Chapter 13

Sugar Versatility—Chemical and Bioprocessing of Many Phytobiomass Polysaccharides Using a Milder Hydrolytic Catalyst: Diluted Thermopressurized Phosphoric Acid

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Additional information is available at the end of the chapter

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Abstract

Phytobiomasses, given the qualitative and quantitative dominance of polysaccharides, are a dominant wealth available in nature. Cellulose and hemicelluloses from softwoods, hardwoods and grasses, starch from tubercles and roots, pectins from fruits and gums from some seeds may be explored as such or following acid or alkaline pretreatments as well enzymatic deconstruction, and even simple chemical derivatization toward more added-value products. A general view in the chemistry of these valuable polymers is here broached, following a sharper focus on acid pretreatments for L(h)C—ligno(hemi) cellulosic materials from sugarcane and other feedstocks. Our particular experience using a gentler proton donor but keeping very advantageous aspects for polysaccharide chemo/biotechnological processing—thermopressurized diluted phosphoric acid (oPA)—is presented with a more detailed description as a result of its validity for the hydrolytic deconstruction of hemicelluloses—heteroxylans and heteromannans, cassava starch, dahlia inulin and mixed glucans from microalgae cell walls. The opportunity of NOs—nutraceutical oligosacchrides—generation from these particular glycopolymers is also shortly commented.

Keywords: phosphoric acid, polysaccharides, sugarcane, ligno(hemi)cellulose, starch

1. Introduction

It is intriguing remarkable the metabolomics of some biomass such as pentose or hexose derivatives. The 2-deoxyribose is the only carbohydrate selected to integrate the most important macromolecule of living organisms; DNA or deoxyribonucleic acid. It is also a component of riboflavin or vitamin B2 and the energetic main coins, ATP and similar coenzyme structures. However, the occurrence in nature of its mother molecule, ribose, is not so common. Crotonoside or isoguanosine, an exotic ribonucleotide is present in croton bean (*Croton tiglium*) and in the wings of the butterfly *Prioneris thestylis* [1]. Its beneficial action against tumors was demonstrated. Ribosyl units in polysaccharides are quite rare and one example is the capsular polymers from some pathogenic bacteria. These short comments lash out us to a pertinent question: why deoxyribose-built DNA and ribose-built RNA (mRNA, rRNA and tRNA) molecules? The answer is rather complex considering the consequences from this single difference: vital metabolic roles, chemical stability, rate of degradability facing ribonucleases and deoxyribonucleases, etc.

Conversely, glucose is the most widespread carbohydrate and the main energetic fuel in any organism besides the homocomponent in the most common nature polysaccharides such as starch (plants, molds, bacteria and microalgae) and glycogen (mammals); while 2-deoxyglucose inhibits organisms growth blocking glycolysis, although some controversial benefits for the treatment of epilepsy and also has been proposed as therapeutic tool for some types of cancer [2].

Monosaccharides are the major source of fuel for cell metabolism, bioconversion processes and structural materials [3–5]. D-glucose is the most universalized carbohydrate occurring in the nature under polymerized forms. In these natural polymers, the dominant glycosidic linkage is 1,4 connecting the anhydropyranose residues, as α or β anomeric configurations as is the respective cases of cellulose and starch. However, besides these examples of homopolyglucoses, glucopyranosyl units may integrate the whole structure of important heteropolymers like glucogalactomannans in conifers or softwood hemicelluloses. Also, worth of mention is that both ionic forms of glucose—glucuronic acid and glucosamine—make portion of other important natural polymers such as acidic xylans from hardwoods and chitin from marine crustacean and even aquatic mold cell walls.

Disaccharides are produced naturally and in abundance in plants such as sugar beet and sugarcane. Sugarcane comprises several grass species of the genus *Saccharum* chosen as feedstock in tropical and subtropical countries to produce sucrose which in turn can be fermented to produce bioethanol, notably by the Brazilian sugarcane industry. The remaining sugarcane biomass (bagasse and straw) can be burnt to electricity production, left in the field (straw) for agronomic purposes, or, more recently, applied in industrial scale for the production of bioethanol after deconstruction by a pretreatment of choice followed by enzymatic hydrolysis and a fermentation step.

Polysaccharides, as natural polymers, are by far the most renewable resource in the Earth [6]. They are the products of a natural carbon-capture process, namely photosynthesis, that

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follow further biosynthetic modifications to carry out various specific functions in plants and other organisms. Examples include structural polymers such as cellulose, chitin, pectin and storage polysaccharides such as starch and inulin. Because of their huge structural diversity (e.g. pentoses, hexoses, aldo- and keto-sugars, deoxi-derivatives like fucose and rhamnose, D and L-configurations, other glycosidic type linkages other than 1,4) and functional diversity, polysaccharides and monosaccharides are expected to play a progressive role in industry, either in their native or chemically modified forms. As some polysaccharides (such as cellulose, starch and chitin) are produced on a very large scale in nature, the interest in their hydrolytically or non-hydrolytically processing is strongly associated with a variety of applications in the food, paper, pharmaceutical, cosmetic and biofuel industries.

As one stringent example, considering how much purified cellulose (e.g. from textile weaving yarns) is simply discarded: a large-size textile factory in a daily operation with cotton treads can accumulate as much as >1.5 ton of cotton dust waste (CDW) around the loom machines. The collected cellulosic residue may be just burned to generate vapor and additional energy supply thus aggravating the local greenhouse environmental problem. We are partially alleviating this situation by, through one pot reaction, transforming the mercerized cellulose residue in ionic forms (CDW-Carboxymethyl and CDW-diethylaminoethyl positively charged—DEAE+) and efficiently utilizing these insoluble ion exchange matrices to, respectively, sequester/remediate a large volume of residual cationic and anionic dyes from the factory wastewater [7, 8].

This chapter will cover two major polysaccharides, cellulose and starch, and their deconstruction from different substrates with emphasis to the advantages and wide applicability of aqueous moderately thermopressurized phosphoric acid pretreatment for bioethanol production as for other applications such as oligosaccharides with nutraceutical properties.

2. Brazilian sugarcane industry in brief and environmental issues

Brazilian agribusiness is the strongest arm of its whole economy. The contribution from sugarcane business and derived products is outstanding for local consumption and exportation, financial incomes and, in our view, overcoming concurrent activities such as soy, corn and coffee commodities. About 1 m^3 of sugarcane juice may contain around 200 kg of sucrose, easily splitable into their valuable counterparts: glucose and fructose. These monosaccharides are equally and promptly hydrolyzed into the same monosaccharides by *Saccharomyces* spp. hyperactive yeast cell wall-bound invertase, capable to quickly pave the metabolic pathway of this precious aldo- and keto-aldoses to ethanol and other useful fuels.

Brazil, secondly followed by India, is the world leading processing sugarcane to first generation ethanol, table sugar and other goods as corroborated by its huge year-crop (2016–2017) in the range of 657.2 million tons with a final production of 11 billion liters of anhydrous ethanol and around 39 million ton of table sugar (sucrose) [9]. It is a good time to focus on the Brazilian sustainability scenario and actors: economically feasibility, environmentally correctness and

social fairness. There are gladness and sadness criticisms from both sugar mills entrepreneurs and capitalism lovers versus poorer workers at cane plantations, respectively. Let us emphasize the thoughts and opinions from the closer national teacher and researcher on environmental sciences, recalled his graduation title as a social scientist, too—Prof. Dr. Valdir Fernandes [10]. His comments and Strengths, Weaknesses Opportunities, Threats (SWOT) guidelines were built in a partnership with other three other publication colleagues and was summarized below:

(a) Brazil is committed from many decades ago with sugarcane ethanol as a mandatory surrogate for petrochemical derived fuels; (b) given the huge figures for production and processing, when examining the sustainability, it is necessary to build tools which allow to assess an integrated conception of the sugarcane matter, prospects, goals and subjects, about everything to help and to influence decision-makers to establish public policies for a sustainable development; (c) the complexity may be captured from economic and social indicators without no reduction in the significance of each system component; (d) taking the State of São Paulo—the major Brazilian producer and more developed state federation unit—and the trustable indicators raised by its Environmental Secretariat—sounded as one pertinent strategy for the current evaluation; (e) water supply and its quality regarding environmental implications is a valuable cornerstone; (f) the evaluation of environmental indicators encompasses the application of extensive interviews allied to experts workshop pointing out to a set of benchmarks; in the present case, 16 respectable experts were involved. These interviews established three main focus: water, soil and atmosphere. Each focus considering, respectively, 11, 12 and 2 relevant aspects/opinions input. As illustrative examples, reduce availability, oxygen-deprivation as biochemical oxygen demand (BOD) and chemical oxygen demand (COD) parameters, eutrophication of surface sheets by NPK and respective leaching intensity in the case of water, loss of soil nutrients nitrification and acidification by low-pH vinasse, microbiota flora reduction for the soil focus and some possibly unchanged/unchangeable indicators such as photochemical formation of tropospheric ozone and atmospheric acidification (permanent greenhouse gases release). Discussions and conclusions drawn for other mentioned topics in water and soil derived from the expert's team suggested opinions and additions. A strengths /weaknesses/opportunities and threats, namely, a SWOT analysis was built. (g) conclusively, a better guide for the people taking decisions—sugarcane industrial managers, union leaders, politicians, governmental authorities in agricultural, health, economic and social fields—all them committed with the whole society benefits on safety, welfare and progress—is to consider and refine, *inter allia*, environmental indicators to feed the discussion and legal decisions to support the so needed sustainability in the giant sugarcane business.

To the authors understanding this remarkable contribution of this environmentally proactive scientists quartet from USP—University of São Paulo, UTFPR—Federal Technological University of Paraná and UFPE—Federal University of Pernambuco (prudly and proudly Brazilian scientists!) deserves a complete reading of their corresponding 27 pages full report for any reader interested in the profits and negative implications of any giant agribusiness as well as other related industrial and highly polluting factories on commodities (e.g., pulp/paper, timber/saw mills).

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3. Structure of lignohemicellulosic biomass

Cellulosic materials are the most abundant renewable polymer resource available in nature as the main component of plant cell walls, which in turn is subdivided into primary wall and secondary parts. Unlike other homopolysaccharides encountered anywhere, cellulose occurs in close association with hemicelluloses and lignin, then named ligno(hemi)cellulose or shortly, L(h)C (**Figure 1**). Together, these three biomolecules are the main components of plant biomasses corresponding, respectively, as 40–60% for cellulose, 20–40% for hemicelluloses and 10–25% for lignin in any L(h)C biomass [11]. The distribution of cellulose, hemicelluloses and lignin varies considerably among cell wall layers. L(h)C biomass also can contain some pectin and xyloglucan along with minor amounts of minerals (ash) and various other compounds, which are called extractives.

Exceptions for this statement seldom occur in the plant kingdom but is the case of cotton caps and kapok ripen fruits where cellulose fibers are almost pure, meaning free of hemicellulose and lignin. Mention to some polysaccharide bacterial anabolism is mandatory here: some species of acetogenic bacteria, specially species such as *Gluconacetobacter xylinus*, formerly known as *Acetobacter xylinum* and since reclassified as *Komagataeibacter xylinus* [12], biosynthesize effectively pure cellulose ribbons of special architecture as soft biofilm gels. There are now a plenty of medical and other biotechnological applications for this noble cellulose occurrence and intensive production.

Southeastern Asian countries (Thailand, Malaysia, the Philippines and Indonesia) consume it as appreciated food known as "nata-de-coco". We have been consolidating other biotech products, one of them its covalently died derivative (Remazol Brilliant Blue R (RBB)-bacterial cellulose) for cellulolytic enzymes detection and measuring [13], following our pioneering

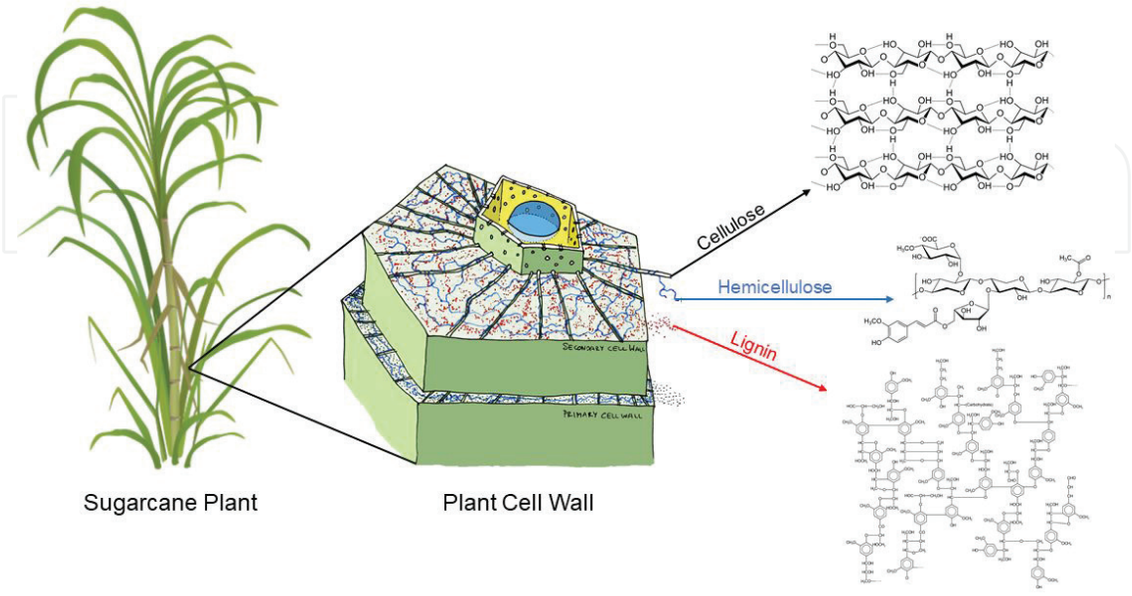

Figure 1. General ligno(hemi)cellulose structure of the plant cell wall.

report of its application just after a quick cleanliness for entrapped cells (although known as Generally Recognized as Safe-GRAS bacterium) as a temporary skin substitute in the case of human skin burns and other dermal injuries [14].

Cellulose consists of a collection of linear chains of β-(1,4)-linked D-glucopyranosyl units. L(h)C biomass include agricultural and agroindustrial residues (cane bagasse, cereal straws, cornstover, cobs or husk and similar polysaccharide-rich materials); wood materials (branches, bark, stumps, wood wastes from sawmills and paper mills) and dedicated energy crops (*Miscanthus* sp., switchgrass, etc.) including energy cane, a hybrid lineage of sugarcane that has been bred and selected for fiber production over sucrose production. In Brazil, pioneer hybrids of energy cane were produced by CANAVIALIS, a private sugarcane breeding company that obtained 138% more total biomass (green matter) per area than a good conventional sugarcane variety and 235% more fiber [15].

The cellulose chains are packed in layers that interact with each other by van der Waals forces with intramolecular and intermolecular hydrogen bonds to form microfibrils [16, 17]. Because of these interactions, cellulose has a recalcitrant crystalline nature that makes it generally resistant to degradation by any mold or bacterial cellulose complexes (endoglucanases + cellobiohydrolases I and II and β-glucosidases). However, some reports have shown that a class of oxidative enzymes, the lytic polysaccharide monoxygenases, have the capacity to degrade recalcitrant crystalline cell wall components, including cellulose [18, 19]. This is, undoubtly, a remarkable progress in biochemical technology.

Hemicelluloses are a diverse group of polysaccharides generally characterized by having a β-(1,4)-linked sugar backbone with the main function to reinforce the cell wall by interaction with cellulose and lignin. In xylans (angiosperms or hardwood and grasses), mannans (conifers and hardwoods) and xyloglucans (predominant in the primary bed of dicot and monocot/non-gramineous monocot cell walls), the backbone sugars are β-1,4-D-Xyl, β-1,4-D-Man, and β-1,4-D-Glc, respectively, while in glucomannans, the backbone comprises of randomly distributed β-1,4-D-Man > β-1,4- D-Glc units [20]. Xylans are the major constituent in secondary plant cell wall comprising a backbone of repeating β-(1,4)-D-Xyl residues most often substituted by L-arabinosyl (Ara*f*) and D-glucuronic acid (GlcpA)/4-O-Methyl-D-glucuronic residues. O-acetyl substituents are also present in the main xylopyranosyl units.

The L-arabinofuranosyl residues can contain ferulic acid groups esterified to the O-5 position of the carboxyl group which in turn can be oxidatively cross-linked to lignin incorporating xylans into the lignin reinforcing even more the network [21]. This feature particularly explains why rye bread hardens as compared with the softer wheat bread. Softwoods and hardwoods can have different hemicellulose content [22]. Hardwood hemicelluloses are composed typically by highly acetylated heteroxylans (4-O-methyl glucuronoxylans) with low amounts glucomannans. Instead, softwoods are common in the presence of partly acetylated galactoglucomannans and glucomannans with xylans corresponding to a minor fraction of their hemicellulose content [22].

Lignin is a phenolic polymer mainly deposited in secondary cell wall coating cellulose and generally combined with hemicelluloses, and built up almost entirely an intervening layer

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L(h)C feedstocks	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Sugarcane bagasse	42	25	20
Hardwood	40-55	24-40	18-25
Softwood	45-50	25-35	25-35
Corn stover	38	26	19
Corn cobs	45	35	15
Rice straw	32	24	18
Wheat straw	29-35	26-32	16-21
Grasses	25-40	25-50	10-30

Table 1. Biomass composition in common L(h)C feedstocks (source: Adapted from [24]).

called middle lamella that acts like a cementing agent biding primary cell walls together. Lignin also provides rigidity, enhances mechanical strength, reinforce vascular cells and acts as a pathogen and water-impermeable barrier for the plant tissue [22]. Lignin is built from monolignols based on three monomeric precursors (coniferyl, sinapyl and *p*-coumaryl alcohols) which appear to be incorporated into the lignin polymer in a non-predictable way [23].

It is worth to mention that oppositely to hemicelluloses (in fact always heteropolysaccharides) with a plenty of secondary substituents and also displaying a covalent connection with part of lignin, cellulose is a pure homopoly-*β*-glucan. Its interaction with other polymers are solely based on physical rather than chemical linkages.

A determining factor when selecting biomass for biochemical processing to recover their respective sugars is the different physico-chemical properties of various L(h)C materials, what is based, at first glance, in their major chemical composition. **Table 1** presents how L(h)C can be diverse depending on the origin and part of the plant. As the enzymatic, or even chemical hydrolysis of cellulose is greater than that of lignin, the complete conversion of the carbon-containing plant material present as cellulose is greater for plants with a lower proportion of lignin.

4. L(h)C biomass deconstruction to sugars

L(h)C biomass, as said before, can be obtained at relatively low cost in different forms, representing a potential sugar source for the fermentative production of renewable fuels as well as other materials in modern biorefineries. Most of these potential applications rely on the predominant cellulose fraction susceptibility to enzymatic hydrolysis/acid hydrolyses or other structural changes.

However, the intrinsic nature of L(h)C materials is completely different from that found in starch. Starch granule serve as a temporary energy storage polymer with glycosidic linkages that can be readily hydrolyzed to supply glucose for germination and plant growth.

Conversely, L(h)C has been projected by nature to function as a resistant structural material of carbohydrates and lignin that resist assault on cellulose through enzymatic deconstruction from a vast hydrolytic and/or oxidative enzymatic machinery secreted by various microorganisms, be fungal or bacterial. Besides to the protection conferred to cellulose by lignin and hemicellulose, factors such as cellulose crystallinity, low surface availability cellulolytic enzymes and degree of acetylation of hemicellulose prevent or hinder the whole polysaccharide deconstruction by microorganisms [25].

Therefore, more aggressive chemical and/or physical environments are required for the dismantlement of the L(h)C matrix, reducing the cellulose crystallinity, and increasing the porosity to a better accessibility to cellulose complex and more importantly, removing the hemicellulose and lignin barriers, which can be addressed to other technological goods. By doing this, the biomass become suitable for the production of second generation biofuels as ethanol, butanol, ABE (Acetone-butanol-ethanol) mix and other compounds like lactic acid, which can serve as a feedstock for the production of polylactic acid (PLA) to replace the petrochemical packaging materials such as polyethylene terephthalate (PET). In the case of cellulosic ethanol production four main steps must be taken in account: (1) biomass pretreatment; (2) enzymatic hydrolysis; (3) monosaccharide fermentation and (4) ethanol distillation.

There are several conditions for the selection of an appropriate L(h)C pretreatment (pT) method: (1) this should avoid the excessive size reduction of biomass particles, (2) preferably hemicellulose portion must be preserved as such (by alkaline pT), (3) converted do free xylose or xylo-oligosaccharides (acid pT) minimizing in this second case, the co-generation of degradation products like furfuraldehyde, a known inhibitor to microbial growth, (4) reduce the energy requirements, (5) explore a low-cost pT catalyst and/or inexpensive catalyst recycle and (6) preferably regenerating of any form of high-value lignin co-product [24].

Pretreatment can be performed by biological, physical and chemical processes and even combination of them. However, there are several possible combinations or operation modes. The choice of a particular method has to be based on a number of considerations, for example, which biomass will be used, the organism used for fermentation of the released sugars, and the costs implied. Common to all of these methods is that the L(h)C materials must be first mechanically pretreated (ground, chipped or milled) to increase the surface area.

In biological pretreatments, microorganism such as brown, white and soft rot fungi are used for degradation of lignin and hemicelluloses from biomass [26]. The efficiency of the process generally depends on the action of lignin-degrading enzymes such as peroxidases—lignin peroxidase (E.C. 1.11.1.7), manganese peroxidase (E.C. 1.11.1.7) and laccases (E.C. 1.10.3.2), generally copper-containing oxidases produced mainly by basidiomycetes. Supplementation with accessory enzymes like hemicellulases (endo/exoxylanases, β-D-xylosidases and α-L-arabinofuranosidase) increase hydrolysis yields but also enzyme costs and dosages [27]. Although eco-friendly and without production of inhibitory compounds, biological pretreatment is not suitable for a pilot scale process mainly due energy demand and to the long incubation time for effective delignification [28]. However, biological pretreatment was found to be more effective if it is combined with another chemical or physical pretreatment [29].

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Dilute and hot aqueous acid pretreatment (e.g., H_2SO_4, H_3PO_4 and organic acids) is the most widely employed method on industrial scale [24]. Acid pretreatment is efficient in promoting hemicellulose removal where a rich C5 liquid fraction is generated, leaving a solid material with high content in cellulose and less lignin, being the residual cellulose fraction more stable in the acidic regime due to its crystalline structure. This pretreated material can be further submitted to enzymatic hydrolysis with an increased cellulase accessibility. Two general approaches in acid pT can be applied; high temperature (above 180°C) during less residence time (1–5 min) and lower temperature (<120°C) for long duration (30–90 min), respectively [24]. The most conventional commercially used acid is dilute sulfuric acid, mainly to its low cost. However, use of mainly strong acids such as sulfuric, hydrochloric and nitric acids, even diluted, presents various drawbacks. These are mainly related with an increased production of inhibitory compounds (furans like HydroxyMethylFurfuraldehyde (HMF) or its progressive degradation species such as levulinic and formic acids if more parameters severity is applied) and corrosion of reaction vessels [24, 25, 30]. Therefore, various other acids have been used to circumvent the harsh conditions imposed by strong acids, such as maleic acid [31], fumaric acid [31], oxalic acid [32] and, in our repetitive experiences, phosphoric acid, that will be discussed later in the chapter.

In the alkaline pretreatments, L(h)C biomass is treated with alkali, normally sodium hydroxide or lime, $Ca(OH)_2$, at normal temperature and pressure. The main advantage is the efficiency in lignin removal [24, 33, 34]. It also demands relatively low capital costs, allows lower inhibitors formation and ensures high glucose yields in the subsequent enzymatic hydrolysis step, besides the possibility of a better lignin recovery by co-addition of air or oxygen. In the case of lime use, the mild catalyst may be recoverable injecting CO_2 in the liquefied alkaline stream [33, 35]. Despite the inexpensiveness of lime and other hydroxides are inexpensive, downstream processing costs are high because the process requires a large quantity of water for the appropriate washing of the residual cellulose [24].

Physico-chemical pretreatments include steam explosion pretreatment which is one of the most used methods for pretreatment of L(h)C biomass. In this approach, biomass is submitted to a high pressure (0.7–4.8 MPa) with saturated steam at high temperatures between 160 and 260°C for a few seconds followed by an explosive decompression, that cause separation of the fibers [24, 33, 36]. It can be carried out with or without (autohydrolysis) addition of an acid catalyst [37]. In autohydrolysis also named solvolysis, the high temperatures (160–250°C), an endogenous catalyst, acetic acid, is delivered from the O-Acetylated xylan and catalyzes the break of part of the xylo-backbone of hemicellulose thus producing of xylooligosacchrides along with some free D-xylose. The few side chain L-arabinofuranose substituents are completely released since their native α-configuration (allied to the furanosyl ring) are more prone to any acid hydrolysis [36]. Any alkaline pT can be performed with high total solids, providing higher yields, and a cellulose fraction with improved accessibility to enzymatic hydrolysis [38]. Some drawbacks of steam explosion pretreatment are the partial degradation of hemicelluloses, production of fermentation inhibitors (e.g. aromatic compounds, furfural and HMF) demanding a washing step for detoxification of the pretreated biomass [39, 40].

Most of the demonstration plants installed worldwide aiming cellulosic ethanol production is based on steam explosion pretreatment, or its variations. One example is POET-DSM Advanced Biofuels that constructed a cellulosic biorefinery alongside the POET Biorefining - Emmetsburg plant. The company contracted ANDRITZ Inc. to supply a two-step biomass treatment process that includes a vertical reactor and a continuous steam explosion (SE) technology to pretreat corn residues (stalks, husks, leaves and cob). In Brazil, GranBio began operations in 2014 with 22 million gallons per year cellulosic ethanol facility, Bioflex 1, using Beta Renewables' PROESA pretreatment process, Novozymes' cellulase enzymes, and DSM's yeasts. In PROESA technology plant, the biomass is pretreated with steam (high temperature and pressure) without chemical addition followed by enzymatic hydrolysis (viscosity reduction and hydrolysis). Some companies have reported difficulties regarding biomass feeding/transport and high degree of equipment wear due to the frictional effect of abrasive materials present in biomass [41]; signaling that this kind of pretreatment still remains as a challenge.

Other important pretreatments comprise ammonia freeze explosion (AFEX) [42], liquid hot water [43], organosolv and ionic liquids [44], the last one implying in higher costs despite the alternative of catalyst recycling. Also, new pretreatment technologies are constantly being developed, such as sub-/supercritical water [45] and supercritical carbon dioxide [46].

4.1. Thermopressurized aqueous phosphoric acid pretreatment for partial or total depolymerization of L(h)C biomass

Thermopressurized aqueous phosphoric (oPA) as pretreatment of L(h)C from different biomasses followed by enzymatic hydrolysis is an efficient approach to further provide free glucose from residual cellulose and immediate free xylose, mannose and the other minor pentose (L-arabinose) and some hexose from the hemicelluloses portion. As phosphoric acid have a higher pKa value than strong acids (pKa: 2.1 against pKa: −3 from H_2SO_4, pKa: −7 from HCl and pKa: −1.3 from HNO_3) it effectively more attractive appeal due to the generation of less carbohydrate dehydration than stronger acids, being possible to carry out the pretreatment of the L(h)C substrate over a wide variety of temperature and pH values. Conversely, the past drawback of phosphoric acid being more expensive than sulfuric acid is now under overcoming considering the aggressive entry of China in the production of several commodities including oPA. A detail, however, is to be taken in account: the moisture content of bulk mineral acids, ranging from 2 to 4, 15, 35 and 63%, respectively for sulfuric, phosphoric, nitric and hydrochloric acids. Nitric acid, due the oxidant action and threat to DNA, has been never considered as a biomass pretreatment. Hydrochloric acid and its toxic fumes offer more serious risk for the labor. Sulfuric acid is a risk for storing, serious burns even by short contact and faster equipment corrosion. So, *inter allia*, we historically from 1980 till nowadays, have been elected phosphoric acid, a gentler proton donor, for all our polysaccharide targets: chronologically from sugar and sorghum bagasses, cassava starch, dahlia tubercles inulin and more recently for microalgae cell wall polysaccharides for the special prospect of nutraceutical oligosaccharides preparation. Except for inulin, the most labile substrate, given its unusual β-1,2-furanofructoside linkage (when 10–30 min heating at 70–80°C in open vessels is

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enough to produce the precious FOS—FructoOligoSacchrides), xylan, mannan and microalgal glucans are pretreated under more severe parameters (thermopressurized reactor) in the range of 2 atm (120°C) to 15.5 (200°C) for a short peaking time (1–2 min) but always keeping the effective oPA concentration with a narrow pH 3.0–1.5 range and more often, at pH 2 or its pKa value (2.1).

Almost four decades ago, initial studies with L(h)C biomass (rye grass straw) treated with phosphoric acid was carried out to verify the amount of sugar and yeast fermentability after treatments with various concentrations of H_2SO_4, HCl and H_3PO_4 aiming to study the feed acceptability by rodents [47]. They have shown that both fermentability (after neutralization with ammonia) and rodent palatability were highest when the straw was treated with a combination of 0.23 N HCl and 0.15 N H_3PO_4 (30 min at 121°C), which produced 0.25 g of sugar per g of straw. They also verified that if straw were treated with higher concentrations (>0.5 N) of H_2SO_4 or HCl, yeast yield declined probably due to the higher concentration of toxic degradation products of monomeric sugars, such as furfural and HMF.

Prof J.D. Fontana's group at LQBB—Biomasses Chemo/Biotechnological Laboratory formerly at UFPR (Federal University of Paraná) and now at UTFPR (Federal Technological University of Parana, Curitiba-PR, Brazil) has been consolidating for a long time the phosphoric pretreatment technology using very diluted H_3PO_4, alone and under moderated thermopressurization. An initial study focused on the pretreatment of sugarcane and sorghum bagasses with H_3PO_4 for the production of bioethanol [48]. The main results from this pretreatment using optimized conditions (0.065% v/v H_3PO_4 and 200°C, 3 atm, during 2 min) was a complete or partial hydrolysis of hemicellulose fraction to xylose>xylo-oligosaccharides>arabinose depending on the variation of the severity parameters just before mentioned. Moreover, it was observed improved fermentation of the solubilized pentoses to ethanol and acetic acid by *Pachysolen tannophilus* and to ethanol by *Fusarium oxysporum*. *Pachysolen tannophilus* was the first yeast shown to be capable to convert xylose directly to ethanol under anaerobic conditions (with the concomitant production of xylitol and acetic acid) [49], while *F. oxysporum* is one of the few fungal species reported to ferment plant carbohydrate polymers to ethanol in just one-step process [50]. It was observed that fermentation capability was related to lignin solubilization followed by its removal using ethyl acetate or activated charcoal. Most importantly, phosphoric acid pretreatment on sugarcane and sorghum bagasses allowed almost complete conversion of cellulose to glucose using commercial cellulases produced at that time by Biobras (BIOFERM, Brazil), a Brazilian company that operated a 2G ethanol plant in the end of 1970s and was further acquired by Novo Nordisk's in 2003.

Interestingly, even past >30 years, our oPA technology for sugarcane processing has been used with industrial proposals, as, for example, in the CANEBIOFUEL (Conversion of sugar cane biomass into ethanol) Project, that was funded by the European Commission (FP7-Energy) which planned to obtain a deeper knowledge and a scientific and technological platform for converting sugarcane biomass into fermentable sugars. The project concluded that, in general, lower severity during pretreatment, with lower temperatures and shorter times, result in better glucose yield than the opposite. Primarily based on ease of enzyme hydrolysis it was also found that H_3PO_4 is superior to H_2SO_4 for the acid catalyzed pretreatment. However,

some Brazilian partners in this project applied steam explosion to the pretreatment of sugarcane biomass with almost exactly the same kinetic conditions of our oPA treatment [48]., omitting, unfortunately and consciously, to mention our pioneering publication, as it is completely clear from their below report at Italy.

> *"Steam explosion of cane bagasse using phosphoric acid catalysis", IBS2010 – 14th Intl. Biotechnology Symposium and Exhibition, Palacongressi, Rimini, Italy; 14–18 Sept, 2010."*

In our another work, aqueous H_3PO_4 was used to increase the nutritional value of sugar cane bagasse for cattle feeding [51]. Enhanced ruminal degradability (almost 70%) was obtained by adding 2.9% (w/w) in comparison to 60% achieved with solvolysis with water (197°C,13.5 atm, 4:1 w/w of water). Furthermore, H_3PO_4 generates less carbohydrate dehydration and does not have to be washed out prior to fermentation because phosphate can act as an important micronutrient, after partial neutralization with ammonia, for the subsequent fermentation step [51, 52].

Steam treatment of sugarcane bagasse with a low level of phosphoric acid (1% of bagasse dry weight) at elevated temperatures (160–190°C) during 10 min resulted in a total sugar yield ranging from 215 to 299 g/kg bagasse (untreated dry weight) and lower levels of products from sugar degradation (furans and organic acids) in all treatment temperatures (140–190°C) as compared to sulfuric acid [53]. Hemicellulose hydrolysates from treatment temperatures below 180°C could be fermented (slowly) by ethanologenic *E. coli* without the need of purification [53]. This demonstrated low level of potential inhibitors.

In another study, hemicelluloses from sugarcane bagasse were efficiently solubilized (96% and 98% after 8 and 24 min, respectively) using a low concentration of phosphoric acid (0.20%) at 186°C [54]. Enzymatic cellulose conversion of pretreated bagasse using 20 filter paper cellulase units (FPU) g^{-1} of Novozymes Celluclast® (a commercial cellulase preparation produced by a selected strain of the fungus *Trichoderma reesei*) treated under these conditions of pretreatment produced the highest cellulose conversion of 56.38%. In general low levels of degradation products were achieved; however, minor increase of these products were observed when temperature was elevated to 186°C that can be explained by the high solubilization of hemicellulose fraction at this condition [54].

Mild phosphoric pretreatment has been also adopted with stream treated substrates. Preimpregnation of *Eucalyptus benthamii* with diluted phosphoric acid followed by steam explosion resulted in an improved selectivity towards hemicellulose hydrolysis (xylose yields of 50–60%), yielding substrates readily susceptible to saccharification with Novozymes Cellic® CTec2 (a commercial enzymatic blend to produce cellulosic ethanol) at relatively high solids (10%) [55].

Results obtained on sugarcane bagasse through a central composite design comparing steam explosion carried out in the absence (autohydrolysis) and presence of phosphoric acid showed that phosphoric acid catalysis (19 mg g^{-1}) resulted in better glucan yields under milder conditions (180°C, 5 min) [56]. Phosphoric acid catalysis produced steam-treated substrates with good susceptibility to enzymatic hydrolysis (30 mg g^{-1} Cellic® CTec2, at 8% of substrate consistency) yielding in average 75% of glucose.

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Current wheat based bioethanol production (first generation) depends significantly on DDGS (distillers dried grains with solubles), a common byproduct that is sold separately as animal feed [57]. Phosphoric acid has been shown as a viable option to maintain substrate quality without contaminating the feed residues with high sulfur levels encountered if H_2SO_4 as used. In a recent study, dilute phosphoric acid pretreatment was optimized for wheat straw in laboratory scale and the results validated for the first time in a Biorefinery Demo Plant (BDP), operated by SP (Technical Research Institute of Sweden) at Örnsköldsvik, Sweden [58]. Optimal pretreatment conditions were determined in the laboratory as an acid concentration of 1.75% (w/v) at a temperature of 190°C for 15 min, based on the maximum enzymatic digestibility with the minimum inhibitor release. Enzymatic polysaccharide hydrolysis reached 36% for untreated straw and 86% for straw pretreated with dilute phosphoric acid. Based on this, scale up of the acid phosphoric pretreatment was applied at the biorefinery demonstration plant and an improved efficiency of polysaccharide hydrolysis was obtained (95% of the theoretical maximum). Further sugar fermentation by the Ascomycete *Neurospora intermedia* showed an improvement in the ethanol yield from 29% (with untreated straw) to 94% (with dilute phosphoric acid pretreated straw) of the theoretical maximum.

5. Starch hydrolysis with diluted phosphoric acid

Starch is the second most abundant polymer in the world [59]. Starch granules are biosynthesized reserve polysaccharide in a broad array of plant tissues and within many plant species. Potatoes and cassava are outstanding starch sources. They are composed of two types of α-linked glucans: amylose, a straight chain of α-1,4-linked glucopyranosyl units and amylopectin, which has besides α-1,4-linked glucopyranosyl units various branch points with α-1,6-linkages. A linear polymer of amylose (around 20% of whole starch) can have up to 6000 glucose units, whereas amylopectin (around 80% of the whole starch) is composed of α-1,4-linked chains of 10–60 glucose units with α-1,6-linked side chains of 15–45 glucose units. Both building blocks represent approximately 98–99% of the starch dry weight [60].

Starch may be chemically, enzymatically or physically modified to produce a broth rich in glucose that possess potential use in biotechnological processes, such as fermentation substrate for microorganisms to produce bioethanol, enzymes and other biomolecules. It can be also modified to present novel characteristics, creating innumerous applications, as for example in the food industry, as sweetener or thickening and gelling agent. Enzymatic conversion of starch to free glucose requires the concurrence of two enzymes: α-amylase, that yields malto-oligosaccharides and dextrins of varying chain length, and α-(1,4)-glucosidase (maltase), which hydrolyses terminal, non-reducing α-1,4-linked D-glucose residues with release of free D-glucose. These two enzymes can be replaced by amyloglucosidase (glucoamylase), a single enzyme able to break simultaneously the α-D-(1-4) and the α-D-(1-6), glycosidic bonds of both poly- and oligosaccharides. Efficient amylase-producing species include those bacteria of genus *Bacillus* (e.g. *B. licheniformis, B. subtilis, B. stearothermophilus, B. amyloliquefaciens*)

and fungi of genus *Aspergillus* (e.g. *A. niger, A. oryzae, A. awamori, A. fumigatus*). Amylases makes up today up to 25% of the world enzyme market (personal communication, August, 2017), and are together with proteases, the most versatile enzymes in the industrial enzyme sector because of the abundance of substrates, raw materials and variety of applications as bakery goods, sugar products, biofuel industry, and many others.

Starch can be modified by chemical methods and an example are those termed "acid-thinned", normally used for food and beverage applications that involve an existing high starch content. Both α-1,4 and α-1,6 glucosidic linkages are moderately resistant to acid hydrolysis, with the amorphous regions of the granule more susceptible to chemical treatment than the crystalline regions. Acid modified starches are prepared industrially by treating the starch slurry (40%) with varying concentrations of mineral acids and hydrolysis time at temperatures below that of gelatinization (25–55°C) [61]. Acid treatment increases the gelatinization parameters (gelatinization temperature and enthalpy), reduces the molar mass and viscosity, increases the solubility of the granules, minimizes syneresis (separation of liquid from a gel caused by contraction), and causes gel thermo-reversibility when subjected to cooling after melting [61].

Acid Hydrochloric and sulfuric acid, more often the second, are the generally used mineral/inorganic acids for starch hydrolysis, but they can present several problems. When using hydrochloric acid, in downstream step is necessary to desalinate the syrup using high cost ion exchange resins. Additionally, undesirable byproducts are produced even when syrups of an average dextrose equivalents are produced, because free glucose is converted to dehydration products such as hydroxymethylfurfural (HMF), levulinic and formic acids, which in turn can inhibit microbial growth if a subsequent fermentation step is required [62]. Furthermore, these mineral acids can easily produce toxic gases in the course of the process [63]. In the food and beverage industry, for example, another problem arises from the Maillard reactions between reducing sugars and R-NH2 groups from amino acids and proteins. This negative occurrence is designed as "mud" in the starch-processing factories of glucose-enriched syrups. Its worse properties are brown color and bitter taste [63].

The use of phosphoric acid instead of the stronger mentioned acids presents several advantages: safer handing because is a non-volatile acid, reduced byproducts formation and if the hydrolysate is to be used in a subsequent fermentation step, there is no need to remove or eliminate the phosphoric acid catalyst. Instead, neutralization with ammonia leads the formation of ammonium phosphate, a convenient supplement for growth as P and N-source. Taken together, these various considerations create the assumption of phosphoric acid as the preferred acid catalyst. From a strict biochemical stand point, let us to recall how much phosphoric acid is a "body friend" molecule: it is present in DNA, ATP, casein and phosphoric-esters (Gluc- and Fruct-phosphates feeding the universal glycolytic pathway).

Our study comparing hydrolysis with phosphoric acid and hydrochloric acid on cassava starch paste (30%, w/v) has shown that at 160°C (*ca.* 6 atm), the desired dextrose equivalent (DE) was obtained with both acids: DE value of 85 at pH = 1.6 and pH = 1.8 using hydrochloric acid and a DE value of 83 at pH = 1.4 with phosphoric acid (**Figure 2**) [63]. Higher

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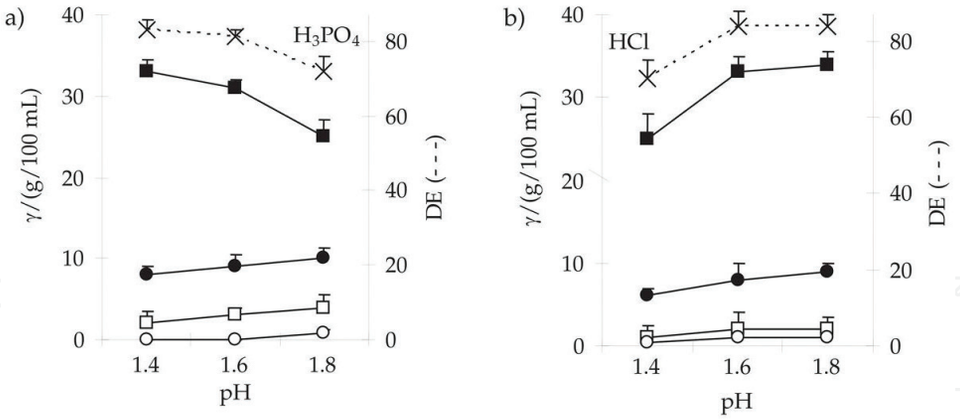

Figure 2. Effect of pH on the hydrolysis of cassava starch (30%) by phosphoric acid (a) and hydrochloric acid (b) at the temperature of 160°C (5 bar) during 10 min. Final concentrations of hydrolysis products and the final DE are shown. Key: (– x –) dextrose equivalents (DE), (■) glucose, (●) maltose, (□) maltotriose, (○) maltotetraose and higher.

Figure 3. Example of sweeteners and GRASE status (generally regarded as safe and effective) of phosphoric and/or citric acids or their salts. (A) Cola soft drink containing HFCS or steviol glycosides and phosphoric acid; (B) fermented milk containing sweeteners from corn (high-fructose corn syrup - HFCS and/or modified corn starch) phosphoric and/or citric acids or their salts (Source: Personal photo, 2015).

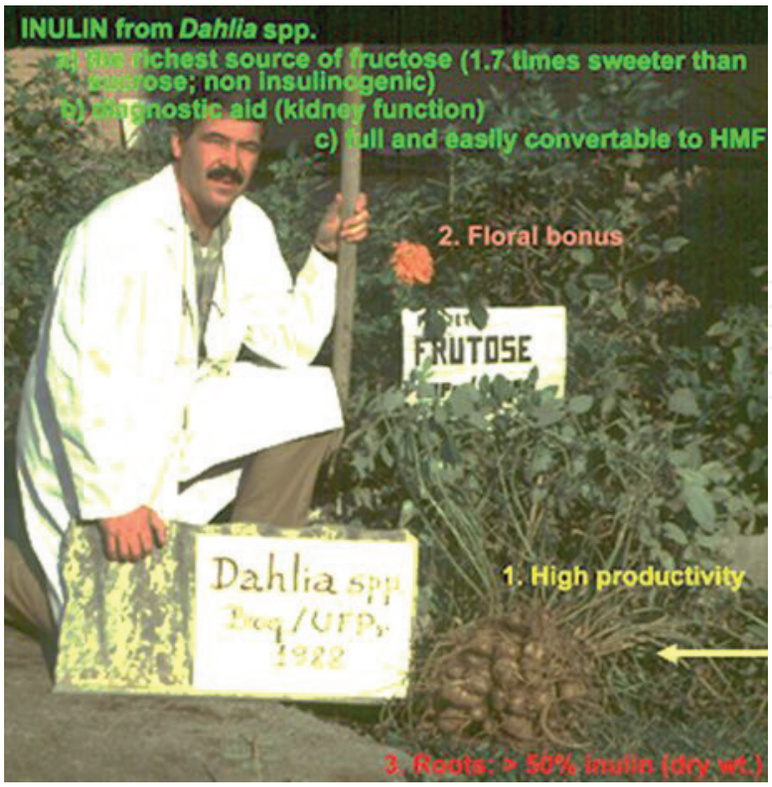

Figure 4. *Dahlia* sp. garden cultivation offers the most productive source for inulin which through a quick extraction of decorticated and sliced tubercles with pH 7 buffered hot water, followed by polymer retrogradation in a cold (ca. 8°C) environment. (Source: Prof. J. D. Fontana private auto-photo album).

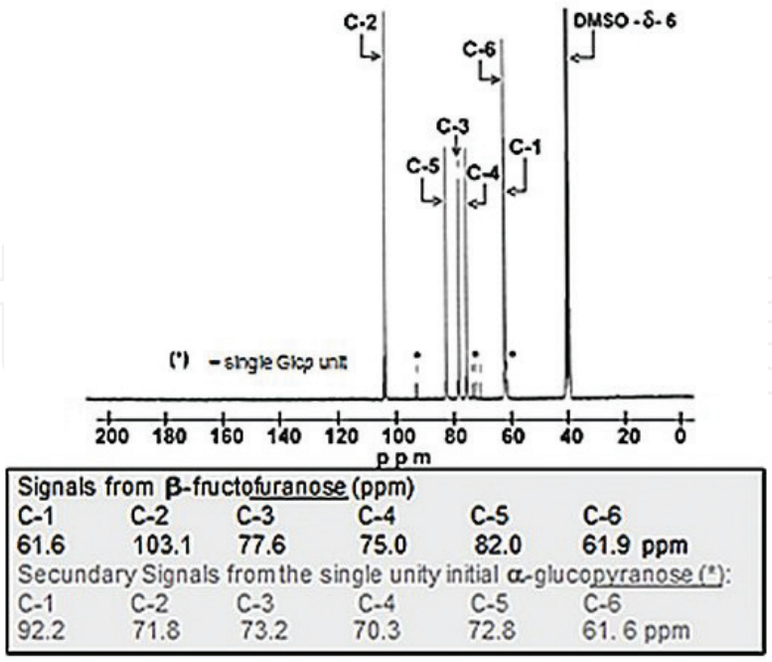

Figure 5. 13C-Nuclear Magnetic Resonance (NMR) of purified *Dahlia* sp. tubercles inulin. The presence of minor spectroscopy signals – asterisk labels – correspond to the single glucopyranosyl of each whole inulin molecule, thus revealing that extraction and purification steps were carefully carried and preserving the polysaccharide native chemical structure. (Source: authors lab associate).

temperatures and lower pH values led to higher concentrations of HMF and formic acid with both acids, but these quantities were always lower when hydrolysis was carried out with phosphoric acid (e.g. for HMF at pH = 1.5 and 152°C, 4 bar with a holding time of 5 min: 0.185 mg/mL using HCl against 0.075 mg/mL with H_3PO_4) [63].

Our oPA-mediated cassava starch hydrolysate allowed biomass growth and astaxanthin production by the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* (formerly: *Phaffia rhodozyma*) with parallel consumption of all maltosugars from G2 to G6 from an initial 64% of reducing sugars) reaching a maximum 3.34 mg/L of astaxanthin in a culture medium containing 6.5% w/v starch hydrolysate with supplementation (0.05 g/L yeast extract, and 25 to 50 mg/L of NH_4NO_3 [63]. Results have shown that that diluted thermopressurized phosphoric acid can be used as alternative catalyst to produce high DE syrups from cassava and other starch sources, residual phosphate being left in the final hydrolysate (after a light neutralization with ammonia or other alkalis) as a fermentation co-nutrient feedstock to produce biomolecules [63].

It may be remembered that sweetening of soft drinks and fermented milk (lacteous beverages and yogurts) can be attained with several alternatives of natural sugars or artificial sweeteners. Any of them are always accompanied with some phosphoric or citric acid and/or their salts, as shown in the following illustration with two worldwide sold products (**Figure 3A** and **B**).

oPa-catalyzed partially starch hydrolysates (and even better if fructo-oligosaccharides, commonly known as FOS, the nutraceutical and anti-tumor oligosaccharides of inulin) along with the retained oPA catalyst within the hydrolysates may be a clever strategy to these product industrial formulas. Our group is waiting for the patent request PI 0703206-4 grant from the Brazilian INPI—National Institute for Industrial Property. Its claim includes the protection of the utilization of phosphoric and citric acids as mild catalysts for the production of FOS from *Dahlia* sp. tubers inulin. One example of such occurrence and some properties of inulin are shown in the **Figures 4–7**.

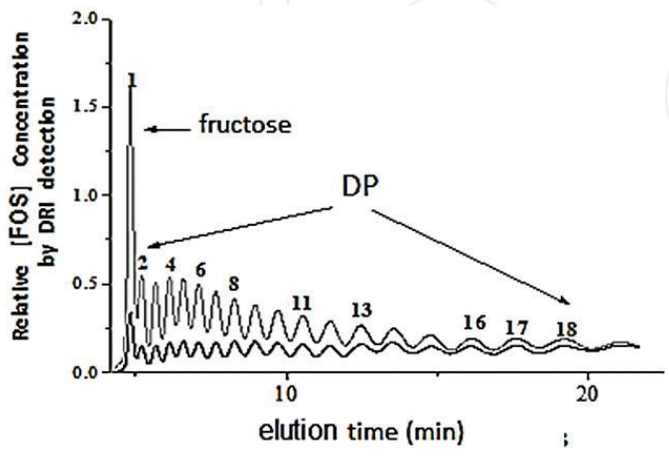

Figure 6. High performance liquid chromatography (HPLC) monitoring of the degree of polymerization (DP) of oPA-partially hydrolyzed 10% inulin with pH 2.0 at 85°C for 5 min (bottom line) or 15 min (upper line). (Source: Authors lab). FOS: fructo-oligosaccharides; RID: refractive index detector.

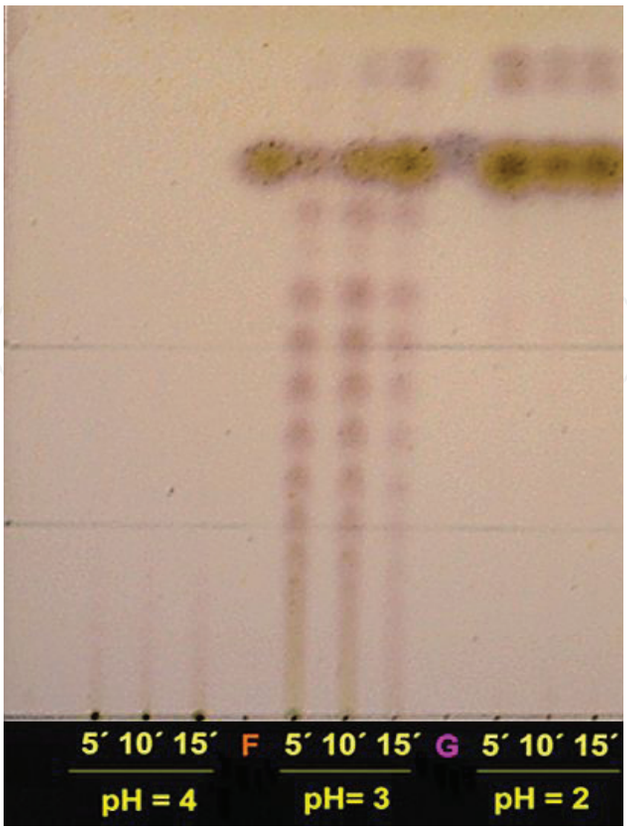

Figure 7. Thin-layer chromatography (TLC) monitoring of inulin (poly-D-fructofuranose) by oPA-catalyzed partial or total hydrolysis along 15 min of incubation at 75°C: pH 4 (*left*), pH 3 (*center*) and pH 2 (*right*). Revelator: Hot 0.5% orcinol in MeOH:H_2SO_4 9:1. The major spot at Rf = 0.8 is free fructose (F´). The multiband profile (*right*) are FOS (Fructo-oligoSaccharides) with degree of polymerization (DP) from 2 till 10. The two spots ahead fructose are HMF (HydroxyMethylFurufuraldeyde) and probably some DFA (DiFructose anhydride) due to acid reversion of free fructose.

6. Nutraceutical oligosaccharides products obtained by diluted thermopressurized phosphoric acid treatment of microalgae cell walls

Our more recent application of the oPA-mediated catalysis of polymeric sugars has been recently published [64]. The hydrolysis substrates, initially, were microalgae cell walls. This is a very convenient approach to be coupled to microalgae whole biomass once extracted with hot organic solvents (e.g., anhydrous ethanol) to preliminary redeem the lipid material for biodiesel production. In fact, our prospection in these so intensively explored marine unicellular microorganism has gone far: the microalgae (*Chlorella vulgaris*) and cyanobacteria or blue-green algae (*Arthrospira platensis*; formerly *Spirulina platensis*) biomasses, coming from photobioreactors or large open bowls installed in the open roof of local steak house were permanently bubbled with the whole but filtered gases and other volatile components of the hot stream arising from the grills and driven to the bottom of photobioreactors and bowls with the help of a fan. Finalizing, the following chromatographic illustration – variable series of oligosaccharides – confirms the validity of this technology novelty (**Figure 8**).

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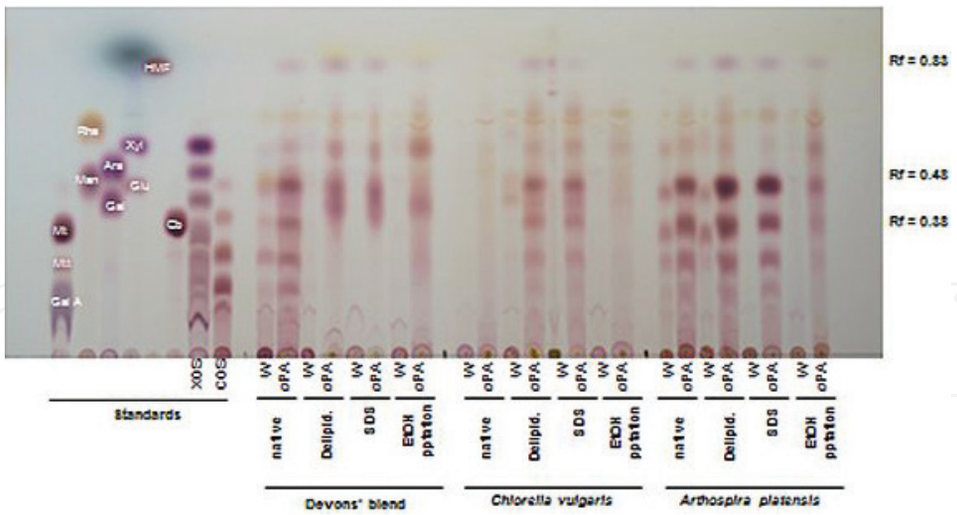

Figure 8. Thin-layer chromatography (TLC) of Nutraceutical oligosaccharides (NOs) arising from diluted thermopressurized oPA-catalyzed treatment of two microalgae and one cyanobacterium cell walls. Real effective pH 2.0 (after equilibration and complete wetting of each microorganism cell mass) and then a thermopressurization at 4.5 atm (156°C) till the peak condition for 2 minutes. Mobile phase: Acetonitrile:Isopropanol:Water (15:3:5). Chromogenic reagent: Orcinol in sulfuric acid. Standards: (Gal A) galacturonic acid, (Rha) rhamnose, (Man) mannose, (Ara) arabinose, (Gal) galactose, (Xyl) xylose, Rf = 0.48 - (Glu) glucose, Rf 0.38 - (Cb) cellobiose and (Mt) maltose, Rf = 0.29 - (Mtt) maltotriose, (XOS) xylo-oligosaccharides, (COS) cello-oligosaccharides, Rf = 0.83 - (HMF) hydroxymethylfurfural. (Source: Authors; picture abstracted from Bruna Leal master dissertation, supervised by Prof. Marcelo R. Prado and Adéia Grzybowski, 2015).

7. Conclusions

Great potential is observed in the deconstruction of phytobiomass polysaccharides to its component sugars. Resulting monomers and/or oligomers can be used for the production of a plethora of products, with application to biofuels, food, fine chemicals and other industries. As mentioned before, due to the inherent characteristics of phosphoric acid, it can be used as an advantageous catalyst for the depolymerization of polysaccharides from a great variety of phytobiomass. Pretreatment technology using very diluted phosphoric acid, alone and under moderated thermopressurization for the bioprocessing of a sugarcane and other L(h)C substrates can possess important advantages over the use of mineral/inorganic acids, despite its relatively higher cost when compared to sulfuric acid, for example. We have shown the potential for using phosphoric acid hydrolysates to fermentation processes using different microorganisms, to the production of bioethanol, to increase nutritional value in animal feed, for starch modification, biomass growth and in the production of prebiotic/alternative sweetener (fructo-oligosaccharides). A continuous study in the use diluted phosphoric acid on different biomass could improve strategies that can be further used in industry and biorefinery processes.

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