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Combining hydrothermal pretreatment with enzymes de-pectinates and exposes the innermost xyloglucan-rich hemicellulose layers of wine grape pomace

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ABSTRACT

Chardonnay grape pomace was treated with pressurized heat followed by enzymatic hydrolysis, with commercial or pure enzymes, in buffered conditions. The pomace was unfermented as commonly found for white winemaking wastes and treatments aimed to simulate biovalorization processing. Cell wall profiling techniques showed that the pretreatment led to depectination of the outer layers thereby exposing xylan polymers and increasing the extractability of arabinans, galactans, arabinogalactan proteins and mannans. This higher extractability is believed to be linked with partial degradation and opening-up of cell wall networks. Pectinase-rich enzyme preparations were presumably able to access the inner rhamnogalacturonan I dominant coating layers due to the hydrothermal pretreatment. Patterns of epitope abundance and the sequential release of cell wall polymers with specific combinations of enzymes led to a working model of the hitherto, poorly understood innermost xyloglucan-rich hemicellulose layers of unfermented grape pomace.

1. Introduction

Enzymes are important processing tools when utilizing plant wastes and/or residues from the food, beverage (e.g. winemaking) and feedstock industries in biorefining and biovalorization (Sieiro, García-Fraga, López-Seijas, Da Silva, & Villa, 2010). These plant by-products usually need to be made accessible (i.e. opened up using physiochemical and/or enzymatic methods) for the extraction of valued compounds (e.g. succinic acid, anthocyanins etc.) and/or to assist processing of the biopolymers to form new products (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010). Plant polymer waste streams are inherently complex; as plant cell walls have higher order network architectures in addition to their biochemical constituents (i.e. various sugars, phenolics, proteins and lipids). Plant cell walls are also dynamic structures being under both developmental and environmental control (Keegstra, 2010) which means incoming substrate/waste streams show inherent batch and/or seasonal variability for biorefining and related processing (Ortega-Heras, Pérez-Magariño, & González-Sanjosé, 2012). A common observation is that enzymes do not always deliver the desired outcome(s) in industrial applications where unprocessed plant tissue is used as a raw source material. Often the complex intermeshed plant cell wall layers present require a careful choice of pretreatments with selected enzyme combinations and dosages leading to stepwise biomass deconstruction resulting in the sequential production of multiple value-added products from specific fruit and crop waste streams.

A by-product of the winemaking process is grape pomace, essentially made up of berry skins with some pulp, seeds and

Abbreviations: AGPs, arabinogalactan proteins; AIR, alcohol insoluble residue (extracted cell walls); Ara, arabinose; CBM, carbohydrate binding modules; CDTA, diamino-cyclohexane-tetra-acetic acid; Cellc, Celluclast® 1.5L; CoMPP, Comprehensive Microarray Polymer Profiling; EA, endo-arabinase; EG, endo-1,4-β-glucanase; EPG, endo-polygalacturonase; ExCol, Rapidase®ExColor; Expr, Rapidase®Expression; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; GC–FID, Gas Chromatography–Flame Ionization Detector; Glc, glucose; HMDS, 1,1,1,3,3,3-hexamethyldisilane; HG, homogalacturonan; Man, mannose; mAbs, monoclonal antibodies; Pect, pectolytic enzymes; pPME, plant pectin methylesterase; PCA, principle component analysis; RG-I, rhamnogalacturonan-I; Rh, rhamnose; TMCS, trimethylchlorosilane; XG, xyloglucan-specific endo-1,4-β-glucanase;

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stalks. Pomace has several known uses such as animal feed, producing grape seed oil and most commonly as compost fertilizer (Khan et al., 2015). However, enzymes have significant potential in supporting the production of more lucrative revenue-generating compounds from grape pomace such as natural organic colorants, novel antimicrobial agents, by-product source chemicals (e.g. organic acids) as well as antioxidants and nutraceuticals for use as dietary supplements. In addition, a rich source of potentially fermentable carbohydrates is locked into the grape pomace as residual free sugars and generally inaccessible polysaccharides. Red winemaking yields fermented pomace containing ca. 4% (w/w; dry weight) residual free carbohydrates; however white winemaking leaves unfermented pressed grape pomace with ca. 37% (w/w; dry weight) free sugars (Corbin et al., 2015); in addition to the 6% (w/w; dry weight) glucose equivalents from cell wall polymers which have the potential to be enzymatically liberated for use as fermentable substrates (Corbin et al., 2015; Rodríguez et al., 2010) for bioethanol production. However, a significant limitation to the optimal use of grape pomace for generating various valuable side-products (e.g. tartaric acid, flavonoids etc.) and bioethanol in a biorefinery valorization scheme is our general lack-of-understanding of grape berry cell wall structure and composition. Developing a more comprehensive understanding of the various grape pomace cell tissue layers and their corresponding cell wall compositions would permit the selection of an optimal combination of industrial pretreatments and enzyme mixtures to achieve stepwise deconstruction of winery waste streams in a renewable-sustainable manner.

It has been shown that pretreatment of the grape pomace (e.g. typically heating) improves enzymatic hydrolysis and delivers a higher extraction yield (e.g. of polyphenols) (Alvira et al., 2010; Corbin et al., 2015; Kammerer, Claus, Scieber, & Carle, 2005; Maier, Göppert, Kammerer, Schieber, & Carle, 2007). The ability of hydrolytic enzymes (e.g. pectinases) to reach their targets is believed to be limited by the pore sizes (range 4–10 kDa) proposed to be common in grape cell walls (Hanlin, Hrmova, Harbertson, & Downey, 2010) thereby preventing enzyme action (Fleischer, O'Neill, & Ehwald, 1999; Jarvis, 2011). The use of steam treatment on wheat straw has shown a positive effect by increased porosity and the average pore size in these cell walls (Hendriks & Zeeman, 2009) for bioprocessing. However the effect of hydrothermal pretreatment on grape pomace cell walls has not been investigated to any extent, limiting the information needed for developing an efficient biorefinery model and optimal valorization procedure for grape wastes.

Most previous studies have evaluated the efficacy and action of enzymes in winemaking and pomace bioprocessing by focusing on indirect measurements e.g. the release of signature (i.e. measurable) compounds such as pigments, tannins and sugars (Arnous & Meyer, 2010; Xu et al., 2014). This indirect approach is then used to infer information on the grape polymer matrix and how it is constructed and thereafter deconstructed. A weakness of such an approach is that the actual substrates the enzymes act on are often not measured or known. Comprehensive Microarray Polymer Profiling (CoMPP) where monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) are used to probe the cell wall residues before and after treatments (e.g. enzymes etc.) allows direct evaluation of constituent polysaccharides by virtue of their epitope composition and abundance. In conjunction with classical monosaccharide compositional analysis; CoMPP has been used in a number of grape and wine contexts (Fangel, 2013; Moore, Fangel, Willats, & Vivier, 2014; Nguema-Ona et al., 2012; Pedersen et al., 2012). Recent studies have significantly improved our understanding of grape berry cell wall matrix composition and deconstruction in red winemaking (Gao, Fangel, Willats, Vivier, & Moore, 2015, 2016; Zietsman, Moore, Fangel, Willats, & Vivier, 2015; Zietsman et al., 2015). Firstly, a study on Pinotage demonstrated the importance of ripening levels at harvest over two vintages on skin cell wall degradation and the lessened impact of maceration enzymes on the more ripe berries (Zietsman et al., 2015). Then a thorough fractionation and enzymatic deconstruction of whole Cabernet Sauvignon berries during a standard red wine fermentation revealed a two layered pomace structure (i.e. a pectin-rich outer layer followed by a hemicellulose-rich pectin-coated inner layer) (Gao et al., 2015). A second study on isolated Pinotage skin cell walls in buffered conditions revealed the synergistic impact of selected combinations of pectinases and hemicellulases in successfully degrading the berry polymer matrix (Zietsman et al., 2015). A further study on Cabernet Sauvignon processed for red wine investigated the use of recombinant pectinases in the sequential deconstruction of whole berry cell walls (Gao et al., 2016). This study presented the first evidenced-based grape berry cell wall model for linking enzyme action and polymer degradation during winemaking.

Given this context it is now possible to use these earlier approaches to evaluate white wine grape pomace (i.e. Chardonnay), which is unfermented, as a source (waste) stream for potential valorization. This study therefore follows the changes of the matrix upon hydrothermal pretreatment of the pomace and subsequent application of different combinations of enzymes (commercial and purified 'polysaccharide degrading' enzymes). The application of combinatorial experimental design tools with CoMPP and monosaccharide composition analysis (as cell wall profiling methods) have been used to characterise the pomace matrices before and after treatments in this study.

2. Materials and methods

2.1. Preparation of unfermented Chardonnay grape pomace

Grapes (*Vitis vinifera* cv. Chardonnay) were harvested at 21°B in the Boland region of South Africa and the pomace was obtained after the grapes were pressed with a hydraulic press (pressure not exceeding 1.5 bar). This unfermented pomace was the matrix for all further experiments as outlined in Fig. 1. The moisture content of the Chardonnay pomace was determined to be 97.4%, using the method described in (Özcan, 2006). The pomace was thoroughly mixed and randomly divided into three batches and kept at −20 °C until processed for sample preparation. For analyses, sample preparation entailed manual seed removal and blending of the pomace with a stick blender. To evaluate the impact of hydrothermal pretreatment on the cell wall composition, five 10 g (wet weight) samples were freeze-dried (as controls) and another five were autoclaved (121 °C, 15 min, 100 kPa) (as test samples) before freeze-drying (Fig. 1, procedure 1). These samples were stored until AIR (alcohol insoluble residue) isolation. For preparation of hydrothermal pretreated pomace (Fig. 1, procedure 2), the deseeded and blended pomace pulp was mixed with a 0.12 M citrate phosphate buffer (pH 5 for purified enzymes and pH 3 for commercial enzymes) to form a 15% (wet w/v) suspension. This suspension was autoclaved (121 °C, 15 min, 100 kPa) and used as the standard hydrothermally pretreated matrix for all subsequent experiments. The autoclaving also ensured inactivation of any endogenous grape and microbial enzymes present.

2.2. Optimising commercial enzyme treatment of Chardonnay pomace

Factorial experimental design (MODDE software, MKS Data Analytics Solutions, Umeå, Sweden) was used to design a preliminary experiment to optimise the pH (choice of pH 3, pH 4.5 or pH 6), temperature (choice of 15 °C, 32.5 °C or 50 °C), enzyme loading