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β-1,3-Glucanase production from vinasse and its application in degradation of biofilms adhered in stainless steel

Produção de β-1,3-Glucanase utilizando vinhaça e sua aplicação na degradação de biofilmes aderidos a aço inoxidável

Bruna Letícia Martins[1], Luana do Amaral Bovi[1], Cynthia Barbosa Rustiguel[2], Fernando Tozze Alves Neves[1], Flávio Augusto Sanches Politi[3], Geisiany Maria de Queiroz[4]*

[1] Pró-Reitoria de Pesquisa e Pós-Graduação – PRPPG, Universidade do Sagrado Coração – Unisagrado, Bauru, SP, Brasil.
[2] Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo – USP, Ribeirão Preto, SP, Brasil.
[3] Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Araraquara, SP, Brasil.
[4] Departamento de Medicina, Faculdade São Leopoldo Mandic de Araras, Araras, SP, Brasil. *Corresponding author: geisyq@gmail.com

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ABSTRACT

The production of β-1,3-glucanase has been optimized using vinasse, analyzing the ideal concentration, incubation time, pH, temperature, and agitation. The biofilms were formed on stainless steel coupons for 48 hours and treated with 21.3 U/mg of enzyme for 30 minutes. The degradation efficiency of the β-1,3-glucanase produced was evaluated by the reduction of total biomass, using crystal violet staining. The enzyme showed significant biomass degradations of microbial biofilms and had the best action against *Candida albicans* biofilms, with total biomass reduction of 48.76%. These results are important to provide a practicable alternative for elimination of biofilms, employing agro-industrial residues that accumulate in environment.

Keywords: antibiofilm, agroindustrial residue, *Candida albicans*, enzyme, optimization.

RESUMO

A produção de β-1,3-glucanase foi otimizada a partir da vinhaça, avaliando sua concentração ideal, tempo de incubação, pH, temperatura e agitação. Os biofilmes foram formados sob corpos de prova de aço inoxidável por 48 horas e tratados com 21,3 U/mg de enzima por 30 minutos. A eficiência de degradação da β-1,3-glucanase produzida foi avaliada quanto a redução da biomassa total, utilizando coloração com cristal violeta. A enzima produzida apresentou significativa degradação de biomassa de biofilmes microbianos e teve melhor ação contra biofilmes de Candida albicans, com redução total de 48,76% da biomassa. Esses resultados são importantes pois, fornecer uma alternativa viável para eliminação de biofilmes, empregando resíduos agroindustriais que se acumulam no meio ambiente.

Palavras-chave: antibiofilme, resíduo agroindustrial, *Candida albicans*, enzima, otimização.

INTRODUCTION

The β-1,3-glucanases (EC 3.2.1.39) are hydrolytic enzymes able to degrade laminarinase. Are enzymes of great biotechnological interest, as they cleave bonds of β-1,3-glucan, an important cell wall polysaccharide widely distributed in various organisms such as microalgae, bacteria, yeast and fungi (Zerva et al., 2023).

β-1,3-glucanases can be synthesized by saprophytic fungi (such as *Aspergillus*) in the fermentation process using residues as substrate (Scafati et al., 2022). The agro-industrial waste are generated in large amounts and they have high content of organic matter that can be reused as energy nutrients for microorganisms. Statistical planning techniques are widely applied in the production of enzymes, mainly because they result in increased productivity and allow the simultaneous study of multiple variables that influence the fermentation process (Sharma et al., 2022).

Biofilms are communities of microorganisms that grow attached to biotic or abiotic surface and involved by exopolymeric matrix (EPS), an essential structure that delimit a unique microenvironment and protects this community (Pandey et al., 2022). It is important to highlight that β-glucan is part of the composition of polysaccharides constituents the EPS of the biofilms, being functionally responsible for its integrity. The occurrence of biofilms in some surfaces can be a problem and generate negative impacts causing economic losses by the food and equipment spoilage, food intoxication (Camargo et al., 2018).

Currently there are no completely effective means to remove biofilms, being carried out mainly by mechanical removal, treatment based on chemicals or treatments that inhibit cell adhesion to surfaces. The processes most used in industries and hospital sectors to remove biofilms are chemical treatments, however these do not perform well and contribute to the generation of toxic waste, validating the search for alternatives (Craigen et al., 2011). The possibility of using enzymes to degrade biofilms appears to be an interesting strategy, since enzymes can cleave structural macromolecules in biofilm matrix and degrade the bonds present in the cell wall of the biofilm micro-organisms (Birk et al., 2021). Examples of enzymes with anti-biofilm capacity include proteases, DNases, polysaccharide degrading enzymes and anti-quorum sensing (Nett et al., 2007; Flemming and Wingender, 2010).

In this context, this study analyzes the capacity of the vinasse to induce *Aspergillus niger* to produce β-1,3-glucanase and to evaluate the ability of this enzyme to degrade biofilms, adhered to stainless steel, causer of damage, especially, in food industry environment.

MATERIAL AND METHODS

Fungal Strain, Inoculum and Fermentation Medium

A. (ATCC 3998) was used to produce β-1,3-glucanase. Cell suspensions containing 10^7 spores/mL were inoculated into Erlenmeyer flasks for production by liquid submerged fermentation in orbital shaker. The initial conditions of fermentation were pH 5.5, 30 °C and 150 rpm. The culture medium was adapted from El-Katatny et al. (2000), composed of (in grams per liter) 0.2 $MgSO_4.7H_2O$, 0.9 K_2HPO_4, 0.2 KCl, 1.0 NH_4NO_3, 0.002 $FeSO_4.7H_2O$; 0.002 $MnSO_4$, 0.002 $ZnSO_4$, 0.5 casein peptone, 0.5 xylose, 0.5 sucrose and 0.25 laminarin. Vinasse was added in decrease of culture medium. The vinasse employed in this study was donated by an ethanol industry in the region of Bauru - State of São Paulo, Brazil.

Determination of the Protein's Concentration and Enzyme Activity

Protein concentration was determined by bicinchoninic acid (BCA) method, and the enzyme activity was determined according to Marco and Felix (2007), with modifications. It was used as substrate 250 µL of the 1% laminaria solution prepared in sodium acetate buffer (50 Mm and pH 5.0) added to 125 µL of enzymatic extract. The reactions were incubated in a water bath at 37 °C for 30 minutes. After, the same volume of 3,5-dinitrosalicylic acid (DNS) was added and following to incubation at 100 °C for 5 minutes. Absorbance readings were done in a spectrophotometer at 550 nm. One unit of β-1.3-glucanase was defined as the amount of enzyme which liberates 1 µmol of glucose per minute in the experimental conditions described.

Optimization of Vinasse Concentration and Production Period by Factorial Design

The Rotational Central Composite Design (RCCD) was used to optimize β-1,3-glucanase production. The concentration of vinasse and the production period was optimized by factorial design (2^2) with central (0) and

axial (-1.41 and +1.41) points defined through the analysis of preliminary results regarding the initial controls (data not shown). Table 1 lists the specifications of levels of statistical variables used in experimental design.

Optimization of β-1,3-Glucanase Production Conditions

The pH, temperature and agitation conditions have been evaluated individually in sequence. Each condition that best induced the production of β-1,3-glucanase was fixed for the next test. At first, the pH was evaluated: 4.0; 5.0; 6.0; 7.0 and 8.0; after, the temperatures 28 °C, 37 °C and 45 °C, and finally, the agitations of 120 rpm, 150 rpm and 200 rpm. These values were defined based on data previously described in literature (Marco and Felix, 2007; Dewi et al., 2016).

Production, Partial Purification of β-1,3-Glucanase for Application

The best fermentation conditions defined for optimization were used to obtain larger volume of the enzyme for application in biofilm, for this step were 14% of vinasse added to the medium, 12 days of production, pH 6.0, 28 °C and 150 rpm.

β-1,3-glucanase produced was partially purified using 80% ammonium sulphate (Dawson et al., 1969). The clarified extract was freeze-dried and resuspended in acetate buffer (50 mM, pH 5.0) at 10 g/L (m/v) concentration. Extract β-1,3-glucanase after precipitation and freeze-drying activity showed specific activity of 21.33 specific activity (U/mg), corresponding to an increase of 7.30 folds compared to the untreated supernatant.

Biofilm Formation

Biofilms were formed on surface of stainless-steel coupons sterilized made with 1.0 cm^3 simulating stainless steel structures commonly found in food industries.

For biofilm formation, *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 25619) and *C. albicans* (ATCC 10231) was cultivated in orbital shaker at 37 °C and 75 rpm for 18 hours in 15 mL of specific culture medium for biofilm formation. *S. aureus* and *C. albicans* cells in RPMI medium and *P. aeruginosa* cells in trypticase soy broth (TSB). The cells were then recovered by centrifugation at 3,000 x*g* for 6 minutes, washed twice and resuspended in saline (0.9% NaCl). The inoculum was spectrophotometrically standardized at OD600 nm and adjusted to OD600 0.1 for *S. aureus* (Zago et al., 2015), OD600 0.05 for *P. aeruginosa* (Borlee et al., 2010) and OD600 0.2 for *C. albicans* (Al-Fattani and Douglas 2004), respectively, corresponding to a final concentration of 10^7 cells/mL.

The single species adhesion and biofilms assays were carried out on 24-well microplates. One stainless-steel coupons were placed per well and each cellular suspension was added added shortly thereafter. The microplates were incubated at 37 °C for 90 minutes at 75 rpm for the biofilm adhesion. After, non-adhered cells were removed through two washes in 0.9% NaCl. Subsequently being placed on a new plate containing 1 mL of medium for each microorganism and incubated for 48 hours at 37 °C and 75 rpm to the biofilm development (Zago et al., 2015).

Degradation of Microbial Biofilms by β-1,3-Glucanase

The coupons with biofilm were washed twice by immersion in 1 mL of 0.9% NaCl to remove superficially attached cells. The lyophilized β-1,3-glucanase was resuspended in sodium acetate buffer 50 mM pH 5.0 in 21.3 U/mg and coupons were immersed in 1 mL of this solution for 30 minutes at 37 °C and 75 rpm. These conditions were defined according to El-Katatny et al. (2000). Biofilms subjected to 0.9% NaCl were used as a control.

Quantification of Total Biofilm Biomass

The total biomass of biofilm was quantified by crystal violet staining. Brifely, after β-1,3-glucanase application,

Table 1. Specification of variables, production period and concentration of vinasse, used in the factorial design for optimization of production of β-1,3-glucanase.

Variables	Axes	-1.41	-1	0	+1	+1.41
Production period (days)	X	2	4	8	12	14
Concentration of vinasse (%)	Y	2	14	42	70	82

Analysis of variance (ANOVA) of results was performed by program Statistix 9, with values of $p = 0.05$ and $p = 0.1$. The best conditions of vinasse concentration and production period defined by the factorial design was fixed for other variables evaluated.

the coupons were washed once by immersion in 0.9% NaCl to remove possible remnants of the enzyme that could interfere with tests of quantification and the biofilm was fixed in methanol for 15 minutes. Then, coupons were maintained at room temperature until complete methanol evaporation. The crystal violet (0,1% v/v) staining step was carried out for 5 minutes. Coupons were then washed three times by immersion in 0.9% NaCl and after were immersed in 80% ethanol for stain dilution. The absorbance was measured at OD590 nm. The tests were performed in triplicate and statistically analyzed using ANOVA and Tukey test with $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of β-1,3-Glucanase Production

The experimental design for optimization of production of β-1,3-glucanase employing vinasse was evaluated as to specific activity (U/mg) obtained in end of fermentation process. The factorial design developed for vinasse presented $R^2 = 0.83$ ($p < 0.1$) and the equation which describes the model for this planning is*: $Z = 3.30 + 2.70x – 0.80y – 0.37xy$*.

The Figure 1 shows the relationship between concentration of vinasse and the production period. This graph demonstrates that long periods of production associated with low volumes of vinasse favored highest enzyme production.

The conditions pointed to by factorial design for the production of β-1,3-glucanase include production periods ranging from 8 to 14 days and concentrations between 2% and 42% of vinasse. The validation of the factorial design indicated that enzyme production reached higher levels of enzyme activity when held for 12 days and incorporating 14% of the vinasse to medium, confirming the predicted by factorial design.

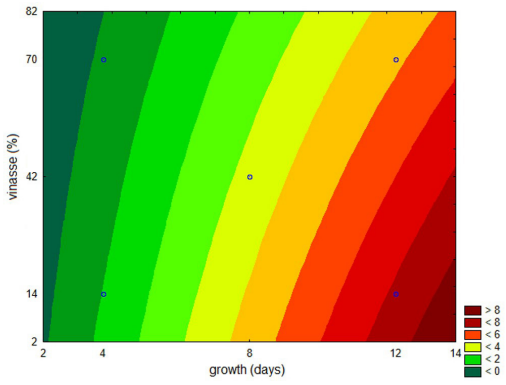

Figure 1. Contour graph representing the influence of concentration of vinasse and period of production in β-1,3-glucanase activity. The warm colors represent the variables that better favored the production of enzyme.

It is highlighted also that the vinasse was able to induce production of β-glucanase for *A. niger* even when employed alone, indicating strong potential of biotechnological application. Veana et al. (2014) also used cane molasses and bagasse of sugar cane, for invertase production by *A. niger* and suggested the possible reduction of costs in production of enzymes, which can be profitable to the industry that adhere to this biotechnological proposal besides generating the sustainability process.

Thus, the bioprocessing industrial surplus of the sugar cane industry can will be reuse as carbon source in biotechnological processes because the ability of certain microorganisms to degrade compounds of this waste and of to secretary biological catalysts, reducing yet, of the disposal in the environment and also the costs of production of enzymes (Sánchez et al., 2015; Diaz et al., 2016).

Figure 2 shows the influence of different pH (Figure 2a), temperatures (Figure 2b) and agitations (Figure 2c) in production of β-1,3-glucanase after defining the vinasse concentration and production period.

Our results showed that the enzymatic production by *A.niger* was not highly influenced between the different evaluated pHs (Figure 2a). β-1,3-glucanase production was higher when the initial pH of the medium was adjusted to 6.0. Minimal enzyme production was observed at pH 4, suggesting that β-1,3-glucanase production is not favored in a very acid medium. Mallikharjuna Rao et al. (2016) stated that the production of β-glucanase is favored by slightly acidic pH. The pH of the culture medium is described as an important factor in the production of enzymes, as it directly affects the biosynthesis of secondary metabolites and the activity and stability of enzymes (Boukaew et al., 2016). *A. niger* is a fungus that tolerates wide pH ranges and can develop in very acidic and even alkaline environments. Such characteristic may justify the production of β-glucanase in the wide range of pH used in this study (Schuster et al., 2002).

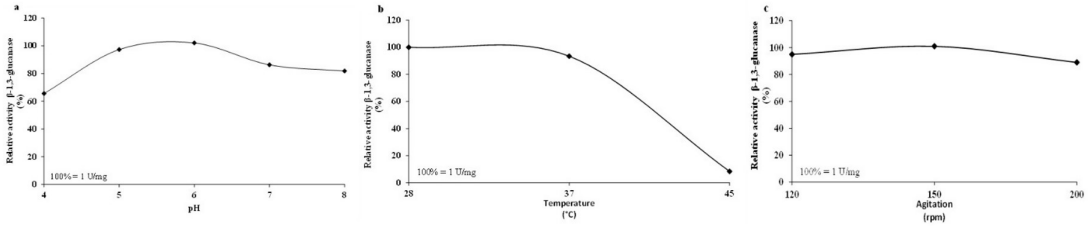

Figure 2. Optimization of parameters of β-1,3-glucanase production. Effect of pH in specific activity (a). Effect of temperature in specific activity (b). Effect of agitation in specific activity (c).

The temperature ranges already described for the production of β-glucanase vary between 28-37 °C (Dewi et al., 2016). In this study, the ideal temperature was 28 °C, observing a sudden reduction in β-glucanase activity at 45 °C, possibly due to conformational denaturation of the enzyme due to high temperature. Furthermore, the optimum temperature for production reflects the mesophilic characteristic of the fungus used, in general the optimum temperature for *A. niger* is between 35-37 °C (Schuster et al., 2002).

The agitation was the parameter that least affected the production of β-glucanase. Agitation of 150 rpm was defined as the optimum production value. Optimization studies by factorial design indicate that stirring speed does not exert a significant negative effect on β-glucanase production, corroborating the data of this study.

The literature describes a wide variety of microorganisms and conditions for the production of β-1,3-glucanase. The composition of culture medium and the production period associated with the investigation of the great bands of pH, temperature and agitation are indispensable analyses in the process of getting analysis of enzymes because it can influence directly the ability of microbial enzyme secretion.

Degradation of Microbial Biofilms by β-1,3-Glucanase

Enzymes are notable because your availability, biodegradability, low toxicity and specificity consequently anti-biofilme strategies based on enzymatic degradation of EPS matrix components may be useful in removing biofilms that bring damage to several industrial or hospital sectors (Leroy et al., 2008). Close to 90% of the total biofilm biomass is represented by its EPS matrix, while the microorganisms represent just 10% of the volume (Flemming and Wingender, 2010). The main benefit of EPS matrix degradation is the reduction of

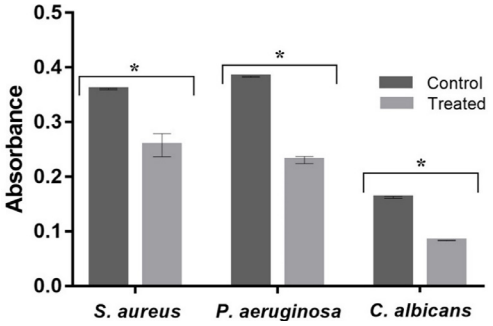

Figure 3. Total biomass quantification of *S. aureus, P. aeruginosa* and *C. albicans* biofilms attached on stainless steel. The error bars represent standard deviation. The asterisks show statistically significant difference compared to the control group ($p < 0.05$).

microenvironment and coherently, the vulnerability of active cells of a biofilm that are susceptible to microbicides compounds (Rasamiravaka et al., 2015).

The Figure 3 shows the quantification of total biomass of biofilm of *S. aureus*, *P. aeruginosa* and *C. albicans* attached to stainless steel before and after treatment with β-1,3-glucanase produced in this study. Based on the difference between the treated and control biofilms observed a significant reduction ($p<0.05$) of the biofilm biomass formed in stainless steel for all microorganisms. After treatment reached 28.33% biofilm biomass reduction of *S. aureus*, 39,94%, of *P. aeruginosa* and 48.76% of *C. albicans*.

In this study, in case of *P. aeruginosa* biofilm, the enzyme can act significantly degrading the total of biofilm biomass compared to the control. The study conducted by Mah et al. (2003) showed that periplasmic β-glucans were present in *P. aeruginosa* biofilms, justifying the action of β-glucanases in reduction of total biomass noted in this study. It is further highlight ted that there is no previous report of degradation of *P. aeruginosa* biofilm biomass by β-glucanase obtained from vinasse.

This effect can contribute to strategies aimed at the EPS matrix degradation in the fight against biofilms, since the EPS matrix contributes significantly to the protection of active cells of the inner layers of the community (Elchinger et al., 2014).

S. aureus biofilm also the treatment with β-1,3-glucanase have degraded significantly the total biofilm biomass compared to the control. This could be explained by the constitution of biofilm matrixes formed by *S. aureus* include mainly components such as cytoplasmic proteins, DNA and intracellular adhesion polysaccharide, embedded within a glycocalyx, it can be assumed that these characteristics contributed (Dengler et al., 2015). Not was found other evaluation studies of β-1,3-glucanase activity against *S. aureus* biofilm, however there are studies evaluating the potential antibiofilme of other enzymes. Vaikundamoorthy et al. (2018) produced a bacterial amylase through a marine strain of *Bacillus cereus* and observed the ability of the enzyme prevent biofilm *S. aureus* and *P. aeruginosa* adhered to glass surface suggesting the enzyme as a potential antibiofilme molecule.

The statistics analysis showed significant degradation of the *C. albicans* biofilm compared to the control biofilm. This result can be justified by the composition of cell wall of *C. albicans* that is composed of an inner layer of β-glucans, that are sub-classified in β-1,3-glucan e β-1,6-glucan and account for about 60% of the cell wall of this microorganism (Galán-Díez et al., 2010). In relation to the amount of β-glucan in biofilms *C. albicans* it is consensus there is an increase in content of this polysaccharide on cells that compose the biofilm possibly because of the transfer of this molecule for EPS matrix composition (Nett et al., 2007).

Tan et al. (2018) evaluated the effect of a β-1,3-glucanase commercially acquired against planktonic cells of *C. albicans*. They evaluate the anti-biofilme activity of enzyme, applied at concentrations of 5 and 10 µg/mL, during a period of 24 hours, on 48 hours *C. albicans* biofilm, formed on poliestirene, and observed that the reduction in biomass by β-glucaanase is dose-dependent, and the maximum concentration (10 µg/mL) reduced the total biomass in 55.96%. The present study reached biomass reduction rates of 48.7% for *C. albicans* biofilm adhered to stainless steel with less treatment time (30 minutes), in addition to applying an enzyme produced with reduced costs to compared with a β-1,3-glucanase commercially acquired.

Our study used the crystal violet assay to quantify the total biofilm biomass (Figure 3). This method quantifies the dead and living cells of the biofilm matrix, not allowing the evaluation of the metabolic activity. Therefore, it is pointed out that quantification methods based on evaluating metabolic activity, such as 2,3-bis reduction assay (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazoliumhydroxide) (XTT), are important additional investigations to demonstrate whether the enzyme has action on the viability of microorganisms in the biofilm or only acts on the degradation of EPS (Zago et al. 2015).

The quantification of the total biomass of the control biofilms showed less adhesion and formation of the *C. albicans* biofilm under stainless steel compared to the biofilm of *S. aureus* and *P. aeruginosa*. Despite studies on biofilm adhesion, the effects of factors that influence microbial adhesion have not yet been fully clarified. *C. albicans* is a hydrophobic yeast and stainless steel has a hydrophilic surface, explaining the lower amount of biofilm biomass produced. In addition, according to Tomičić et al. (2017), temperature influences the adhesion of *C. albicans* to stainless steel, showing better adhesion to the material kept at 43 °C than at 37 °C.

In this context, new alternatives, with lower cost, capable of degraded microbial biofilms, such as enzymes obtained from agro-industrial residues, suggesting that this biotechnological product is promising for the degradation of biofilms commonly found in environments different.

CONCLUSION

Vinasse incorporated into the growth medium encouraged the production of 1,3-β-glucanase by *A. niger* in the best production conditions: 14% of waste, 12 days of production to 28 °C, pH 6.0 and 150 rpm. In addition, β-1,3-glucanase has antibiofilm potential against different microbial biofilms with emphasis for the biofilm of *C. albicans* adhered to stainless steel, where the enzyme exerted betther degradation on biomass total (reduction of 48.76%).

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