Effect of growth conditions on β -glucosidase production using *Flourensia cernua* leaves in a solid-state fungal bioprocess

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Abstract *Flourensia cernua* foliage was used in a solid-state fungal bioprocess to identify factors that could affect β -glucosidase production such as growth medium components and partial identification of molecules from the plant material. Under an exploratory experimental design, each variable had their distinctive result on conditions, which affects and could further improve β -glucosidase production. Under the experimental design, 1482 U/L of β -glucosidase were detected, which marks an improvement in production compared to levels obtained in a control treatment with an activity of 1092 U/L. It was shown that inoculum, water content and pH were the factors with the greater effect on β -glucosidase production. Polyphenolic content and cellulosic fiber in the form of raw fiber were measured to assess compound degradation of the plant material. Although fiber content was apparently unaffected, polyphenolic content decreased; β -glucosidase was produced by *A. niger* GH1. This behavior could be associated with fiber level and polyphenolic content because molecules of this type can be hydrolyzed by β -glucosidase. According to our results, *F. cernua* biomass can be used as a carbon source for β -glucosidase production in a short culture time.

Keywords *Aspergillus* · Cellulases · Tarbush · Solid culture · Experimental design

Introduction

Biological processes have been used by humans for thousands of years. In recent years, the use of these bioprocesses for biomass degradation and recovery of useful metabolites has rapidly been increased (Buenrostro-Figueroa et al. 2017). Natural resources are being sought to obtain compounds of agroindustrial interest. Regarding this matter, lignocellulose, the main component of plants, is the most abundant complex compound in our planet where cellulose is its main component (Gonçalves et al. 2015). This polysaccharide is composed of glucose monomers with β -1,4 glycosidic bonds and it is highly resistant to degradation (Kuhad et al. 2016). As previously stated, cellulose is widely available and it represents raw material for industrial purposes (Lavoine et al. 2012) and biotechnology has an important role in cellulose exploitation. Bioprocesses are being constantly developed to exploit cellulose, relying on the use of microorganisms, particularly fungi, to produce cellulose-degrading enzymes (Castro et al. 2015). In the Mexican deserts, *Flourensia cernua* is an abundant shrub whose foliage can be biotransformed to obtain high added-value compounds (De León-Zapata et al. 2015). This plant is widely distributed in the Chihuahuan Desert and may represent an important source of raw material. One of the most favored practices in biological processes is the fungal production of cellulolytic enzymes (Farinas 2015), which are responsible for hydrolyzing the β -1,4 glycosidic bonds in cellulose. Cellulolytic enzymes have three catalytic activities which are endo- β -1,4-endoglucanase, exo- β -1,4-glucanase and the β -

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1,4-glucosidase, where the latter is considered as the most important of the complex (Singh and Vinod 2016). For β -1,4-glucosidase production, researchers have developed solid-state fermentation (SSF) processes, defined as bioprocesses with absence or near absence of free water, but with enough moisture to favor fungal growth and the production of metabolites (Soccol et al. 2017). β -Glucosidase can be produced by fungi in the presence of phenolic compounds with or without cellulosic fibers in the medium (Ascacio-Valdés et al. 2014). To establish the most significant factors in the growth medium for fungal β -glucosidase, an exploratory experimental design can be chosen to decrease experimental units and identify their effect on the enzyme production (Buenrostro-Figueroa et al. 2017). The main objective of this study was to identify the most relevant factors in the production of fungal β -glucosidase by *Aspergillus niger* GH1 including mineral salts, growth conditions and partial identification of phytochemicals with possible effect on β -glucosidase production.

Materials and methods

Fungal strain, biological material and conditioning

The strain of *A. niger* GH1 was kindly provided by the Autonomous University of Coahuila, Mexico from the DIA-UAdeC microbe collection. The fungal strain was stored in a cryoprotective suspension (1% skim milk and 10% glycerol) at -20°C . For spore harvest, *A. niger* GH1 was propagated in sterile PDA medium for several hours at 30°C . *F. cernua* branches and stems were collected from a rangeland in northern Mexico (25°N). *F. cernua* leaves were placed in trays and were dried in an oven at 50°C for 72 h.

Culture conditions and experimental design

The fermentation process considered as control treatment (CT), and as a basis for further exploratory experimental design is shown in Table 1. Dehydrated *F. cernua* leaves (15 g) were placed in aluminum trays (12.5 cm diameter $\times$ 3 cm depth). The mineral base medium contained (g/L) 2.5 NaNO_3 , 1.0 KH_2PO_4 , 0.5 MgSO_4 , and 0.5 KCl. This medium was sterilized in an autoclave at 121°C during 15 min. Subsequently, the plant material was added 35 mL of the medium with 70% humidity. A total of 2×10^7 fungal spores were added per gram of substrate. Spore count was made with a hemacytometer (Neubauer-ruled bright line chamber). After 36 h of fermentation, 35 mL of 0.1 M citrate buffer, pH 4.6, was added to the fermented material. Filtration of extracts was

carried out with a vacuum pump using Whatman No. 1 filters. The Plackett–Burman design was used for identification of factors that most influenced enzyme production. This type of design favors a linear effect between two levels of a factor (coded as + and $-$) to observe its effect on the response variable. For this methodology, it is necessary to consider at least seven factors. Table 1 shows the factors evaluated coded as -1 and $+1$ with their respective levels where Table 2 shows the specific conditions used in each treatment.

Analytical methods

Measurement of β -glucosidase activity

A volume of 800 μL of 0.1 M citrate buffer pH 4.6 was placed in test tubes. Subsequently, 100 μL of *p*-nitrophenyl- β -D-glucopyranoside 0.009 M were added. The tubes were incubated at 50°C and 100 μL of diluted 1:10 enzyme extract was added. After 10 min, 100 μL of 0.1 M Na_2CO_3 were added to stop the reaction. The released *p*-nitrophenol absorbance was measured in a spectrophotometer at 400 nm. β -Glucosidase unit was defined as the amount of enzyme required to release one micromole of *p*-nitrophenol per time unit (minutes). To carry out the measurement of the activity of interest, enzyme and substrate blanks and a reaction mixture were prepared.

Phenolic compounds and raw fiber content

Phenolic compounds were quantified by spectrophotometry according to Wong-Paz et al. (2015). Raw fiber content was measured by the AOAC (1990) methodology.

HPLC–MS analysis

For the partial identification of phenolic compounds from *F. cernua*, a method reported by Ascacio-Valdés et al. (2016) was used. This method involved extract concentration using Amberlite XAD-16. After concentration, samples were filtered through a 0.45 μm nylon membrane and injected to a Varian ProStar chromatographer. The column used for separation was a Denali C18 column (3.1 μm ; 150×4.6 mm, Grace, USA) with a photodiode array detector at 280 nm. The mobile phases were ethanol as wash phase, CH_3CN and formic acid 0.2%. The solvents were used in gradients with a 1 mL/min with an injection volume of 10 μL . For the analysis of mass spectrometry, a Varian 500/MS equipment with ion trap, electrospray ionization (ESI), negative mode ($\text{M} - \text{H}^-$), capillary voltage 90 V, mass range of 100–2000 m/z was used.

Table 1 Factor levels established on the Plackett–Burman designs with their standard effects and percentage of significance for β -glucosidase production

Factors	Low (–)	Control	High (+)	Standard effect	%
Inoculum size (spores/g substrate)	2×10^6	2×10^7	2×10^8	6.82	33.33
Moisture (%)	65	70	75	2.98	14.58
pH	4.5	5	5.5	2.98	14.58
KH_2PO_4 (g/L)	0.5	1	1.5	–2.56	6.25
MgSO_4 (g/L)	0.25	0.5	0.75	1.28	12.50
NaNO_3 (g/L)	2	2.5	3	1.28	6.25
KCl (g/L)	0.25	0.5	0.75	–2.56	12.50

Table 2 Conditions used in the treatments in the exploratory experimental design for production of *Aspergillus niger* GH1 cellulolytic enzymes and their levels

Treatment	NaNO_3	MgSO_4	KH_2PO_4	KCl	pH	Water content	Inoculum size
A	–	+	+	+	+	+	+
B	–	–	–	–	+	–	+
C	+	–	+	–	+	+	–
D	+	+	–	+	+	–	–
E	–	–	–	+	–	+	–
F	+	–	+	+	–	–	+
G	–	+	+	–	–	–	–
H	+	+	–	–	–	+	+

Data analysis

The mean, standard deviation, and probability “*P*” values of two replicates of the investigated parameters and the control were calculated using the Statistica 7.0 software and Microsoft Excel. Means were compared with SAS 9.0 software using the LSMEANS statement and the Tukey option. Results were considered significant at $P < 0.05$.

Results and discussion

Inoculum size, moisture content, pH and concentrations of KH_2PO_4 and KCl significantly affected the production of β -glucosidase (Fig. 1). From the established treatments, the highest β -glucosidase activity was 1482 U/L, whereas the lowest was 1092 U/L. At CT conditions for fungal growth registered an activity of 1092 U/L (Fig. 2). The design used in the present trial allowed us to identify the most important factors influencing enzyme activity. For the inoculum, the results show a positive tendency in its effect. If the inoculum were to increase, the response variable is likely to show higher β -glucosidase production. The results suggest that β -glucosidase will improve its production if

Fig. 1 Pareto chart of β -glucosidase production ($R^2 = 0.91$ /adjusted $R^2 = 0.83$) showing the significant factors at 95% of confidence level ($p < 0.05$) obtained by the Plackett–Burman design

moisture content is increased. In the case of pH, which is very important in the production of enzyme activity, it is necessary to maintain adequate levels of this parameter. The values used in this trial were 4.5–5.5, where according to the statistical analysis, higher pH values are needed for higher production of β -glucosidase. This factor showed a positive effect in the process. In the case of KH_2PO_4 and

Fig. 2 Effect of the conditions established by the experimental design treatments on β -glucosidase activity by *Aspergillus niger* GH1 using leaves of *Flourensia cernua* as substrate compared with a control treatment (CT). Values are mean $\pm$ SD. Bars with different letters differ ($p < 0.05$)

KCl salts, the effect was negative, indicating that these salts should be reduced, to improve the production of the enzyme. The ions of these salts can function as cofactors required for the biological activities of enzymes or for structure formation of fungal cells. According to the results of the exploratory design, factors can be manipulated to increase the response variable. This implies that higher levels of this factor positively increase the response variable. In this case, a higher content of spores of *A. niger* GH1 promoted further degradation of the fibers present in the plant material. Thus, the production of β -glucosidase can be improved via increasing the amount of fungal cells. Since this enzyme is responsible for the release of glucose from cellulose, the higher production of β -glucosidase may have increased the susceptibility of the fibers to be degraded. The moisture content is crucial to promote the fungal growth and subsequent degradation of available substrate (Hansen et al. 2015). To find the best conditions of humidity in the bioprocess, one must establish the appropriate moisture content considering the microorganism and substrate. Another equally important aspect was the fact that viability of filamentous fungi decreases in $\text{pH} > 7$ and that low pH promotes the production of cellulolytic enzymes (Lisboa-García et al. 2015). In Table 3, is shown the production of enzyme per gram of fermented substrate. The levels of water content had an effect in calculating the enzymatic activity. No difference was observed with treatments A and H. Regarding volumetric activity, a similar behavior was observed between treatments A, B and H. Considering that the main variable for this work was volumetric activity, treatment B had the higher results, although the most adequate β -glucosidase production conditions were not met in this report. Considering induction, it is possible that the presence of fibers, the

evaluated factors and glycosylated compounds play a significant role in enzyme induction (Kumar et al. 2008; Lu et al. 2010; Ascacio-Valdés et al. 2014). It is worth mentioning that, the modifications in the growth conditions could be added while keeping the solid bioprocess conditions viable for improvement of β -glucosidase production.

Table 3 shows fiber and phenolic content in the evaluated treatments. There was a little change in fiber content and phenolic compounds degraded by *A. niger* (Ascacio-Valdés et al. 2016). Along with the molecules shown in Fig. 3 that were detected in *F. cernua* aqueous extract, it is possible that β -glucosidase production can take place with no apparent fiber degradation, as research reports of fungal bioprocesses suggest. Vattem and Shetty (2003) attributed degradation of a certain type of polyphenols to a β -glucosidase. In the study of Ascacio-Valdés et al. (2016), β -glucosidases were produced by *A. niger* GH1 on a substrate composed by polyphenols. This kind of compounds include tannins, which are glycosylated phenolic compounds (Aguilera-Carbó et al. 2008; Kumar et al. 2008) which could induce cellulolytic enzyme production. Microbial growth in phenolic-containing substrates produces aglycones from compounds such as hydrolyzable phenolics or flavonoids (Dey et al. 2016). Production of β -glucosidase was relatively low, which agree with other authors such as Ascacio-Valdés et al. (2014) who obtained 525.53 U/L approximately, although enzyme production can be higher (Hansen et al. 2015), but as previously stated, the most adequate conditions were not attained.

Species of *Aspergillus* have been reported to produce hydrolytic enzymes in solid-state fermentation, β -glucosidase among them, which improved phenolic release (Dey et al. 2016). In the bioprocess, compounds detected such as quercetin-3-glucoside could be deglycosylated by a β -glucosidase to release quercetin and glucose. This process may be attributed to glycosyl-hydrolases produced by microorganisms, such as β -glucosidase (Huynh et al. 2014). Lu et al. (2010) describe cellulolytic enzyme production using xylose as carbon source where, among other enzymes, β -glucosidases were produced by an *A. niger* strain, suggesting that hemicellulose sugars can induce β -glucosidase production. In addition, it could be attributed to compounds of other nature. In Fig. 3 are shown possible molecules which could have an effect on β -glucosidase production in the present bioprocess. In the case of compound dimethyl epigallocatechin gallate, according to Kumar et al. (2008), this molecule could induce cellulase production from gallic acid associated with this compound, which would include β -glucosidase. It is possible that degradation of compounds such as dimethyl epigallocatechin gallate could be done by a combined effect of enzymes such as tannase, cellulases and β -glucosidase (Ascacio-Valdés et al. 2016).

Table 3 Additional parameters obtained from the bioprocess

Treatments	Raw fiber content (%)	Phenolic content (mg/g)	Enzymatic activity per gram of substrate (U/g)
A	12.80 ± 0.444 ^{ab}	1.27 ± 0.181 ^b	3.63 ± 0.000 ^a
B	12.99 ± 1.034 ^{ab}	0.60 ± 0.034 ^{cd}	3.21 ± 0.092 ^{ab}
C	12.57 ± 0.908 ^{ab}	1.02 ± 0.190 ^{bc}	3.03 ± 0.212 ^{abc}
D	12.18 ± 0.795 ^{ab}	0.50 ± 0.083 ^d	2.50 ± 0.000 ^c
E	12.36 ± 1.333 ^{ab}	1.13 ± 0.033 ^b	2.88 ± 0.000 ^{bc}
F	12.36 ± 0.541 ^{ab}	0.55 ± 0.100 ^d	2.50 ± 0.368 ^c
G	12.97 ± 0.758 ^{ab}	0.61 ± 0.014 ^{cd}	2.37 ± 0.000 ^c
H	12.95 ± 0.373 ^{ab}	1.33 ± 0.105 ^b	3.71 ± 0.106 ^a
CT	13.54 ± 0.500 ^a	0.69 ± 0.014 ^{cd}	3.04 ± 0.297 ^{abc}
N	10.93 ± 0.312 ^b	4.62 ± 0.499 ^a	–

Values are mean ± SD. Means with different superscripts differ ($P < 0.05$).

Fig. 3 Molecules detected by HPLC–MS where **a** quercetin-3-*o*-glucoside-7-*o*-glucoside [$M - H^-$] 713.4, **b** luteolin-7-*o*-rutinoside [$M - H^-$] 593.1, **c** apigenin-6-arabinoside-7-glucoside [$M - H^-$] 562.9 and **d** dimethyl epigallocatechin gallate [$M - H^-$] 487.2

Conclusions

The ability of *A. niger* to utilize *F. cernua* leaves to produce β -glucosidase was demonstrated. Thanks to the Plackett–Burman design, the bioprocess to produce these enzymes was improved and could be further optimized. The fungus used was capable of biodegrading polyphenols, which is noteworthy because these compounds could obstruct the isolation of these enzymes. Moreover, the apparent absence of biodegradation of the fiber components present in the plant material did not prevent the production of the enzymes of interest. In addition, there

could be mechanisms for fungal cellulolytic enzyme production that are interesting for further research. Considering that *F. cernua* is an abundant plant of the Chihuahuan Desert and it is sparingly ingested by wild and domestic ruminants, it has a potential use for enzyme production by biotechnological means. The use of fungal bioprocesses on this type of plant material shows the potential for exploiting renewable sources for added-value compound production.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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