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Filamentous Fungal Biomass as Inducer of Extracellular B-1,3-Glucanases and B-Glucosidases by *Aureobasidium Pullulans* 1WA1

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Abstract

Microbial enzymes capable of hydrolyzing β-glucosidic linkages have increasingly been applied to wine oenology. β-1,3-Glucanases can lyze oenological yeasts to release mannoproteins responsible for organoleptic characteristics in wines. β-Glucosidases too can increase flavours and aromas in wines through release of monoterpenes. The aim of this paper was to evaluate the production of β-1,3-glucanases and β glucosidases by the yeast *Aureobasidium pullulans* 1WA1 grown on fungal mycelium from *Botryosphaeria rhodina* MAMB-05 as inducing substrate. β-glucanolytic enzyme production by *Aureobasidium pullulans* 1WA1 was examined by a statistical design, and showed maximal β-1,3-glucanase (5.96 U/mg) and β glucosidase (1.77 U/mg) activities at four days growth on media containing 1.5 % (w/v) of fungal mycelium at an initial pH of 2.5. Good agreement was obtained between the experimental values and the corresponding values predicted by the mathematical model. Scanning electron microscopy revealed structural damage to the fungal hyphae by the β-Glucanolytic enzymes from *Aureobasidium pullulans* 1WA1 during growth on the fungal mycelium. The results improved the production of both enzyme types, and demonstrate the potential of a cheaper raw material (fungal mycelium) as inducer of β-1,3-glucanases and β-glucosidases by *Aureobasidium pullulans* 1WA1.

Keywords: *Beta-Glucanolytic Enzymes; Botryosphaeria rhodina MAMB-05; Scanning Electron Microscopy; Response Surface Methodology*

Abbreviations

LFM: Lyophilized Fungal Mycelium; RSM: Response Surface Methodology; SD: Standard Deviation; ECF: Extracellular fluid; p-NP: p-nitrophenol; SEM: Scanning Electron Microscopy; SmF: submerged fermentation; VMSM: Vogel Minimal Salts Medium

Introduction

The β-glucanases constitute a large class of enzymes that are responsible for the hydrolysis of glucosidic linkages present in microbial and plant polysaccharides. Among this group of specific hydrolases are the β-1,3-glucanases of both exo (EC 3.2.1.58) and endo (EC 3.2.2.39) activity that specifically promote the lysis of terminal and internal (1→3)-β-D-O-glucosidic bonds respectively, in linear

(1→3)-β-D-glucans or mixed-linked branched (1→3;1→6)-β-D-glucans [1]. In parallel, β-glucosidases (EC 3.2.1.21) hydrolyze the β-D-O-glucosidic linkages between glucose units in disaccharides and oligosaccharides, as well as cleavage of aglycon groups of glycoconjugates [2].

Microbial enzyme preparations of β-glucosidases and β-1,3-glucanases are utilized extensively in winemaking to facilitate the processing of wines through filtration and clarification, as well as to enhance flavor and aroma development, and augment the liberation of grape skin pigments such as the anthocyanins [3,4]. In particular, the addition of enzymatic preparations to botrytized grape musts affected by the fungus, *Botrytis cinerea*, which produces an extracellular (1→3)-β-D-glucan [5] and affects processing of these Noble-rot wines. These hydrolytic enzymes can also be employed post-fermentation (wines left to age on the lees, sur lie) to promote the lysis of the *Saccharomyces* yeast cell walls releasing into the wine constituents such as mannoproteins and oligomeric fragments that impart organoleptic characteristics to the wine [6].

The production of oenological β-glucanolytic enzymes has been described from different microbial sources; fungi [7], bacteria [8], and non-*Saccharomyces* yeasts [3]. Fungal branched (1→3)-β-D-glucans are the main substrates reported as inducing microbial β-1,3-glucanase activities and they can include schizophyllan from *Schizophyllum commune* [9], scleroglucan from *Sclerotium glucanicum* [10], and botryosphaeran from *Botryosphaeria rhodina* [11,12]. Yeast cell walls and fungal mycelia have also been investigated as inducing β-1,3-glucanase activities [13,14].

β-D-Glucan, mannoprotein and chitin are the mainly components of fungal and yeast cell walls. The β-D-glucans are the major structural polysaccharides and comprise mostly linear chains of (1→3)-and (1→6)-linked β-D-glucose residues, but can also be branched as in the (1→3; 1→6)-β-D-glucans. Other glucans such as (1→3; 1→4)-β-D-glucans and α-D-glucans also have been reported as constituents of fungal cell walls [15,16]. The microbial production of extracellular β-1,3-glucanases increases significantly when nutrient media is supplemented with fungal cell wall material (mycelium) to produce specific hydrolases that can utilize the components in fungal biomass as an energy source [17,18].

Considering that investigations into β-glucanolytic enzyme production by yeast species are important in grape must fermentation (vinification), evaluation of conditions that influence the production of β-1,3-glucanases and β-glucosidases are significant for application in the winemaking industry. In this work we examined the production of β-1,3-glucanases and β-glucosidases by *Aureobasidium pullulans* 1WA1, a yeast strain isolated from Brazilian grape microbiota. The yeast was grown on nutrient medium containing fungal mycelium produced by *Botryosphaeria rhodina* MAMB-05 that was grown on glucose as sole carbon source [19]. We report on the optimization of the β-glucanolytic enzymes produced by *A. pullulans* 1WA1 using the response surface methodology. The structural changes in native and autoclaved fungal mycelium by *A. pullulans* 1WA1 during growth on this substrate were examined by scanning electron microscopy (SEM).

Material and Methods

Fungal mycelium biomass

Botryosphaeria rhodina MAMB-05 was grown on glucose [20] and the mycelium was collected by centrifugation (1,500 g/15 min at 4°C) and freeze-dried without pre-washing the mycelium. The lyophilized fungal mycelium (LFM) was pulverized to a powder in a blender at maximum speed, and stored dried in sealed plastic bags at -20°C until required.

Growth conditions and production of β-glucanolytic enzymes by *Aureobasidium pullulans* 1WA1

Cells of *A. pullulans* 1WA1 were grown on YEPD plates for 48 h according to Bauermeister., *et al*. (2015) [19]. Inoculum was prepared by taking a loopful of yeast cells and adding these to isotonic saline solution to a final concentration of 1 x 107 cells. One-mL was used to inoculate 25 mL of nutrient medium (minimum salts medium, VMSM) [21] in 125-mL Erlenmeyer flasks containing LFM as substrate

(concentration to be determined by RSM, see below). Cultures were incubated at 28°C on a rotary shaker at 180 rpm for 96 h. All experiments were carried out in triplicate and the results represent the means ± SD.

Statistical design for β-1,3-glucanase and β-glucosidase production by *Aureobasidium pullulans* 1WA1 grown on mycelial biomass from *Botryosphaeria rhodina* MAMB-05

A full 3^2 factorial experimental design [22] with three replicates at the central-point summarizing 12 experimental runs conducted in triplicate (Table 1) was used to optimize the conditions for the production of β-1,3-glucanase and β-glucosidase by *A. pullulans* 1WA1 evaluating the fermentation variables: concentration of LFM as the growth substrate, and the initial pH of the nutrient medium.

Run Nº	Variables		β-1,3-glucanase (U/mg)	β-glucosidase (U/mg)
	x_1	x_2		
1	-1	-1	3.88 ± 0.28	1.32 ± 0.13
2	-1	0	1.52 ± 0.08	0.45 ± 0.03
3	-1	1	0.72 ± 0.09	0.17 ± 0.02
4	0	-1	4.88 ± 0.14	1.63 ± 0.02
5[a]	0	0	1.15 ± 0.01	0.39 ± 0.05
6	0	1	0.59 ± 0.03	0.17 ± 0.02
7	1	-1	5.96 ± 0.08	1.77 ± 0.07
8	1	0	1.77 ± 0.01	0.31 ± 0.02
9	1	1	0.60 ± 0.01	0.16 ± 0.00
10 [a]	0	0	1.25	0.32
11 [a]	0	0	1.05	0.40
12 [a]	0	0	1.13	0.38
Factors	**Real levels**			
	-1		0	+1
x_1, LFM [b] (%, w/v)	0.5		1.0	1.5
x_2, Initial pH	2.5		5.0	7.5

Table 1: *Matrix 32 full factorial experimental design defining conditions for the production of β-1,3-glucanase and β-glucosidase by Aureobasidium pullulans 1WA1 by investigating the effects of concentration of mycelial biomass (B.rhodina MAMB-05) and initial pH. [a]central point; [b]lyophilized fungal mycelium*

The mathematical model function used was given by a polynominal equation (1) below:

$$Y_n = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 \sum_{i<j} \beta_{ij} X_i X_j + \in \quad (1)$$

where Yn represents the response function of the experimental data (enzyme activity), xi and xjare the coded independent variables corresponding to the LFM concentration and initial pH, respectively; β is constant coeficient; and ε is the observed error. Regression coefficients and analysis of variance were determined using STATISTICA v.9.0 software.

Enzyme assays

The ECF obtained after centrifuging the yeast-grown cultures was used as the source of enzyme. β 1,3 Glucanase activity was measured against laminarin (4 g/L) as substrate, in 0.025 M sodium acetate buffer (pH 5.0) [23]. The mixture was incubated at 37°C for 60 min followed by measuring the reducing sugars produced by the cupro-arsenate method [24,25]. The unit of β-1,3-glucanase activity was defined as the number of μ mol reducing sugars produced per min under the standard assay conditions.

β-Glucosidase activity was measured using p-nitrophenyl-β-D-glucopyranoside (4 g/L) in 0.025 M citrate phosphate buffer (pH 4.0), and was incubated at 37°C for 30 min [23]. β-Glucosidase activity was determined by measuring the amount of p-nitrophenol (*p*-NP) liberated (A_{410}; extinction coefficient, 18,300 M.cm 1) after addition of 0.25 M Na_2CO_3. The unit of β-glucosidase activity was defined as the number of μ mol *p*-NP liberated per min under the standard assay conditions.

Analytical methods

Reducing sugars were determined by the cupro-arsenate method [24,25]. Total sugars were measured by the phenol–sulfuric acid method [26]. D-glucose was used as the standard in both sugar determinations. Protein was determined by a modified Lowry procedure according to Hartree (1972) [27] using bovine serum albumin as standard.

Scanning Electron Microscopy (SEM)

SEM was performed on: (a) *A. pullulans* 1WA1 cells obtained by centrifugation following submerged fermentation (SmF) on nutrient medium (VMSM and glucose, 10 g/L); (b) hyphae of *B.rhodina* MAMB-05 taken from freshly-grown mycelium colonizing a potato-dextrose-agar plate; (c) the same hyphal sample of *B. rhodina* MAMB-05 after autoclaving; and (d) *A. pullulans* 1WA1 cells grown for 96 h at 28°C (180 rpm) on media comprising VMSM and mycelial biomass (LFM; 10 g/L).

The yeast and fungal samples were harvested by centrifugation and the supernatants discarded. The pellets contained in a centrifuge tube were fixed by immersion in a solution comprising 2.5% (v/v) glutaraldehyde and 2% (w/v) p formaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C. On the following day, the samples were carefully washed (3x) in sodium phosphate buffer, post-fixed for 1 h with 1.0% (w/v) osmium tetroxide in the same buffer (pH 7.2), and then gently washed (3x) in sodium phosphate buffer. The fixed material was dehydrated by passing the sample material through a series of ethanol solutions (70, 80, 90 and 100%) in water.The samples were then dried by critical point drying in CO_2 (BALTEC DCP 030 Critical Point Dryer), and coated with a layer of gold (BALTEC SDC 050 Sputter Coater). The surface morphologies were observed using a FEI Quanta 200 Scanning Electron Microscope (FEI Company, Hillsboro, OR, USA). A large number of images were obtained on different areas of the samples to validate the reproducibility of the results.

Results and Discussion

The ascomyceteous fungus, *B. rhodina* MAMB-05, secretes an exopolysaccharide of the (1→3;1→6)-β-D-glucan type, named botryosphaeran, into the culture medium when grown on glucose and other some sugars [20,28]. Botryosphaeran exists in a triple helix conformation [29] and possesses interesting rheological properties [30], exhibited hypoglycemic and hypocholesterolemic activities [31], and showed strong antioxidant activity [32]; all of which characterize a broad range of promising applications for this exocellular β-D-glucan in pharmaceuticals, cosmetics and food products.

The production of botryosphaeran by *B. rhodina* MAMB-05 results in large amounts of mycelial biomass being produced [28], which presents a rich source of a mixture of cell-wall β-D-glucans [33] that can serve as a suitable and cheap substrate to induce specific microbial β-1,3-glucanases, rather than using more-expensive extracted β-D-glucans for this purpose [11,12,23]. Bauermeister., *et al*. (2015) [19] showed that *A. pullulans* is able to growth and produce β-glucanolytic enzymes using botryosphaeran and also mycelium (LFM) from

B. rhodina MAMB 05 as substrate, besides, the enzymes produced exhibited optimum physicochemical properties applicable in wine oenology to augment aroma and taste of wines.

A 3^2 factorial design was used to evaluate the potential of mycelium (LFM) from *B. rhodina* MAMB 05 as an inducer of β-1,3-glucanases and β-glucosidases by *A. pullulans* 1WA1 under submerged fermentation (Table 1). Multiple regression analysis of the experimental data resulted in a second-order polynomial equation for β-1,3-glucanase production shown by equation (2).

$$Y_1 = 1.219 + 0.368x_1 - 2.135x_2 + 0.278x_1^2 + 1.368x_2^2 - 0.550x_1x_2 \quad (2)$$

The variable more important for β-1,3-glucanase production by *A. pullulans* 1WA1 was the linear term of initial pH (p= 1 x 10-6), while the squared effect term of LFM concentration was the unique term of equation (2) that was not significant (p= 0.10). Besides that, the intercept was significant (*p*= 2.8 x 10-5) indicating that the central-points (LFM, 1% (w/v) and initial pH 5.0) were correctly chosen. The R-squared value implies 98% of the variability of the observed response values can be explained by the model, or by experimental factors and their interactions. The pure error was low, indicating good reproducibility of the experimental data. According to the experimental data, maximum β-1,3-glucanase production (5.96 ± 0.08 U/mg) by *A. pullulans* 1WA1 occurred at initial pH of 2.5 and 1.5% (w/v) LFM as substrate (Table 1, run 7), and (Figure 1a, Table 2). The optimum β-1,3-glucanase value estimated by the model was 5.92 U/mg. The experimental value obtained was therefore statistically in agreement with equation (2) under the conditions as described above.

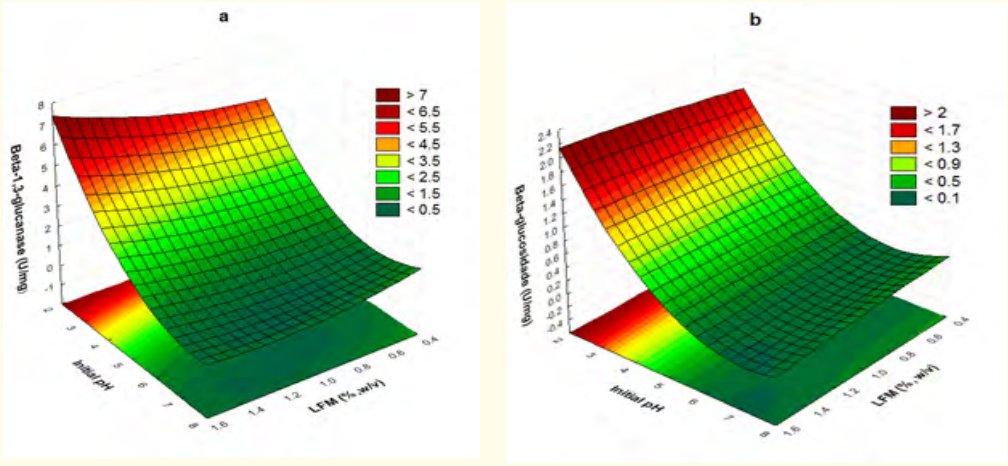

Figure 1: *The 3-D and contour plots showing the expected responses (Y_1) of (a) β-1,3-glucanase, and (b) β-glucosidase production by Aureobasidium pullulans 1WA1 as a function of Botryosphaeria rhodina MAMB-05 mycelium biomass concentration (x_1) and initial pH (x_2).*

Noronha and Ulhoa (2000) [34], obtained highest enzyme titres when *T. harzianum* was grown on fungal cell wall material (10.8 U/ml) and laminarin (4.0 U/ml) as sole carbon sources after 3 days of growth. These findings suggested that the β-1,3-glucanase activity levels were strongly influenced by the levels of β-D-glucan present as inducer, and that the type of glucosidic linkages present in the β-D-glucans used as carbon source were important for β-1,3-glucanase production [12].

Factors	S.S.	D.F.	M.S.	F-value	*p-value*
β-1,3-Glucanase					
x_1 linear	0.8140	1	0.8140	14.7891	0.0090
$x_1$quadratic	0.2054	1	0.2054	3.7308	0.1016
x_2 linear	27.3494	1	27.3494	496.8845	$1x10^{-6}$
$x_2$quadratic	4.9868	1	4.9868	90.6008	$1.7x10^{-5}$
$x_1.x_2$	1.2100	1	1.2100	21.9833	0.0034
Error	0.03303	6	0.0550		
Total	36.3038	11			
β-Glucosidase					
x_1 linear	0.0150	1	0.0150	1.7631	0.2325
$x_1$quadratic	0.0009	1	0.0009	0.1102	0.7512
x_2 linear	2.9681	1	2.9681	348.8708	$2x10^{-6}$
$x_2$quadratic	0.6700	1	0.6700	78.7532	0.0001
$x_1.x_2$	0.0529	1	0.0529	6.2179	0.0469
Error	0.0510	6	0.0085		
Total	3.8230	11			

Table 2: *ANOVA data obtained from the experimental design defined conditions for optimizing the production of β-1,3-glucanases and β-glucosidases by Aureobasidium pullulans 1WA1.*

x_1: mycelial biomass; x_2: initial pH; S.S: Sum of squares; D.F: Degree of freedom; M.S: Mean Square

Statistical analysis using RSM was also employed to optimize the production of β-1,3-glucanase by *T. harzianum* Rifai in submerged cultures using botryosphaeran as sole carbon source, and combined the effects of the variables, substrate concentration and time of growth [23]. These parameters were further employed to optimize β-1,3-glucanase production by this fungus when cultured in a fermenter resulting in enhanced enzyme titres [11], and also to evaluate the production of β-1,3-glucanase by *T. harzianum* Rifai on *Agaricus blazei* extract as inducer under shaking conditions [18]. The production of β-1,3-glucanase from another *T. harzianum* strain by SmF on glucose was optimized by analyzing the effects of temperature and initial pH [35].

Aureobasidium spp. and strains have been described that produce β-glucosidases [36,37], and these enzymes can be induced on (1→3;1→6)-β-D-glucans [38]. Optimization of β-glucosidase production by A. pullulans 1WA1 using RSM resulted in a second-order polynomial equation (3).

$$Y_1 = 0.381 + 0.050x_1 - 0.703x_2 - 0.019x_1^2 + 0.501x_2^2 - 0.115x_1x_2 \quad (3)$$

The variable more important for β-glucosidase production by *A. pullulans* 1WA1 was also the linear term of initial pH, while both linear- and squared-effect terms of LFM concentration were not significant. Highest β-glucosidase activities (1.77 ± 0.07 U/mg) were obtained using 1.5% (w/v) LFM at initial pH of 2.5 (Table 1, run 7). The R-squared value implies that 97% of the variability of the observed response values can be explained by the model, or by experimental factors and their interactions. The pure error was low, indicating good reproducibility of the experimental data. The response surface plots are depicted as a contour and a 3-dimensional surface (Figure 1b, Table 2). The optimum value estimated by the model for β-glucosidase was 1.73 U/mg, indicating that the experimental value obtained was statistically in agreement with equation (3) under the conditions described above. Initial pH was also found to be an important variable for β-glucosidase production by SmF for the wine yeast, *Debaryomyces pseudopolymorphus* UCLM-NS7A, when cultivated on cellobiose as C source [39].

Scanning electron microscopy (SEM) was employed to visualize the degradation of the mycelium of *B. rhodina* MAMB-05 by *A. pullulans* 1WA1 cells when grown on the mycelium (LFM). Figure 2a shows *A. pullulans* 1WA1 cells cultivated on nutrient media. Figure 2b shows hyphal structures of *B. rhodina* MAMB 05 taken from freshly-grown mycelium on agar plates, and (Figure 2c) shows hyphae that had been autoclaved to assess whether autoclaving caused any structural damage to the fungal hyphae. The micrograph shows that the hyphae remained intact. SEM analysis (Figure 2d) showed evidence (rough surface, perforations) of degradation of the fungal hyphae following treatment with *A. pullulans* 1WA1 cells. This was due to the yeast producing β-glucanolytic enzymes that attacked the β-glucan components [33] present in the fungal cell wall. A similar phenomenon showing evidence of degradation of *Schizosaccharomyces pombe* cell walls by a bacterial β-1,3-glucanase preparation has been reported [8]. (Figure 2)

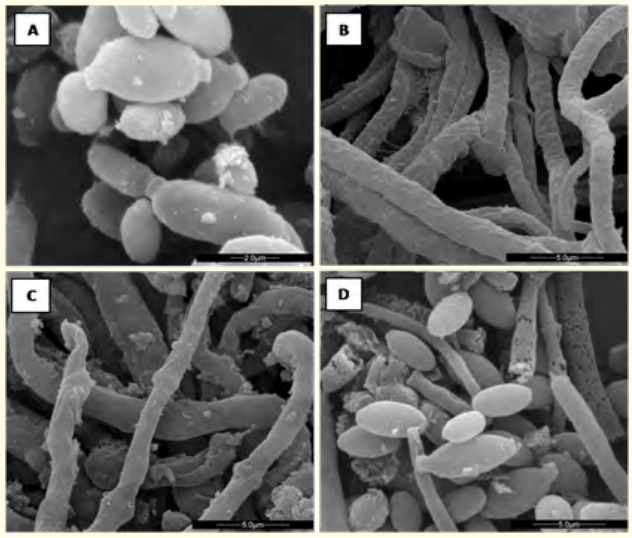

Figure 2: *Scanning electron micrographs of (a) Aureobasidium pullulans 1WA1 cells grown on glucose (10 g/L) (20,000x), (b) hyphae of Botryosphaeria rhodina MAMB-05 taken from mycelium colonizing an agar plate (12,000x), (c) autoclaved sample of the same hyphal sample (12,000x), and (d) B. rhodina MAMB-05 mycelium on which A. pullulans 1WA1 cells were grown for 96 h (12,000x).*

Conclusion

In conclusion, we demonstrated that *A. pullulans* 1W1A grew on *B. rhodina* MAMB-05 mycelium as substrate and produced β-glucanolytic enzymes under these conditions. Enzyme production by *A. pullulans* 1WA1 was optimized by the response surface method resulting in specific activities of 5.96 U/mg (β-1,3-glucanase) and 1.77 U/mg (β-glucosidase) when cultivated on fungal mycelium. SEM revealed the yeast cells attacked the fungal mycelium leaving the hyphal surface perforated, indented with broken remnants indicative of enzymatic degradation. Furthermore, the mycelium of *B. rhodina* MAMB-05 proved to be a good and suitable substrate for yeast β-glucanolytic enzyme production.

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