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Optimization of dilute acid-based pretreatment and application of laccase on apple pomace

Indu Parmar, H.P. Vasantha Rupasinghe *

Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, P.O. Box 550, Truro, Nova Scotia, Canada B2N 5E3

HIGHLIGHTS

- Apple pomace is a polysaccharide-rich under-utilized substrate for bio-processes.
- Optimization of the key pretreatment conditions using surface response methodology.
- Laccase treatment degraded 85% of inhibitory phenolic compounds of the hydrolyzate.
- The mild acid pretreatment produced low amounts of inhibitory furans and acetate.
- Enhancements of digestibility of apple lignocellulose allow efficient bioconversion.

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ABSTRACT

The present study was aimed to optimize acid-based pretreatment of apple pomace in relation to acid concentration, temperature and reaction time using response surface method with glucose as response variable. In addition, laccase (EC. 1.10.3.2) from *Trametes versicolor* was applied for degradation of polyphenols in apple pomace that could inhibit the further bioconversion steps involving enzymes and fermenting micro-organisms. The optimized conditions were: 1.5 g/100 mL acid concentration, 16 min reaction time and 91 °C reaction temperature, producing 13.9 g glucose/100 g on a dry matter basis. Subsequent application of laccase to hydrolyzates degraded most of the phenolic compounds in apple pomace by more than 85%. The optimized pretreatment conditions resulted in lower concentrations of other inhibitors such as furan compounds and acetic acid. Therefore, dilute acid pretreatment in combination with laccase application can be used for enhancing subsequent hydrolysis of polysaccharides and fermentation of apple pomace.

1. Introduction

The world's total apple production in the year 2008–2009 has been reported to be more than 69.5 million t (FAO, 2008), of which Canada contributed 455,361 t (Statistics Canada, 2010). Every year apple processing industries produce more than 5500 t of apple processing by-products, mainly pomace (residue left after juice extraction) in Nova Scotia, Canada (Rupasinghe, 2003). Apple pomace presents a serious disposal challenge for the industry and is considered as an under-utilized biomass. Nevertheless, it is rich in both soluble (fructose, glucose, sucrose) and insoluble (cellulose, hemicellulose, pectin) carbohydrates; therefore, is suggested to be one of the excellent substrates for bio-processes (Vendruscolo et al., 2008). Apart from carbohydrates, apple pomace also contains lignin, which along with other factors such as cellulose crystallinity affects the use of this biomass for bio-conversion (Hendriks and Zeeman, 2009). The complex physico-chemical associations between lignin, cellulose and hemicelluloses hinder the accessibility of cellulose for hydrolytic enzymes. High lignin to cellulose ratio (Villas-Bôas et al., 2003) and presence of pectin-cellulose, hemicellulose-cellulose, hemicellulose–lignin interactions in apple pomace also limit enzymatic hydrolysis of polysaccharides of apple pomace.

Cellulose could be made accessible to cellulolytic enzymes by pre-treating the plant-based substrate with milling, steam (heat), acid, alkaline, sulfur dioxide, ionic liquids etc. (for review, see Hendriks and Zeeman, 2009). An effective pretreatment is expected to decrease the cellulose crystallinity and degrade the lignin–cellulose network and increase the surface area, thereby allowing the cellulose conversion into glucose by cellulases. Furthermore, pretreatment of fruit-based biomass is also known to release phenolic compounds, essential oils, pectin, and carotenoids (Galanakis, 2012). Pretreatment is considered to be one of the most crucial and expensive steps due to high input involved in terms of heat, acid/base and reaction time. Dilute acid pretreatment is an

* Corresponding author. Tel.: +1 902 893 6623; fax: +1 902 893 1404.
E-mail address: vrupasinghe@ndal.ca (H.P.V. Rupasinghe).

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effective and relatively inexpensive method for polysaccharide hydrolysis and lignin disruption. However, acid pretreatment can also give rise to by-products such as furfural, hydroxymethylfurfural (HMF), phenolic compounds, acetic acid, formic acid and levulinic acid at high temperatures ($\geqslant$121 °C) (Ibbett et al., 2011). In addition to reduced yield of sugars, the secondary reactions could also produce recalcitrant or inhibitory products that limit the suitability of hydrolyzates for subsequent bio-conversion (for review, see Hendriks and Zeeman, 2009). Apple pomace is already known to contain high amounts of specific polyphenols such as chlorogenic acid, epicatechin and phloridzin (Rupasinghe and Kean, 2008). Therefore, an additional step for the removal of enzyme and fermentation inhibitors could be advantageous while enhancing the fermentable sugar yields.

Method of inhibitor removal needs to be selected based on the type of inhibitor, substrate and micro-organism used for bio-conversion. Laccase (*p*-diphenol oxidase; E.C. 1.10.3.2) is an extra-cellular polyphenol oxidase from fungi, which catalyzes degradation of polyphenols and ring cleavage of aromatic compounds. The mode of action consists of loss of electron from substrate forming a free radical that could undergo further oxidation and non-enzymatic reactions including polymerization, disproportionation and hydration. It catalyzes lignin bio-degradation by acting on wide range of aromatic compounds including polyphenols, methoxy-substituted monophenols and aromatic amines. In addition, laccase from *Trametes versicolor* has also been studied for fruit juice and wine stabilization (Minussi et al., 2007).

Studies have shown that dilute acid pretreatment as a suitable process for producing sugar monomers from cellulosic waste such as citrus pomace (Oberoi et al., 2010), banana and mango fruit waste (Arumugam and Manikandan, 2011). However, to the best of our knowledge, no research effort has been undertaken on dilute acid and laccase pretreatment of apple pomace. Therefore, the current study was conducted to optimize the dilute acid-based pretreatment of apple pomace using response surface method with the long-term aim of enhancing the digestibility of lignocelluloses to increase fermentable sugars. In addition, commercial laccase was investigated for degradation of phenolic compounds present in acid hydrolyzed apple pomace. The effects of applied pretreatments on production of inhibitory products have also been assessed.

2. Methods

2.1. Raw materials

The apple pomace was collected from a commercial apple juice manufacturer, J.W. Mason and Sons Ltd., Windsor, NS, Canada during the year 2008–2009 and was from 'McIntosh' cultivar, which was stored at −20 °C until use. Apple pomace, contained flesh, peel, core, seeds and rice husks. Rice husks are added by commercial juice manufacturers to facilitate recovery of juice and could be present up to 10% of the total weight. Apple pomace was homogenized into puree by grinding for two min using a food processor.

Reagent grade sulfuric acid (72%), potassium ferrocynide [$K_4Fe(CN)_6{\cdot}3H_2O$], zinc acetate [$Zn(CH_3COO)_2{\cdot}H_2O$], anhydrous sodium carbonate, sodium bicarbonate, sodium sulfate, copper (II) sulfate, Folin Ciocalteu reagent and gallic acid were acquired from Sigma Aldrich (Oakville, ON, Canada). Potassium sodium tartrate, ammonium molybdate, disodium arsenate, ethanol, methanol, ethyl acetate (reagent grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Ottawa, ON, Canada). The liquid chromatography standards were purchased as follows: furfural, hydroxymethylfurfural (HMF), glucose, acetic acid, phloridzin, chlorogenic acid, ferulic acid and caffeic acid from Sigma Aldrich (Oakville, ON, Canada); catechin, quercitin and quercitin-3-*O*-glucoside from ChromaDex, Inc. (Santa Ana, CA, USA); quercitin-3-*O*-rhamnoside and quercitin-3-*O*-galactoside from Indofine Chemical Company (Hillsborough, NJ, USA). Laccase (isolated from *T. versicolor*) was obtained from Cedarlane (Burlington, ON, Canada).

2.2. Composition

Proximate composition and mineral analysis of apple pomace were carried out using methods of the AOAC (2006) at Laboratory Services of Nova Scotia Department of Agriculture, Harlow Institute, Truro, NS, Canada; while soluble and insoluble fiber determinations were conducted at Maxxam Analytics Inc., Mississauga, ON, Canada, an ISO 17025 registered analytical laboratory. Total reducing sugar content was determined using the Nelson Somogyi method (Somogyi, 1952). The cellulose content, lignin content and monosachharide composition of the raw material were carried out at the Cell Wall Analytical Facility, Great Lakes Bio-energy Research Center, Michigan State University, East Lansing, MI, USA, according to procedures standardized by Foster et al. (2010a,b). For determining the monosaccharide composition, the samples were simply hydrolyzed by trifloroacetic acid (TFA), without including any washing steps; thereby, it was assumed that any free sugars or starch will still be present. The results are reported as g/100 g dry matter (DM).

2.3. Dilute sulfuric acid pretreatment

Dilute acid pretreatment of apple pomace was carried out in 250 mL Wheaton Magna-flex spinner flasks (Fisher Scientific, Ottawa, ON, Canada) by mixing the apple pomace puree with sulfuric acid at different concentrations (Table 1). The puree (25 g fresh apple pomace/100 mL) was loaded into the flasks and subjected to selected temperature and time. The acid pre-treated samples were cooled to room temperature and used for further analysis. A sample of 1 mL was taken out after each pretreatment and analyzed for glucose by HPLC.

The values for sulfuric acid concentration were selected based on the range obtained from the reported levels of fruit-based biomass (Oberoi et al., 2010). As compared to high lignin containing biomass sources (corn stover, hardwood and softwood) that needs higher pretreatment temperature (150–200 °C), lignin present in apple pomace could be disrupted at moderate temperatures of 80–100 °C. This would unravel the underlying cellulose fibers, thereby enhancing the enzyme accessibility of cellulose. Moreover, moderate pretreatment temperatures could limit heat induced sugar degradation and formation of inhibitory products of fermentation. For reaction time, a range of 5–30 min was selected in order to reduce substrate degradation and promote economic feasibility of the process. Coding was applied as a part of the response surface methodology (RSM) and coded values are given in brackets: acid concentration 0.5 (−1) to 2 (+1) g/100 mL, reaction time 5 (−1) to 30 (+1) min, reaction temperature 80 (−1) to 100 (+1) °C as shown in Table 1.

Table 1
The acid pretreatment variables and their experimental design levels.

	Levels				
Coded value	−1.68	−1	0	+1	+1.68
Uncoded variables					
Acid concentration (g/100 mL)	0.7	1.0	1.5	2.0	2.3
Reaction time (min)	7.0	10.0	15.0	20.0	23.0
Reaction temperature (°C)	81.3	85.0	90.0	95.0	98.7