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Effect of steam treatments on the availability of various families of secondary metabolites extracted from green sweet sorghum

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ABSTRACT

Both free carbohydrates and hemicelluloses from green sweet sorghum [*Sorghum bicolor* (L.) Moench] were extracted using a steam process to produce combined first and second generation ethanol. Once the carbohydrates removed, Soxhlet extraction was used to extract the lipophilic secondary metabolites from the plant fibers and the results were compared to those obtained with the biomass extracted without prior steam treatments. This allowed assessing if the carbohydrate removal process had an impact on the secondary metabolites that could be recovered from the biomass. Soxhlet extracts where characterized using several techniques such as gas chromatography-mass spectrometry, liquid chromatography-triple quadrupole mass spectrometry and liquid chromatography-UV detection followed by high-resolution mass spectrometry. A total of 13 compounds were detected and quantified in the non-polar soxhlet fraction and were mainly fatty acids (6), phenyl glycosides (5) and sterols (2) at concentrations ranging between 0.015 g kg^{-1} and 1.5 g kg^{-1} (dry biomass). Comparison of the extractive content before and after steam process showed that most of the compounds were found in a lesser extent (up to 71%) although still recoverable after the steam process while other compounds (namely phytosterols) were as concentrated as they were prior to steam treatments. These results show that in the case of sweet sorghum, the preliminary extraction of the carbohydrate content with a steam process may not completely hinder the possibility to extract high value secondary metabolites, which would in this case come second in a biorefinery approach.

1. Introduction

Large-scale production of second generation biofuels, although depending on a cheaper feedstock than first generation biofuels (Lee and Lavoie, 2013), is still relying on more expensive technologies, especially in regards to the hydrolysis of cellulose. Thus, in order to be economically realistic, it is now widely accepted that second generation biofuels have to rely on a biorefinery model, which requires a complete utilisation of the carbon structures found in lignocellulosic biomass. In addition to the numerous researches made on cellulose hydrolysis (Lavoie et al., 2010; Sun and Cheng, 2002), hemicelluloses (Fuente-Hernández et al., 2013; Matsushika et al., 2009) and lignin (Beauchet et al., 2012; Shabtai et al., 1999), secondary metabolites (Naik et al., 2010) have to be considered as well in order to provide an added value to lignocellulosic biomass. Secondary metabolites are in some cases high value compounds such as taxol (Rowinsky et al., 1992), betulin (Alakurtti et al., 2006) and phenyl glycosides (Althwab et al., 2015) to name only a few. One of the most common secondary metabolite is salicylic acid (a component in pharmaceuticals) that can reach a market value up to 1900 USD/tonne. Such high value compounds, if compared to ethanol (approx. 500 USD/tonne − Sept 2016) could really contribute to the overall economics of biorefineries which would be beneficial both for the refiners themselves as well as the biomass producers, which will be able to expect more incomes for their crops. Whilst conversion technologies are struggling to reduce their production prices, growers in North America are, on the other hand, facing a paradox. Although many are willing to grow biomass for energy purposes, they can hardly meet the conversion industry pricewise (Lee and Lavoie, 2013). Furthermore, while the energy sector is adjusting, some biomass producers develop new markets to liquidate their production and often at better price than energy, like mulch, bedding or forage (Lalonde et al., 2015). Thus, second generation biofuels at this point target residual biomass such as forest or agricultural biomass or even municipal solid wastes (Lavoie et al.,

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2011) which are often cheaper and easier to find (Lee and Lavoie, 2013). Nevertheless, energy crops represent a potential that has to be considered since the total available residual carbon sources in one location may not be sufficient to cope for the increasing demand in liquid fuels. In order to support the biorefinery economy, an optimal utilisation of all the carbon structures of biomass is thus essential.

Of the many crops that are currently investigated as potential raw materials for energy production, sweet sorghum [*Sorghum bicolor* (L.) Moench] is getting increased attention especially in light of its water-soluble carbohydrate content (WSC) requiring a lower energy for processing (Bennett and Anex, 2009). Sweet sorghum, an annual C4 species used for forage or grain production, is well adapted in North America since hybrids were adapted to temperate climates (dos Passos Bernardes et al., 2016). The potential of sweet sorghum has been recently investigated in eastern Canada, where mean dry matter yields of 15.8 t/ha have been reported by both Thivierge et al. (2015) and dos Passos Bernardes et al. (2015) while WSC concentrations of 272.5 and 231 g/kg dry mass have been respectively reported. However, conservation and extraction of the WSC found in the plant is often a challenge and classical extraction techniques such as maceration, cold pressing and hot water extractions may not allow the extraction of a maximum content of WSC.

Furthermore, in order to avoid as much as possible plant degradation, WSC should be rapidly extracted after harvesting, thus making secondary metabolites extraction very difficult. Our group has recently investigated the use of steam treatments for the extraction of WSC from sweet sorghum (unpublished results) and found that, when using steam processes at a severity index of 2.061, it was possible to extract most of the WSC as well as part of its hemicellulosic content. Furthermore, steam processes could be applied directly on green biomass without any impregnation, hence making the sugar recovery process very efficient, close to 100%. In comparison, extractions using roller mill process, allow extraction of the juices at a rate varying from 50 to 60% (Bryan et al., 1985; Propheter et al., 2010; Putnam et al., 1991; Weitzel et al., 1989). Reports from open literature also mentioned the possibility to extract sweet sorghum using a screw press allowing juice recovery varying from 63 to 75% (Crépeau et al., 2013). Since WSC are extracted in parallel with hemicellulosic sugars, such extraction is at the interface between first and second generation biofuels and could contribute to an easier commercialisation of second generation technologies.

In addition to its high sugar content, sweet sorghum contains higher lipid levels than most other grains (Hwang et al., 2005). The wax is composed of fatty aldehydes, policosanols, fatty acids, hydrocarbons, wax esters and triacylglycerol (Hwang et al., 2002). Fatty acids have been widely observed in *S. bicolor* kernels in different proportions in other works (Althwab et al., 2015; Hwang et al., 2002; Mehmood et al., 2008; Abugri et al., 2013). In the oil fraction, triacylglycerols are the most abundant lipid group while linoleic, oleic and palmitic acids are reported to be the most abundant fatty acids although linolenic, stearic and arachidic acids have also been observed (Lee et al., 2014; Neucere and Sumrell, 1980). Sweet sorghum also contains other lipidic fractions such as phytosterols, which are comparable to cholesterol in terms of molecular structure and are located in the oily fraction of the plant. Stigmasterol and β-sitosterol have been widely observed in *S. bicolor* kernels surface, particularly in the oil fraction, as one of the most abundant phytosterols (Althwab et al., 2015; Awika and Rooney, 2004; Leguizamón et al., 2009). Phytosterols have been reported to reduce plasma LDL cholesterol levels (Carr et al., 2005; Hoi et al., 2009; Lee et al., 2014) and have been approved by regulatory agencies to be sold as over-the-counter natural products that reduce risks of heart disease (European Food Information Council (EUFIC), 2005; Food and Drug Administration (FDA)). Policosanols are also present in the wax fraction of the plant and have also been reported to be linked to the reduction of plasma LDL cholesterol (Arruzazabala et al., 1997; Berthold et al., 2006; Gouni-Berthold and Berthold, 2002; Kato et al., 1995), but such effects are still under debate (Dulin et al., 2006; Greyling et al., 2006; Lin et al., 2004).

Sweet sorghum phenolic compounds are as diverse as numerous, however they are essentially found within the phenolic acids and flavonoids families. The formers are mostly benzoic acids and include derivatives such as gallic, salicylic, coumaric (*o*- and *p*-) or cinnamic acids (such as ferulic acid). Flavonoids, commonly found in sweet sorghum (Awika and Rooney, 2004), include a large variety of subgroups such as anthocyanins, 3-deoxyanthocyanins, flavones, etc. The concentration of these metabolites in the plant's tissues were reported to vary according to variety and environmental conditions surrounding the crops (Althwab et al., 2015).

In this work, the lipophilic secondary metabolites from sweet sorghum were extracted after a preliminary steam process intended to recover the free sugars. Sorghum fibers were then extracted with ethanol, ethanol/toluene and water using a classical Soxhlet apparatus. After calculation of mass balances, extracts were both characterized and quantified using gas chromatography-mass spectrometry (GC–MS) for phytosterols, liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS) for fatty acids and liquid chromatography-UV followed by high resolution mass spectrometry (LC-UV-HRMS) for flavonoids in order to verify if the secondary metabolites could still be recovered after steam treatments and to estimate the economic potential of the identified molecules as integrated products of a biorefining approach. The secondary metabolites were then compared prior and after steam treatments to assess the impact of the process on the extractive recovery.

2. Material and methods

2.1. Sorghum biomass production conditions

Sweet sorghum was grown at the CEROM research center in Saint-Mathieu-de-Beloeil (Québec, Canada, 45.58° N, 73.24° W) and accumulated between 2901 and 3100 CHU (corn heat units) during the growing season (CRAAQ, 2012). Soil type was a St. Urbain clay loam (very-fine clayey, mixed mesic Typic Humaquapt). Sweet sorghum hybrid CSSH45 (AERC Inc., Delhi, ON, Canada) was used for a production of 200 kg (dry mass) for the project. Seedbed preparation consisted of mouldboard ploughing in the fall, harrowing in the spring to stimulate weed germination, and a final harrowing to kill weeds prior to seeding. The seeding rate was 320 m^{-2} pure live seeds. Seeding was performed at a depth of 2.5 cm, as soon as soil temperature reached 12 °C (AERC, 2016), using a Fabro plot seeder (Fabro Enterprises Ltd., Swift Current, SK).

Bentazone [3-isopropyl-1H-2.1.3-benzothiadiazin-4 (3H)-12.2-dioxide] was applied at a rate of 1.08 kg active ingredient per ha between the three- and six-leaf stage to suppress dicotyledon weeds. Hand weeding of the sweet sorghum plots was done at the ten-leaf stage. Nitrogen fertilization using nitrate (27–0–0) was performed at a rate of 80 kg N ha^{-1}, with 40 kg N ha^{-1} broadcast at seeding and the remaining N side-dressed at the four-leaf stage. Phosphorus was applied as triple superphosphate (0–46–0) and potassium as potassium chloride (0–0–60) based on soil analyses and local recommendations (Centre de référence en agriculture et agroalimentaire du Québec (CRAAQ), 2015).

Sweet sorghum stems were hand-harvested using a sickle the day before a killing frost corresponding to 121 days after seeding. Full size green stems were shipped the same day they were harvested in order to ensure integrity of water-soluble carbohydrates (WSC) and secondary metabolite content.

2.2. Reagents

2.2.1. Soxhlet extraction

Toluene (ACS Grade) was obtained from Fisher Scientific (Fair Lawn, NJ), ethanol (anhydrous) was obtained from GreenField Specialty Alcohols Inc. (Toronto, Canada) and water was obtained with