CBCTA 2018
XXXVI CONGRESSO BRASILEIRO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
O USO CONSCIENTE DA BIODIVERSIDADE: PERSPECTIVAS PARA O AVANÇO DA CIÊNCIA E TECNOLOGIA DE ALIMENTOS
13 a 16 de Agosto de 2018
Belém . Pará . Hangar

CORRELATION BETWEEN HYDROLYTIC ENZYMES PRODUCED BY SOLID-STATE FERMENTATION AND BIOACTIVE COMPOUNDS RELEASE FROM GRAPE POMACE

Aline Soares Cascaes Teles[1*], Davy William Hidalgo Chávez[2,3], Leda Maria Fortes Gottschalk[4], Renata Valeriano Tonon[4]

[1]Universidade Federal do Rio de Janeiro, Instituto de Química – Rio de Janeiro
[2]Universidade Federal Rural do Rio de Janeiro, Departamento de Ciência e Tecnologia de Alimentos – Rio de Janeiro
[3]Instituto de Educação, Ciência e Tecnologia do Rio de Janeiro, Campus Valença, Departamento de Engenharia de Alimentos – Rio de Janeiro
[4]Embrapa Agroindústria de Alimentos – Rio de Janeiro
* Corresponding author: aline_cascaes@yahoo.com.br

ABSTRACT

Hydrolysis of the cell wall components of grape pomace by hydrolytic enzymes may be an alternative for the recovery of conjugated bioactive compounds from a matrix. In this sense, a pool of enzymes was produced by solid-state fermentation (SSF) using wheat bran as a standard medium and a mixture of grape pomace and wheat bran as substrate. The aim of this work was apply the principal component analysis (PCA) to evaluate the activity of the enzymes produced and the release of bioactive compounds. The enzymes synthesized with the addition of grape pomace were more efficient in the extraction of compounds with higher proanthocyanidins content and higher antioxidant potential. A significant correlation between the bioactive compounds and the enzymes activity was observed.

Keywords: Bioactive compounds, enzyme-assisted extraction, antioxidant capacity.

1. INTRODUCTION

Grape pomace is the main residue of wine production consisting of skins, stalks and seeds. This co-product is highly rich in phenolic compounds, mainly proanthocyanidins, anthocyanins, flavonols, stilbenes and phenolic acids that are associated with several human health benefits with antioxidant potential. However, phenolic compounds may have strong connections with the cell wall of the plant, which may hinder their recovery through conventional extraction methodologies (Pinelo, *et al.*, 2006). Many researches has studied the application of hydrolytic enzymes for release these compounds (Puri *et al.*, 2012; Xu *et al.*, 2014).

The use of solid-state fermentation may be a good alternative to obtain these enzymes. However, in this process several enzymes responsible for the hydrolysis of the lignocellulosic biomass are secreted. Therefore, most authors use several steps to purify the enzymatic pool until obtaining the enzyme of interest for the release of bioactive compounds from plants. However, the hydrolysis of the polysaccharides of the plant cell wall requires the action of several enzymes (Brijwani *et al.*, 2010; Martins *et al.*, 2016).

In this study, the use of solid-state fermentation of grape pomace and wheat bran was used for the induction of the synthesis of an enzymatic pool with potential for the extraction of bioactive compounds from grape pomace. In addition, the correlation between the activity of the enzymes present in the pool and

the phenolic compounds released was evaluated using the principal component analysis.

2. MATERIAL AND METHODS

2.1. Raw material

The grape pomace of variety Alicante bouschet from red wine production was provided by Rio Sol winery (Lagoa Grande, PE, Brazil) and wheat bran was supplied by Bunge Alimentos S.A. (Rio de Janeiro, Brazil). The experiments were carried out at Embrapa Food Agroindustry.

2.2. Fermentative processes

A mutant strain *Aspegillus niger* 3T5B8 was used for hydrolytic enzymes production by solid-state fermentation on two substrates – one containing a mixture of wheat bran and grape pomace (1:1) (mixed medium) and other containing only wheat bran (standard medium) - over time (24, 48, 72 and 96 hours). The experiments were carried out in Erlenmeyers flasks containing 10 g of substrate adjusted to a moisture of 60% (with the addition of 0.91% (w/v) ammonium sulfate solution) in a chamber at 32°C.

The enzymatic pool was recovered from the fermentation medium according to the methodology described by Couri *et al.* (2000).

2.3. Enzymatic activity

The activity of the enzymes xylanase, Carboximetilcellulase (CMCase), β-glucosidase, polygalacturonase and tannase were determined according to the methodology proposed by Miller (1959), IUPAC method and expressed in international units (Ghose *et al.*, 1987), Couri & Farias (1995) and Sharma *et al.* (2000), respectively.

2.4. Extraction of bioactive compounds

The enzyme-assisted extraction was conducted using 0.5 g of grape pomace and 4 mL of sodium acetate buffer (0.02 M, pH 5.0) incubated with 0,555 mL (aliquot based on previous laboratory studies) of enzymatic pool at 50°C for 2 hours (adapted from Xu *et al.*, 2014). The calculation of the activity used was performed based on the aliquot taken of the enzymatic pool from the SSF and the corresponding activity observed in the fermentation process.

2.5. Bioactive compounds and antioxidant capacity

Total phenolic compounds, total anthocyanins, proanthocyanidins, $ABTS^{+}$ cationic radical scavenging activity and Oxygen Radical Absorbance Capacity (ORAC) were determined by Folin-Ciocalteu's according to Singleton & Rossi (1965), pH differential methodology (Giusti & Wrolstad, 2001), vanillin acidified method (Broadhurst & Jones, 1978), $ABTS^{+}$ antiradical activity (Re *et al.*, 1999) and ORAC assay (Zulueta *et al.*, 2009), respectively.

3. RESULTS AND DISCUSSION

3.1. Enzymatic activity and extraction of bioactive compounds

The enzymatic pool used for extraction bioactive compounds from grape pomace, presented different activities profiles according to the fermentation medium used and to fermentation time (Table 1). Standard medium favored xylanase (31 IU mL-1 at 48 hours) and polygalacturonase (22 IU mL-1 at 24 hours) activities, while β-glucosidase (25 IU mL-1 at 96 hours) and tannase (0.21 IU mL-1 at 48 hours) activities was observed in higher levels in the Mixed Medium.

Table 1. Enzymatic activities of the different pool used for release of bioactive compounds from grape pomace.

Time (hours)	Xilanase activity (IU mL^{-1})	CMCase activity (IU mL^{-1})	Polygalacturonase activity (IU mL^{-1})	β-glucosidase activity (IU mL^{-1})	Tannase activity (IU mL^{-1})
24 MD	9	1	4	6	0.08
48 MD	11	3	5	24	0.09
72 MD	5	4	4	20	0.07
96 MD	6	5	13	25	0.21
24 SM	30	2	22	6	0.09
48 SM	31	3	17	15	0.02
72 SM	21	4	21	19	0.02
96 SM	22	4	19	23	0.01

MD. mixed medium. SM. Standard medium.

Xu et al (2014) used similar values of β-glucosidic activity (25 IU mL^{-1}) and polygalacturonase activity (7 IU mL^{-1}) for hydrolysis and recovery of bioactive compounds from grape pomace using commercial enzyme.

According to Table 2, the enzymatic pool produced at 96 hours by SSF were more efficient for the extraction of bioactive compounds with antioxidant potential, except for anthocyanins and ORAC antioxidant capacity, which had higher quantifications in 24 hours (presenting degradation over the time) and 72 hours (MD), respectively.

According to Arnous & Meyer (2010), anthocyanins are susceptible to enzymatic hydrolysis and can be degraded, mainly by the action of β-glycosidase, which catalyzes the deglycosylation of anthocyanins in aglycones.

The values of total anthocyanins was higher than those found by Xu *et al.* (2014)(1.6 mg Cyn.$100g^{-1}$). Total phenolic compounds and proanthocyanidins were higher than those reported by Martins *et al.* (2016)(60 mg GAE.$100g^{-1}$ and 0.131 g CE.$100g^{-1}$, respectively). However, the antioxidant capacity (ORAC) obtained by these authors was higher (approximately 400 µmol TE.g^{-1}).

Table 2. Bioactive compounds and antioxidant capacity in extracts obtained for extraction enzyme-assisted.

Sample	TP (mg GAE.100g^{-1})	PAs (g CE.100g^{-1})	TA (mg Cyn.100g^{-1})	ABTS^{+} (µmol TE.g^{-1})	ORAC (µmol TE.g^{-1})
Control	506.09 ± 3.32^{e}	2.60 ± 0.04^{d}	29.00 ± 0.32^{b}	36.65 ± 0.09^{b}	127.85 ± 9.49^{e}
24 MD	719.90 ± 3.35^{c}	3.27 ± 0.04^{c}	34.93 ± 0.36^{a}	39.09 ± 0.71^{b}	163.09 ± 3.28^{c}
48 MD	733.80 ± 7.31^{c}	5.49 ± 0.01^{a}	17.36 ± 0.55^{c}	18.66 ± 0.34^{c}	173.24 ± 3.59^{b}
72 MD	670.96 ± 3.33^{d}	5.30 ± 0.02^{a}	12.61 ± 0.75^{d}	13.68 ± 0.25^{c}	192.33 ± 12.80^{a}
96 MD	910.56 ± 2.22^{a}	5.76 ± 0.02^{a}	11.57 ± 0.01^{d}	52.14 ± 0.83^{a}	161.95 ± 16.80^{c}
24 SM	847.02 ± 7.84^{b}	2.27 ± 0.01^{d}	34.45 ± 1.21^{a}	36.75 ± 0.18^{b}	175.84 ± 3.11^{b}
48 SM	909.98 ± 4.45^{a}	3.59 ± 0.01^{c}	12.68 ± 0.66^{d}	34.00 ± 0.63^{b}	158.27 ± 0.84^{c}
72 SM	747.57 ± 5.58^{c}	4.35 ± 0.01^{b}	8.47 ± 0.36^{d}	32.86 ± 0.16^{b}	140.73 ± 5.78^{d}
96 SM	923.43 ± 2.17^{a}	4.77 ± 0.04^{b}	7.51 ± 0.24^{d}	36.90 ± 0.03^{b}	172.54 ± 2.95^{b}

Control. Control without enzyme addition; TP. phenolic compounds; AT. Total anthocyanins. Different lowercase letters in the same column indicates statistical differences ($p< 0.05$) through in the quantification of the compound released by the enzymatic cocktail produced by SSF over time.

These differences in the values of bioactive compounds and antioxidant capacity may be related to the grape variety, extraction time and loss of compounds responsible for the antioxidant potential Lafka *et al.* (2007).

3.2. Principal component analysis

The PCA was performed to evaluate the correlation between enzymatic pool activity obtained over time by SSF and the quantification of bioactive compounds and antioxidant capacity (Figure 1). The two principal components explain 73.17% of the total variance in the data evaluated (51.0% to PC1 and 22.17% to PC2).

(a) (b)

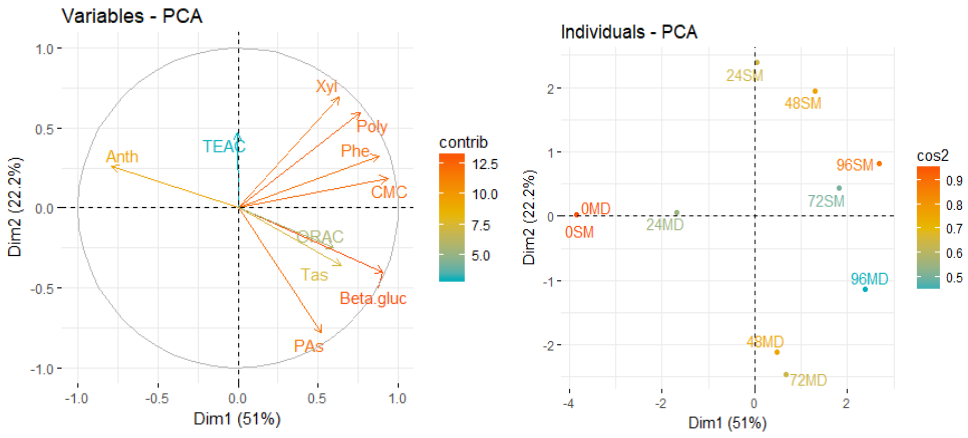

Figure 1 – Two principal components analysis of enzymatic activity and bioactive compounds. Left and right are the variables and samples maps respectively. MD: Mixed Medium and SM, Standard Medium (Fig.3a and b). Xyl: xylanase, CMC: carboxymethilcellulase, Poly: polygalacturonase, Beta.gluc: β-glucosidase, Tas: tannase, Phe: phenolics, PAs: proanthocyanidins, Anth: anthocyannins.

The activities of the enzymes xylanase, CMCase and polygalacturonase showed a strong correlation with total phenolic compounds and β-glucosidase and tannase a moderate correlation with the proanthocyanidins and phenolic compounds. The anthocyanin content showed a negative correlation with the other parameters, being located on the opposite vector. All variables were well explained by the PCA, with the exception of the activity of the antioxidant capacity by ABTS and ORAC.

These results can be visualized in Figure 2, through the Pearson correlation, according to which the activity of enzymes xylanase, CMCase, polygalacturonase and β-glucosidase demonstrated high correlation with total phenolic compounds content. This result is in agreement with the study carried out by Kessy *et al.* (2018)), which obtained high concentrations of phenolic compounds from litchi pericarp extracts using polygalacturonase, cellulase, pectinase and β-glucosidase. Therefore, demonstrating the efficiency of these enzymes in the release of these compounds.

According to Figure 2 the correlations between the activities of the enzymes and the bioactive compounds extracted from the grape pomace confirm the dependence between the responses suggested by the PCA. According to Mukaka (2012), the activities of xylanase vs CMCase and polygalacturonase enzymes showed very high correlations with r values of 0.72 and 0.91, respectively; CMCase vs polygalacturonase and β-glycosidase, with r = 0.83 and 0.76, respectively (both with strong correlations), and β-glycosidase vs tannase, r = 0.69 (moderate correlation), indicating a synergism between these enzymes, as also described by Singhania *et al.* (2013). The synthesis of these enzymes by *Aspergillus niger* during the SSF of the lignocellulosic complex matrix may explain this synergism (Krogh *et al.*, 2004).

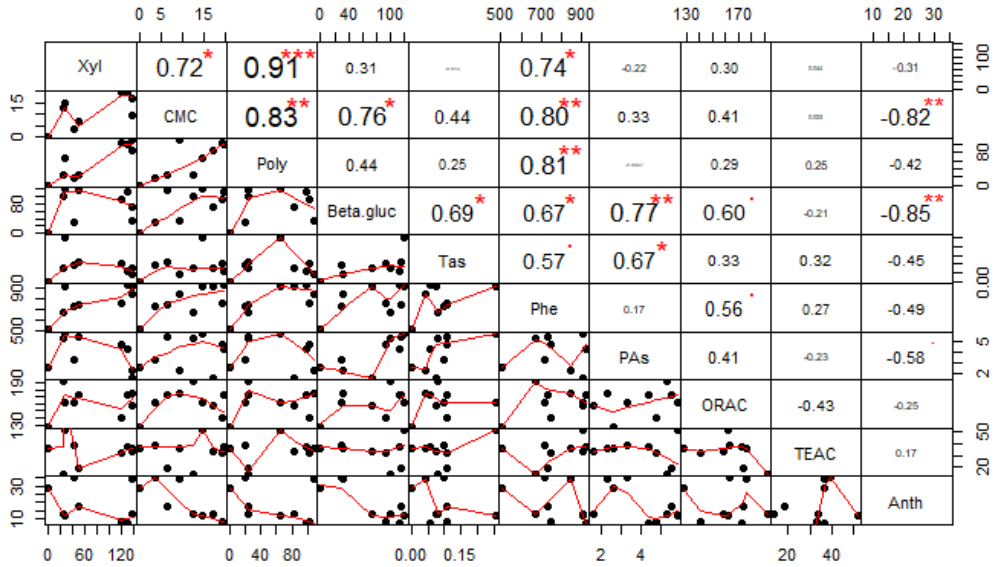

Figure 2 – Correlogram between enzyme activity (MD and SM) and bioactive compounds from grape pomace extracts. Xyl: xylanase, CMC: carboxymethilcellulase, Poly: polygalacturonase, β-gluc: β-glucosidase, Tas: tannase, Phe: phenolics, PAs: proanthocyanidins, Anth: anthocyannins. *** p<0.0001, ** p<0.001, *p<0.01, p<0.05.

In relation to the bioactive compounds, a high correlation was found between the enzymatic activities of xylanase, CMCase, polygalacturonase and total phenolic compounds (Figure 4), with r values of 0.74, 0.80 and 0.81, respectively. This correlation was lower for β-glucosidase activity (r = 0.67). Singhania *et al.* (2013), reported results that demonstrated the action of these enzymes through the study of the release

of phenolic compounds from extracts of grape skin and lychee pericarp using polygalacturonase, cellulase, pectinase and β-glycosidase, respectively. According to Zhang & Sang (2015), the high activity of xylanase is related to the hydrolysis of the hemicellulose present in the cell wall of the plants, which facilitates the release of the bound phenolic compounds.

Although β-glycosidase evidenced a lower correlation with total phenolic compounds, it showed a higher correlation with the proanthocyanidins content in the extracts (r = 0.77), demonstrating that it was involved in the release of this compound. Previous studies Martins *et al.* (2017) also reported this relationship during extraction of phenolic compounds from grape pomace.

A strong negative correlation between CMCase and β-glycosidase enzymes was observed in relation to anthocyanin content (r values were -0.82 and -0.85, respectively). This result can be explained since these enzymes catalyze the breakdown of glycosidic binding of anthocyanins (Yuan *et al.*, 2016).

From the results, it is possible to observe that the highest enzymatic activity was found for xylanase, the highest correlation occurred between the polygalacturonase, and the phenolic compound content, demonstrating that possibly the activity of the enzymes was not necessarily proportional to the release of the bioactive compounds of grape pomace. Moreover, even with low activity, the enzyme tannase showed a good correlation with proanthocyanidin content.

4. CONCLUSIONS

The substrates for SSF promoted the synthesis of enzymes with high activity and, consequently, the ability to release the bioactive compounds from the grape pomace, mainly from the enzymatic pool produced after 96 hours of SSF. This result was confirmed by the strong correlation between the enzymes and the released compounds, suggesting the use of enzymatic complexes without the need for enzymatic purification as an efficient alternative for the release of bioactive compounds from grape pomace.

Acknowledgements

Authors thank CAPES, UFRJ and Embrapa Food Agroindustry for their support.

5. REFERENCES

Arnous, A. & Meyer, A. S. (2010). Discriminated release of phenolic substances from red wine grape skins (Vitis vinifera L.) by multicomponent enzymes treatment. *Biochemical Engineering Journal* 49(1), 68-77.

Broadhurst, R. B. & Jones, W. T. (1978). Analysis of condensed tannins using acidified vanillin. *Journal of the Science of Food and Agriculture* 29(9), 788-794.

Couri, S., Da Costa Terzi, S., Pinto, G. a. S., Freitas, S. P. & Da Costa, A. C. A. (2000). Hydrolytic enzyme production in solid-state fermentation by Aspergillus niger 3T5B8. *Process Biochemistry* 36(3), 255-261.

Couri, S. & Farias, A. (1995). Genetic manipulation of Aspergillus niger for increased synthesis of pectinolytic enzymes. *Revista de Microbiologia* 26(4).

Ghose, T., Panda, T. & Bisaria, V. (1987). Effect of culture phasing and a polysaccharide on production of xylanase by mixed culture of trichoderma reesei D1-6 and aspergillus wentii Pt 2804. *Biotechnology and bioengineering* 30(7), 868-874.

Giusti, M. M. & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current protocols in food analytical chemistry*.

Kessy, H. N. E., Wang, K., Zhao, L., Zhou, M. & Hu, Z. (2018). Enrichment and biotransformation of phenolic compounds from litchi pericarps with angiotensin I-converting enzyme (ACE) inhibition activity. *LWT - Food Science and Technology* 87(Supplement C), 301-309.

Krogh, K. B., Mørkeberg, A., Jørgensen, H., Frisvad, J. C. & Olsson, L. "Screening genus Penicillium for producers of cellulolytic and xylanolytic enzymes." In *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO*, 389-401: Springer, 2004.

Lafka, T.-I., Sinanoglou, V. & Lazos, E. S. (2007). On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chemistry* 104(3), 1206-1214.

Martins, I. M., Macedo, G. A., Macedo, J. A., Roberto, B. S., Chen, Q., Blumberg, J. B. & Chen, C.-Y. O. (2017). Tannase enhances the anti-inflammatory effect of grape pomace in Caco-2 cells treated with IL-1β. *Journal of Functional Foods* 29, 69-76.

Martins, I. M., Roberto, B. S., Blumberg, J. B., Chen, C. Y. O. & Macedo, G. A. (2016). Enzymatic biotransformation of polyphenolics increases antioxidant activity of red and white grape pomace. *Food Research International* 89, Part 1, 533-539.

Miller, G. (1959). Modified DNS method for reducing sugars. *Anal. Chem* 31(3), 426-428.

Mukaka, M. M. (2012). A guide to appropriate use of correlation coefficient in medical research. *Malawi Medical Journal* 24(3), 69-71.

Pinelo, M., Arnous, A. & Meyer, A. S. (2006). Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends in Food Science & Technology* 17(11), 579-590.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine* 26(9-10), 1231-1237.

Sharma, S., Bhat, T. & Dawra, R. (2000). A spectrophotometric method for assay of tannase using rhodanine. *Analytical Biochemistry* 279(1), 85-89.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C. & Pandey, A. (2013). Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource technology* 127, 500-507.

Singleton, V. & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture* 16(3), 144-158.

Xu, C., Yagiz, Y., Borejsza-Wysocki, W., Lu, J., Gu, L., Ramírez-Rodrigues, M. M. & Marshall, M. R. (2014). Enzyme release of phenolics from muscadine grape (Vitis rotundifolia Michx.) skins and seeds. *Food chemistry* 157, 20-29.

Yuan, B., Yang, X.-Q., Xue, L.-W., Feng, Y.-N. & Jiang, J.-H. (2016). A novel recycling system for nano-magnetic molecular imprinting immobilised cellulases: Synergistic recovery of anthocyanin from fruit and vegetable waste. *Bioresource technology* 222, 14-23.

Zhang, H. & Sang, Q. (2015). Production and extraction optimization of xylanase and β-mannanase by Penicillium chrysogenum QML-2 and primary application in saccharification of corn cob. *Biochemical engineering journal* 97, 101-110.

Zulueta, A., Esteve, M. J. & Frígola, A. (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry* 114(1), 310-316.