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Evaluation of Macerating Pectinase Enzyme Activity under Various Temperature, pH and Ethanol Regimes

Andrew G. Reynolds *, Anthony Knox and Frederick Di Profio

Cool Climate Oenology and Viticulture Institute (CCOVI), Brock University, St. Catharines, ON L2S 3A1, Canada; ajknox55mail@gmail.com (A.K.); fdiprofio@yahoo.ca (F.D.P.)

* Correspondence: areynolds@brocku.ca; Tel.: +1-905-688-5550 (ext. 3131)

Academic Editors: Matteo Marangon and James Harbertson

Received: 29 September 2017; Accepted: 13 January 2018; Published: 1 February 2018

Abstract: The polygalacturonase (PGU), hemicellulase (mannanase) and protease enzyme activities in commercial macerating, pectinase-enzyme preparations commonly used by wineries in Ontario (Scottzyme Color X and Color Pro) were measured under various simulated process conditions (temperature, pH, and ethanol concentration). Treatments included three temperatures (15, 20 and 30 °C; pH = 3.0, 3.5, 4.0 and 5.0; ethanol = 0%), four pH levels (3.0, 3.5, 4.0 and 5.0; temperature = 15, 20, 30 and 50 °C; ethanol = 0%), and four ethanol concentrations ((2.5, 5, 7.5 and 10%); temperature = 20 °C and pH = 3.5.) Polygalacturonase enzyme activity in Color X increased linearly with temperature at all pH levels, and increased with pH at all temperature regimes. Polygalacturonase activity decreased with increasing ethanol. Color X mannanase activity increased with temperatures between 15 and 40 °C, and decreased with increased pH between 3.0 and 5.0. Response of mannanase to ethanol was cubic with a sharp decrease between 8 and 10% ethanol. Protease activity increased linearly with temperatures between 20 and 40 °C. These data suggest that the PGU, mannanase and protease components in these enzyme products provide sufficient activities within the ranges of pH, temperature, and ethanol common during the initial stages of red wine fermentations, although low must temperatures (<20 °C) and presence of ethanol would likely lead to sub-optimal enzyme activities.

Keywords: red wine; polygalacturonase; hemicellulase; mannanase; protease

1. Introduction

Enzymes have been widely used for decades in the beverage industry to enhance clarification, color extraction, and overall juice yield, including applications on apple [1], cherry [2], currant [3], kiwifruit [4,5], and raspberry [6]. Use of enzymes is likewise common for red wine making, primarily for enhancement of color and phenolic extraction [7]. Early trials in California indicated that pre-fermentation use of pectinases on red wine grapes led to enhanced wine color and phenols [8,9]. Two enzymes, Scottzyme Color X and Color Pro (manufactured by Lyven, Colombelles, France; http://www.lyven.com/; distributed by Scott Laboratories, Pickering, ON, Canada), were assessed for extraction efficacy in terms of anthocyanins and phenolic compounds in Merlot, Cabernet Franc, and Cabernet Sauvignon over a three-year period [10]. The efficacy of both enzymes was variable and highly dependent upon cultivar and season. In Merlot, enzyme treatments increased concentrations of five phenols, and Color X increased epicatechin relative to the control, but most acylated and non-acylated pigments diminished in enzyme-treated wines, with the exception of cyanidin (both enzymes) and peonidin coumaryl glucoside (Color Pro only). In Cabernet Franc, enzyme treatments exceeded an untreated control for several phenols, three non-acylated pigments, and three acylated pigments, but had lower delphinidin coumarate. The following year, enzyme treatments were not particularly effective other than increases in caffeic and *p*-coumaric acids plus

petunidin coumarate (both enzymes) and delphinidin coumarate (Color X). However, in a third season, both enzymes increased gallic acid, catechin, epicatechin, and quercetin, and decreased *p*-coumaric acid. Color Pro increased all anthocyanin monoglucosides and most acylated anthocyanins (acetates and coumarates). Color X increased petunidin, peonidin and malvidin plus numerous acylated anthocyanins. In Cabernet Sauvignon, enzyme treatments were less efficacious than several viticultural treatments. Color Pro increased gallic acid and both enzymes increased catechin, epicatechin, and caffeic acid as well as two coumarates. The following season, enzyme treatments had potentially negative effects: e.g., although catechin increased in Color X-treated wines, caffeic acid and *p*-coumaric acid, peonidin and malvidin monoglucosides, plus two acylated compounds decreased in both enzyme treatments. Decreases also occurred in epicatechin, peonidin acetate (Color X) and malvidin coumarate (Color Pro). The only pigment increased by both enzymes was peonidin coumarate. In the third season, Color X increased concentrations of gallic acid and two coumarylated pigments.

These somewhat inconsistent observations caused us to question the efficacy of these two enzyme products, and strongly suggested that these enological enzymes, and perhaps others, have limited activity under some winemaking conditions, which include relatively low pH (<3.50), low temperatures (<10 °C pre-fermentation), and eventually, presence of ethanol. It was in this context that a series of in vitro treatments were conceived with the objective of explaining whether pH, temperature, and ethanol might significantly affect the activities of the main enzyme components of Color X and Color Pro.

Color X and Color Pro are partially purified commercial enzyme preparations, produced by culture of the fungus *Aspergillus niger* on sugar-beet solids. Color X contains a mixture of polygalacturonase (PGU; >1050 units/g), pectinmethylesterase (PME; >320 units/g), pectinlyase (>62 units/g) and hemicellulases (640 units/g), while Color Pro contains a mixture of PGU (>1000 units/g), PME (>170 units/g), pectinlyase (>25 units/g), and proteases (>120 units/g) (Lyven, pers. comm. 2005).

PGU (E.C. 3.2.1.15) is one of many pectic enzymes (pectinases) that cleave glycosidic bonds in polygalacturonic acid residues, leading to release of oligosaccharides (oligogalacturonates) [11–13]. They are formed by several species of fungi, bacteria, and yeasts [14]. PME (E.C. 3.1.1.11) are responsible for de-esterifying pectins with the release of methanol [13,15,16]. Pectinlyases (E.C. 4.2.2.10) depolymerize pectins by cleavage of glycosidic linkages adjacent to methylated galacturonic acid residues [11–13]. Pectinases have been widely used in the beverage industry [17,18], particularly apple [19], citrus [20] and pear [21]. The wine industry has likewise made use of pectinases for increasing juice yields, clarity, color and aroma [7,22,23].

Hemicellulase is the common name for a mixture of carbohydrase enzymes derived from *Aspergillus niger* and other fungi that hydrolyze hemicellulose. A hemicellulose can be any of several heteropolymers (matrix polysaccharides) present in almost all plant cell walls along with cellulose [24]. In contrast to cellulose, which is crystalline, strong, and resistant to enzymatic hydrolysis, hemicellulose has a random, amorphous structure, little strength and is easily hydrolyzed by hemicellulase enzymes [25]. The carbohydrase enzymes in fungal hemicellulase mixtures are: α-L-arabinofuranoside arabinofuranohydrolase (3.2.1.55); β-1,4-D-mannan mannanohydrolase (3.2.1.78); β-1,3-D-xylan xylanohyrolase (3.2.1.32); α-1,5-L-arabinan arabinanohydrolase (3.2.1.99) [26]. On suitable hemicellulose substrates, the products of hydrolysis of these enzymes are oligosaccharides and simple hexose and pentose monosaccharides [27]. For example, the extended reaction of β-1,4-D mannan mannanohydrolase enzyme with a suitable glycomannan polysaccharide substrate such as locust bean gum results in the formation of oligosaccharides and monosaccharides D-mannose and D-galactose [28]. D-mannose is an aldohexose sugar similar to D-glucose and D-galactose. β-1,3-D-xylan xylanohydrolase incubated with a suitable polysaccharide substrate such as wheat bran results in the production of oligosaccharides and the five-carbon monosaccharide, D-xylose [29].

Proteases are a large group of enzymes sourced from fungi, bacteria, viruses, plants and vertebrates [30,31] that perform proteolysis, i.e., protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain [31,32]. Different classes of protease can

perform the same reaction by completely different catalytic mechanisms. They have been widely used in the beverage industry including those used for reduction of turbidity in beer [31], fruit juice [3–5,33], whey-based beverages [34], as well as wines [35,36]. In addition to haze elimination, it has been suggested that proteases also act to increase yeast-assimilable nitrogen and as aroma enhancers by increasing concentrations of volatile phenols [36–38].

There have been frequent questions with respect to the efficacy of various enzymatic components within commercial enzyme products under typical winemaking conditions; i.e., relatively low pH (2.80 to 3.50), low temperatures (<15 °C), and presence of ethanol. It is possible that slight modifications to temperature and must pH might optimize the activity of these enzymes under industrial conditions. The PGU, hemicellulase, and protease enzyme activities in two commercial macerating, pectinase-enzyme preparation commonly used by wineries in the Niagara region (Color X, Color Pro) were measured under a range of various treatments that included typical processing conditions (pH, temperature and alcohol concentration). The objective of this study was to evaluate the efficacy the main enzymatic components of these commercial macerating pectinase enzyme preparations used for potential release of grape varietal, aroma-precursor and color compounds during red wine fermentation under a series of temperature, pH, and ethanol regimens.

2. Materials and Methods

2.1. Polygalacturonase

2.1.1. Assay Method and Treatments

Following validation of the Lyven assay procedure (http://www.lyven.com/), PGU enzyme activity in Color X was measured in triplicate at three temperatures (15, 20 and 30 °C; pH = 3.0, 3.5, 4.0 and 5.0; ethanol = 0%), four pH levels (3.0, 3.5, 4.0 and 5.0; temperature = 15, 20, 30 and 50 °C; ethanol = 0%) and four ethanol concentrations (2.5, 5, 7.5 and 10%; pH = 3.5, temperature = 30 °C). A modified colorimetric assay method [39,40] was used to determine PGU enzyme activity in pure samples and in enzyme products. The basic principle of this assay is as follows: Samples containing PGU activity are incubated with a polygalacturonic acid substrate. Excess, unconverted substrate is removed from the reaction mixture by centrifugation. Reducing sugars in the supernatant are oxidized by addition of an alkaline cupric solution. The copper precipitate is then converted to a chromophore by the addition of an arsenomolybdate solution. The absorbance of the resulting blue-colored solution is measured by spectrometer and the concentration of reducing sugar degradation products is proportional to the PGU activity in the sample [41]. One unit of PGU activity will release 1.0 µmole of reducing sugar measured as D-galacturonic acid from polygalacturonic acid per minute at pH 3.5 and 30 °C.

Specific conditions were temperature: 30 °C; pH: 3.5 (varied according to treatment); substrate concentration: 0.50% *w*/*v*; enzyme concentration: 0.2–1.0 unit/mL; incubation time: 40 min.; detection: spectrophotometer (Ultrospec 2100 Pro UV/VIS, Biochrom Ltd., Cambridge, UK), 10 mm pathlength cuvette, 540 nm. A standard solution containing a known amount of reducing sugar (expressed as µmoles D-galacturonic acid) was used as a control sample during analysis of samples (i.e., Color X) containing unknown PGU enzyme activity. A control sample was included at the beginning and at the end of each sample analysis series. When analyzing a large number of samples, a control sample was included with every 20 samples. The most suitable enzyme stock solution containing 0.2 to 1.0 PGU units/mL was used to determine the unknown PGU enzyme activity in the test sample. Samples containing unknown PGU enzyme activity were diluted on the basis of anticipated activity, to prepare an enzyme stock solution containing 0.2 to 1.0 PGU units/mL (PGU/mL). All reagents were obtained from Sigma (St. Louis, MO, USA).

2.1.2. Standard Curve

The absorbance (540 nm) measurements for the series of duplicate standard solutions were used to plot a standard curve, with μmoles D-galacturonic acid as the x-axis and the corresponding absorbance values as the y-axis. The data were fitted by linear regression analysis. The resulting regression equation was then used to calculate the μmoles of reducing sugar (as D-galacturonic acid) produced during the reaction that corresponded to absorbance values measured for samples containing unknown PGU activity.

Standard solutions for preparation of the standard curve were made by pipetting (μL) the volume of D-galacturonic acid stock solution and volume of sodium acetate buffer in Table S1 into 20-mL test tubes. Duplicate standard solution blanks were prepared by pipetting 2.00 mL of acetate buffer into each of 18 test tubes. Each standard solution and standard solution blank test tube was mixed for 10 s. using a Whirlmixer, and tubes were incubated at 30 °C in a water bath (Fisher Scientific, Mississauga, ON, Canada) for 40 min. Following incubation, 2.00 mL of alkaline copper reagent were pipetted into each standard solution and standard solution blank test tube. Each test tube was covered with a glass marble and placed in a boiling (100 °C) water bath for 15 min. Test tubes were thereafter removed from the bath and cooled to room temperature, after which 2.00 mL of arsenomolybdate reagent were pipetted into each test tube. Each test tube was mixed for 15 s on a Whirlmixer until the foaming stopped and all bubbles on the surface of the reaction mixture disappeared. The tubes were then centrifuged (IEC Centra CL2, International Equipment Company, Needham Heights, MA, USA) at 3000 rpm for 10 min. to clarify the reaction mixture. A suitable volume of clear supernatant was transferred to 10-mm cuvettes and the absorbance of each standard solution was read at 540 nm on an Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.1.3. Samples

Duplicate test solutions and test solution blanks for the determination of PGU enzyme activity were prepared by pipetting 1.90 mL of polygalacturonic acid solution substrate into each of four test tubes. Test and blank tubes were equilibrated at each individual temperature (15, 20 and 30 °C) for each aforementioned pH value (3.0, 3.5, 4.0 and 5.0) in a water bath for exactly 10 min. After 10 min., 0.10 mL of suitable enzyme stock solution were pipetted to duplicate test solutions, and 0.10 mL of sodium acetate buffer to test solution blanks. Each test solution and test solution blank test tube were immediately mixed for 10 s. using a Whirlmixer. Test and blank tubes were incubated at each temperature and pH in a water bath for 40 min., after which 2.00 mL of alkaline copper reagent were pipetted into each test solution and test solution blank test tube. Each test tube was covered with a glass marble and placed in a boiling (100 °C) water bath for 15 min., after which the test tubes were removed from the water bath and cooled to room temperature. When at room temperature, 2.00 mL of arsenomolybdate reagent were pipetted into each test solution and test solution blank test tube. Each test tube was mixed for 15 s using a Whirlmixer until the foaming stopped and all surface bubbles disappeared. Tubes were centrifuged at 3000 rpm for 10 min. to clarify the test solutions and test blank solutions, then 3.00 mL of clear supernatant were transferred from each of the test solutions and test blank solutions to suitable glass culture tubes containing 12.00 mL of distilled water and the tubes were mixed by inversion two times. A suitable volume of each diluted test solution and test solution blank were transferred to 10-mm cuvettes and absorbance was measured at 540 nm with the spectrophotometer. PGU activity was expressed in μmoles/mL D-galacturonic acid released per min. The procedure was thereafter repeated for four pH levels (3.0, 3.5, 4.0 and 5.0) for each of four temperatures (15, 20, 30 and 50 °C) and for four ethanol concentrations (2.5, 5, 7.5 and 10%) at pH 3.5 and 30 °C.

2.2. Hemicellulase (Mannanase)

2.2.1. Assay Method and Treatments

Following validation of the Lyven assay procedure (http://www.lyven.com/), endo-1,4-β-D-mannanase (hemicellulase) enzyme activity in Color X (Lot 3YE1303) was measured in triplicate at four temperatures (15, 20, 30 and 40 °C; pH = 4.0; ethanol = 0%), four pH levels (3.0, 3.5, 4.0 and 5.0; temperature = 40 °C; ethanol = 0%), and four ethanol concentrations (2.5, 5, 7.5 and 10%; temperature = 20 °C, pH = 3.5). Following evaluation of three commonly used hemicellulase assay procedures, a modified Nelson–Somogyi reducing sugar assay procedure was selected to measure the endo-1,4-β-D-mannanase enzyme activity in Color X (endo-1,4-β-D-mannanase is one of six major endo-hydrolytic enzyme activities included in the group of enzymes identified as hemicellulases). Endo-1,4-β-D-mannanase is classified as a hemicellulase enzyme because of its hydrolytic action on galactomannan polysaccharide substrates. The substrate used in the assay procedure was a 2.0% (*w*/*v*) solution of purified, high-ratio mannose to galactose, Remazol Brilliant Blue-dyed, locust bean (carob) gum (LBG-galactomannan; S-ACGLM, Megazyme, Bray, Ireland) dissolved in 0.2 M sodium acetate buffer solution (pH 4.0). β-Mannanase samples were incubated for 20 min. with the LBG, and unhydrolyzed substrate was precipitated with ethanol. The blue color of the supernatant, due to soluble blue-dyed oligosaccharide products, is proportional to the β-mannanase activity.

2.2.2. Standard Curve

Pure enzyme (E-BMANN, Megazyme, Bray, Ireland), sodium acetate buffer and LBG substrate were prepared. To validate the assay for endo-1,4-β-D-mannanase enzyme activity, two standard curves and regression equations (0 μg/mL to 350 μg/mL reducing-sugar as mannose) vs. spectrophotometer absorbance (590 nm) were prepared (Table S2).

2.2.3. Samples

Twelve assays were completed at a range of Color X dilutions between 1 and 1000 fold. Preliminary assays conducted at pH 4.0 and 30 °C demonstrated lower (≈10 units/mL) of endo-1,4-β-D-mannanase activity compared to the manufacturer's declared enzyme activity specification of 640 hemicellulase units (HCU)/g for Color X. Thereafter, 14 assays of β-mannanase enzyme activity in Color X were run. Each assay run consisted of a series of seven Color X dilutions, giving an apparent range of hemicellulase activities between 0.014 HCU/mL and 18 HCU/mL based on the manufacturer's specification of 640 HCU/g. Using the modified Nelson-Somogyi assay procedure, the β-mannanase activity in Color X was thereafter measured at pH 4.0 and at temperatures of 15, 20, 30 and 40 °C. The procedure was thereafter repeated at pH 3.0, 3.5, 4.0 and 5.0 at 40 °C and at ethanol concentrations of 2.5, 5, 7.5 and 10% at pH 3.5 and 20 °C. An additional two assays of β-mannanase enzyme activity were completed for Color X pre-treated with 2% (*w*/*v*) polyvinylpyrrolidone (PVP) to remove any potentially inhibiting phenolic compounds and thereby enhance endo-1,4-β-D-mannanase enzyme activity. β-mannanase activity was expressed in nkatals (nanomoles) reducing sugar released per second. To further investigate and to explain initial results whereby the β-mannanase activity of Color X, as measured by the modified Nelson–Somogyi reducing-sugar assay, consistently decreased with increasing enzyme concentration, assay results were compared to those of β-mannanase activity measured in another commercial *A. niger* hemicellulase enzyme preparation (Sigma H2125).

2.3. Protease

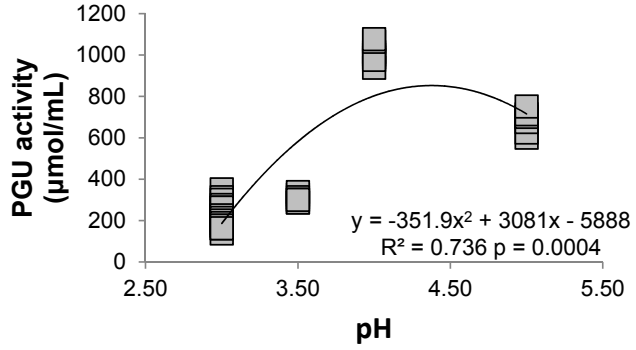
Following validation of the Lyven spectrophotometric casein assay procedure (http://www.lyven.com/), protease activity in Color Pro was measured at four temperatures (15, 20, 30 and 40 °C), combined with four pH levels (3.0, 3.5, 4.0, 5.0) and four ethanol concentrations (2.5, 5, 7.5 and 10%; temperature = 20 °C, pH = 3.5). Four protease assay procedures, including the method used by Lyven (manufacturer of Color Pro), and the widely-used hemoglobin Food Chemical Codex method,

were evaluated to determine their efficacies over a range of pH 3.0–5.0. Each assay method specified the use of either casein or hemoglobin protein substrates, four different buffer systems (phosphoric acid potassium salt, lactic acid sodium salt, acetic acid sodium salt, phthalic acid potassium salt), a range of assay conditions between pH 2.8–7.5, and temperatures between 37–40 °C. Following evaluation of the assay procedures, the Lyven method, modified to incorporate the phthalic acid potassium salt buffer system rather than a lactic acid sodium salt buffer, was selected to measure protease enzyme activity in Color Pro. Examination of the pKa values (ionization equilibrium pH) of the weak acid buffer systems used in each of the four aforementioned assay procedures ultimately suggested that the phosphoric acid potassium salt and the lactic acid sodium salt buffer systems not be used since these weak acids do not provide adequate buffering capacity at pH 3.0—the typical pH of grape juice and wine. Consequently, based on the pKa, the phthalic acid potassium salt was selected as most suitable for the protease enzyme assay in Color Pro, since it provided useful buffering capacity over a pH range of 2.2–6.2.

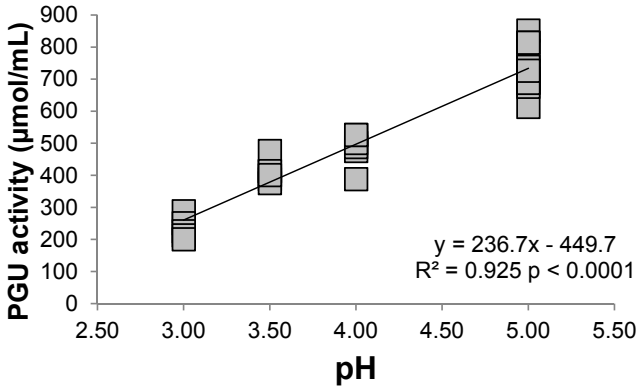
3. Results

3.1. Polygalacturonase

Increased pH led to increases in Color X PGU activity at all three temperature regimes (15, 20 and 30 °C; Figure 1). Responses at pH 3.0 were quadratic in nature while those at 20 and 30 °C were linear. Enzyme activity increased more rapidly at higher temperature regimes. Response of PGU in Color X to temperature was quadratic at pH 3.0, and linear at pH 3.5, 4.0 and 5.0, with greater increases due to temperature observed at pH 3.0 to 4.0 (Figure 2). Increased ethanol concentration in the medium led to a linear decrease in Color X PGU activity (Figure 3).

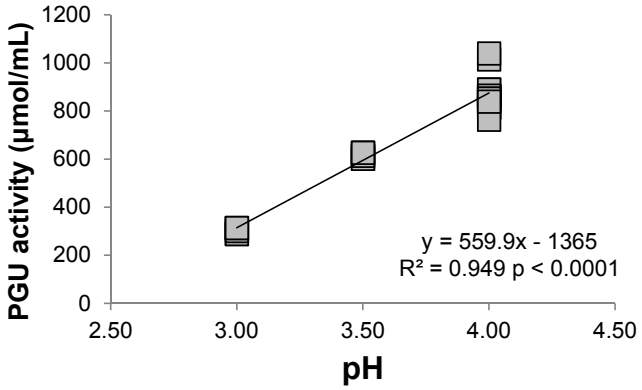


(**A**) 15 °C



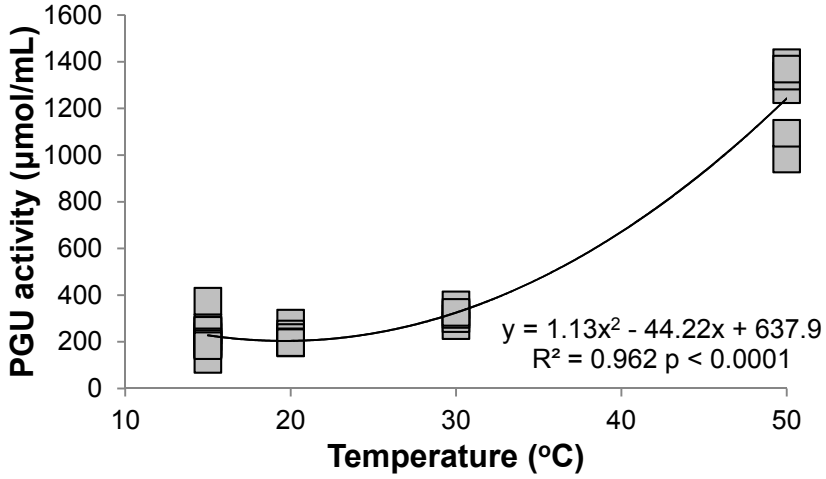
(**B**) 20 °C

Figure 1. *Cont.*

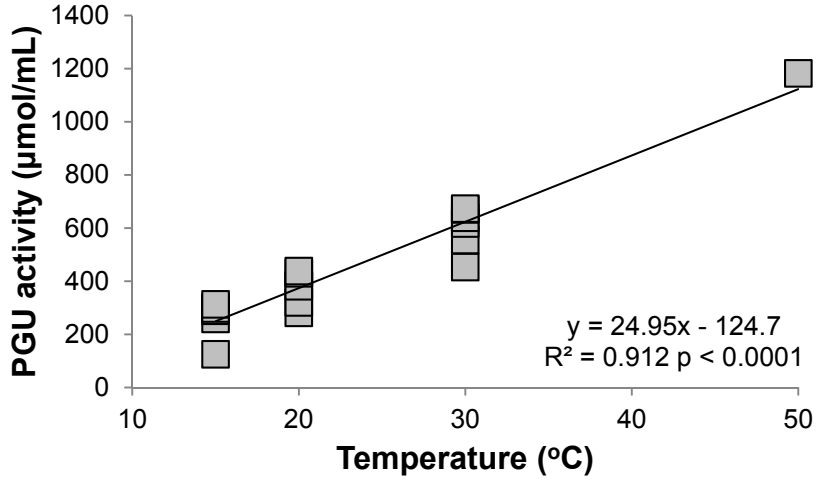


(**C**) 30 °C

Figure 1. Impact of pH on polygalacturonase (PGU) activity in Scottzyme Color X (temperature = 15, 20, 30 °C; ethanol = 0%), measured in sodium acetate buffer solution.

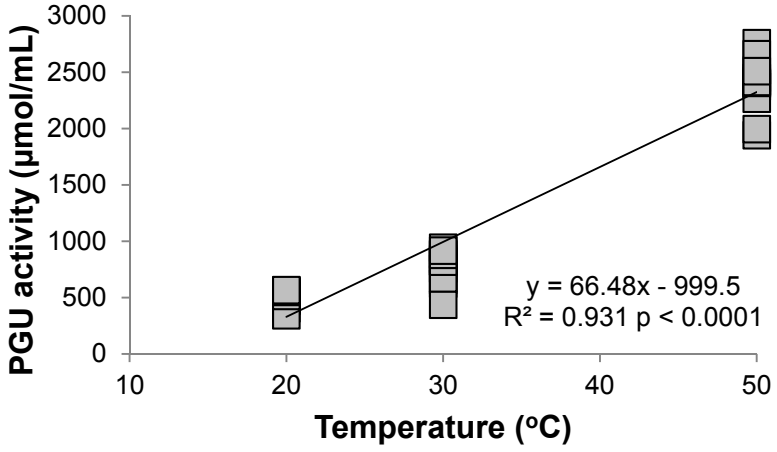


(**A**) pH = 3.0

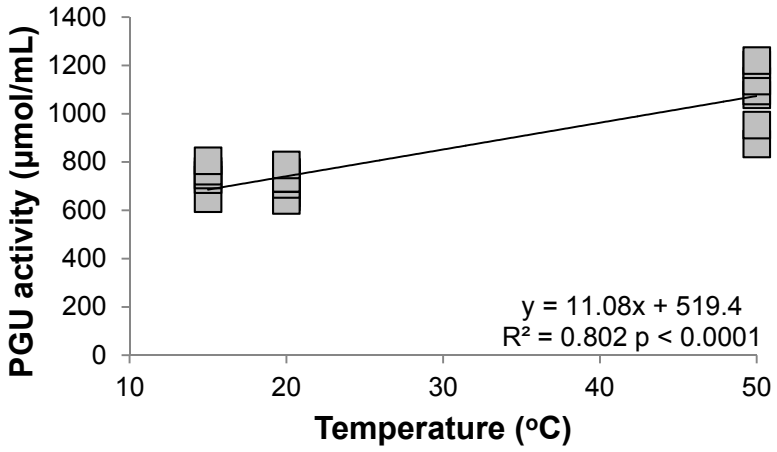


(**B**) pH = 3.5

Figure 2. *Cont*.



(**C**) pH = 4.0



(**D**) pH = 5.0

Figure 2. Impact of temperature on polygalacturonase (PGU) activity in Scottzyme Color X at four pH values; ethanol = 0%, measured in sodium acetate buffer solution.

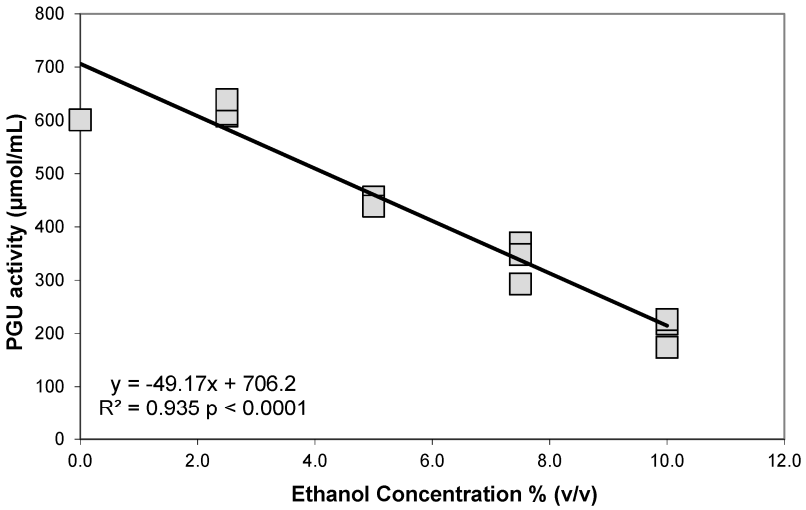


Figure 3. Impact of ethanol on polygalacturonase (PGU) activity in Scottzyme Color X (pH = 3.5; temperature = 30 °C), measured in sodium acetate buffer solution. Each PGU value associated with each individual ethanol value is based upon four determinations.

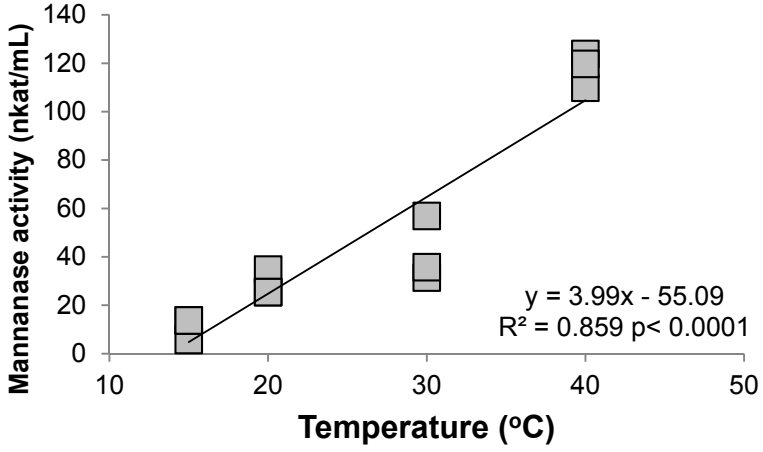
3.2. Hemicellulase (Mannanase)

All initial assay results showed decreasing endo-1,4-β-D-mannanase activity with increasing enzyme concentration (μg/mL). This suggests that the endo-1,4-β-D-mannanase enzyme activity in Color X were inhibited by unknown compounds in the liquid enzyme preparation or by the LBG substrate. Liquid pectinase enzyme preparations, derived from solid fermentation of the fungus *A. niger*, contain significant concentrations of culture by-products such as sugar residues, non-enzyme proteins and phenolic compounds released from lignocellulosic substrates. Due to the manufacturing processes, both the Color X liquid enzyme and the LBG substrate may contain enzyme-inhibiting phenolic compounds.

In PVP-treated assays, all the β-mannanase assay results, including the results of the PVP-treated Color X, showed decreasing β-mannanase activity with increasing Color X enzyme concentrations between 22.5 μg/mL and 28 mg/mL (data not shown). Overall, β-mannanase activity in the samples of Color X pre-treated with PVP was reduced by ≈40%, at pH 4.0 and 30 °C compared to β-mannanase activity measured in untreated samples of Color X at the same pH and temperature. These results suggest that the Color X enzyme preparation does not contain significant quantities of enzyme-inhibiting phenolic compounds. Furthermore, it appears that treating Color X with PVP may have bound or otherwise inactivated some of the enzyme protein.

When assayed at 15 and 30 °C, at pH 3.5, Color X exhibited ≈40 and 60% less β-mannanase activity than when assayed at 40 °C (Figure 4A). Color X β-mannanase exhibited maximum activity (82 nkat/mL) at 30 °C when assayed at pH 3.0 for 20 min. (Figure 4B). Mannanase activity relative to ethanol in the medium displayed a cubic trend with a slight increase between 2% and 8% ethanol, and thereafter a clear decrease occurred (Figure 4C). These data suggest that Color X contains significant β-mannanase enzyme activity that catalyzes the hydrolysis of soluble galactomannan polysaccharides at temperatures typically used during red wine fermentation.

Repeated measurement of the β-mannanase activity in the Sigma hemicellulase, using 0.5% (*w*/*v*) LBG-galactomannan substrate at similar pH and temperature conditions, showed consistent β-mannanase activity (12.5 nkat) over a series of 10-fold to 350-fold dilutions. When compared, the rate of reducing sugar liberation by the hemicellulase showed a direct and a linear increase with increasing enzyme concentration. Whereas, the relationship between the rate of reducing sugar liberation and the concentration of Color X, was not linear, and exhibited a decreasing rate of reducing sugar liberation with increasing enzyme concentration (data not shown). These results suggest that hydrolysis of the LBG-galactomannan substrate by the β-mannanase or by some other glycosidase-enzyme activity, in Color X, was inhibited by one or more of the reaction products.



(**A**)

Figure 4. *Cont.*

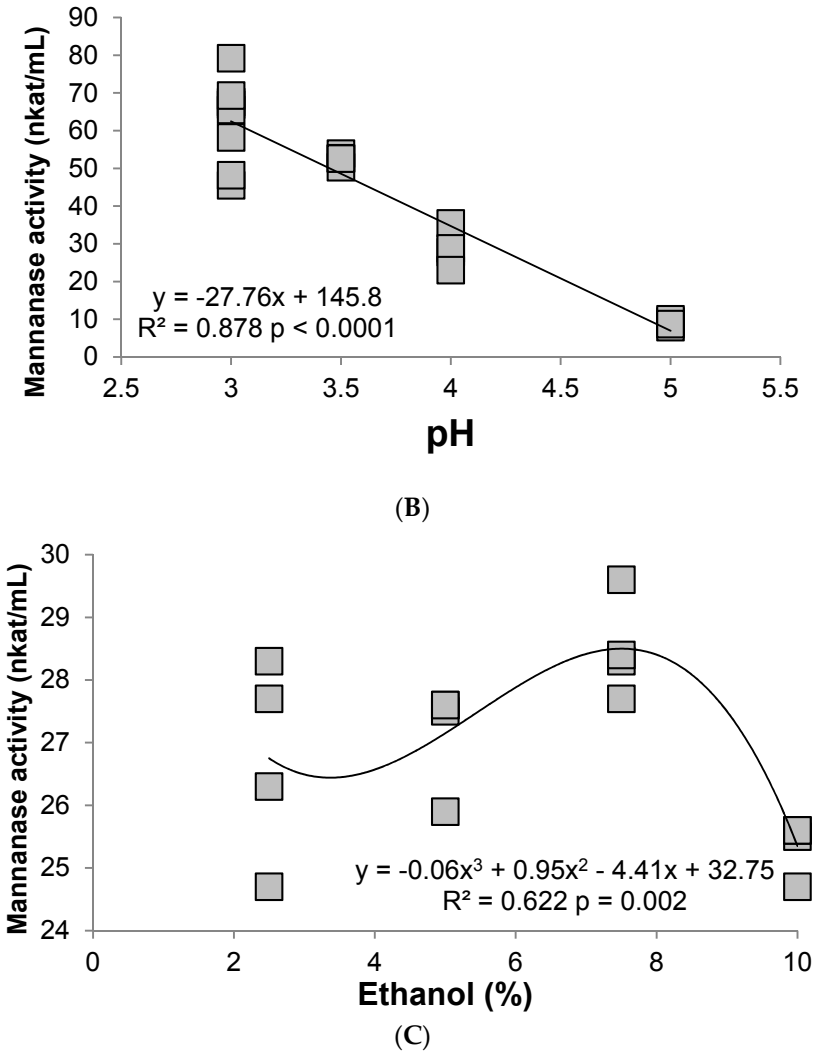


Figure 4. Scottzyme Color X β-mannanase (hemicellulase) activity on azo-carob galactomannan, measured in sodium acetate buffer solution. (**A**) Impact of temperature (pH = 3.5; ethanol = 0%); (**B**) Impact of pH (temperature = 30 °C; ethanol = 0%); (**C**) Impact of ethanol (pH = 3.5, temperature = 20 °C).

3.3. Protease

Protease activity in Color Pro was tested under various temperature regimes. An apparent linear increase in protease activity was observed in the medium between 20 and 40 °C (Figure S1). Responses to pH and ethanol were similar to those of PGU (data not shown).

4. Discussion

Enzyme products may vary considerably in terms of their PGU, hemicellulase, protease and other enzymatic components. Guerin et al. [42] demonstrated a wide range among 41 products in terms of pectolytic, cellulolytic, and hemicellulolytic activities. In this study, PGU activity in Color X was successfully measured under several temperature, pH, and ethanol regimes using a modification of the standard Lyven protocol. Enzyme activity increased linearly with pH at all temperatures (Figure 1), and increased with temperature at all pH levels (Figure 2). Polygalacturonase activity decreased with increasing ethanol. A positive response of PGU relative to temperature has been previously reported for apple [43], grape [44], mango [45], pomegranate [44] preparations. The response of PGU to pH suggested a linear function whereby highest activity occurred at pH values between 4–5

(Figure 1), while others have described parabolic functions with similar pH optima [46] and others have reported ranges of pH optima for *Aspergillus*-based PGU between 3.0 and 5.5 [47]. These responses are attributable to conformational changes in enzymes over a range of pH values that may affect substrate binding and ionization state of the individual amino acid residues. Ethanol will inhibit activities of some enzymes such as catalase [48], however, and its action was attributed to the binding of ethanol with the enzyme, thereby inhibiting the reaction. Others have shown no effect of ethanol on pectinases in fermentations, suggesting wide variability of tolerance [49].

A review of the international enzyme research database and relevant journal articles suggests that β-mannanase enzymes produced by *A. niger* and other fungi are not normally inhibited by galactose, mannose, glucose or by various oligosaccharides liberated during the hydrolysis of galactomannan, galactoglucomannan or mannan substrates [50]. However, partially purified *A. niger* enzyme preparations such as Color X, besides containing varying concentrations of hemicellulase enzyme activities (e.g., endo-1,4-β-mannanase activity), also contain varying concentrations of α-galactosidase (EC 3.2.1.22; α-D-galactoside galactohydrolase) enzyme activity depending on the *A. niger* strain and culture substrates used induce enzyme production [51]. *A. niger* α-galactosidase enzymes can hydrolyze galactomannan and galactoglucomannan polysaccharides by removing side-chain galactose sugar residues from the backbone mannan polysaccharide [52]. Literature also suggests that some *A. niger* strains can produce large quantities of three or more different α-galactosidase enzyme proteins, when grown in cultures containing galactomannan polysaccharide substrates [53]. Furthermore, these data also suggest that α-galactosidase enzymes are strongly inhibited by galactose and mannose liberated during the hydrolysis of galactomannan, and galactoglucomannan polysaccharide substrates [54].

Previously published data, these assay results, and the fact that Color X is a partially purified enzyme preparation, suggest that Color X contains significant α-galactosidase-enzyme activity. It appears that galactose liberated by the action of α-galactosidases and mannose and/or mannan oligosaccharides liberated by the action of β-mannanase enzyme on LBG-galactomannan are all measured by the modified Nelson–Somogyi reducing-sugar assay procedure. The fact that the assay results consistently showed decreasing reducing sugar liberation with increasing Color X enzyme concentration suggests that galactose and mannose sugars liberated during the hydrolysis of LBG-galactomannan were inhibiting α-galactosidase enzyme activity and thereby reducing the rate of galactose liberation with increasing Color X concentration over the 20-min assay period.

It should also be noted that α-galactosidase enzyme activities in crude or partially purified *A. niger* enzyme preparations such as Color X will also affect the accuracy of any viscometric assay procedure used to measure hemicellulase or β-mannanase activity with LBG-galactomannan as substrate [54]. Purified *A. niger* α-galactosidases, when incubated with LBG-galactomannan substrate, rapidly remove galactose sugar residues from the LBG galactomannan polymer, thereby reducing mannan polysaccharide solubility and the viscosity of LBG substrate solution. Since the manufacturer of Color X uses a viscometric assay procedure with LBG-galactomannan substrate to measure hemicellulase activity, the actual hemicellulase enzyme activity declared on the Lyven finished-product certificate-of-analysis/conformation is probably high due to action of contaminating α-galactosidases in the partially purified enzyme preparation. The actual concentration of β-mannanase and α-galactosidase enzyme activities in any crude or partially purified commercial enzyme preparation will therefore depend on the *A. niger* fungal strain used, the culture conditions—particularly the substrate(s) used to induce enzyme production, the enzyme recovery and purification process, and any blending employed.

Previous experience with Color X and Color Pro [10] suggested that, under typical winemaking conditions (20–25 °C fermentation temperature; pH 3.60–3.80; ethanol 12–13%), both enzymes tended to increase the total phenol concentrations in Cabernet Franc and Cabernet Sauvignon wines. Individual phenolic compounds increased in Merlot and Cabernet franc, particularly gallic acid, catechin, epicatechin, caffeic acid, *p*-coumaric acid, and quercetin. The efficacies of the enzymes were clearly cultivar and vintage-related, with Cabernet Franc and Merlot very responsive and Cabernet Sauvignon

nearly unresponsive over three vintages. A few individual anthocyanin compounds were increased by either or both enzyme products; e.g., cyanidin-3-monoglucoside increased in Merlot, while in Cabernet Franc, three non-acylated and four acylated pigments increased in response to Color Pro, and two non-acylated and three acylated pigments increased in response to Color X. The following season, Cabernet Franc was minimally responsive with respect to anthocyanins, but one year later, both enzymes increased nearly all non-acylated compounds, as well as seven of 10 (Color Pro) and six of 10 (Color X) acylated compounds, respectively. These results demonstrate that the efficacy of these enzymes is not substantially negatively impacted under conditions that may not include optimum temperature and pH.

5. Conclusions

Polygalacturonase activity in Scottzyme Color X was measured under several temperature, pH, and ethanol regimes using a modification of the standard protocol. Enzyme activity increased with temperature at all pH levels, and increased with pH at all temperatures. Polygalacturonase activity decreased linearly with increasing ethanol. The modified Nelson-Somogyi reducing-sugar assay procedure was used to measure β-mannanase enzyme activity in Color X. Protease activity in Color Pro was temperature dependent. These data suggest that the PGU, mannanase, and protease components in these enzyme products provide sufficient activities within the ranges of pH, temperature, and ethanol common during the initial stages of red wine fermentations, although low must temperatures (<20 °C) and the presence of ethanol would likely lead to sub-optimal enzyme activities.

Supplementary Materials: The following are available online at www.mdpi.com/2306-5710/4/1/10/s1, Table S1: Reagents used in preparation of the galacturonic acid standard curve, Table S2: Reagents used in preparation of the β-mannanase standard curve. The 0.2 M sodium acetate buffer volume was 25 mL for each standard, Figure S1: Impact of temperature on acid protease activity in Scottzyme Color Pro (pH = 3.5; ethanol = 0%).

Acknowledgments: Funding was provided by the National Research Council of Canada's Industrial Research Assistance Program and the Grape and Wine Research Foundation.

Author Contributions: A.G.R. Conceived and designed the experiments and secured funding; A.K. and F.D.P. performed laboratory analyses.

Conflicts of Interest: The authors declare no conflict of interest.

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