Industrial Crops and Products 59 (2014) 228–233

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Comparative degradation of hydrothermal pretreated winery grape wastes by various fungi

Avinash V. Karpe, Ian H. Harding, Enzo A. Palombo*

Department of Chemistry and Biotechnology, Faculty of Science, Engineering and Technology, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia

ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form 14 April 2014
Accepted 18 May 2014
Available online 5 June 2014

Keywords:
Hydrothermal pre-treatment
Lignins
Sugars
Cellulase
β-glucosidase
Xylanase

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.

© 2014 Elsevier B.V. All rights reserved.

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.

* Corresponding author. Tel.: +61 3 9214 8571.
E-mail addresses: akarpe@swin.edu.au (A.V. Karpe), iharding@swin.edu.au (I.H. Harding), epalombo@swin.edu.au (E.A. Palombo).

http://dx.doi.org/10.1016/j.indcrop.2014.05.024
0926-6690/© 2014 Elsevier B.V. All rights reserved.

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

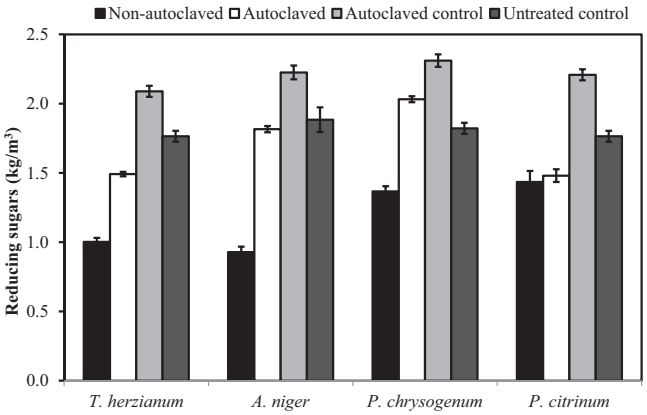

Fig. 1. Reducing sugar content (kg/m^3) in fungal cultures of non-autoclaved, autoclaved and untreated Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different to untreated samples ($p < 0.05$).

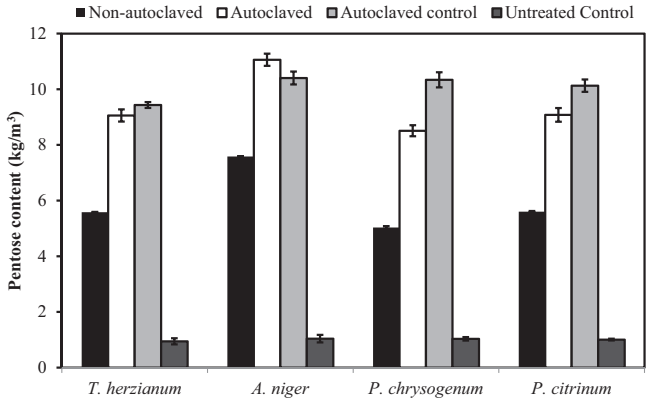

Fig. 2. Pentose sugar content (kg/m^3) in fungal cultures of non-autoclaved, autoclaved and untreated Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different to untreated samples ($p < 0.05$).

increases from 0.64×10^{-14} at 18 °C to 54.6×10^{-14} at 100 °C and 268×10^{-14} at about 150 °C. K_w reaches its highest value at about 250 °C, when it is 634×10^{-14}. This increase in the ionic product is important as it is directly proportional to the solubility of water. Therefore, the solubility of substrates (such as cellulose and hemicelluloses) increases with the rise in K_w value. This relationship is always taken into account when applied to several hydrothermal processes like hot-compressed water (HCW), steam explosion (SE) and supercritical water (SW) treatments. Therefore, temperatures around 250 °C or above are applied in these processes (Ando et al., 2000; Goto et al., 2004). As compared to HCW or SE processes, K_w value during autoclaving is nominal, but is generally sufficient to ensure a substantially high substrate solubilisation without yielding strong microbial inhibitors such as 2-furfural and 5-hydroxymethyl furfural (5-HMF) which are generated during hydrothermal treatments, especially during SE treatment (Sanchez and Bautista, 1988; Klinke et al., 2004). This allows an efficient subsequent microbial degradation without requiring the multiple rinsing of substrate with deionised water to remove these inhibitors, thereby saving important time and resources.

3.2. Utilization of reducing sugars and pentoses

Reducing sugars such as glucose are the primary carbon sources for microbial growth. Cellulose is the most abundant polysaccharide and makes up the basic structure of plant cell walls. It is an almost linear molecule made up of the reducing sugar β-D-glucopyranose, linked by β-1, 4-polyanhydroglucose with cellobiose (also a reducing sugar) as the smallest repetitive unit, thus is a β-glucan (Fengel, 1971).

It was observed that the process of autoclaving released considerable amounts free sugars in the filtrate. The final sugar concentration suggests complete utilization of these sugars by growing fungi. The subsequent degrading process of cellulose and hemicelluloses generated further free sugars (2.2 kg/m^3). Noticeable amounts of these sugars were then consumed by fungi during their growth. It was observed that substantial amounts of reducing sugars accumulated in the autoclaved samples. The highest amount of sugar utilization was observed for *T. herzianum* and *P. citrinum* cultures, where the concentration of reducing sugars decreased from 2.2 kg/m^3 to 1 kg/m^3 and 1.5 kg/m^3, respectively (a decrease of 1.2 kg/m^3 and 0.7 kg/m^3, respectively). *A. niger* also showed noticeable degradation amounting to about 0.4 kg/m^3 utilization as the reducing sugar concentration decreased from 2.2 kg/m^3 to 1.8 kg/m^3 (Fig. 1), whereas *P. chrysogenum* did not show significant utilization. The fungal degraded non-autoclaved grapes accumulated lower amounts of reducing sugars, ranging from 0.9 kg/m^3 (*A. niger*) to 1.4 kg/m^3 (*P. citrinum*). Noticeable amounts of sugars were utilized by *T. harzianum* as the sugar concentration decreased by 0.8 kg/m^3 (from 1.8 kg/m^3). A similar result was seen in the *A. niger* culture, where the reducing sugars decreased to 0.9 kg/m^3 from 1.9 kg/m^3 (untreated control). Contrasting to this, *P. chrysogenum* and *P. citrinum* cultures both accumulated 1.4 kg/m^3 sugars, decreasing from 1.8 kg/m^3 in untreated control. They were able to utilize only 0.4 kg/m^3 of sugars. Overall, proportionally higher amounts of reducing sugars were observed to be utilized in autoclaved substrate as compared to non-autoclaved substrate.

It was observed (Fig. 2) that high amounts of pentose sugars accumulate in the medium due to the process of autoclaving. The pentose sugar content of non-autoclaved substrate cultured with *T. harzianum*, *A. niger*, *P. chrysogenum* and *P. citrinum* was 5.6 kg/m^3, 7.6 kg/m^3, 5.1 kg/m^3 and 5.6 kg/m^3, respectively. These values increased significantly in the autoclaved samples where the pentose content increased to 9.1 kg/m^3, 11.1 kg/m^3, 8.5 kg/m^3 and 9.1 kg/m^3 in the same order. This possibly indicates higher hemicellulosic degradation in autoclaved samples as also reflected in the higher xylanase activities in those substrates as compared to the non-autoclaved substrates (see below).

Hydrothermal heating weakens the lignin crosslinks within cellulose and hemicellulose, thus exposing them to physical, chemical or biological degradation (Hassan et al., 2013; Papadimitriou, 2010). Further, the hydrothermal process, as it occurs in an autoclave, enhances the breakdown of hemicellulosic polymers to form its constituent pentose and hexose sugars in addition to other derived products such as galacturonic and/or glucuronic acids. The highly heterogeneous composition of hemicelluloses makes them more susceptible to physical and chemical reactions compared to cellulose. The noticeable content of hexoses and minor amount of heptoses might explain the extraordinary utilization of reducing sugars in *T. harzianum* and *P. citrinum* cultures (Hassan et al., 2013; Papadimitriou, 2010).

The initial low pH (5.6) probably intensified the degradation process of hemicelluloses and cellulose in both cultures (Hassan et al., 2013; Takashima and Tanaka, 2008). The further release of sugars and their ultimate utilization explains the degradation ability of the fungal cells. Additionally, *T. harzianum*, *A. niger* and *P. citrinum* are known to produce a significant amount of hemicellulose-hydrolyzing enzymes such as xylanases, arabinases and pectinases among many others (Brink and Vries, 2011; Kumar et al., 2008).

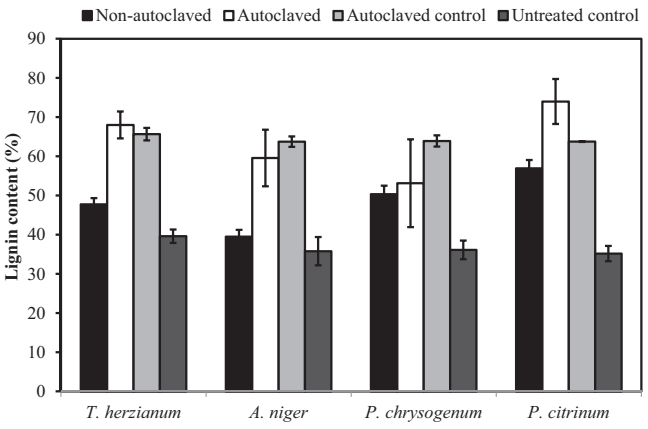

Fig. 3. Lignin content (%) in fungal cultures of non-autoclaved, autoclaved and untreated Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different to untreated samples ($p < 0.05$).

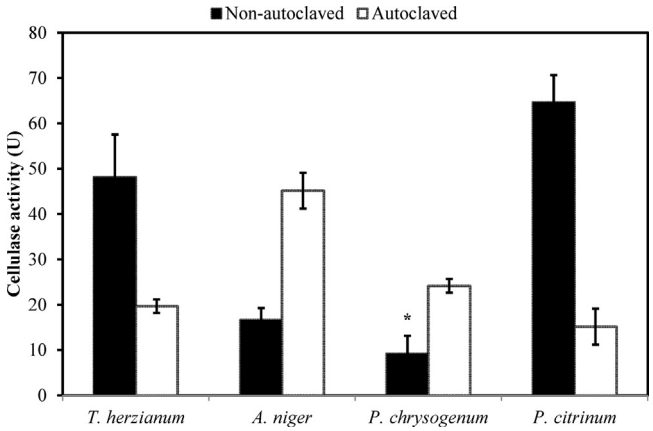

Fig. 4. Cellulase activity (U) of the fungal cultures in non-autoclaved and autoclaved Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different between the samples ($p \leq 0.05$), except for the value marked with an asterisk.

3.3. Change in the lignin content

Lignins are abundant biopolymers, second only to cellulose. In most plants, especially, in higher plants, they function as the prominent components of xylem. They are composed of hydroxycinnamyl alcohols i.e. p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, and their methoxy derivatives. Due to their hydrophobic nature, they aid in efficient water transport throughout the plant system. However, this very nature also makes them tolerant towards biodegradation (Boerjan et al., 2003; Vanholme et al., 2010). Lignins not only act as the physical barriers to microbial degradation, but also as non-productive binders of cellulases, thus acting as cellulase inhibitors (Duarte et al., 2012b)

The total lignin (Acid Soluble Lignin and Acid Insoluble Lignin) was observed to increase in every sample when compared to the untreated control (Fig. 3). This was expected since *T. harzianum* and *A. niger* do not have the ability to degrade lignin. *Penicillium* sp. also has limited ability to mineralize lignin (Rodríguez et al., 1994).

The process of autoclaving was observed to degrade considerable amounts of sugars, thereby substantially increasing the lignin content of the substrate from an average of about 36% in untreated control to about 63% in autoclaved substrate. Subsequent fungal growth consumed the sugars, but their inability to mineralize lignin resulted in further lignin accumulation in the growth media. This increase was especially noticeable in *T. harzianum* and *P. citrinum*, where the lignin content spiked from 65.7% to 68% and 63.8% to 74%, respectively, thus, showing inability of these fungi to degrade lignin. However, interestingly, *P. chrysogenum* was able to mineralize some lignin in autoclaved substrate as total lignin in the culture showed a decrease of about 9%, from 64% to 53.1%. On the other hand, the non-autoclaved substrate from the *P. chrysogenum* culture displayed an increase of about 14% lignin from 36.1% to 50.3%, thus, suggesting the inability of this fungus to degrade lignin in non-pretreated substrates.

Similar changes were observed with autoclaved substrate cultured with *A. niger*. Similar to *P. chrysogenum*, a decrease in lignin content by 4.2% (from 63.7% to 59.6%) was observed in this culture. This was unexpected since *A. niger* has not been reported to have any lignin degrading properties. The observation encourages further study on the degradation pattern of *A. niger* on winery wastes. One of the other interesting observations was the limited increase in lignin content in non-autoclaved substrate cultured with *A. niger*. In this culture, the lignin content rose slightly from 35.8% in the untreated control to 39.5% in the non-autoclaved sample. One of the reasons for this might be a lower cellulase activity of *A. niger* (Fig. 4), which resulted in minimal substrate degradation, thereby, almost conserving the original lignin composition.

Although all fungi used in this study generated substantial amounts of cellulases and hemicellulases, all of them except *P. chrysogenum* do not have the ability to synthesize lignases. *P. chrysogenum*, however, generates lignases which degrade the low molecular weight lignin components such as cinnamic acid, ferulic acid and vanillic acid (Rodríguez et al., 1994). In the case of *A. niger*, the fungus might possess minor lignin mineralization ability for its major substrates, cellulose and hemicellulose. Indeed, there are little data available regarding the quantification of lignin degradation/transformation by these fungi on wooden substrates (Hassan et al., 2013; Singh Arora and Kumar Sharma, 2010). To our knowledge, the current study is the first to investigate the degradation of the lignin component of winery biomass waste.

3.4. Enzyme activities

Cellulose is the main source of carbon in lignocellulose complexes, as it is the richest source of glucose, the primary source of carbon for most microorganisms. However, cellulose exists in the core of lignocellulosic complexes, surrounded by hemicellulose and lignins. To achieve access to cellulose, fungi have to penetrate through these layers (Fengel, 1971). Aerobic ascomycetes such as *T. harzianum* and *Aspergillus* sp. have cellulases composed of endoglucanases and exoglucanases, reportedly of varied individual enzyme composition depending on substrate availability and specificity (Kovacs et al., 2009; Liu et al., 2012; Sipos et al., 2010). Cellulase activity is thus effectively measured by enzyme activity (Ghose, 1987).

The highest cellulase activity of about 45 U was observed in the *A. niger* culture, significantly higher than the enzyme activities of 19.6 U, 24.1 U and 15.1 U observed in *T. harzianum*, *P. chrysogenum* and *P. citrinum* cultures, respectively (Fig. 4). This is in contrast to reportedly higher activities in *Trichoderma* with respect to *Aspergillus* (Kovacs et al., 2009; Liu et al., 2012). One of the reasons for this might be the limited requirement for enzyme production due to the availability of large amounts of free sugars which result from the process of autoclaving the substrate. Another reason might be significant product inhibition of the fungal cellulases by free sugars produced, most of which are known to be product inhibitors of cellulolytic enzymes (Brink and Vries, 2011; Duarte et al., 2012a).

The cellulase activities of the non-autoclaved samples were found to be 48.1 U, 16.6 U, 9.2 U and 64.6 U in *T. harzianum*, *A. niger*, *P. chrysogenum* and *P. citrinum* cultures, respectively. Most

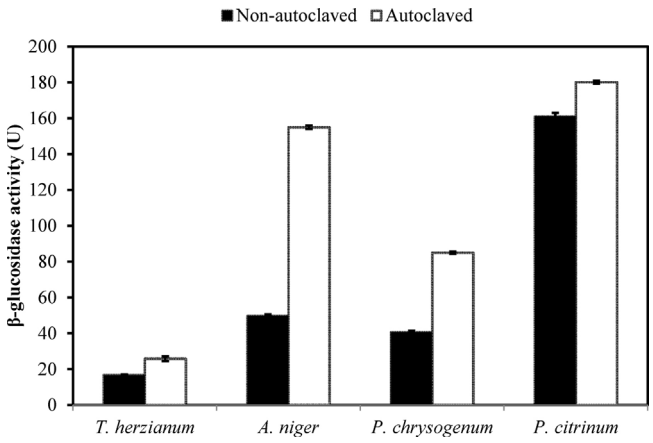

Fig. 5. β-glucosidase activity (*U*) of the fungal cultures in non-autoclaved and autoclaved Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different between the samples ($p < 0.05$).

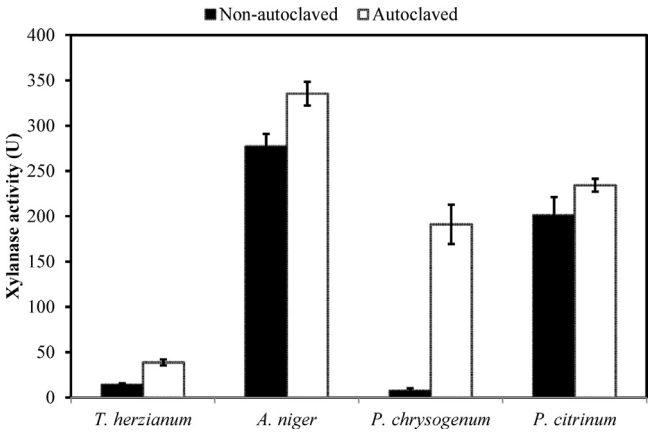

Fig. 6. Xylanase activity (*U*) of the fungal cultures in non-autoclaved and autoclaved Shiraz grape waste. Values for non-autoclaved and autoclaved samples are significantly different between the samples ($p < 0.05$).

contrasting differences of activities were seen in *T. harzianum* and *P. citrinum* cultures where the cellulase activities were about 3–4 times higher in non-autoclaved samples as compared to autoclaved samples (Fig. 4). However, this was expected in *T. harzianum* cultures as the species is known (and was observed) to have a higher cellulase activity, but very low β-glucosidase activity. This resulted in product inhibition of *Trichoderma* cellulase by glucose and cellobiose thus lowering the activity, as seen in the autoclaved samples, which had considerable amounts of those free sugars. However, the considerable difference of cellulase activity in the *P. citrinum* culture is more difficult to interpret, since it displayed higher β-glucosidase and xylanase activities in autoclaved and non-autoclaved substrates. Higher cellulase activity was observed in autoclaved substrate further degraded by *A. niger*. This was expected since the species, although having comparatively lower cellulase activity, possesses high β-glucosidase activity which prevented product inhibition of its cellulase enzyme.

Product inhibition is a well-known phenomenon which contributes to the difficulties observed in lignocellulose degradation. It has been reported by numerous authors, especially in relation to cellulose degradation, that cellobiose (the chief product of cellulose degradation) and glucose (the minor product) act as inhibitors for cellulases. The main reason for this is the exceptionally low catalytic activity of β-glucosidase as compared to the other enzymes present in the cellulase enzyme complex (Duarte et al., 2012a; Kovacs et al., 2009; Liu et al., 2012; Sipos et al., 2010).

The activity and secreted amount of β-glucosidase is also one of the important contributing factors towards the cellulase activities. β-glucosidase hydrolyses the β-(1-6) glycosidic bonds in cellobiose (the smallest functional unit of the cellulose molecule) to glucose units which can be readily utilized by chemical or microbial actions (Brink and Vries, 2011; Kim et al., 2012). However, numerous previously reported works and our own findings suggested very low activity (26 U) of these enzymes in *Trichoderma* sp. (Baldrian and Valášková, 2008; Juhász et al., 2005; Kovacs et al., 2009) as compared to other fungi (Fig. 5), especially *A. niger* (Singhania, 2012), which displayed enzyme activity of 155 U in our work. This might explain the overall lower cellulase activity in the *T. harzianum* culture. *Penicillium* cultures also displayed significant β-glucosidase activities, which probably resulted in their overall high cellulolytic activities (Fig. 5).

The β-glucosidase activity of *T. harzianum* from the non-autoclaved substrate (16.8 U) did not vary greatly from the autoclaved substrate, although, it showed a decrease of about 9 U over the fermentation period. Similar results were seen in the *P. citrinum* culture where β-glucosidase activity in non-autoclaved substrate was 160.9 U as compared to 180.1 U in autoclaved substrate. However, considerably lower activities were found in the non-autoclaved substrates cultured with *A. niger* and *P. chrysogenum*. The β-glucosidase activity of the autoclaved substrate cultured with *A. niger* was 154.9 U, which was more than 3 times the value of the non-autoclaved substrate (49.6 U). Similarly, the activity in the *P. chrysogenum* culture reduced from 84.9 U in autoclaved substrate to 40.6 U in non-autoclaved substrate, a greater than two-fold decrease in activity. Overall, the higher β-glucosidase activity in autoclaved substrate as compared to non-autoclaved substrate indicates the hydrolyzing of a major part of cellulose and hemicelluloses to disaccharides such as cellobiose, which is the substrate for enzymes such as β-glucosidase (Fig. 5).

Xylanases are a group of differential hemicellulases responsible for the degradation of various xylans by hydrolysing their β-(1, 4) linked D-xylopyranoside units. Comprising up to 1% of total fungal lignocellulolytic enzymes, xylanases may work symbiotically with other hemicellulases and cellulases, thereby facilitating a consortial mix for degradation of biomass substrates (Dashtban et al., 2009; Sweeney and Xu, 2012).

Xylanase activities (Fig. 6) across all the fungi were lower than expected. The highest activity of 335 U was found in *A. niger* followed by *P. citrinum* and *P. chrysogenum* at 234 U and 191.1 U, respectively (Fig. 6). *T. harzianum*, by contrast, displayed a lower enzyme activity of 38.7 U. The lower activity of xylanase reflects the presence of very low amounts of hemicellulose substrates in the medium following thermal hydrolysis. The xylanase activities in non-autoclaved substrates were lower than that of the autoclaved substrate. The enzyme activities of 13.8 U, 277.3 U and 201.1 U were observed in *T. harzianum*, *A. niger* and *P. citrinum* non-autoclaved cultures, respectively. The most significant decrease was seen in *P. chrysogenum*, where the xylanase activity decreased dramatically from 191.1 U in autoclaved substrate to 7.2 U in non-autoclaved substrate. The activities reported here are significantly lower than those reported previously (Betini et al., 2009; Hideno et al., 2011). The higher activity of xylanase in autoclaved substrate indicates a possible hydrolyzing of hemicelluloses to oligomers rather than mono-saccharides, which acted as the substrates to xylanases.

4. Conclusions

In this study, degradation of winery discarded grape waste was assessed after hydrothermal treatment, followed by further treatment with *T. harzianum, A. niger, P. chrysogenum* or *P. citrinum*. Variation in the percent composition of cellulose, hemicelluloses

and lignin in the grape waste was determined by various assays after an incubation period of 5 days. Significant amounts of reducing sugars and pentoses were secreted in the medium after the hydrothermal treatment, presumably resulting from the partial and/or total degradation of cellulose and hemicelluloses, respectively. Subsequent fungal treatments not only utilized the free sugars in the medium, but also enhanced further degradation of the grape waste. The total lignin was noticeably mineralized by *P. chrysogenum* and to a minor level by *A. niger* over the incubation period. The other fungi did not show any lignin degrading abilities. *A. niger* displayed substantial xylanase, β-glucosidase and xylanase activities, marginally higher than *P. citrinum* except for β-glucosidase. Although *T. harzianum* showed comparable cellulase activities, it displayed lower xylanase and β-glucosidase activities with respect to other fungi. Overall, a significant increase in the efficiency of fungal enzyme activities and subsequent degradation of winery waste was observed after hydrothermal treatment compared to that seen in a normal fermentation process.

Acknowledgements

The authors would like to thank Dr. Jacqui McRae, The Australian Wine Research Institute, South Australia for providing the winery grape waste and Dr. Mary Cole, Agpath Pty. Ltd., Victoria for providing the fungal cultures. The authors also acknowledge the contribution of the Faculty of Science, Engineering and Technology, Swinburne University of Technology, for the provision of finances and resources towards this research.

References

Ando, H., Sakaki, T., Kokusho, T., Shibata, M., Uemura, Y., Hatate, Y., 2000. Decomposition behavior of plant biomass in hot-compressed water. Ind. Eng. Chem. Res. 39, 3688–3693.

Arvanitoyannis, I.S., Ladas, D., Mavromatis, A., 2006. Potential uses and applications of treated wine waste: a review. Int. J. Food. Sci. Tech. 41, 475–487.

Baldrian, P., Valášková, V., 2008. Degradation of cellulose by basidiomycetous fungi. Fems Microbiol. Rev. 32, 501–521.

Betini, J.H.A., Michelin, M., Peixoto-Nogueira, S.C., Jorge, J.A., Terenzi, H.F., Polizeli, M.L.T.M., 2009. Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioproc. Biosyst. Eng. 32, 819–824.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546.

Bravo, L., Saura-Calixto, F., 1998. Characterization of dietary fiber and the in vitro indigestible fraction of grape pomace. Am. J. Enol. Viticult. 49, 135–141.

Brink, J., Vries, R., 2011. Fungal enzyme sets for plant polysaccharide degradation. Appl. Microbiol. Biot. 91, 1477–1492.

Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int. J. Biol. Sci. 5, 578–595.

Duarte, G., Moreira, L., Gómez-Mendoza, D., de Siqueira, F.G., Batista, L., Amaral, L., et al., 2012a. Use of residual biomass from the textile industry as carbon source for production of a low-molecular-weight xylanase from *Aspergillus oryzae*. Appl. Sci. 2, 754–772.

Duarte, G.C., Moreira, L.R.S., Jaramillo, P.M.D., Filho, E.X., 2012b. Biomass-derived inhibitors of holocellulases. Bioenerg. Res. 5, 768–777.

Fengel, D., 1971. Ideas on the ultrastructural organization of the cell wall components. J. Polym. Sci. Pol. Sym. 36, 383–392.

Ghose, T., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

González-Centeno, M., Rosselló, C., Simal, S., Garau, M., López, F., Femenia, A., 2010. Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. Lwt-Food Sci. Technol. 43, 1580–1586.

Goto, M., Obuchi, R., Hirose, T., Sakaki, T., Shibata, M., 2004. Hydrothermal conversion of municipal organic waste into resources. Bioresour. Technol. 93, 279–284.

Hassan, O., Ling, T.P., Maskat, M.Y., Illias, R.M., Badri, K., Jahim, J., et al., 2013. Optimization of pretreatments for the hydrolysis of oil palm empty fruit bunch fiber (EFBF) using enzyme mixtures. Biomass Bioenerg. 56, 137–146.

Hideno, A., Inoue, H., Tsukahara, K., Yano, S., Fang, X., Endo, T., et al., 2011. Production and characterization of cellulases and hemicellulases by Acremonium cellulolyticus using rice straw subjected to various pretreatments as the carbon source. Enzyme Microb. Tech. 48, 162–168.

Juhász, T., Szengyel, Z., Réczey, K., Siika-Aho, M., Viikari, L., 2005. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem. 40, 3519–3525.

Kim, E., Choi, H., Kang, S., Song, K., Han, S., Park, C., et al., 2012. Enhanced production of cellobiose dehydrogenase and β-glucosidase by Phanerochaete chrysosporium. Korean J. Chem. Eng. 29, 77–81.

Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26.

Kovacs, K., Macrelli, S., Szakacs, G., Zacchi, G., 2009. Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with *Trichoderma atroviride* enzymes produced in-house. Biotechnol. Biofuels. 2, 14.

Kumar, R., Singh, S., Singh, O.V., 2008. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. J. Ind. Microbiol. Biot. 35, 377–391.

Liu, H.-Q., Feng, Y., Zhao, D.-Q., Jiang, J.-X., 2012. Evaluation of cellulases produced from four fungi cultured on furfural residues and microcrystalline cellulose. Biodegradation 23, 465–472.

Papadimitriou, E., 2010. Hydrolysis of organic matter during autoclaving of commingled household waste. Waste Manage. 30, 572–582.

Ping, L., Brosse, N., Sannigrahi, P., Ragauskas, A., 2011. Evaluation of grape stalks as a bioresource. Ind. Crop. Prod. 33, 200–204.

Plummer, D.T., 1987. An introduction to practical biochemistry, 3 ed. McGraw-Hill Book Company, London.

Pramod, S.N., Venkatesh, Y.P., 2006. Utility of pentose colorimetric assay for the purification of potato lectin, an arabinose-rich glycoprotein. Glycoconj. J. 23, 481–488.

Rodríguez, A., Carnicero, A., Perestelo, F., de la Fuente, G., Milstein, O., Falcón, M.A., 1994. Effect of *Penicillium chrysogenum* on lignin transformation. Appl. Environ. Microb. 60, 2971–2976.

Sanchez, B., Bautista, J., 1988. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. Enzyme Microb. Technol. 10, 315–318.

Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnol. Adv. 27, 185–194.

Singh Arora, D., Sharma, R.K., 2010. Ligninolytic fungal laccases and their biotechnological applications. Appl. Biochem. Biotech. 160, 1760–1788.

Singhania, R.R., 2012. Beta-Glucosidase from Aspergillus niger NII 08121-Molecular characterization and applications in Bioethanol production. Cochin University of Science and Technology, Cochin, India.

Sipos, B., Benkő, Z., Dienes, D., Réczey, K., Viikari, L., Siika-aho, M., 2010. Characterisation of specific activities and hydrolytic properties of cell-wall-degrading enzymes produced by Trichoderma reesei Rut C30 on different carbon sources. Appl. Biochem. Biotech. 161, 347–364.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., et al., 2011. Determination of structural carbohydrates and lignin biomass. National Renewable Energy Laboratory, 1–18.

Strong, P.J., Burgess, J.E., 2008. Treatment methods for wine-related and distillery wastewaters: A Review. Biorem. J. 12, 70–87.

Sweeney, M.D., Xu, F., 2012. Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2, 244–263.

Takashima, M., Tanaka, Y., 2008. Comparison of thermo-oxidative treatments for the anaerobic digestion of sewage sludge. J. Chem. Technol. Biot. 83, 637–642.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W., 2010. Lignin biosynthesis and structure. Plant Physiol. 153, 895–905.

Vicens, A., Fournand, D., Williams, P., Sidhoum, L., Moutounet, M., Doco, T., 2009. Changes in polysaccharide and protein composition of cell walls in grape berry skin (Cv. Shiraz) during ripening and over-ripening. J. Agr. Food Chem. 57, 2955–2960.