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Comparative degradation of hydrothermal pretreated winery grape wastes by various fungi

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ABSTRACT

Hydrothermal-treated (autoclaved) winery waste was degraded by *Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum* and *Penicillium citrinum*. Quantification of lignins, reducing sugars, pentoses and enzyme assays for cellulase, β-glucosidase and xylanase were performed. Lignin content increased from 36% in untreated waste to 63% in autoclaved substrate. Lignin degradation of 9% and 4.2% was achieved by *P. chrysogenum* and *A. niger*, respectively. Reducing sugars decreased by 1.2 and 0.7 kg/m^3 in *T. harzianum* and *P. citrinum* cultures, respectively. Pentose utilization was also considerable across all cultures. Cellulase and xylanase activities were higher in *A. niger* cultures at 45 U and 335 U, respectively. It also showed high β-glucosidase activity of 155 U, marginally less than *P. citrinum* (180 U). Autoclaving hydrolyzed hemicelluloses and crystalline cellulose, converting the latter to a more degradable amorphous form. The results suggest that successive hydrothermal and fungal treatments produce greater lignocellulose degradation than regular fermentation.

1. Introduction

Fermentation is chiefly used for alcohol production from various sources such as grape, corn and barley. In the fermentation process, microorganisms such as *Saccharomyces cerevisiae* are able to generate up to 12–15% alcohol from the raw material, which is used as either beverage alcohol or an industrial reagent. The rest, and the bulk, of the material is discarded as spent wash, and may now include unused live or dead fermenting organisms. The wine waste also consists of dry matter including crude fibers, grape seeds, skin, waste, marcs, stalk and skin pulp, proteins, ethers and amino acids. Apart from the moisture content, the major parts of grape waste are dietary fibre (30–40%), lipids (0.5–1%), soluble sugars (2–3%), proteins (2.5–3.5%) and ash (2–3%). Dietary fibre consists mainly of cellulose (27–37%), pectins (37–40%) and lignins (33–35%) (Bravo and Saura-Calixto, 1998; Ping et al., 2011; Vicens et al., 2009).

Various fungi such as *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp. have been reported as extensive biomass degraders owing to their ability to generate an array of enzymes such as endo- and exo-glucanases, β-glucosidase, xylanases, arabinofuranosidases and pectinases (Brink and Vries, 2011; González-Centeno et al., 2010). This degradation generates useful industrial and medicinal biomolecules such as ethanol, flavonoids, phenolic compounds, anthocyanins and hydroxybenzoic acid (Arvanitoyannis et al., 2006; Sánchez, 2009; Strong and Burgess, 2008). Additionally, fungi such as *Penicillium* sp. can be used for lignin mineralization during the degradation process (Rodríguez et al., 1994; Singh Arora and Kumar Sharma, 2010).

These fungi, and ultimately the enzymes derived from them, convert the lignocellulose complex to various soluble sugars, which can be converted into other secondary products. However, due to the relatively recalcitrant nature of the lignocellulose complex, pretreatment is usually required to facilitate access of fungal enzymes to the cellulose and hemicelluloses, which are the primary sources of carbon for these organisms. Hydrothermal treatment has been shown to be a comparatively efficient pretreatment technique for the breakdown of these biomolecules (Papadimitriou, 2010).

The experiments described here were performed to study the effects of fungal degradation on the composition of winery grape waste to achieve biodegradation of its lignocellulose components, namely cellulose, hemicelluloses and lignins. The degradation patterns of various fungi including white rot fungi (*P. chrysogenum and P. citrinum*), brown rot fungi (*A. niger*) and *T. harzianum* were compared so as to formulate the ideal fungal/enzymatic combination to yield maximum bioconversion.

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2. Materials and methods

2.1. Grape wastes

Post-fermentation grape wastes of *Vitis vinifera* var. Shiraz were obtained from the Australian Wine Research Institute (AWRI), Glen Osmond, South Australia. The substrate was oven dried at 70 °C for 96 h and ground using mortar and pestle. The dried, ground substrate was further oven dried for 96 h at 70 °C.

2.2. Cultivation of fungal cells

Fungal cultures of *T. harzianum*, *P. chrysogenum* and *P. citrinum* were obtained from Agpath, Victoria, Australia, and *A. niger* was obtained from the culture collection of the Microbiology Laboratory, Department of Chemistry and Biotechnology, Swinburne University of Technology. All the fungi were first cultured in Yeast Malt broth at 30 °C for 48 h and 0.1 mL of each culture (1×10^7 spores/mL) was used for the biodegradation studies.

2.3. Degradation of grape waste

For all the degradation studies, AATCC (American Association of Textile Chemists and Colorists) mineral salts iron medium was used. The medium was composed of (kg/m^3): NH_4NO_3 – 3.0; KH_2PO_4 – 2.5; K_2HPO_4 – 2.0; $MgSO_4.7H_2O$ – 0.2; $FeSO_4.7H_2O$ – 0.1 and crushed-dried grape waste – 30, as the sole carbon source. The pH of this medium was adjusted to 5.6 before autoclaving at 121 °C for 15 min. Fungal inocula were added to the medium and each culture was incubated at 30 °C with shaking at 200 rpm for 5 days. The degraded grape samples were collected and cryopreserved for further analyses.

2.4. Determination of reducing sugars

The quantitative determination of reducing sugars in the filtrate of degraded grape waste was performed by the dinitrosalicylic acid (DNSA) assay. Grape waste filtrate (100 μL sample) was mixed with 1 ml DNSA and incubated in a boiling water bath for 5 min followed by cooling on ice to stop further reaction and bring the sample to room temperature. The absorbance was taken at 540 nm to determine the concentration of reducing sugars. A glucose gradient was used to derive the standard reducing sugar (Plummer, 1987).

2.5. Determination of pentoses

The pentose sugar concentration was quantified by Bial's assay (Pramod and Venkatesh, 2006). Grape waste filtrate (200 μL) was mixed with 1 mL Bial's reagent (80 mg ethanolic orcinol in 40 mL concentrated HCl and 0.1 mL 10% $FeCl_3$) and kept in a boiling water bath for 5 minutes. The samples were then cooled to room temperature on ice to terminate the reaction and the absorbance was taken at 660 nm. The concentration was determined on the basis of the standard curve of arabinose.

2.6. Determination of lignins

Lignins were determined as Acid Soluble Lignin (ASL) and Acid Insoluble Lignin (AIL) by the NREL procedure (Sluiter et al., 2011). 0.1 g dried grape was incubated in 1 mL 72% H_2SO_4 at 30 °C for 1 h. This was followed by dilution of the acid hydrolyzed sample to 4% H_2SO_4 by addition of deionized H_2O. The mixture was then autoclaved at 121 °C for 1 h followed by cooling to room temperature. The supernatant was collected as the ASL fraction after a brief centrifugation. The pellet was rinsed with distilled water and was dried at 105 °C for 4 h to ensure complete drying. The dried sample was weighed as Acid Insoluble Residue and was vaporized in a muffle furnace at 575 °C for 1 h followed by cooling to room temperature. The weight of this sample was considered as ash. ASL in each sample was determined by the absorbance of the centrifuged filtrate at 320 nm using the equation given below:

$$\%ASL = \frac{ABS \times volume \times Df}{\varepsilon \times W_{S1} \times pahlength} \times 100$$

where, ABS = absorbance at 320 nm; Volume = volume of total filtrate (30.35 mL); ε = absorptivity of biomass at 320 nm (30 L/g cm); W_{S1} = oven dried weight of sample (milligrams); Pathlength = pathlength of the cell (1 cm)

AIL was determined by the ratio of difference between dry Acid Insoluble Residue (AIR) and ash to the original dry weight of grape waste as given in the equation below:

$$\%AIL = \frac{W_{S2} - W_{S3}}{W_{S1}} \times 100$$

where, W_{S1} = oven dried weight of sample (milligrams); W_{S2} = weight of AIR (milligrams); W_{S3} = weight of ash (milligrams); the total lignin content was calculated as the cumulative ASL and AIL.

2.7. Enzyme assays

Cellulase activity was measured in terms of Filter Paper Activity (FPA) as per IUPAC protocols (Ghose, 1987) using Whatman no. 1 filter paper as the substrate. One International Unit (IU) of cellulase is defined as the amount of enzyme required to liberate 1 μmol glucose per minute under assay conditions.

Activity of β-glucosidase was determined by the p-nitrophenyl-β-D-glucoside (pNPG) assay according to the method given by Kovacs et al. (2009). One IU of β-glucosidase is defined as the amount of enzyme required to liberate 1 μmol p-nitrophenol per minute under assay conditions.

Xylanase activity was measured by method given by Kovacs et al. (2009). 1.8 mL of Birchwood xylan (1%) in 0.05 M Na-citrate buffer was mixed with 200 μL appropriately diluted enzyme sample and was incubated at 50 °C for exactly 5 min. 3 mL DNSA reagent was added to this mixture before boiling for 5 min. The reaction was terminated by cooling in an ice bath. Absorbance was taken at 540 nm to determine enzyme activity. One IU of xylanase is defined as the amount of enzyme required to liberate 1 μmol xylose per minute under assay conditions.

2.8. Statistical analysis

All data were presented as the mean values of triplicate data samples with their standard error. The consistency and deviations between the data were analyzed by one way ANOVA using IBM® SPSS® 20.0 statistics software.

3. Results and discussion

3.1. Degradation of grape waste

Autoclaving altered the composition of winery grape waste, with an overall decrease in dry mass (data not shown). Pentose content in the filtrate increased from 1 kg/m^3 to 10 kg/m^3. The reducing sugars also increased significantly from 1.76 kg/m^3 to 2.21 kg/m^3. It has been reported by numerous workers that biomass tends to undergo hydrolysis during hydrothermal processing. Although most of the reports have used temperatures around 180 °C, hydrolysis of biomass has been documented to occur at autoclaving temperatures (Papadimitriou, 2010). The molal ionic product of water (K_w) is dependent on the temperature of the system. K_w