 103; 104). In order for substances to be collected after the extraction the isolation of the solvent is taking place. Most common isolation methods of the organic solvent are freeze-drying and vacuum evaporation (95) and in this project the method vacuum evaporation was chosen.

2.7. Folin-Ciocalteu method

Folin-Ciocalteu (FC) is a spectrophotometric assay applied to determine the total phenolic content in liquid samples This assay relies on the colorimetric redox reaction which determines the total amount of phenolics without separating monomeric, dimeric and larger size components (105). Basically, provides a convenient and simple estimation of the phenolic content in liquid samples such as plant extracts, juices, mixtures using a specific reagent. This reagent is a solution of complex polymeric ions formed by Phospholomolybdenum and Phospho-tungsten heteropolymeric acids and it is called FC reagent. More specifically phenolic ions are oxidized by simultaneous reduction of heteropolymeric acids and the product is a complex of tungsten and molybdenum in blue which can be determined spectroscopically at 765 nm (106). The absorbance is proportional to the number of aromatic phenolic groups and for their determination gallic acid, which is a type of phenolic acid, is the most common chemical as standard (107). Generally, the method has been proposed as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products (108), because it is known that polyphenolic compounds can act as antioxidants in fruits and vegetables (109). One disadvantage of this method is that tends to overestimate the total phenolic content of the sample compare to the other methods such as chromatographic methods (HPLC) and solid-phase extraction (110).

2.8. Tannins

Tannins are described as a water-soluble, complex polyphenolic biomolecule with high molecular weight with great structural diversity and wide phylogenetic distribution (111; 112) . Sources of tannins can be fruits, leaves, tea, coffee, wine (112; 113). It is known that the more astringent a fruit is the more tannic content the fruit has (113). In this project, the PE that is being used comes from astringent persimmon fruit, so it should contein tannins. Tannins can be classified in three main categories based on their structures, such as hydrolysable tannins, non-hydrolysable tannins (condensed tannins) and pseudo tannins (114). Each category consists of many different tannins making the identification of tannins in extracts quite difficult. Tannic acid (Figure 9) is one

representative tannin for the hydrolysable tannins and it was used in this project. One of the properties that tannins have is that they can cross link and precipitate proteins with hydrogen bonds as well as ionic bonds (113). Given the fact that enzymes are proteins it is interesting to evaluate the effect that tannins have on the enzymes, which is known that tannins have inhibitory effect to enzymes (115; 116). Kawamoto et al (117) showed that tannins interact with proteins and they can decrease the BGs activity due to irreversible protein denaturation. Tannin-enzyme interaction is not only depended on the source of tannin, but also on the nature and source of the enzyme (118).

In literature has been found (119; 120) that there is an insoluble matrix that called Polyvinyl PolyPyrrolidone (PVPP) and has the ability to bind tannins and impurities from solutions. It is also used in wine treatment in order to reduce phenolic content and organic acids in wine (121; 122). PVPP is a highly cross-linked solid co-particle material, it is insoluble in water and swells very rapidly generating a swelling force (123). Therefore, it can be used as an disintegrant in pharmaceutical tablets. It is a modification of polyvinylpyrrolidone (PVP), which is also known for binding tannins. Like PVP, PVPP can bind phenolic content, especially big polyphenolic molecules, such as tannins, by forming hydrogen bonds with them (124). Polyethylene glycol is also known for binding tannins but in this project only the effect of PVPP was evaluated (123).

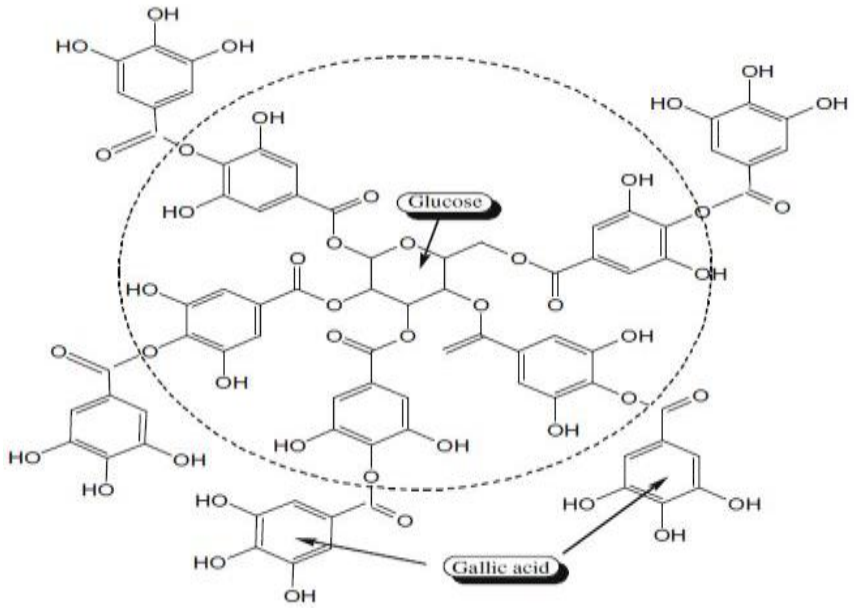


Figure 9. *Chemical structure of tannic acid. Tannic acid is composed of a central glucose molecule esterified at five hydroxyl moieties with two gallic acid molecules each. The shaded circle highlights pentagalloylglucose and the core structure of tannic acid* (125).

3. Experimental part

In order to find modulators for LPMO enzymes, an experimental procedure has been followed that shows the activity of LPMOs and BGs on their saccharification process. BG were chosen as a representative hydrolytic enzyme in the experiments in order to observe if there are different effects on LPMO and BG. Also, LPMOs release relatively small amount of products which are oxidized compounds. Detection and identification of those oxidized compounds is quite laborious. So, by addition of BG it is easier the detection of glucose as the final product. BG without addition of LPMOs is releasing low values of glucose. So, combining LPMO and BG it is possible to observe the action of LPMO.

As a source for modulators a PE was added to the mixture in order to observe a difference on the saccharification results from samples without addition of it. This PE comes from an astringent persimmon fruit and it is based on literature (126) which shows that immature persimmon has an inhibitory effect to the C1 enzymes, that now we know them as LPMOs. This PE alone is quite complex to be analyzed so fractionation of this took place in order to create different and simpler fractions of it with different effects to be tested in enzymatic saccharification experiments. Therefore, LLE was used as a fractionation method to the PE. In general, plant extracts that come from fruit contain a variety of phenolic acids, flavonoids, tannins and carotenes (127; 124). Persimmon fruit especially is known that has high content of tannins (128; 129). For that reason, isolation of tannins from the PE took place using PVPP. Also, the effect on temperature was tested on the PE with the aim to evaluate if there is a protein nature compound that has the inhibitory effect to the enzymes and can be precipitated in those temperatures. The total phenolic content on the PE and the fractions of it was measured using the FC method. Last concentration of tannic acid was added in the saccharification system in order to evaluate the inhibitory effect of tannins to LPMOs and BGs.

3.1. Chemicals and Enzymes

Different chemicals and enzymes were used in the experimental part of this project. All enzymes were kindly provided by Novozymes A/S and prepared as described by Scott et al. (130) . The enzymes were purified BGs with concentration 34 g/L and fungal AA9 purified LPMO enzymes with concentration 73 g/L, with name TaAA9A and from now on throughout the thesis they will be called like this. Ta stands for the name of the microorganism of origin, which is *Thermoascus aurantiacus*, AA9, a fungal class of LPMOs and the last A shows that it is the first LPMO reported from that particular organism.

For PASC production the chemicals that they have been used were Avicell PH-101 (from cotton linters Cellulose powder 50 μm particle size), Phosphoric acid 85 wt % in H_2O, Sodium Carbonate and double distillated water (miliq water) was used. Sodium Carbonate and Phosphoric acid were purchased from Sigma-Aldrich and Avicel from Fluka Analytical. The solution of sodium carbonate was made by adding 5,3 g of sodium carbonate in 250 ml of water.

For the saccharification experiments the chemicals that they were used were gallic acid with concentration 40 mM and triethylammonium acetate with concentration 0,1 M and pH 5,5 which from now on will be called buffer. Gallic acid and buffer were purchased from Sigma-Aldrich. The solution of gallic acid was made by adding 340.6 g of solid gallic acid in 50 mL of miliq water in order to have the requested concentration of 40 mM. Then the solution of 50 mL it was pippeted in 25 2-mL Eppendorf tubes and then it was storage in the freezer at -20oC in a box for light protection. The buffer was produced after ten times dilution of commercial triethylammonium acetate (1 M) with miliq water and then adding solution of acetic acid of 1 M concentration till the pH of buffer became 5,5. Then the solution was added in a glass bottle and it was stored in the fridge at 4ºC which is the solution of buffer that was used in the saccharification experiments. For the fractionation experiments the four solvents ethyl acetate, dichloromethane, diethylamine ,1-butanol were all purchased from Sigma-Aldrich. About the isolation of tannins commercial PVPP (110 μm particle size) was used, which was purchased from Sigma-Aldrich. Tannic acid and FC reagent also used in parts in the experiment process and they were purchased from Sigma-Aldrich.

3.2. Production of phosphoric acid swollen cellulose (PASC)

PASC has been chosen as a model substrate for the enzymes because it is an amorphous form of cellulose with low degree of crystallinity (Table 1). That makes PASC more accessible for the enzymes than other forms of cellulose such as Avicel or cotton. Also, after the hydrolysis process from the variety of simple sugars only glucose is being produced and that makes the system simpler where it is easier to screen many conditions and to have a robust and quick experimental set-up. In case we had a lignocellulosic substrate, more simple sugars would have been produced such as xylose, mannose. In addition, the major issue in case of lignocellulosic substrate would have been the production of complex oxidized products by LPMOs (131; 64). Those oxidized products would have made the quantification of final products more difficult task since standards for them are not available for purchase and they do not produce much amount of those which would have made the results more complicated.

The experimental procedure for PASC production was based on the reference (35). Firstly, 1,4 g of Avicell were mixed with 4,2 ml of water and then 70 ml ice cold Phosphoric acid was slowly added (10 ml each time) to the mixture. The mixture stayed on ice for one hour with occasional stirring. After one hour, 280 ml of ice cold water were added to the mixture slowly with stirring at the same time. Then the mixture of water, Avicel and phosphoric acid was centrifuged for 20 minutes at 5000 rpm and 4^{o}C. After centrifugation, the supernatant was discarded and ice-cold miliq water was added till approximately 350 ml for the pellet in order to wash away the phosphoric acid from PASC. Then the second centrifugation was performed and the same procedure of removal of liquid and addition new ice-cold water was repeated three more times, so five times in total. After the last centrifugation, solution of sodium carbonate with concentration 2 M was added till the pH reaches values between 5-7. Then three more times of centrifugation was performed with the same conditions as before. At the end, the white slurry part (Figure 3.1) was transferred in a glass bottle and some small portion of miliq water was added in order for the PASC to became less viscous.

Figure 3.1. *PASC solution*

To measure the concentration of PASC triplicates were taken from the PASC solution and they were dried in the oven at 105^{o}C for approximately 10-15 hours. After, the weight of the dried samples was measured and the concentration of the solution of PASC was calculated in g/L. For the saccharification experiment, which is going to be described in section 3.3 the initial concentration of PASC should be 10 g/L. So, after the calculation of the concentration from the dry weight measurement, which was usually around 12-15 g/L, the stock solution was diluted in 10 g/L and it was stored in the fridge at 4^{o}C.

3.3. Enzymatic saccharification of PASC

The procedure that was followed in the saccharification experiments was based on the reference (56). In these experiments, all the samples were putted in in 2 mL Eppendorf tubes and they have constant final reaction volume of 1 mL. First 500 μL of stored PASC was added in the Eppendorf tubes so the final concentration of PASC was 5 g/L. Then buffer with initial concentration 0,1 M and pH =5,5 was added to each sample with different volumes, depending the sample. These differences were based on the volumes of the other reactants in order to have final reaction volume 1 mL. The final concentration of buffer in the mixture for these experiments was between 0.0359-0.0372 M. Then solution 125 μL of gallic acid with concentration 40 mM were added to each sample so the final concentration of gallic acid in the reaction volume was 5 mM. At the end, 15 μL of TaAA9A and 15 μL BG were added after they got diluted with buffer 5 times. As it been mentioned the initial concentration of the enzymes were 34 g/L for the BGs and 73 g/L for the TaAA9A so after the 5 times dilution the final

concentration for the enzymes in the reaction volume were 0,219 g/L for TaAA9A and 0.102 g/L for BGs. The pH was tested in samples and it was always between 4.85-5,35 which is in the range of pH that the enzymes can work (56) and the values of pH from representative samples is given at the Appendix (Table 6.3). Last in some samples dilutions of the PE were added to the mixtures. The PE was always diluted first 20 times with buffer and based on the volume that it was added in the mixture, it got different final dilutions. Specifically, four different final dilutions were tested: 1000x, 500x ,100x, 50x. Below (Table 3.1) are the volumes of representative samples that have been used and for every different condition triplicates were made.

Table 3.1. *Reactants volumes at representative samples at the saccharification experiments. In this table, the samples with the PE have 100 times final dilution from the initial PE.*

Samples	**PASC (10 g/L)**	**Buffer (0,1 M)**	**Gallic acid (40 mM)**	**Enzymes**		**PE (20x diluted in buffer)**
				LPMOs (5x diluted with buffer)	**BGs (5x diluted with buffer)**	
1	500 μL	345 μL	125 μL	15 μL	15 μL	-
2	500 μL	360 μL	125 μL	15 μL	-	-
3	500 μL	360 μL	125 μL	-	15 μL	-
4	500 μL	145 μL	125 μL	15 μL	15 μL	200 μL
5	500 μL	160μL	125 μL	15 μL	-	200 μL
6	500 μL	160μL	125 μL	-	15 μL	200 μL

After adding all the reactants in the 2 mL Eppendorf tubes then incubation took place in thermomixer for three days at 50^{o}C and 1000 rpm. After three days, the samples were centrifuged (Figure 3.2) at 10000 rpm and then the supernatant was collected and it was filtrated with 0,2 nm Nylon filters. Finally, the filtrated supernatant of each sample it was putted in freezer (-20^{o}C) in 1,5 mL Eppendorf tubes for storage and then it was measured for its glucose concentration by HPLC as it is being explained in the section 3.6. There were also samples taken out every day out of the three days reaction in order to do a time course saccharification experiment.

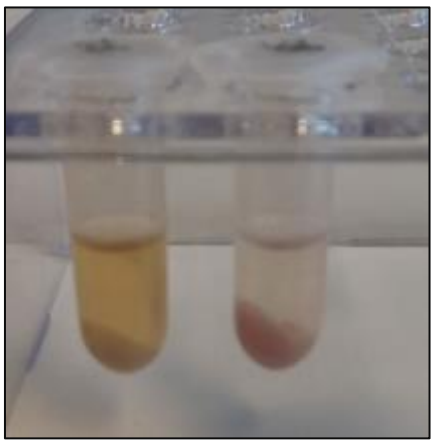


Figure 3.2*. Samples after three days saccharification reaction and after centrifugation. The left sample in photo is without adding PE and on the right sample is by adding the 100x dilution of PE.*

3.3.1. Saccharification experiments adding tannic acid

In order to test the effect of tannins on LPMOs and BG and evaluate if there is a different effect on them, tannic acid was added in the saccharification mixtures at different concentrations. Initially commercial tannic acid was dissolved in water and not in buffer due to the fact that tannic acid was precipitated in buffer. At table 3.2 are given the volumes of reactants at representative samples at the saccharification experiments. Two different saccharification experiments were made adding different range of tannic acid concentrations. Every different dilution of tannic acid was added in the same volume of 200 μL at the mixture. The dilutions that were made were 6, 2.9 ,1.44, 0.36 mM at the first experiment and 1, 0.75, 0.5, 0.36 mM at the second one. Final dilutions of the tannic acid in the mixtures are given in the Table 3.3. In these experiments, the final concentration of buffer for the samples with addition of tannic acid was 0.0169 M for samples with both enzymes (Sample 4 Table 3.2) and 0.0172 M for samples with one of the two enzymes (Samples 5,6 Table 3.2). For the Blank samples (Samples 1,2,3 Table 3.2) the final concentration of buffer was 0.0369 M for the samples with both enzymes (Sample 1 Table 3.2), 0.0372 M for the samples with one of the two enzymes (Samples 1,2 Table 3.2) and they have the same final concentrations with the Blank samples from Table 3.1 (Samples 1,2,3).

Table 3.2. *Reactants volumes at representative samples at the saccharification experiments adding dilutions of tannic acid to the mixture.*

Samples	**PASC (10 g/L)**	**Buffer (0,1 M)**	**Gallic acid (40 mM)**	**Enzymes**		**Tannic acid (diluted with in water)**
				LPMOs (5x diluted with buffer)	**BGs (5x diluted with buffer)**	
1	500 µL	345 µL	125 µL	15 µL	15 µL	-
2	500 µL	360 µL	125 µL	15 µL	-	-
3	500 µL	360 µL	125 µL	-	15 µL	-
4	500 µL	145 µL	125 µL	15 µL	15 µL	200 µL
5	500 µL	160µL	125 µL	15 µL	-	200 µL
6	500 µL	160µL	125 µL	-	15 µL	200 µL

Table 3.3. *Initial and final concentrations of tannic acid at the saccharification experiments.*

Tannic acid initial concentration diluted in water (mM)	**Tannic acid final concentration in the mixture (mM)**
0.36	0.072
0.5	0.1
0.75	0.15
1	0.2
1.44	0.288
2.9	0.58
6	1.2

3.4. Liquid-liquid extraction experiment

In order to create fractions from the PE, LLE was performed, using different organic solvents. The solvents were diethylamine, ethyl acetate, dichloromethane and 1-butanol. Those solvents were chosen for their properties such their polarity, miscibility and solubility with the PE and based on literature (103; 132). In the Appendix, polarities, boiling points and pH of those solvent are given (Table 6.1).

At the beginning a trial set of experiment took place in order to evaluate if this method can create fractions with modulatory effect on the LPMOs and BGs. The experimental process is represented in the Figure 3.3. First, 8 mL of PE were mixed with 32 mL of the solvent in 50 mL plastic tubes (Figure 3.4.i). The volume ratio was chosen based on the reference (132) and afterwards the tubes were mixed thoroughly for approximately 4-5 minutes (Figure 3.4.ii). Then centrifugation took place for 5 minutes at 5000 rpm and room temperature. After centrifugation, the two immiscible liquids which were the solvent layer and the PE layer (F1) (Figure 3.4.iii) were separated using a separation funnel. After the separation, the organic solvent layer was evaporated till dryness in a vacuum evaporator between 30-45^{o}C. The differences in the temperature were because of the differences in volatility of each solvent. At the end, after the isolation of solvent, the respective residue was dissolved in 1 mL of buffer that was used for the saccharification experiments. Then this mixture of the residue and buffer, which is a fraction of the PE (F2), was used in the saccharification experiments instead of buffer like the sample 1 (Table 3.1). The PE after mixing and centrifugation (F1) was tested in the saccharification experiments as samples 4,5,6 (Table 3.1).

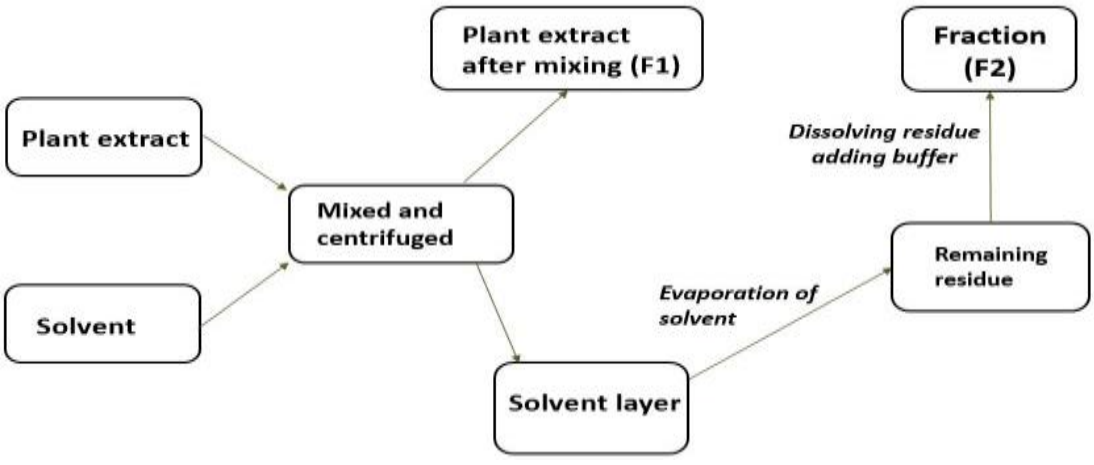


Figure 3.3. *Fractionation experimental process scheme*

Results from this trial experiment are given in the Figure 4.4.1. Then the whole process was repeated in order to achieve higher concentrations of the fractions (F2) and in order to collect more volume of it. The procedure of mixing solvent and PE was repeated 4 more times using each time new solvent and new PE so in total 32 mL of PE were mixed with 160 mL of solvent but for convenience reasons the mixing was done in five different 50 mL plastic tubes. Then the 5 solvent layers were mixed and they were evaporated till dryness to the vacuum evaporator. The final residue was diluted in 5,7 mL of buffer and this mixture of buffer and residue was stored covered with aluminum foil to protect from light at 4^{o}C temperature. The same procedure was done for all four solvents.

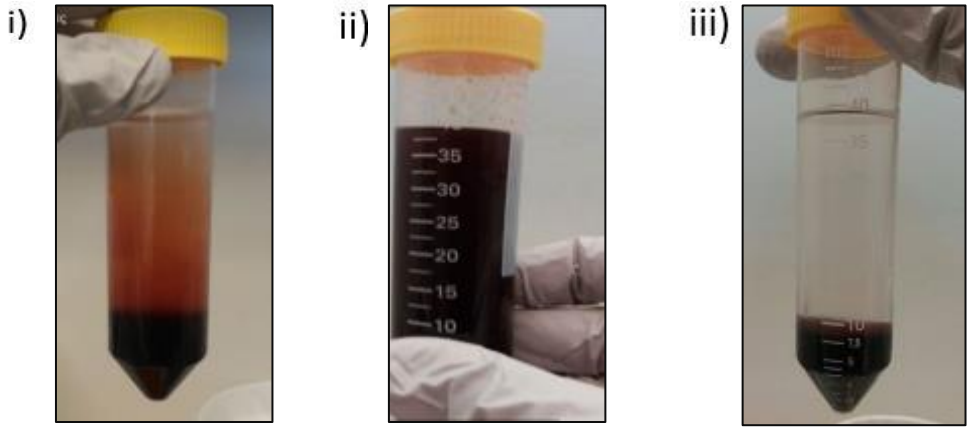
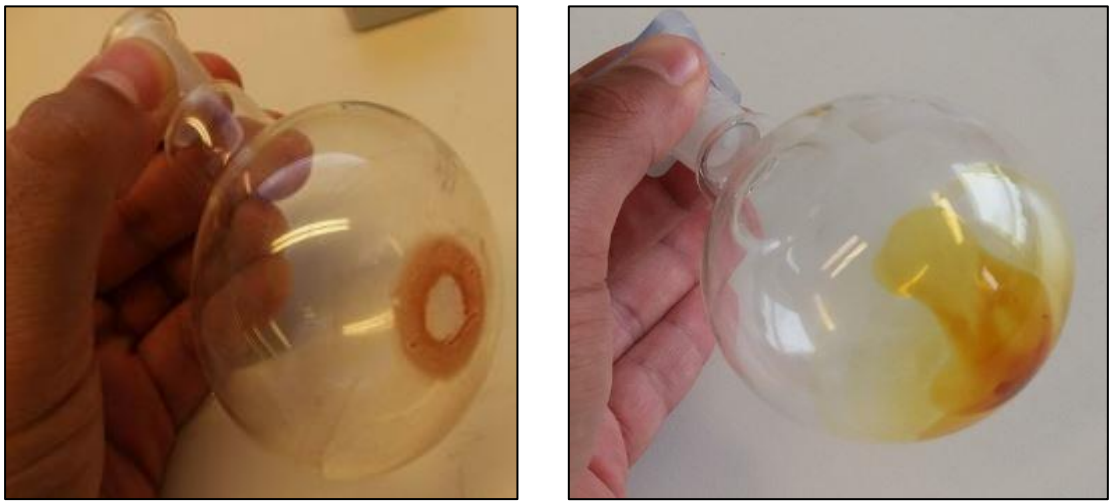


Figure 3.4. *Extraction snapshots using ethyl acetate as solvent. After adding ethyl acetate to the PE (i), right after mixing (ii), after centrifugation (iii)*

Finally, the four mixtures that were stored they were used in the saccharification experiments as the samples 1,2,3 (Table 3.1) but instead of buffer, these mixtures were added in different dilutions. Three dilutions of every mixture were made and were used in the saccharification experiments. The most concentrated mixtures were those in which the remaining residues (Figure 3.5) after the evaporations of the solvents were dissolved in 5.7 mL of buffer (Fraction 1x). Then two and four times dilution of this mixture with buffer took place (Fraction 2x, Fraction 4x). The pH values of the mixtures are given in the Appendix (Table 6.2). Results from these experiments are given to the Figures 4.4.2, 4.4.3, 4.4.5, 4.4.7.



i) ii)

Figure 3.5*. Remaining residue after the evaporation of ethyl acetate at the vacuum evaporator (i) Remaining residue after the evaporation of diethylamine (ii)*

3.5. Isolation of tannins

In order to test if tannins from the PE have an effect on the enzymes an experiment was performed that includes the isolation of them from the PE. First, 1,5 mL of PE was mixed 3 different amounts of commercial PVPP (0,15 g, 0.095 g, 0.04 g) in 2 mL Eppendorf tubes. Then the mixture was putted in the thermomixer (model) for 30 minutes at 20^{o}C and 1400 rpm. After 30 minutes of incubation and mixing the PE and the PVPP were separated after centrifugation in 20^{o}C and 14000 rpm (Figure 3.6.i , Figure 3.6.ii). Then the supernatant was centrifuged again in the same conditions, in case of remains of PVPP in the supernatant. Final the supernatant was added as the sample 4,5,6 from the table 3.1 in the saccharification experiments. Then the experiments were repeated but this time 1,5 mL of PE were mixed with 0,2 g of PVPP and the mixing time was 60 minutes. The rest of the procedure was the same.

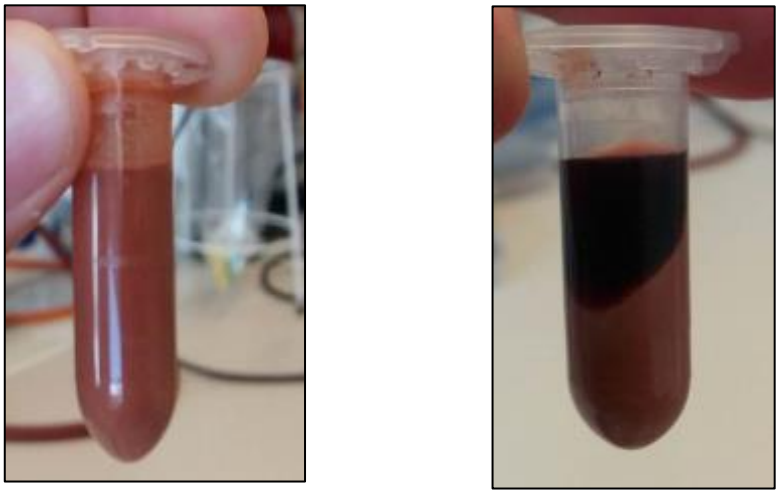


Figure 3.6*. PE and PVPP after mixing and before centrifugation (i). PE and PVPP after centrifugation (ii).*

3.6. Folin-Ciocalteu assay

For the quantification of the total phenolic content of the PE and the fractions after LLE, FC method was applied. The following experimental procedure was based on the reference (106). First, dilutions of PE samples and fractions from LLE were made. Specifically, the all the samples were diluted 200 times in water and one sample of PE was diluted 300 times in water to test the different values between different dilutions. Then 0.1 mL of the diluted sample were pipetted into a 2 mL Eppendorf tube. After 0.2 mL of FC reagent 10 times diluted with miliq water were added and then intensively mixing for 1 minute took place in a Vortex spinning machine. When those two get mixed if the sample contains phenolics then blue color appears and the more phenolic content exists the bluer color the mixture gets. Afterwards 0.8 mL of 70 mM sodium carbonate solution was added in the mixture, and then incubation took place for two hours in a dark place at room temperature. With the sodium carbonate the alkalinity is adjusted which also does not disturb the stability of FC reagent. After the incubation 0,2 mL of each sample were transferred in a 96-well plate. Finally, the absorbance of blue coloration was measured at 765 nm against a blank sample in microplate reader. The samples were compared to a standard curve of prepared gallic acid solutions which

is given in the Appendix (Figure 6.2). The final concentrations of gallic acid that were used in the standard curve were 0 mM, 0.0125 mM, 0.025 mM, 0,05 mM and 0.1 mM. Since the total volume of the mixture is 1100 μL and the initial volume of the sample is 100 μL the final dilutions of the PE and fractions samples that were tested in the final mixtures were 2200 times and 3300 times.

3.7. High Performance Liquid Chromatography (HPLC)

In order to measure the glucose concentration of samples after the three days reaction and after 1 and 2 days in the time course saccharification experiments HPLC as method was applied. HPLC is one of the most used and accurate methods for separation, identification and quantification for each component in a mixture. Identification and quantification of the components depends on the column used and standards available. HPLC is based on the different interaction of each component with adsorbent material that exists in the column, causing different flow rates for the different components and leading to the separation of the components as they flow out the column (133). As it was mentioned in section 3.3, after the three days reaction from the saccharification experiments centrifugation took place at 10000 rpm, 5 minutes and 20^{o}C and then supernatant from the samples was filtrated with 0,2 nm Nylon filters. Then the filtrated supernatants were tested for their glucose concentration. As a standard for glucose concentration, glucose solution was used. Specifically, 5 different concentration of glucose were used 10, 5, 2.5, 0.5 and 0.25 g/L in order to create a calibration curve for the quantification of glucose for the samples. The HPLC analysis was performed using a Rezex ROA Organic Acid H+ (300 mm x 7.8 mm) column with 0.8 mL/min flow at 80°C and a program time of 18 min. The eluent used will be a 5 mM solution of sulfuric acid and the wash buffer is a 5% methanol solution. Data analysis will be performed using Chromeleon software. Figure from HPLC from one representative sample is given in the Appendix (Figure 6.1).

4. Results and discussion

4.1. Effect of PE on saccharification by LPMO and BG

To evaluate the effect that the PE from persimmon fruit has on the enzymes, 4 different dilutions of the PE were added in the saccharification samples (Figure 4.1.1). The different dilutions of the PE were made based on different reaction volumes of the initial 20x diluted PE in the total reaction volume. Most of the results in this section are represented as the graph at the Figure 4.1.1. It is important to mention that in every series of experiments, there were samples without addition of the PE, which from now will be called on Blanks. Blanks are the samples 1,2,3 as they are described in Table 3.1 and they represent the glucose concentration that can be released when there is no addition of PE or something with modulatory effect on the enzymes. New Blank samples were prepared every time when new series of experiments were performed, due to the fact that in the whole experimental period four PASC were produced and it is known that every PASC that is produced is different from each other (35; 32). That is because properties of PASC are very dependent on the procedure that was used to produce it, such as incubation time and mixing. Also in most of the experiments the existing PASC was diluted from its original concentration to 5 g/L, so differences at the glucose release in Blank samples might exists due to errors in dilutions of PASC in addition with errors from the dry-weight measurement of the PASC concentration.

Giving an idea about the total saccharification at these experiments about the blank samples, there were always between 34-38%. That conversion number is calculated based on the final concentration of PASC (5 g/L) in the reaction volume and the average amount of glucose titer from all the blank samples, which was around 1.7-2.1 g/L. The factor to take into account for the increase in molecular mass when cellulose is converted to glucose is 1.111, because glucose molecular mass is 180 g/mol and glucosyl unit in cellulose molecular mass is 162 g/mol. These conversion values are not in line with the percent conversion number of the Quillan et al (56) where with the same enzymes and similar amounts of concentrations of PASC, gallic acid and enzymes they got a conversion of substrate around 14%. This difference could not easily be explained since the quantification of glucose from HPLC was correct and the calculations for the PASC concentration were correct. It might be explained with the fact every PASC that

is produced is different from another one as it was mentioned before. So even though the concentration of PASC was similar is possible that the surfaces of PASC are not similar accessible to the enzymes due to differences in properties and structure of PASC. In addition, there were some parameters at the saccharification experiments that is uncertain if were done with the same way with Quillans et al and they can affect the saccharification such as the final pH of the reaction samples, the difference of buffer pH and the mixing speed.

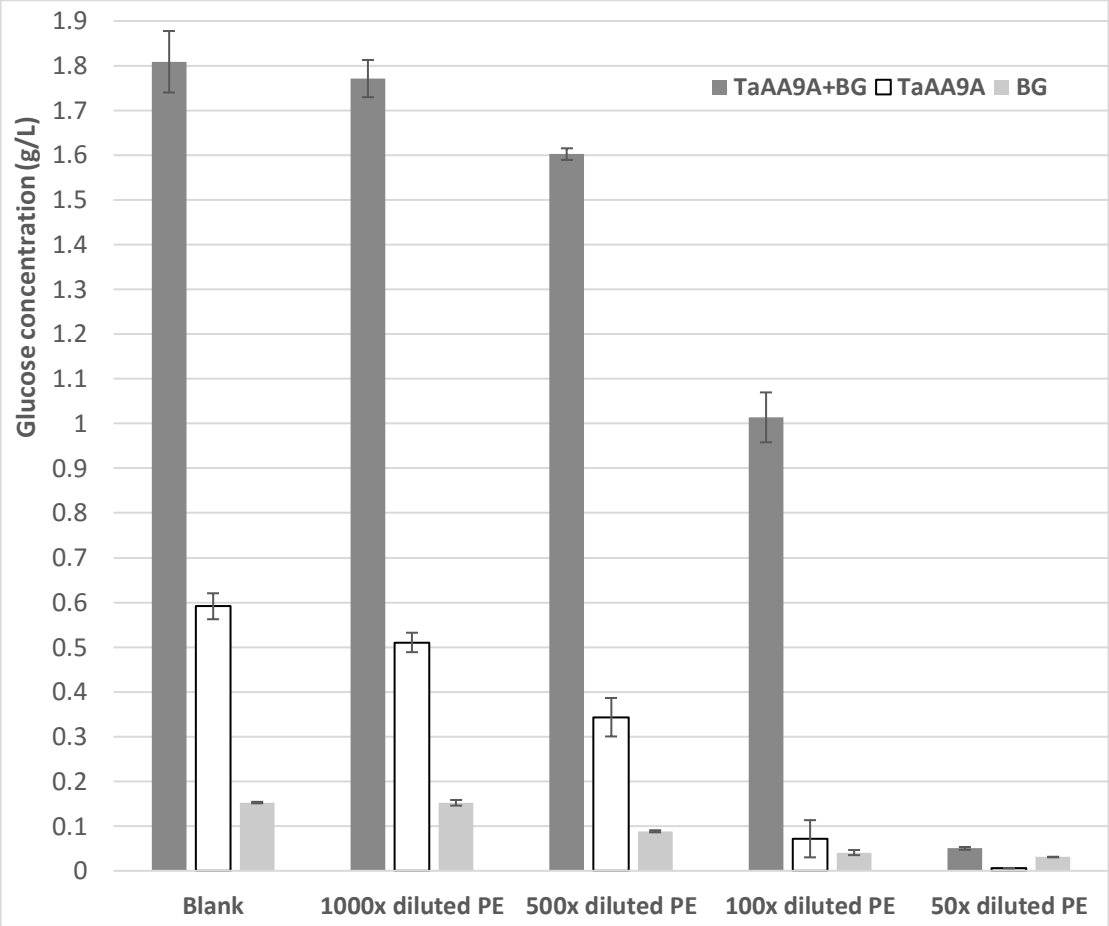


Figure 4.1.1. *Glucose titers after three days of reaction. The columns with dark grey represents the samples that they are loaded with both TaAA9A and BG, while white color columns represents the glucose titers when sample are loaded with TaAA9A and last with less darker grey are the samples loaded with BG. The bars represent average values of three replicates and the error bars show standard deviation of the average.*

Table 4.1. *Reduction of glucose release at the saccharification experiments after adding different dilutions of PE. The values are based on the average glucose release of the samples from the Figure 4.1.1.*

Samples	***TaAA9A + BG***	***TaAA9A***	***BG***
	Reduction in glucose release (%)		
Blank	*0*	*0*	*0*
1000x dilution of PE	*2.5*	*13.7*	*0.1*
500x dilution of PE	*11.6*	*41.9*	*35.5*
100x dilution of PE	*42.1*	*85.1*	*73.2*
50x dilution of PE	*97.3*	*98.4*	*80.1*

From the graph and the table, we can clearly observe that with all the dilutions tested there a decrease in glucose release, so there is an inhibitory effect on both of the enzymes. It is clear from both Figure 4.1.1 and the Table 4.1 that the PE has higher effect on the TaAA9A than the BG, which makes the PE more interested to be further tested for the overall aim of this project, which is to find a modulator for the TaAA9A. Specifically in every dilution that was tested the reduction of the glucose release was always higher than the glucose release from BGs and the most concentrated dilution of the PE, the 50 times dilution, had almost complete inactivation of the TaAA9A unlike the BGs.

In order to understand what are the inhibitory compounds in the PE, it is necessary to think the source that this PE came from. As it mentioned the PE comes from an astringent persimmon fruit and it is basically a fermented tannin juice from an unripe persimmon. Persimmon juice like any fruit juice contain a huge variety of phenolic compounds such as flavonoids, organic acids and tannins (113; 134; 135) and most of these compounds of these compounds can have an inhibitory effect at cellulases (116; 136). Gorinstein, Shela, et al (1994) found using analytical methods in persimmon extracts that *p*-coumaric acid was a main component and also, they measured gallic acid, *p*-coumaric acid and protocatechuic in persimmon fruit-ethanol extracts (137). However, when it comes to TaAA9A, phenolic compounds that might exists in the PE, like gallic acid, can possible act as an electron donor to them (16). For this reason, samples without gallic acid, but with and without addition of the PE were tested, in order to test if the PE can act as an electron donor (Figure 4.1.2.).

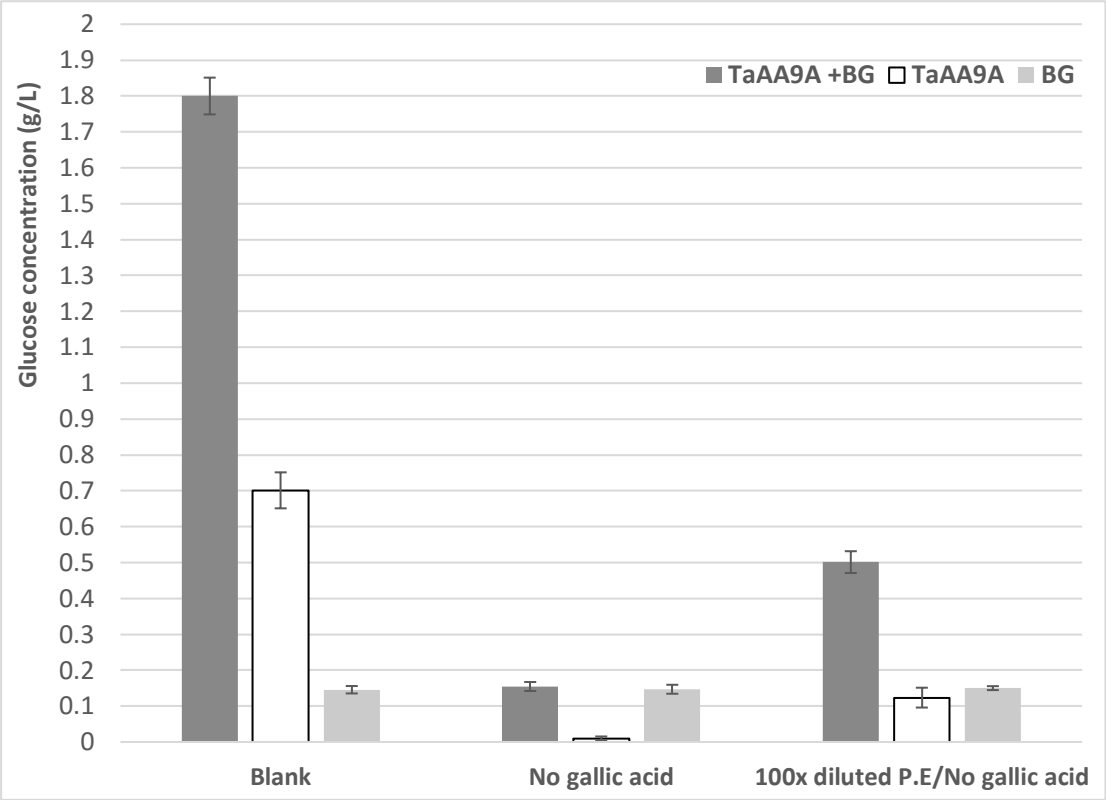


***Figure 4.1.2**. Glucose titers after three days of reaction. The first triad are Blank samples and the second triad represents samples without adding gallic acid but adding 100x diluted PE. The bars represent average values of three replicates and the error bars show standard deviation of the average.*

It can be observed that the PE can act as an electron donor to LPMO. That proves the complexity and the variety of compounds that exists in the PE that can work either as activators or as inhibitors for the LPMO.

Something else that was interesting about the PE was the difference of the effect on TaAA9A and BG that two PE have (Figure 4.1.3), which they have been purchased on different times. Specifically, one PE has been purchased almost a year ago and the other one about 4 months ago. From now on the first one will be called Old PE and the other one New PE.

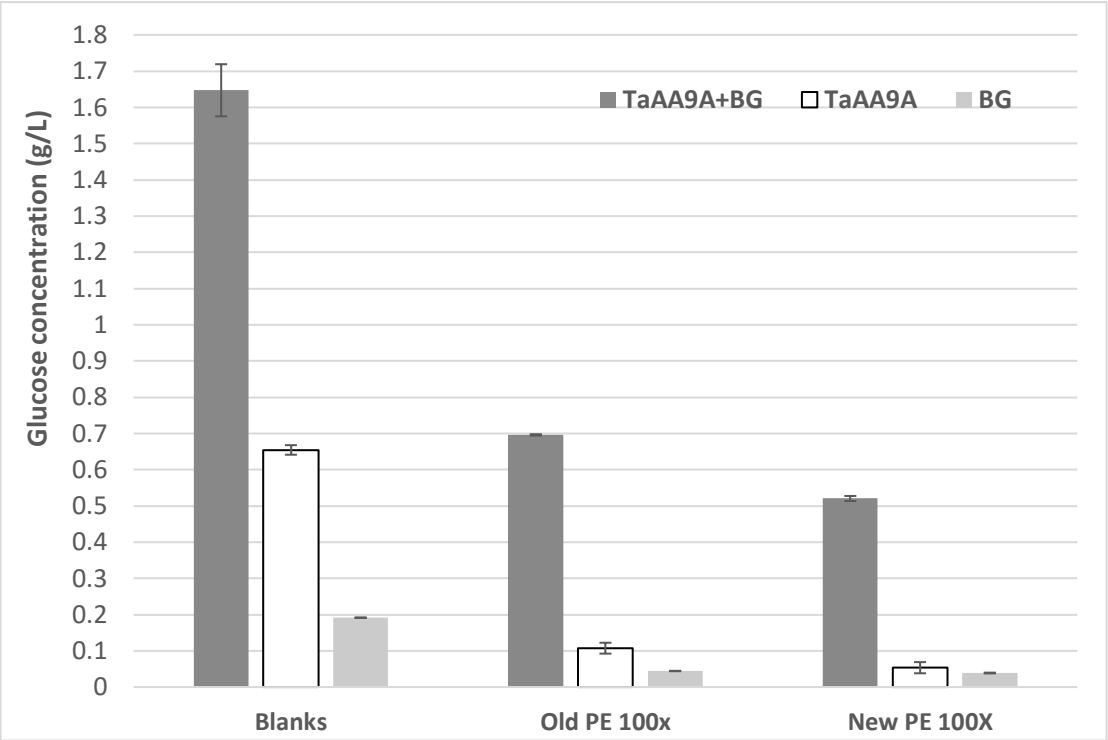


Figure 4.1.3 *Glucose titers after three days of reaction. The bars represent average values of two replicates and the error bars show standard deviation of the average.*

It can be observed that the Old PE has less inhibitory effect than the New PE to TaAA9A. This means that the inhibitory substances can be affected by storage time and/or by atmosphere conditions, such as oxygen or light, due to the openings of the receptacle. That is a useful information about what might be the inhibitory compound, which as it seems it can be affected by time and it can be oxidized. Substances that are known that can be oxidized and they are affected by time storage and they exist in the PE are phenolic compounds and organic acids such as vitamins. Klimczak et .al have tested the effect of storage for 6 months in three different temperatures on the content of polyphenolics in orange juice using F-C method and HPLC and their results show that the total polyphenols are decreasing over time (110). Interesting also is that the Old PE was precipitated from buffer when it was diluted in it. Photos of this precipitation are shown in the Appendix (Figure 6.3.). After observing the results from the previous graph most of the saccharification experiments were repeated using the New PE.

Something else that was also plotted to a graph was the release of glucose concentration during a time course experiment of the 3 days reaction in two cases, one for the Blanks and one adding 100x diluted PE. Specifically, out of the three days reaction, triplicates of samples were taken out every day (Figure 4.1.4, 4.1.5).

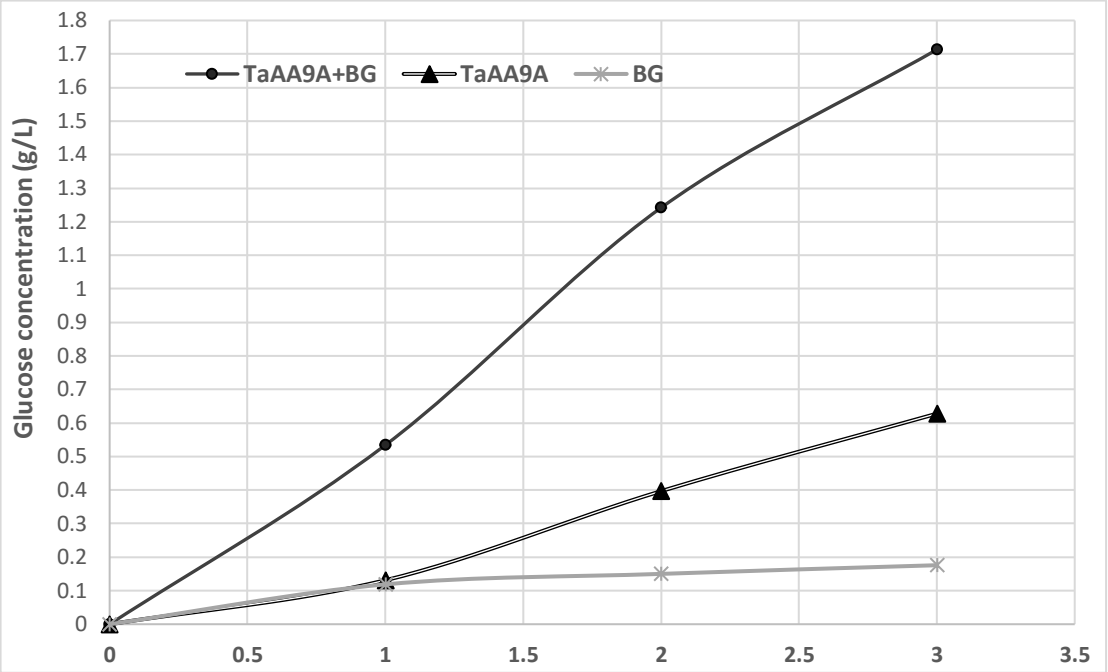


Figure 4.1.4. *Increase of glucose concentration from Blanks samples at the three days saccharification reaction. Samples were taken out every day and triplicates were made.*

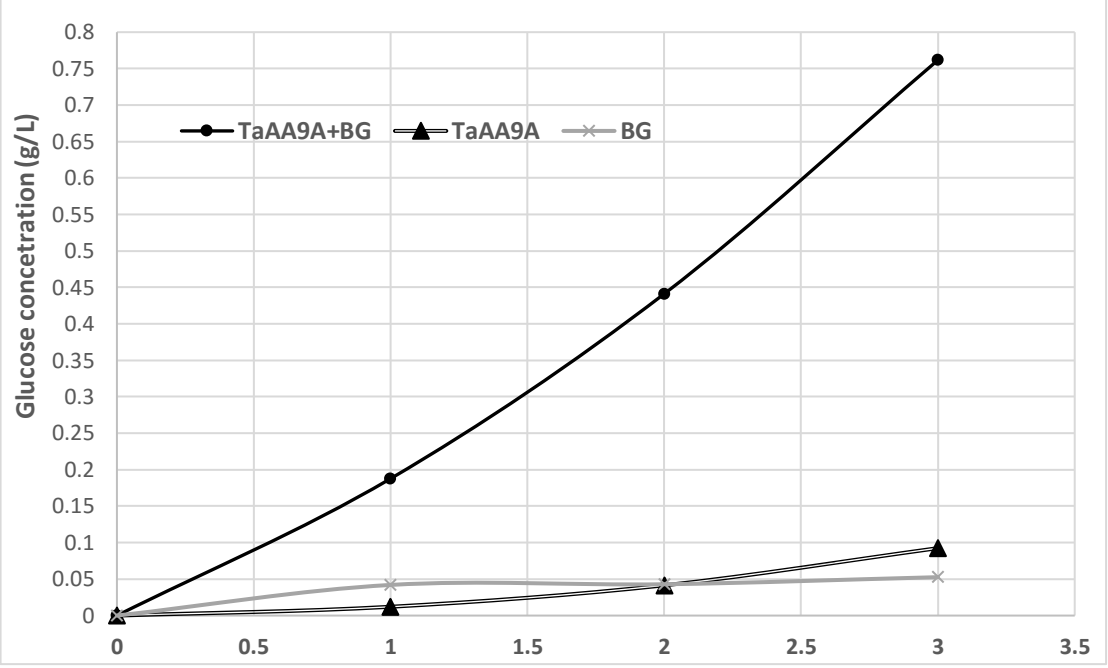


Figure 4.1.5. *Increase of glucose concentration from samples with 100x PE at the three days saccharification reaction. Samples were taken every day and triplicates were made.*

From these two graphs, it can be observed that in case of the PE (Figure 4.1.5) the increase of the glucose concentration for TaAA9A is not similar with the increase in case of the blank samples (Figure 4.1.4). It seems that the PE affect the TaAA9A from the beginning of the reaction in contrast with the BG which from the first day the produce 98% of their total glucose release when PE is added. In case of the blank samples, TaAA9A produce similar amount of glucose with BG but that does not happen when PE is added where TaAA9A reach the similar amount of glucose the second day. Also at the end of the experiment the difference between the TaAA9A and BG final glucose release is different in the two cases. At the Blank samples TaAA9A have 71% difference with BG samples, while when PE is added they have 43%. For the BG the increase stays similar, the first day there is 70% increase from the total glucose release in case of the blank samples and when PE is added the glucose release is 98% of the total which means that the glucose release almost stops from the first day when PE is added. Their synergism seems to be no affected how the glucose release is increasing over the days, but the amount of glucose is changed as in every case when PE is added. Specifically, the glucose release has been reduced about 51.7 %, close to the number of reduction of glucose release at the Table 4.1, which is 46.9 %.

From all of the previous figures and results in this section it can be summarized that the PE has different and more significant effect on TaAA9A than on BG. It is possible that the PE is blocking the surface of the PASC without interacts or inactivates the TaAA9A. This blocking is probably more intense in case of the TaAA9As, because they are about 6 times smaller than BGs, so they might need more time to overcome the potential barrier that the PE is creating to PASCs surface, than BGs. That blocking can be more significant if the PE binds with PASC external surface, creating areas that are no accessible for both enzymes and especially for TaAA9A. However, it seems that there might be potential deactivation of TaAA9A, due to the fact that the most diluted PE (50x) clearly deactivates them, which can be observed also from the values of glucose, when both enzymes are loaded. It can be seen that the glucose release in case of 50x dilution, when both of the enzymes are loaded, is mostly because of the BGs action, due to the fact that they have similar values with the samples were only BGs are loaded. That is something that does not happened in all the other dilutions. However, maybe the rest of the concentrations were not enough to deactivate all of the molecules of TaAA9A so that is why there is still activity from TaAA9A. But from the Figure 4.1.2

it can be observed that there is increase of the activity in case where no electron donor is added (no gallic acid) so if there are substances that deactivates the TaAA9A, it is clear that there are other substances that can activate them. Nevertheless, it is not clear and certain if PE deactivates the TaAA9A or is just blocking surfaces of the substrate.

4.2. Effect of filtrated and centrifugated PE on saccharification by TaAA9A and BG

It could be seen that the receptacle of the juice had solid residues on its cap and also in the description of the PE by the providers it is mentioned that it should be shaken periodically and last when the PE was being centrifuged, small amount of residues exists at the bottom. In order to evaluate if after isolation of small solid particles from the PE there is a different effect on the enzymes, two treatments of the PE were made. One filtration with 0,45 nm Nylon filters and the other was centrifugation. The results are shown in the Figure 4.2.1.

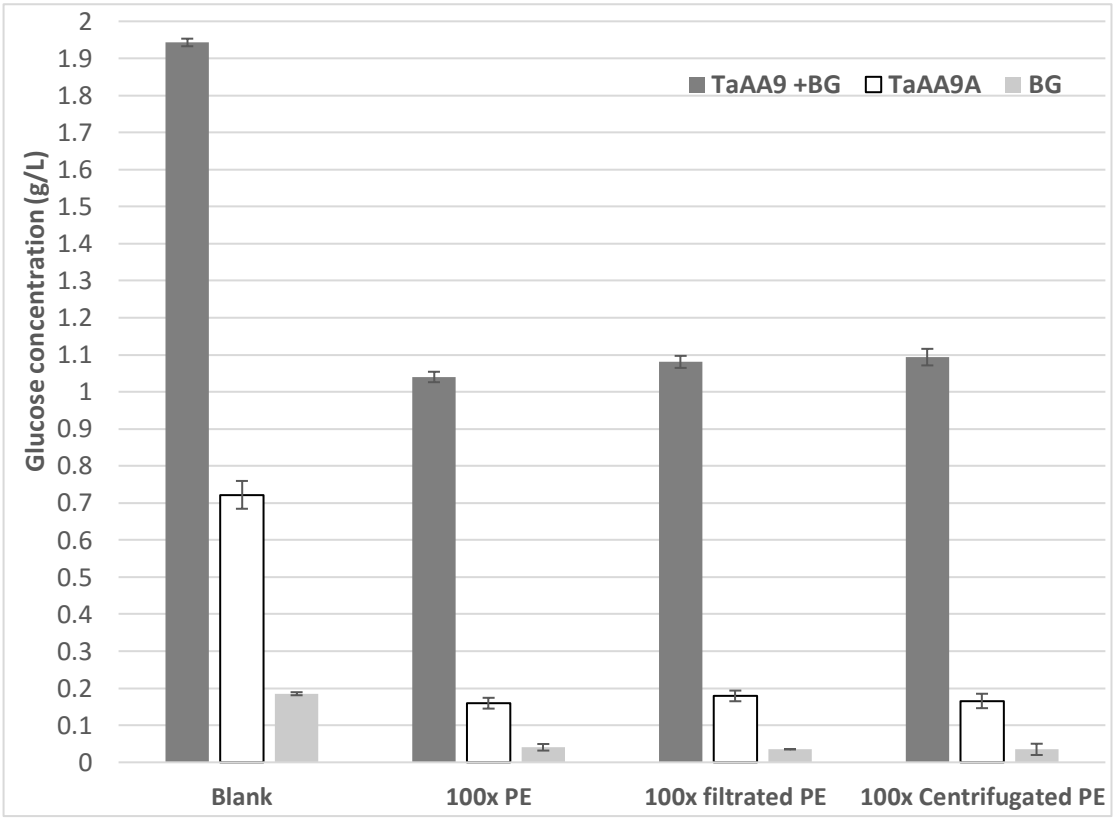


Figure 4.2.1. *Glucose titers after three days reaction with addition of PE with and without filtration with 0,45 nm Nylon filters. For all the samples triplicates were made and the error bars show standard deviation of the average.*

From this graph, it can be observed that there is no significant difference at the glucose concentration between the samples loaded with and without filtrated PE or from the centrifugated samples. However, in case of the TaAA9A+BG there is a slightly increase but is not significant to make conclusions from them. That means that particles that exists in the PE and they can be filtrated from 0,45 nm Nylon filters have no major impact on the enzymes, both to TaAA9A and BG. The same applies for the particles that can be centrifuged. However, this is a small increase and in order to be further tested, more dilutions should be done. It is important to mention that the filtration of the PE wasn't so easy to be done due to fact that the PE is a viscous liquid and the filters were stuck and break due to the pressure of the filtration in most of the tries, so the repeat of the experiment was not so easy.

4.3. Effect of thermal treatment of the PE on saccharification by TaAA9A and BG

This experiment was done in order to evaluate if there is a protein nature compound that has the inhibitory effect to the enzymes and can be precipitated in the tested temperatures. So, if there is a protein nature compound inhibitor in the PE that can be precipitated in the tested temperatures then the glucose release should be higher than the second triad of columns, which are samples with addition of PE at room temperature. To evaluate this, the PE was incubated in seven different temperatures for one hour and then it was added with the same way with the previous experiments at the saccharification experiments (Figure. 4.3.1).

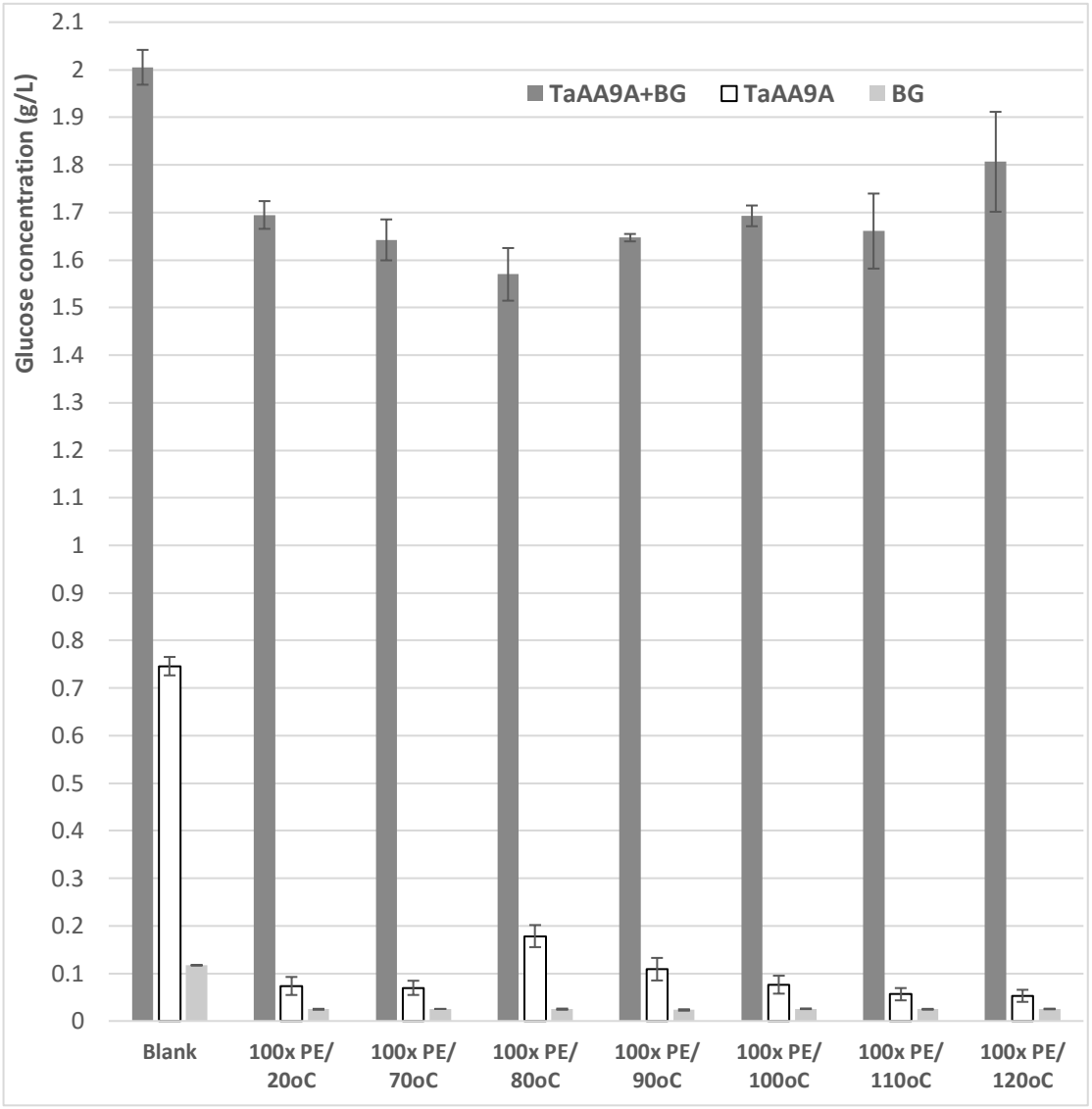


Figure 4.3.1*. Glucose titers after three days reaction with addition of PE after being incubated in seven different temperatures for one hour. The second triad was the PE at room temperature. Each column represents an average of three replicates and the error bars show the standard deviation of the average.*

It can be observed that there is no serial increase or decrease in the glucose concentration with the increase of the temperature. Also, some of the differences in the columns are in the range of the error bars, so it cannot be said confidently that there is difference. In all of the temperatures tested there is no difference between the BG samples. The temperature that seems to have no effect in any enzyme is the 70. The most interesting columns seem to be the 80 and 90 where the glucose concentration was increased in TaAA9A samples. However, the samples with both TaAA9A and BG in

those temperatures have decreased glucose concentration compared to the samples at the second triad. That is not rational due to the fact that if in those temperatures TaAA9A alone can work better and they can release more glucose then the samples with TaAA9A and BG should also release more glucose. This could be explained due to mistakes from pipetting errors in the experimental process. Important to mention is that in this experiment the Old PE was used which means that the experiment should be repeated. However due lack of time for this project two temperatures, the experiment was repeated using the New PE (Figure 4.3.2).

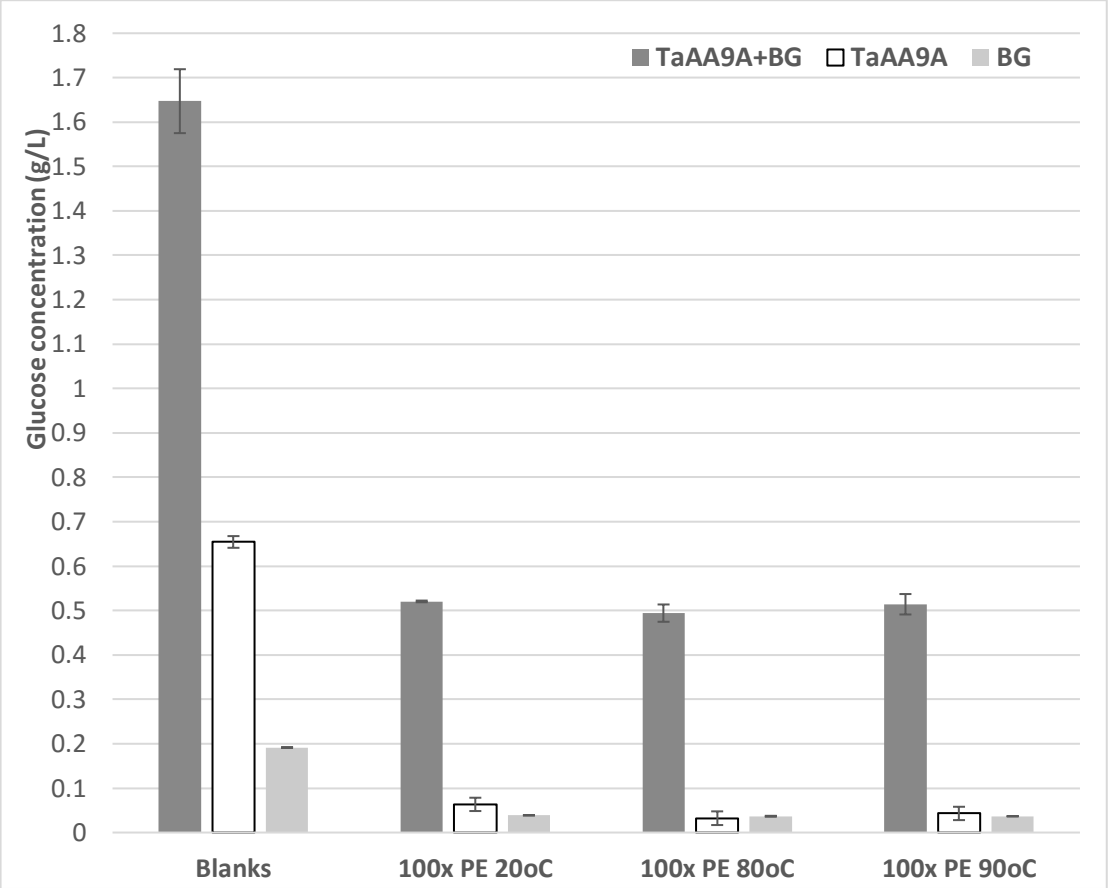


Figure 4.3.2. *Glucose titers after three days reaction with addition of PE after being incubated in two different temperatures for one hour. For all the samples duplicates were made and the error bars show the standard deviation of the average.*

It can be observed that there is no increase in any temperature in those two temperatures, meaning that if the inhibitory compound is a protein nature cannot be precipitated from these temperatures and this incubation time. BGs seem again to be no affected by the different treatment temperatures to PE. However, there is a decrease in the TaAA9A samples which is not in line with the results from the previous graph about these temperatures, meaning that is likely that errors in the experimental process took place in the experiment at the Figure 4.3.1. This decrease is not high enough to conclude information from it but it could be probable due to the fact that compounds in the PE got deconstructed from the temperatures and after the deconstruction could have more inhibitory effect on TaAA9A.

4.4. Effect of the fractions after Liquid-liquid extraction on saccharification by TaAA9A and BG

For the fractionation of the PE, LLE as a method was used using 4 organic solvents, ethyl acetate, dichloromethane, diethylamine, 1-butanol. Methanol and Ethanol were also tested for this method but they were fully miscible with the PE so LLE could not work with these two solvents. Initially a trial of fractionation and saccharification experiments was performed in order to evaluate if this method can create fractions from the PE with modulatory effect on the TaAA9A than the PE itself for example activation or higher decrease. The results from the trial are given in the Figure 4.4.1.

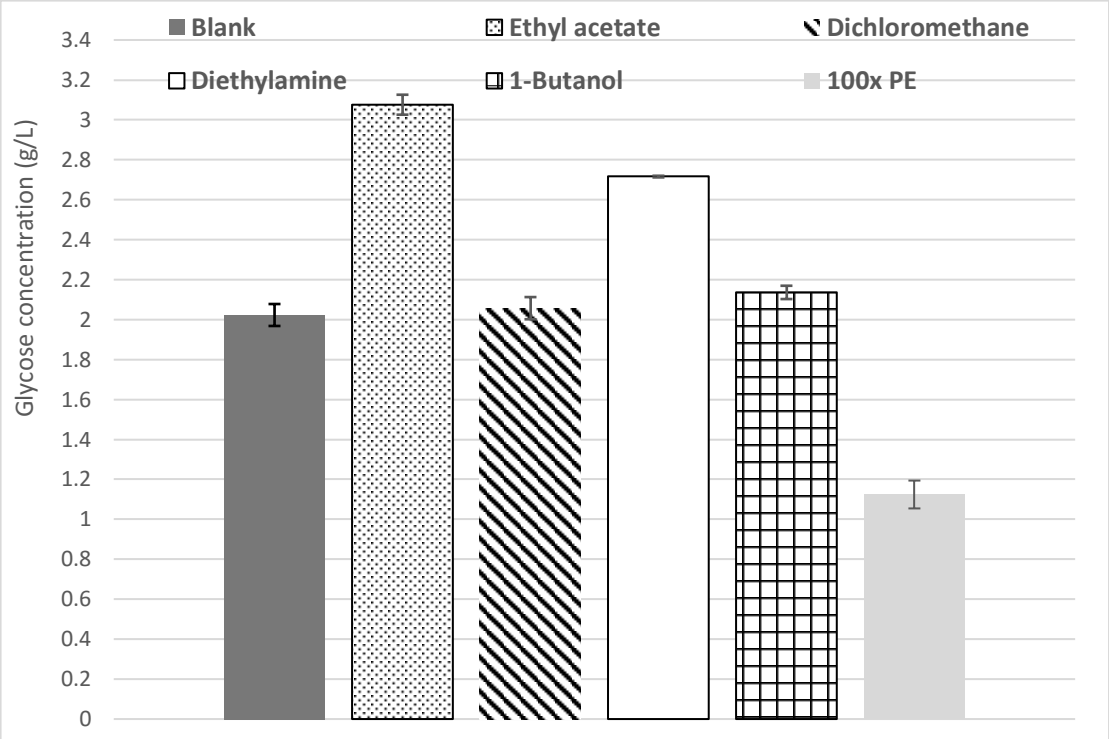


Figure 4.4.1. *Glucose titers after three days reaction with samples loaded with TaAA9A and BG enzymes and instead of buffer they were loaded with residues from the LLE diluted in buffer. Each column represents an average of two replicates and the error bars show the standard deviation of the average*

From the Figure 4.4.1, it can be observed that this method created fractions with different effect from the original PE. In particular, it is clear from the graph that the fractions from the ethyl acetate and the diethylamine have an activatory effect to the enzymes. For 1-butanol the glucose release is a little higher than the blank column and for dichloromethane there is no difference. Especially for the samples with the ethyl acetate fraction there was an increase about 33% from the Blank samples. Furthermore, three of the solvents managed to extract substances from the PE which they can increase the glucose release at the saccharification experiments, while for dichloromethane is not certain if the fraction was not enough concentrated to have an effect on the enzymes.

In the case of the ethyl acetate and dichloromethane the PE after the mixing and centrifugation with the solvents (F1) was tested in the saccharification experiments as the samples 4,5,6 Table 3.1 and the results are shown in the Appendix Figure 6.4. It can be observed that there are no significant differences with the values from the original PE. As it is going to be described in the next sections the F1 from the diethylamine and 1-butanol solvent could not be tested at the saccharification experiments because after the mixing with these two solvent the PE was precipitated.

In order to test different concentrations of the fractions and in order to test the effect of the fractions on samples loaded with TaAA9A and samples loaded with BG the experiments were repeated. This time the fractions were collected using different quantities of PE and solvent as it was described in the section 3.4 and the results are shown in the Figures 4.4.2, 4.4.3 ,4.4.5 and 4.4.7.

4.4.1. Ethyl acetate fractions

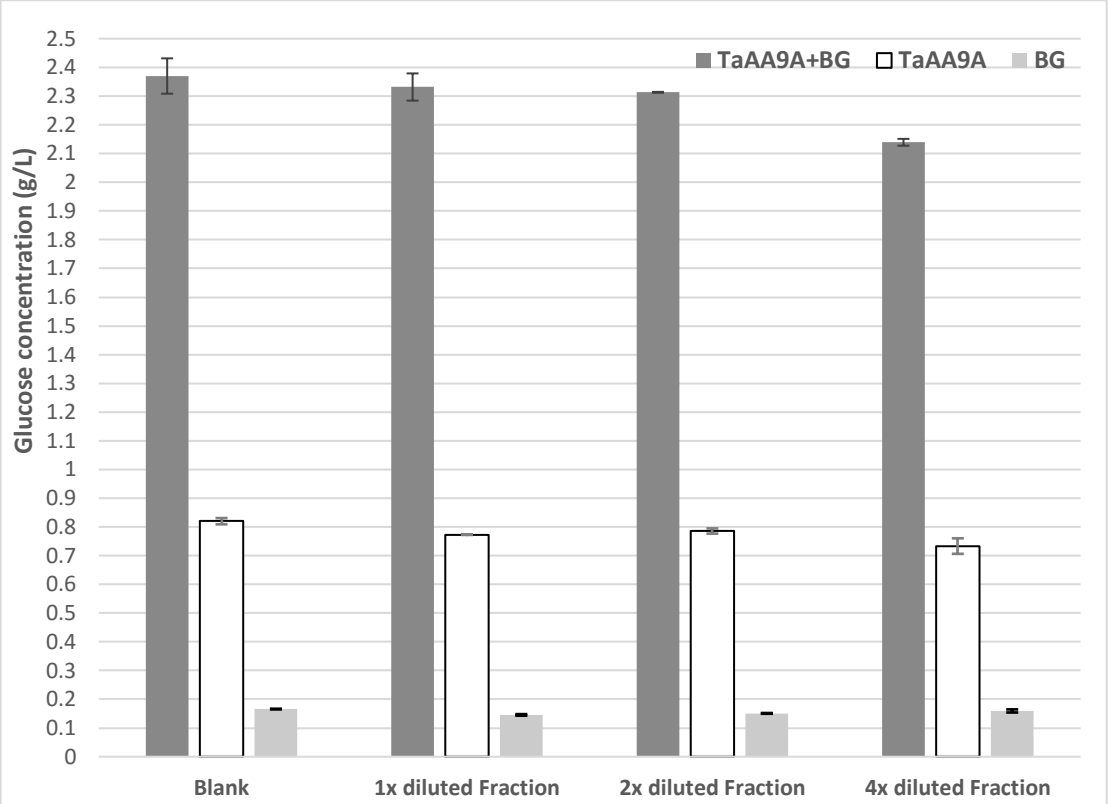


Figure 4.4.2 *Glucose titers after three days reactions. The first triad of columns is the blank samples while the other three triads have instead of buffer the dissolved residue in buffer from the LLE using the ethyl acetate solvent. Three dilutions of the fraction were investigated. First one is the residue after the evaporation of ethyl acetate adding 5,7 m L of buffer and the third triad is two times dilution with buffer of the first one and last triad is four times diluted.*

It can be observed from the Figure 4.4.2 that the ethyl acetate does not have activatory effect on the enzymes in any of the three dilutions, which is not related with the results from the trial experiment (Fig 4.4.1), where the ethyl acetate fraction had the highest increase from all the fractions that have been created from the LLE. Samples with BG seem to be unaffected from the fraction. However, it can be observed that the more concentrated the fraction is, the more glucose concentration we get. In representative samples with the fractions, the pH was tested and it was around 4.9-5.1 (Table 6.3). Generally, it can be summarized from this that the fraction with higher concentrations has positive effect to the glucose concentration. Having different results from the trial

experiments can be explained with the fact that the residue from the ethyl acetate evaporation (Figure 3.4.i) was not easily dissolved in the buffer. The amount of buffer, that was chosen for the dissolving of every residue in those experiments was 5.7 μL. As it was mention at the section 3.4 that number was based on the amount of the fraction needed for the those experiments and for getting similar but a slightly higher concentration of the fractions from the trial experiments. However, in the case of the residue from the ethyl acetate that could not be easily achieved due to the fact that the dissolving part was not easy to be controlled. Specifically, the residue was mixing the buffer for over an hour and still there was amount of the residue that it could not be dissolved in the buffer. So, for the two experiments, it can be assumed that they did not have similar concentrations of the fraction and in case of the trial experiment the fraction was more concentrated which explains the higher than the blank glucose release. That is in line with the results of the Figure 4.4.2 where the more concentrated fraction is, the more glucose release we get. In literature ethyl acetate is a solvent that can extract substances such as alkaloids, for example caffeine from coffee (138) or furanocoumarins from grapefruit juice (132) but there is no information about extraction from persimmon fruit or juice using this solvent. Some observations from this solvent were that during the mixing with the PE, no chemical interaction took place and the color of the residue was dark orange.

4.4.2. Diethylamine fractions

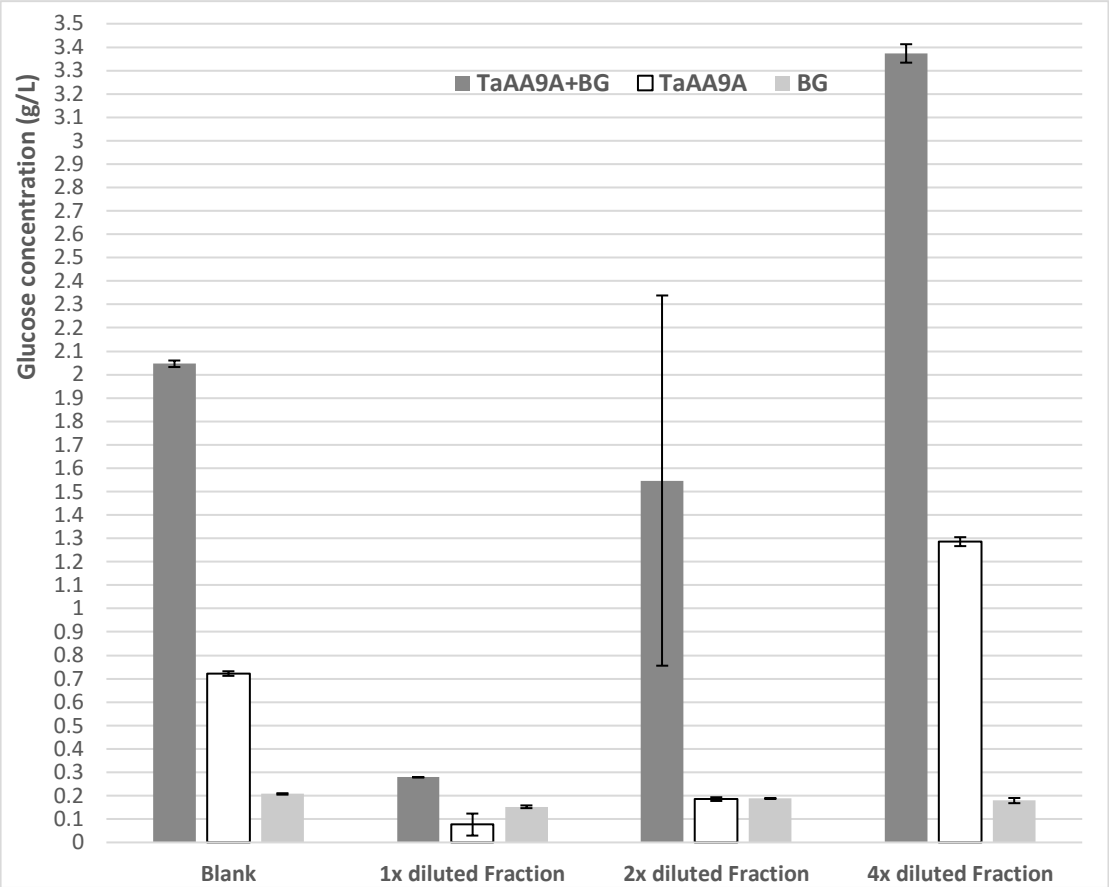


Figure 4.4.3*. Glucose titers after three days saccharification reactions. The first triad of columns is the blank samples while the other three triads have instead of buffer the dissolved residue in buffer from the LLE using the diethylamine solvent. Like the samples the Figure 4.4.2 three concentrations of the fraction were made. For all the samples duplicates were made.*

It can be observed from the Figure 4.4.3. that the fraction that have been created from the diethylamine solvent, based on its concentration has different effect on TaAA9A. The fraction does not affect the BG as much as the TaAA9As. For TaAA9A and for TaAA9A+BG samples, the most concentrated fraction, which are the samples from the second triad of columns has a clearly inhibitory effect. However, when the fraction gets two times diluted with buffer the inhibitory seems to not exist anymore. The big standard deviation that exists in the column loaded with both enzymes is because only one out of the two samples had a lower glucose concentration than the blank one, while

the other one sample of the duplicate had a higher glucose concentration. All TaAA9As samples seems to be still effected in all concentrations of the fraction. The least concentrated fraction (last triad of columns) is the one with the clearest activatory effect to the TaAA9A+BG and TaAA9As samples. Specifically, in that case the increase of the glucose concentration from the blank samples is 39% which is the highest increase in all the samples at the saccharification experiments. In representative samples, the pH was tested and it was around 5.2-5.35 (Table 6.3). Only in case of the most concentrated fraction was about 5.35 which cannot explain that decrease at the glucose concentration. Concluded for the diethylamine fraction is that the concentration of it seems to have a major effect to the TaAA9As. Is also in line with the results from the trial experiment (Fig 4.4.1) that show that there is an activatory effect. In comparison with the ethyl acetate fraction the fraction from diethylamine was easily dissolved in buffer. Also, this time the fraction had a yellow color. Concluded this fraction seems to work both as an inhibitor and an activator and that could be explained from the fact that the fraction is still a complex mixture with both activators and inhibitors to TaAA9A, which they act competitive. It is also important to mention the effect of mixing the PE with dethylamine. The moment that those two liquids were mixed the PE became much darker and solidified to some extent, plus heat also was released so the tube become warmer. So, it seems that there is a chemical interaction between the PE and the diethylamine. This could be acid-base reactions, which there are exothermic reactions and they can create solids. The PE is a slightly acid liquid (pH = 4,4-4,5) and the diethylamine is a strong base chemical (pH= 12,3-12,4) so it is rational for acid-base reaction to take place when those two liquids are mixed.

To test the activatory effect of the 4 times diluted fractions saccharification experiments were done this time without gallic acid, which is the electron donor in our experiment, in order to evaluate if it can still act as an activator. The results are shown in the Figure 4.4.4 and it can be observed that the fraction has a activatory effect to TaAA9A, however it cannot reach the values of Blanks samples but the activatory. Observing the figures 4.1.2 and the 4.4.4, which were experiments where we add the PE and the 4x fraction from diethylamine without the electron donor (gallic acid) seems that the 4 times diluted fraction can activate the TaAA9A more than the PE. It can be seen that the glucose release in the case of samples loaded with both enzymes is the same in both figures.

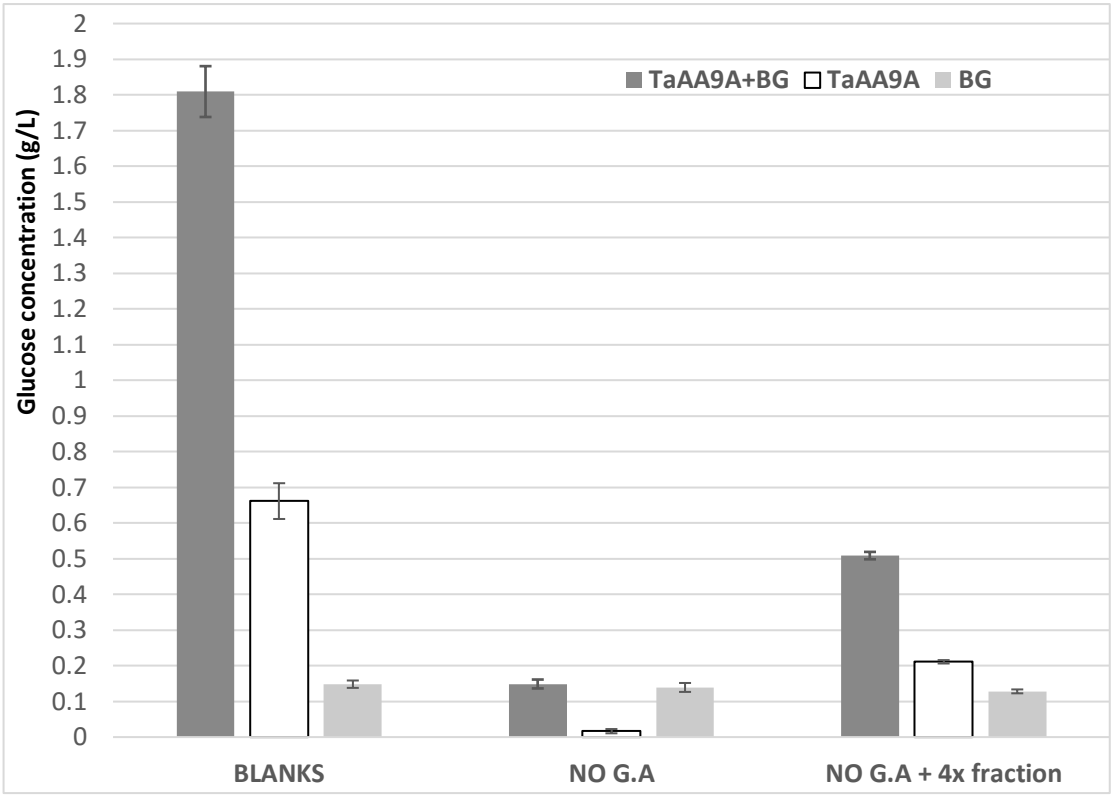


Figure 4.4.4. *Glucose titers after three days saccharification experiments. The first triad of columns is blank samples, the second are samples without adding gallic acid (NO G.A) which is the electron donor for TaAA9A and the last triad are samples adding the 4x times diluted fraction from diethylamine LLE, which in the Figure 4.4.3 showed the most activatory effect. For all the samples duplicates were made and the error bars show the standard deviation of the average.*

4.4.3. 1-Butanol fractions

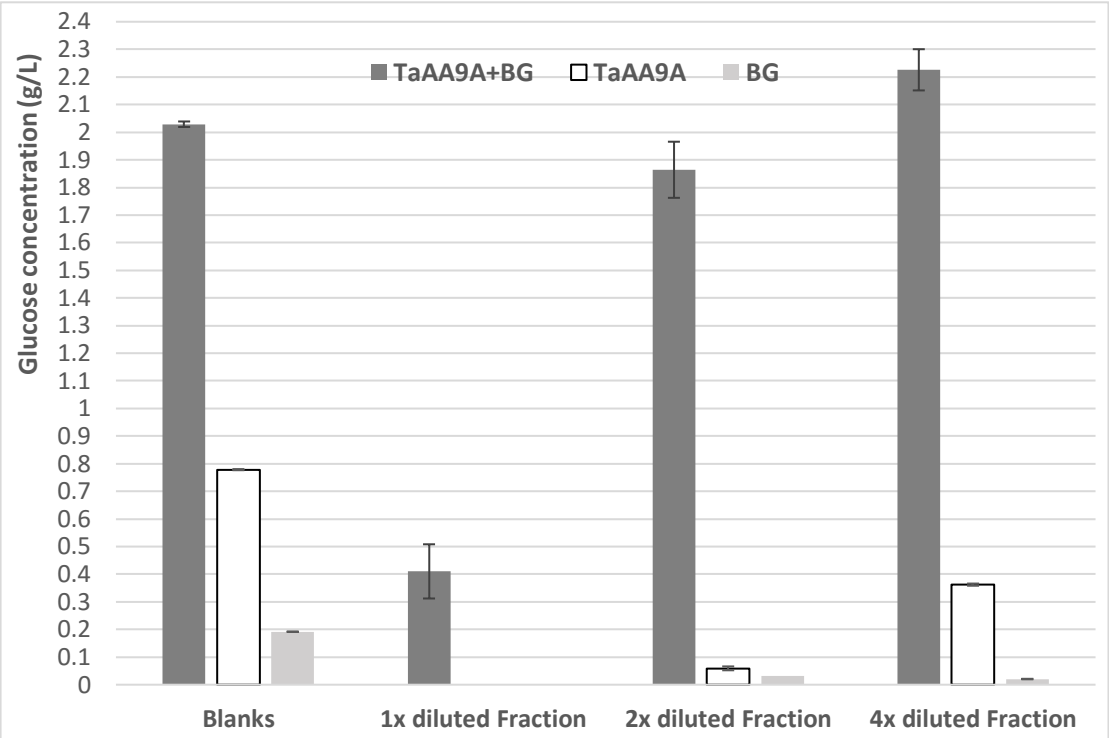


Figure 4.4.5. *Glucose titers after the three days saccharification reactions. The first triad of columns is the blank samples while the other three triads have instead of buffer the dissolved residue in buffer from the LLE using the 1-butanol solvent. Like the samples from the figure 4.4.2. three concentrations were made.*

It can be observed from the Figure 4.4.5 that the butanol fraction has similar effects with the diethylamine fractions. However, the effects are not same as the previous case, for example the increase in the least diluted fraction is not so high as the diethylamine case. One important difference is that in butanol case the BG is also effected by the fraction. Briefly the less concentrated the fraction with is the more glucose concentration but only for to TaAA9A and TaAA9A+BG samples. Observations from the extraction using this solvent were that the butanol has similar effect when it was mixed with the PE but in lesser extent. That means after mixing the PE became darker again and solidified but not as much as in the case of diethylamine and the heat that released was not too much. Also, the fraction was a dark orange fraction. Columns are missing from the graph due to the fact that HPLC had weird peaks that did not gave glucose peaks. That might be explained from remains of butanol in the fraction, since the fraction had a slightly smell of butanol, which indicates the existence of butanol in

it. For that reason, samples were made adding representative amount of 1-butanol that might exists still in the fraction. Specifically, the volume of 1-butanol that was mixed with buffer was 15% of the volume of buffer and the saccharification experiments were repeated (Figure 4.4.6). It can be observed that there is a decrease in the glucose release that can explain the decrease in the most concentrated fractions. However different amounts of 1-butanol should have tested and also it is not certain how much of 1-butanol remained in the fraction. It is important to mention that the fraction from the 1-butanol during the storage got precipitated from the buffer and it could not be tested again for repeats of saccharification experiments the repeat of the LLE using 1-butanol did not took place due to lack of time. The fractions from this solvent have pH from 5.3-5.2 (Table 6.3).

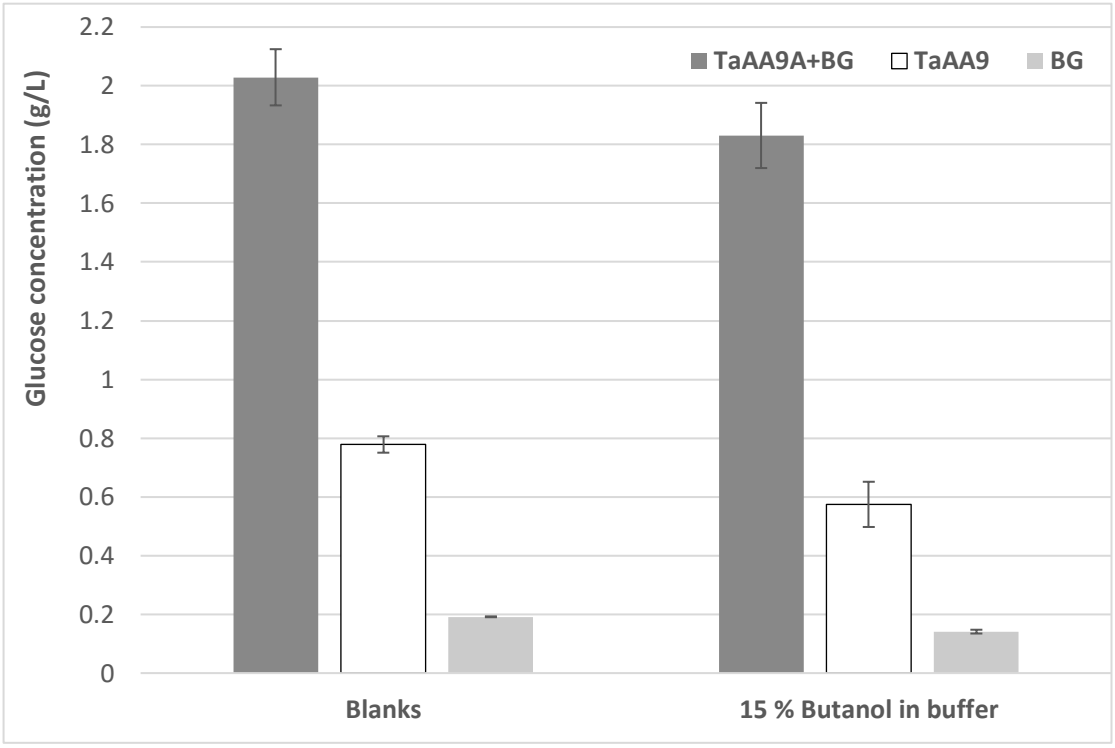


Figure 4.4.6. *Glucose titers after the three days saccharification reactions. The first triad of columns is the blank samples while the other triad is samples loaded with 1-Butanol with volume 15% of the volume of buffer. For all the samples duplicates were made.*

4.4.4. Dichloromethane Fractions

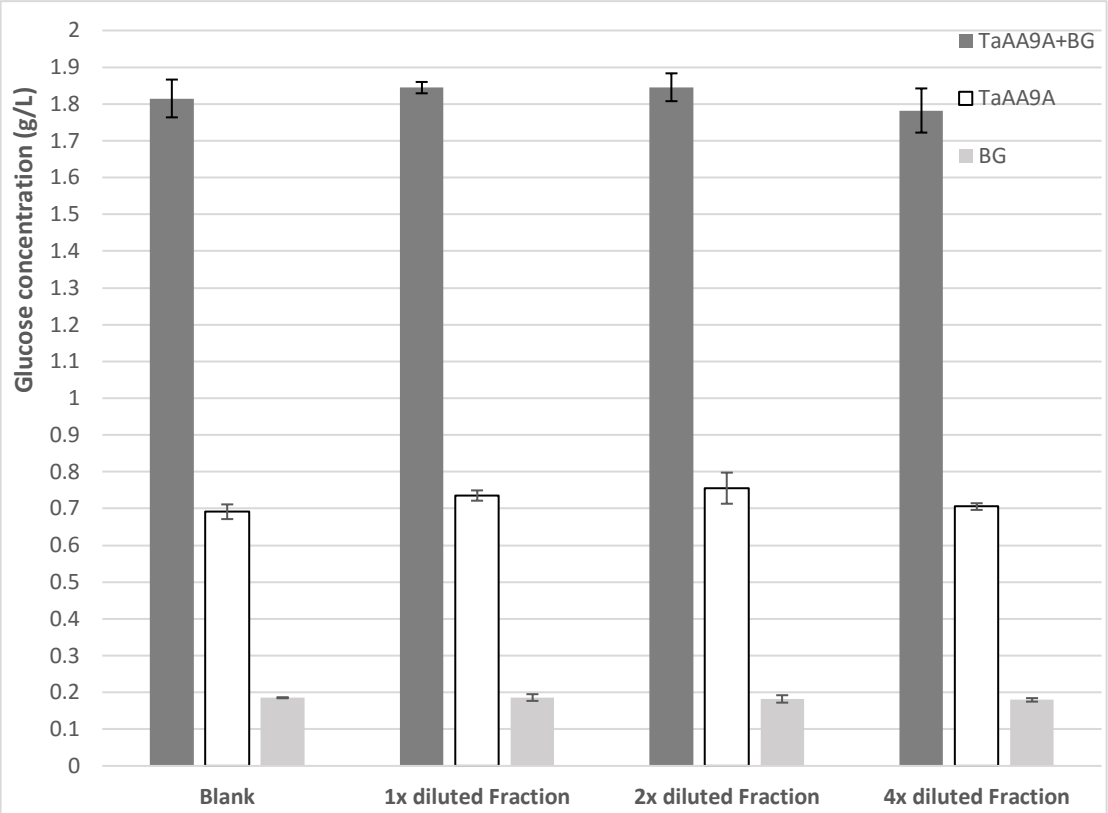


Figure 4.4.7. *Glucose titers after the three days saccharification reactions. The first triad of columns is the blank samples while the other three triads have instead of buffer the dissolved residue in buffer from the LLE using the dichloromethane solvent. Like the samples from the figure 4.4.2. three concentrations were made. For all the samples duplicates were made.*

As it can be observed from the graph, that none of the concentrations of this fraction had any remarkable difference with the blank samples. Also, all the differences in the columns are between the error bars so we can exclude any conclusions from them. So, dichloromethane as a solvent didn't extract substances which have effect to the enzymes. It should also be mentioned that the Dichloromethane was the only chlorinated solvent that was used and seems to be least ineffective from all the solvents. Also, this solvent can be characterized as the solvent with the least solubility with PE than the three others, meaning that the solvent could not be easily mixed with the PE and after mixing it was almost immediately separated. The solubility characteristics of the solvent appears to be an important in determining its ability to fractionate liquids and it is being suggested that highly insoluble solvents have less activity at the extraction (139). The residue after the evaporation of the solvent had a light grey and orange color. The fractions from this solvent have pH from 4.9-5.1 (Table 6.3).

4.5. Effect of the isolated from tannins PE on saccharification by TAAA9A and BG

As it was described in the section a method for the isolation of tannins in the PE was used. This method was used in order to evaluate if the PE after being treated with PVPP will have a different effect on the enzymes. If we observe less inhibitory effect after it would mean that the tannins are important compound to this inhibition. Briefly the PE was mixed and incubated with 0,2 g of PVPP for one hour. Then after two rounds of centrifugation supernatant, which was the treated PE with PVPP was used in the saccharification experiments like the samples 4,5,6 from the Table 3.1. The results are given in the Figure 4.5.1 when three PE were tested, one with no treatment with PVPP, one with treatment and one being centrifugated in order to evaluate if the centrifugation that took place in the PVPP treatment had an effect on PE.

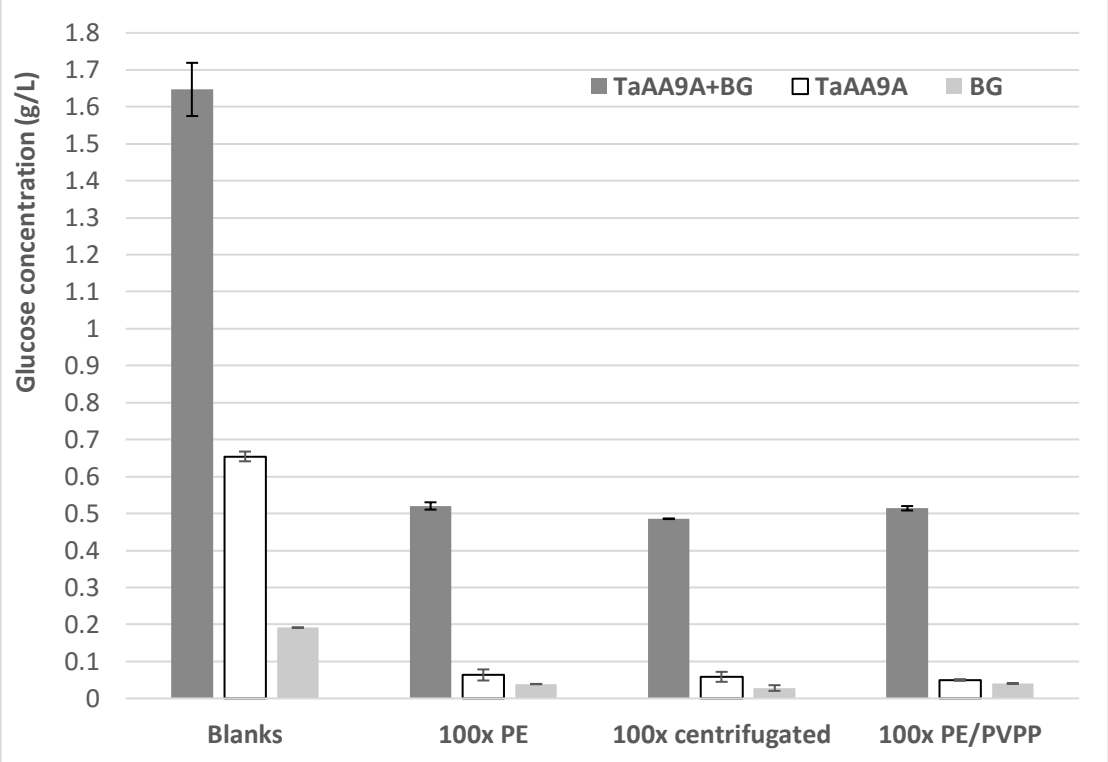


Figure 4.5.1. *Glucose titers after three days saccharification. For all the samples duplicates were made expect from the Blank ones where triplicates were made and the error bars show the standard deviation of the average.*

It can be observed that with this amount of PVPP and this incubation time that was tested the difference in the glucose concentration is not significant. Maybe the amount of tannins that the PVPP binds from the PE are not enough to change the inhibitory effect on the enzymes or maybe the compounds that the PVPP binds are not the ones that are creting this inhibitory effect. However, with the same conditions of treatment the FC showed decrease in the total phenolic content of the treated PE (Figure 4.6.1) meaning that this method had an effect to the PE. The other amounts of PVPP and the half an hour incubation time did not have effect either on the enzymes and the glucose release was the same with blank samples.

4.6. Quantification of phenolic content on the PE and its fractions

The FC method was performed in order to quantify the total phenolic content of the PE and its fractions. Specifically, the samples which they have been tested were dilutions of Old and New PEs and New PE treated with PVPP. Also, three of the fractions after the LLE method were tested, specifically the diethylamine, the ethyl acetate and the dichloromethane fraction (Figure 4.6.1). The butanol fraction was not tested due to precipitation that took place during the two-week storage. As it was described in the section 3.6. the final concentration of the PE and fractions samples that have initial dilutions 200x in water were 2200 times.

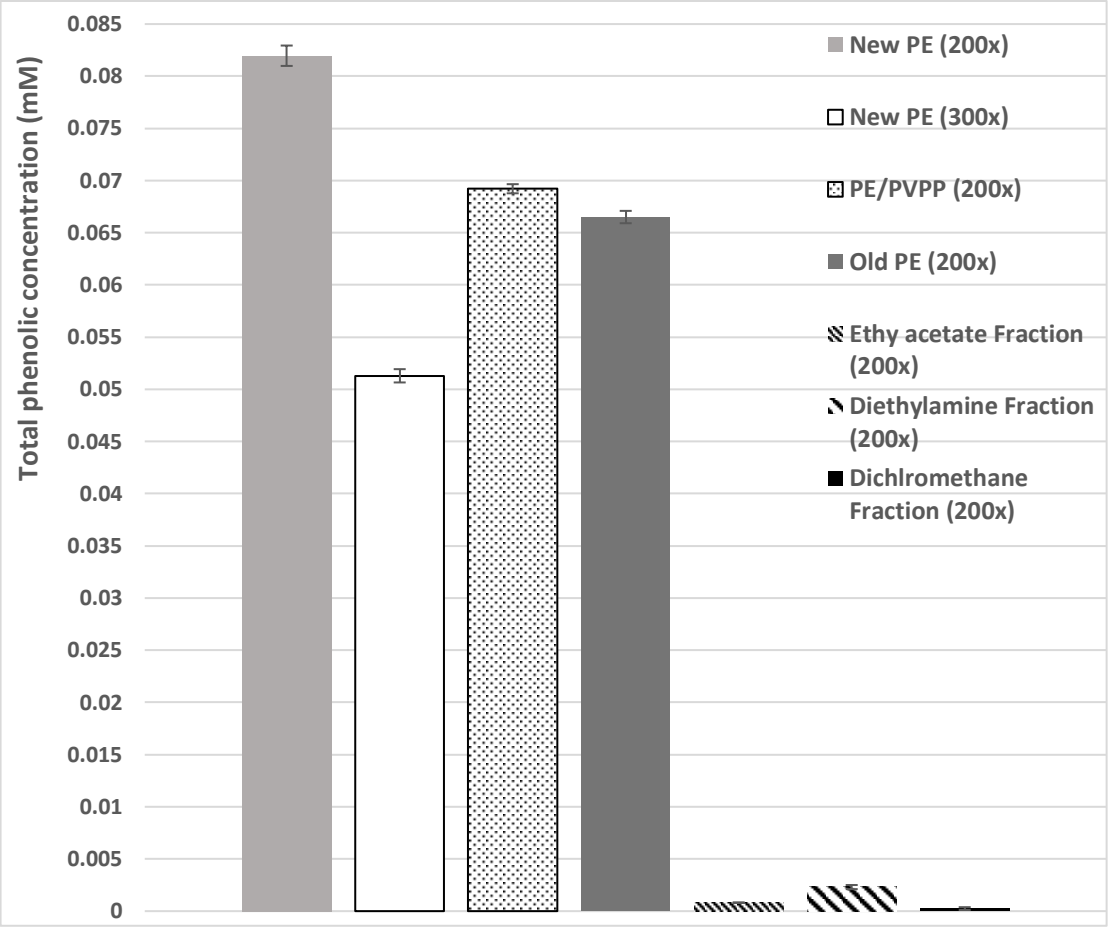


Figure 4.6.1. *Total phenolic concentration measured by F-C method in diluted PE samples and samples with fractions from LLE. The first column is New PE with initial dilution 200x times in water, the second one the same sample with 300x initial dilution in water. The third one is New PE treated with PVPP 200x diluted in water and the fourth one is Old PE with initial dilution 200x in water. The rest of columns are farctions from the LLE with the same initial dilution in water. For all the samples triplicates were made.*

First from the graph it can be observed that there are differences in the total phenolic content between the New and the Old PE. It was expecting that the old PE it would have less phenolics, because time of storage has an effect on the total phenolic content. As it is mentioned in the section 4.1 Klimczak et al (110) have tested the effect of storage using FC and HPLC method for different storage times and temperatures. Specifically, in the case of 18^{o}C which is the closest temperature in our case, because the PE was stored in room temperature, they showed with both methods that after 4 months of storage there is a decrease in the total phenolic content. Interesting results from the method is the reduction in the total phenolic in case of the PE when is treated with PVPP. Specifically, we observe a difference of 0,013 mM of concentration, which could be the isolated tannins since PVPP was used for this reason, which was the isolation of tannins. This difference is almost half of the difference in the concentration between the 200x and 300x diluted PE. The fractions that the have been created from the LLE they do have phenolic compounds. It can be observed that the diethylamine fraction has the highest concentration and the dichloromethane has the lowest one. Based on the final concentration of the samples and the values that were measured from the FC it is possible to quantify the total phenolic content of the PE. Specifically, based on the values from the two New PE samples (200x, 300x) it was calculated that the PE has an average value of concentration of total phenolics 174.8 ± 5,52 mM.

4.7. Effect on tannic acid on TAAA9A and BG

In order to test the effect of tannins on TaAA9A and BG and evaluate if there is a different effect on them, tannic acid was added in the saccharification mixtures at different concentrations. Tannin acid was interesting to be evaluated as an inhibitory compound due to the fact that the PE had tannin content, which is one potential group of compounds that have the inhibitory effect on the enzymes. Two different range of concentrations of tannic acid were tested. In the Figure 4.7.1 results from the first range of final concentrations of tannic acid are shown.

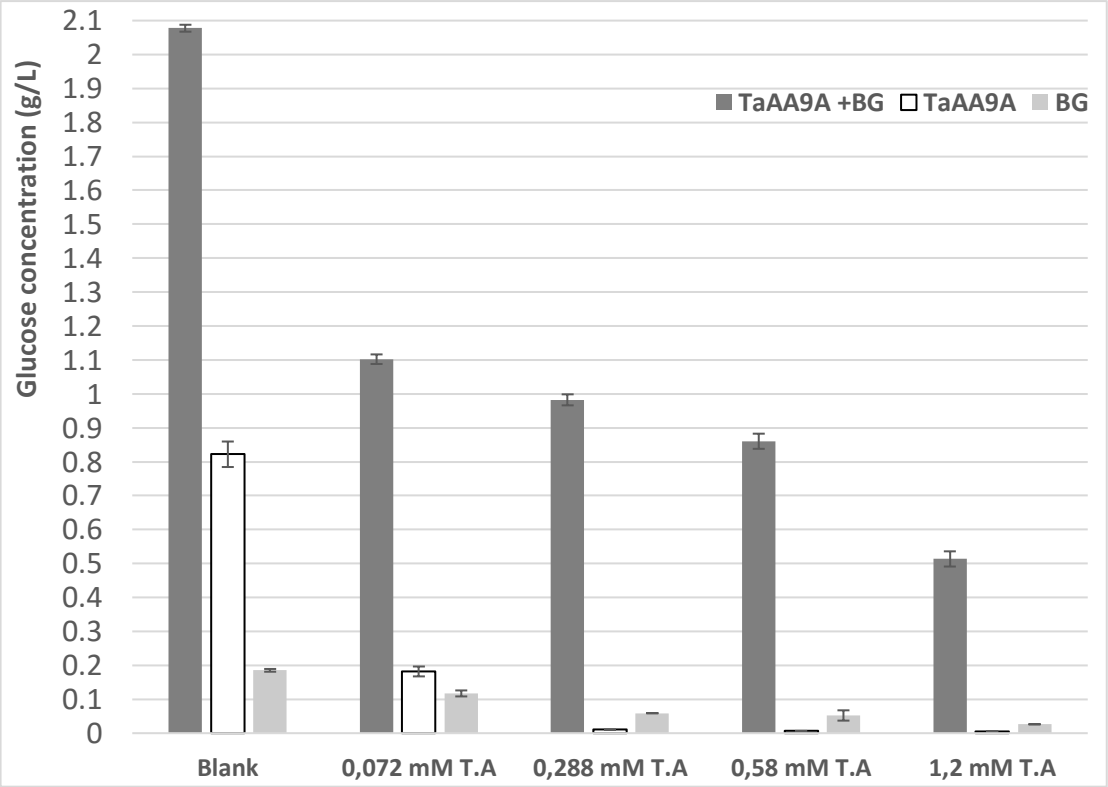


Figure 4.7.1. *Glucose titers after the three days saccharification reaction using Blanks and samples adding different concentrations of Tannic acid (TA). For all the samples triplicates were made and the error bars showed the standard deviation of the average.*

It can be observed that the tannic acid has a clear inhibitory effect to both enzymes. However, the effect on the TaAA9A appeared to be more inhibibitory than on BGs. Interesting is that between the concentrations 0,072 and 0.288 mM the inhibitory effect on TaAA9A is becoming quite significant and the tannin acid with concentration of

0.288 mM and after almost completely deactivates the TaAA9A. However, their synergism levels are not deactivating meaning that the TaAA9A can still act with the BGs but not alone when tannins are existing in the mixture. The concentrations of tannic acid that were tested were based on the difference at the concentration of the total phenolics that was observed from the FC method when the PE was treated with PVPP. When the PE was incubated in a thermomixer with PVPP for one hour, room temperature and mixing at 14000 rpm had a reduction of phenolic about 0.013 mM (Figure 4.6.1). From the Figure 4.5.1 it can be seen that when the PE is treated with the same way with PVPP there is no difference at the glucose release from the non-treated PE. For that reason, the less concentrated tannic acid that was tested was about 5 times more concentrated than the difference of the concentration that was measured in the FC method between the treated and untreated PE with PVPP. So, the range of final concentrations of tannic that was tested 0.072-1.2 mM. To test which concentration is the one that has this change in the inhibitory effect on TaAA9A the experiments were repeated using 3 more concentrations of tannic acid between the 0,072 mM and the 0.288 mM (Figure 4.7.2).

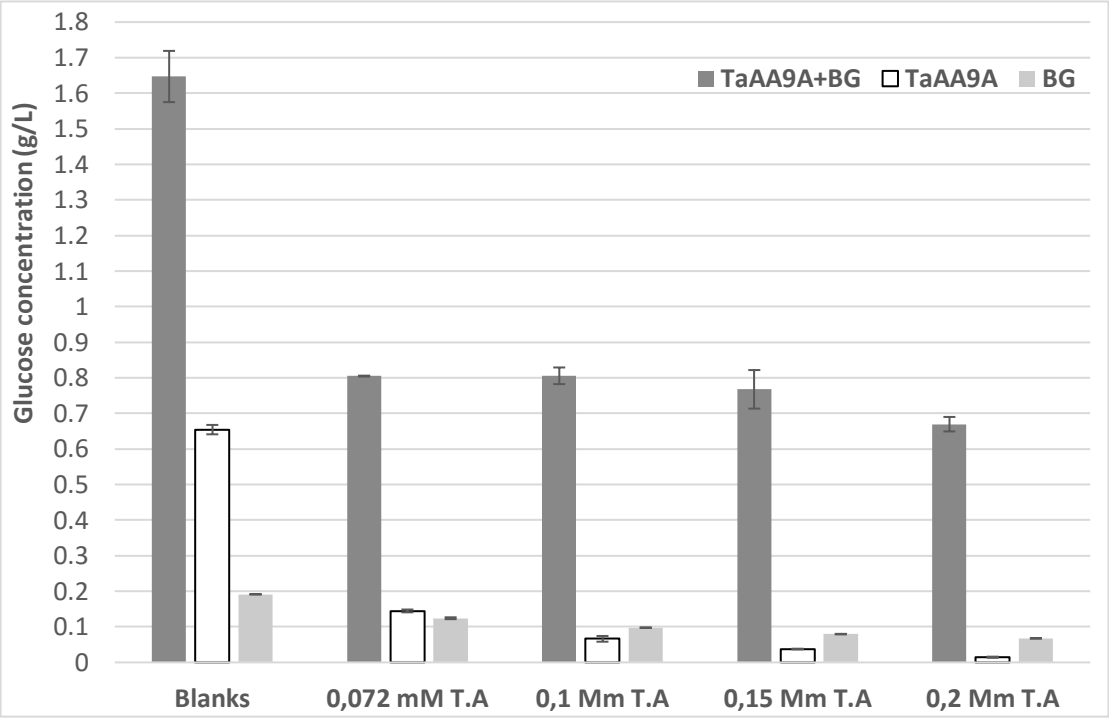


Figure 4.7.2. *Glucose titers after the three days saccharification reaction using Blanks and different concentrations of Tannic acid (T.A). For all the samples triplicates were made and the error bars showed the standard deviation of the average.*

It can be observed from the graph that the glucose release from TaAA9A becomes less than the value of BG samples between 0.072 and 0.1 mM of tannic acid. Like the Figure 4.7.1 it can be seen that tannin acid has more significant inhibitory effect on TaAA9A. Specifically, at 0.2 mM the TaAA9A they seem to be completely inactivate but BG produce almost 40% of the glucose than the Blank samples. However still their synergism seems to be no affected so much as TaAA9A between those concentrations.

Generally, the inhibition that was observed in the Figures it is rational since it is known that tannins have an inhibitory effect on cellulases (140; 141) . As it has been mentioned in section 2.8. tannins are a group of complex polyphenolic compounds and one of their characteristics is that they are able to bind simultaneously at several sites on a protein surface. This inhibitory effect can be different in different enzymes like in the case of TaAA9A and BGs and depends on the source and the type of the enzymes (140). Tannic acid has been used as standard for hydrolysable tannins and it has been shown that it can affect negatively all the major cellulases, CBHs, EGs and BG and that tannic acid inhibition seemed to be independent of cellulose types (141). Since BGs and TaAA9A are quite different protein molecules with differences such as their size and their type of active site, it is not irrational that tannin acid has different effect on each enzyme. BGs active site is a pocket or crater type (142) unlike the TaAA9A which has an extended flat face active site about 40 Å x 30 Å (143). So, it is possible that tannic acid binds to the active site of TaAA9A and blocking them to bind to several glucose units in the cellulose chain. Tejirian and Feng Xu (2011) (141) suggest that when tannic acid was added in cellulase mix the reduction of product release was mainly by inactivation of endoglucanases. Endoglucanases have cleft or groove shape of actives site which they are not very different from the shape of LPMOs active site. Their action is also similar since they both can help cellulase such as CBHs to access cellulose fibers by producing nicks in between the chain. However, TaAA9A have cooper in their active unlike endoglucanases and the metal in their active site it is also possible that can interact with tannins. Tannins and metals interactions can form complex compounds called chelates (144). Chelation is a type of bonding of ions and molecules to metal ions and tannins are known molecules that can form chelates with metal ions such as Iron and Copper (145; 144). So, one possible explanation about the higher inhibition of TaAA9A than BG, it is that tannic acid is forming chelates with the cooper in the active site of TaAA9A and leading to inactivation.

5. Conclusions

The focus of this project was an investigation of the effect of a PE which comes from persimmon fruit on TaAA9As with the overall aim to search for modulators for TaAA9As. The saccharification experiments after three days reaction using PASC as a substrate showed that the glucose titers decreased when different dilutions of PE were added in the system, meaning that this PE has an inhibitory effect both to TaAA9As and BGs. It was observed that the effect was more significant inhibitory on the TaAA9As than the BGs in all of the dilutions that were tested. It was also observed that an old purchased PE had less inhibitory effect than a more recently purchased one meaning that storage time had an effect on PE. The PE was incubated for one hour in different temperatures, from 70 to 120 to test if the inhibitory compound is a protein nature compound and if it can be precipitated in the tested temperatures. The results showed no significant differences in glucose titers from the PE that was stored in room temperature, meaning that there might be no protein nature inhibitor compound that can be precipitated in these temperatures and incubation time. Physical treatments of the PE such as filtration and centrifugation had also no significant differences in the glucose titers from the non-treated PE which means that the inhibitory effect does not come from residues or small particles bigger than 0,45 nm.

The LLE as a fractionation method to PE was used and it created 4 different fractions using ethyl acetate, dichloromethane, diethylamine and 1-butanol as solvents and the fractions were tested for their effect on TaAA9A and BG. The fraction from dichloromethane solvent, did not have any effect in the tested conditions so it is not recommended for extraction of substances from the PE. The ethyl acetate as solvent created a fraction that in the trial experiments had an activatory effect and the repeat of the experiments showed that the fraction could not act again like in the trial as an activator but the concentration of it seem to have an important role. The fraction from the diethylamine solvent, in the trial experiment showed activatory effect, and when it was tested again in different concentrations only the least concentrated fraction showed clear activatory effect, while the other two showed inhibitory effect. That means that the concentration of this fraction can affect its modulation effect to the TaAA9A fraction. Also, the fraction with the activatory effect was tested without addition of gallic acid, which was the electron donor to the experiments, and there was still

activation to TaAA9A. Last the 1-Butanol fraction show no clear results but seems like the fraction of it in least concentration that were tested had activatory effect and in the most concentrated ones had inhibitory effect. The order of the solvents with fractions with the most modulatory effect on TaAA9A in those experiments is diethylamine >1-butanol > ethyl acetate > dichloromethane.

Treatment of PE with PVPP, with the aim of binding tannins from PE, in two different conditions showed no differences in the glucose titers from the Blank samples. That means that the content of tannins or phenolics that was bind in the PVPP could not make a difference in the inhibitory effect. However, with FC method the quantification of total phenolic content of PE took place and showed that the treatment with PVPP could reduce the total phenolic content of PE, proving that this method did have an effect on the total phenolic content of PE. The FC also showed that fractions from the LLE contain phenolics. Based on the amount of reduction at total phenolic content that the PVPP treatment create there was an investigation of the inhibitory effect of tannins to the enzymes using different concentration of tannic acid. Tannin acid was interesting to be evaluated as an inhibitory compound due to the fact that the PE has tannin content. The results showed that tannic acid can act as an inhibitor to both enzymes but more significant to TaAA9A than to BGs. That is also in line with the results from the dilutions of PE where the PE had more significant inhibitory effect on TaAA9 in more of the dilutions that were tested. That means that tannins from the PE could be one potential group of compounds that have inhibitory effect on TaAA9A.

5.1. Future outlook

From this study, the PE that was tested seem to have a higher inhibitory effect on TaAA9A than BGs making this natural PE interesting for further research for discovering a modulator for TaAA9A. Having more time for this project something that is interesting to be searched is what is the type of inhibitor is this PE, which can be either competitive, uncompetitive, non-competitive or mixed inhibitor. Then it could be also searched with the same experiments what type of inhibitor is the tannic acid to TaAA9A and BGs and to compare the results with the PE inhibition and evaluate if the inhibition type is the same or different in these two cases. More concentrations also of tannic acid should be tested in order to evaluate where the inhibitory effect starts for both enzymes. In addition, different group of tannins could be also tested, such as

condensed tannins, to those two enzymes in order to test if different group of tannins has different effect on the enzymes. It is important cellulases which have not been tested in this project and are used in cellulases mixtures to be tested like endoglucanases and cellobiohydrolases. Something also interested to be tested is to use methods for extraction of compounds, such as tannins, from astringent and non-astringent persimmon fruit. Then the extracts could be tested in saccharification experiments aiming to evaluate if the extract that comes from the fruit has different effect than the PE that was used and can it also identify if astringency in fruits plays a role in the inhibition effect. Having more time, it would be also interested to test the effect from other liquid plant extracts coming from high tannin content fruits, for example grapes, combined also with information that can be found in literature about the fruit.

About identification of group of compounds in the PE and in fractions of it, absorption spectroscopy can be used measure the absorption of radiation, as a function of frequency or wavelength, due to its interaction with the sample. Due to trials that were done in this project with this technique in a wavelength range of 300-1000 nm, is suggested that the wavelength range should be between 1000 and less than 300 for having bigger range of wavelength. In addition, with the absorption spectroscopy, Fourier Transformation infrared spectroscopy (FTIR) can be used. It is an analytical method that has been used for identification and characterization of compounds in samples, like plant extracts. Interesting fractionation technique for the PE and its fractions could be the Thin-layer Chromatography (TLC) which is a method for identify compounds present in a given mixture. Another fractionation technique for the PE that can be done is the supercritical carbon dioxide extraction, which is based on the solubility of extracted compounds in carbon dioxide. This method, permits selective extractions because the solubility of extracted compounds varies with pressure that is implied. This method that does not use solvents, which means that there is no chemical interaction with the PE and it is used in food industry so it can be used also for juices. After fractionation, Gas chromatography–mass spectrometry (GC-MS) is an analytical method for identification of substances in the fractions or in the PE itself but it might be quite complex for this method. About the isolation of tannins something that was not tested in this project was the treatment of PE with other tannin-binding agents like polyethylene Glycol and PVP, which they might have different effect on isolated tannins from the PE.

6. Appendix

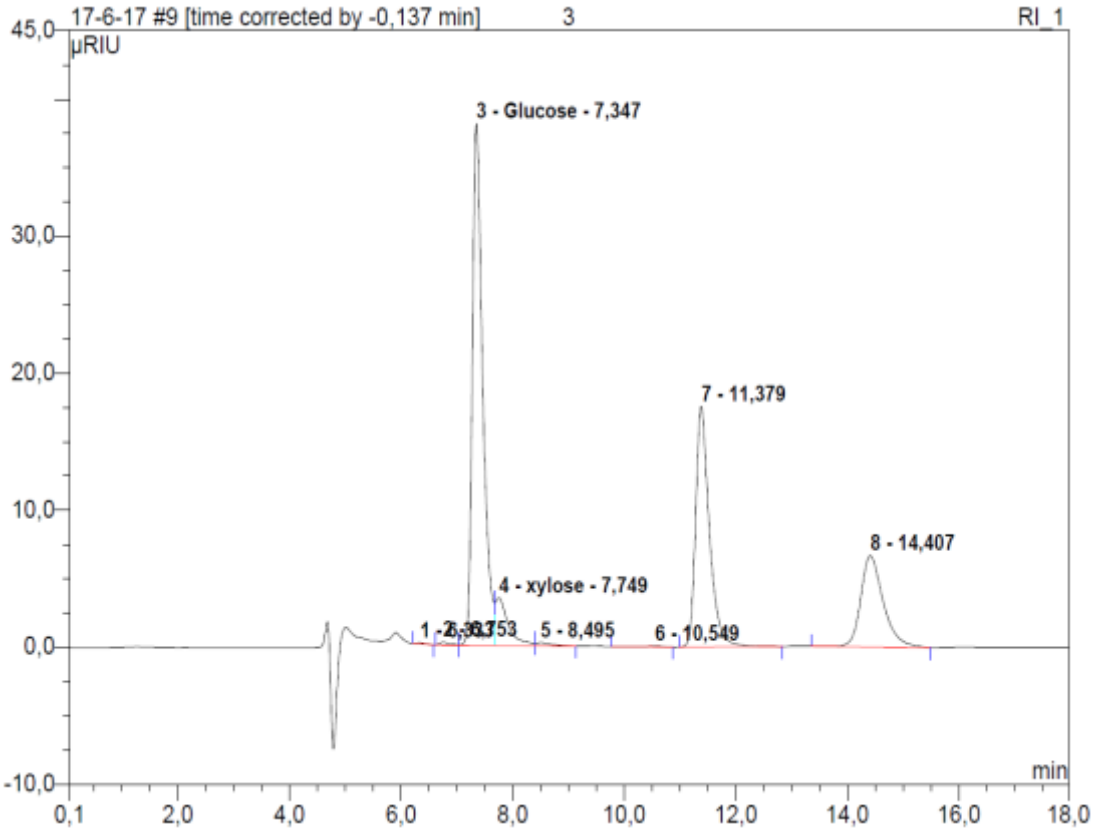


Figure 6.1. *Representative HPLC peaks from Blank samples loaded with TaAA9A and BG. The first peak is glucose and second small peak is xylose which was released always when BG were in the sample. The third peak is the buffer, which is acetate, and the last peak might be oligomers that are creating from the hydrolysis process and they were always in the results in these amounts.*

Table 6.1. *Some properties of the solvent that they were used in the LLE experiments* (146)

Solvents	**Relative polarity**	**Boiling point**	**pH**	**Density (g/mL)**
Ethyl acetate	0.228	77	9.2-9.3	0.894
Dichloromethane	0.309	39.8	9.4-9.5	1.326
1-Butanol	0.586	117.6	8.3-8.4	0.81
Diethylamine	0.145	56.3	12.4-12.5	0.706

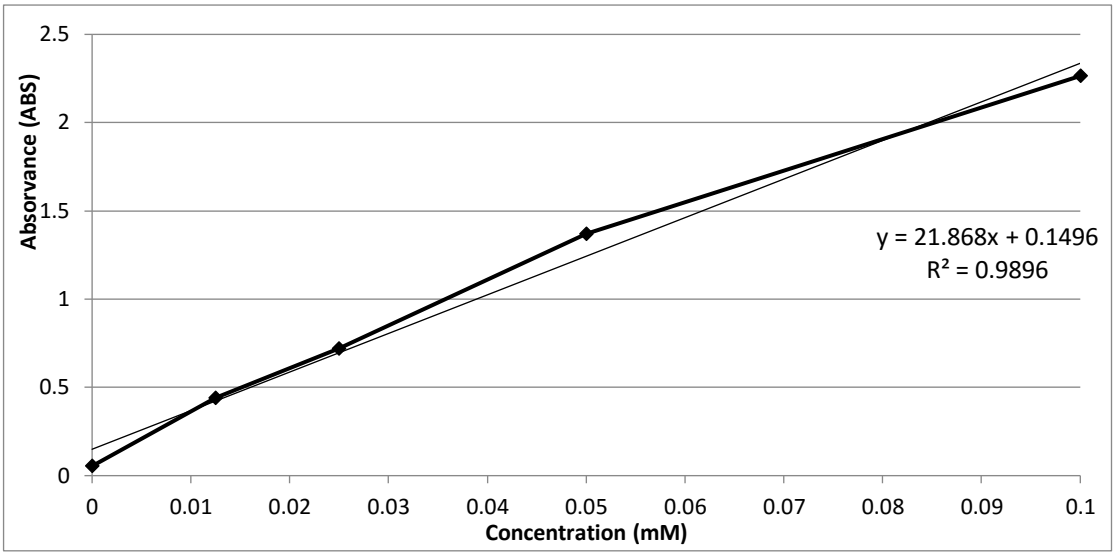


Figure 6.2. *Calibration curve using gallic acid as standard for the FC method. Final concentrations that were chosen are 0 mM, 0.0125 mM, 0.025 mM, 0,05 mM and 0.1 mM.*

Table 6.2. pH values for the mixtures of residue and buffer (fractions) made from the LLE

Samples	**pH**	**Samples**	**pH**
Ethyl acetate Fraction 1x	5.3-5.4	1-Butanol Fraction 1x	5.6-5.7
Ethyl acetate Fraction 2x	5.4	1-Butanol Fraction 2x	5.6
Ethyl acetate Fraction 4x	5.4-5.45	1-Butanol Fraction 4x	5.5-5.6
Dichloromethane Fraction 1x	5.4	Diethylamine Fraction 1x	5.7-5.8
Dichloromethane Fraction 2x	5.45-5.5	Diethylamine Fraction 2x	5.7
Dichloromethane Fraction 4x	5.45-5.5	Diethylamine Fraction 4x	5.6-5.65

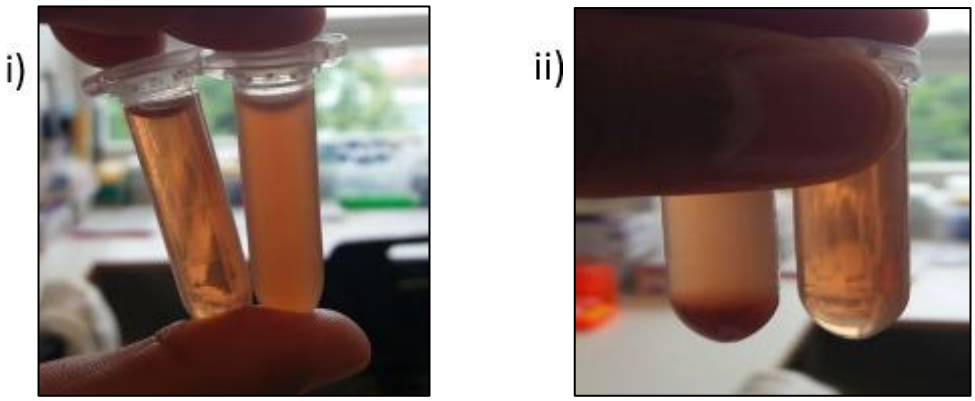


Figure 6.3. *PE after was diluted in buffer, specifically 20x times in both. Right after mixing New PE to the left, Old PE to the right (i), after some time from mixing, Old PE to the left and New PE to the right (ii).*

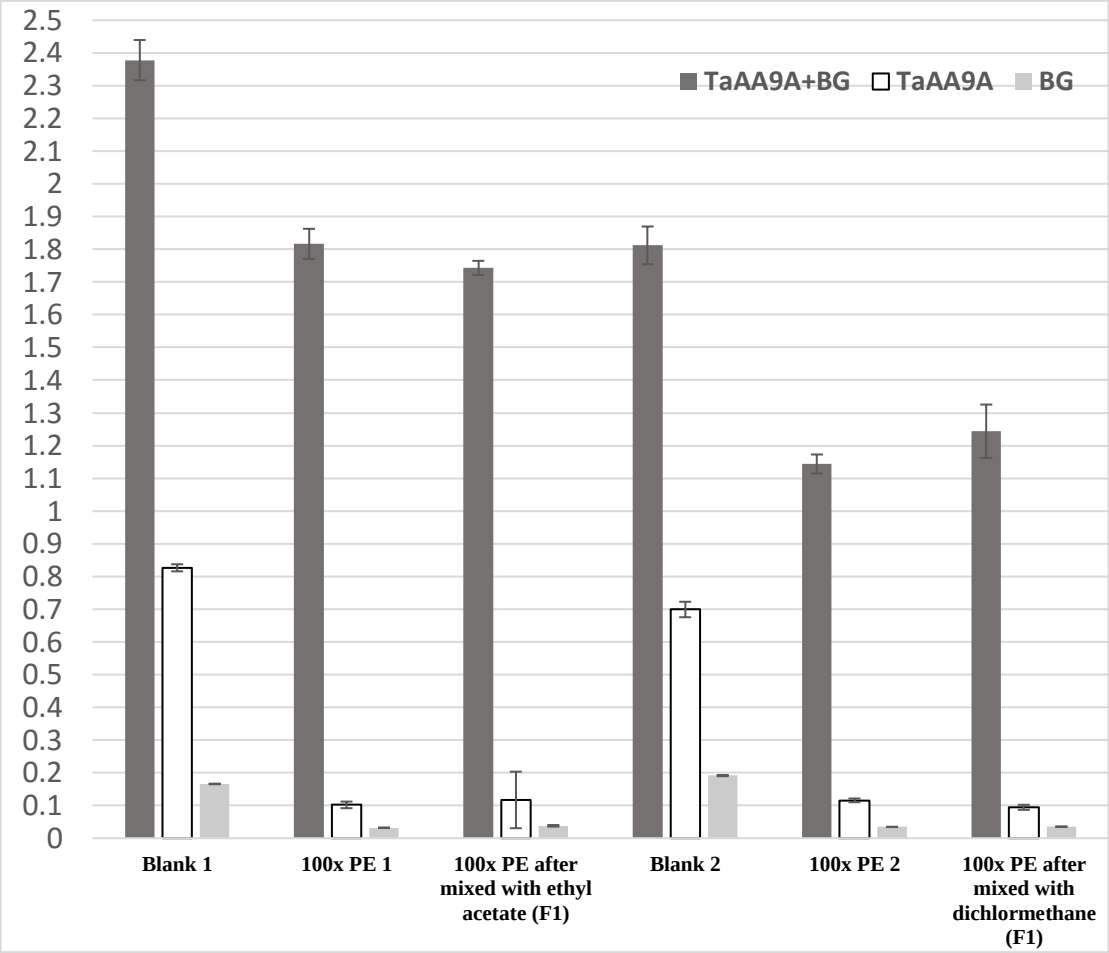


Figure 6.4 *Glucose titers after three days saccharification experiments adding PE after being mixed and centrifuged with ethyl acetate and dichloromethane. Values from adding F1 from ethyl acetate are compared with Blank 1 and 100x PE 1 and F1 from dichloromethane are compared with Blank 2 and 100x PE 2. For the samples triplicates and duplicates were made and error bars show the standard deviation of the average.*

Table 6.3. *pH values from representative samples from the saccharification experiments before the reaction. After the reaction, the pH was did not changed much.*

Number	Samples	pH values
1	Blank	5.0-5.25
2	Samples with addition of 1000x diluted plant extract	5.1-5.2
3	Samples with addition of 500x diluted plant extract	5.0-5.1
4	Samples with addition of 100x diluted plant extract	5.0-5.25
5	Samples with addition of 50x diluted plant extract	4.9-5.0
6	Samples with addition of 1x Fraction after ethyl acetate LLE	4.9-5.0
7	Samples with addition of 2x Fraction after ethyl acetate LLE	4.9-5.0
8	Samples with addition of 4x Fraction after ethyl acetate LLE	5.0-5.1
9	Samples with addition of 1x Fraction after diethylamine LLE	5.3-5.35
10	Samples with addition of 2x Fraction after diethylamine LLE	5.25-5.3
11	Samples with addition of 4x Fraction after diethylamine LLE	5.2-5.25
12	Samples with addition of 1x Fraction after 1-butanol LLE	5.25-5.3
13	Samples with addition of 2x Fraction after 1-butanol LLE	5.2-5.25
14	Samples with addition of 4x Fraction after 1-butanol LLE	5.2-5.22
15	Samples with addition of 1x Fraction after dichloromethane LLE	5.1-5.2
16	Samples with addition of 2x Fraction after dichloromethane LLE	5.1-5.2
17	Samples with addition of 4x Fraction after dichloromethane LLE	5.1-5.2
18	Samples with addition of 0.36 mM Tannic acid	5.0-5.15
19	Samples with addition of 0.5 mM Tannic acid	5.0-5.1
20	Samples with addition of 0.75 mM Tannic acid	4.95-5.05
21	Samples with addition of 1 mM Tannic acid	4.9-4.95
22	Samples with addition of 1.44 mM Tannic acid	4.9-4.95
23	Samples with addition of 2.9 mM Tannic acid	4.9-4.95
24	Samples with addition of 6 mM Tannic acid	4.85-4.9
25	Samples with addition of 15% in buffer 1-butanol	5.0-5.1

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