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Evaluation of cellulase activity of fungi isolated from vanilla beans (*Vanilla planifolia* Jacks. ex Andrews)

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ABSTRACT

The main effects of fungal survival and infections of vanilla beans are related to their production of extracellular enzymes including cellulases. The aim of this study was to analyze the cellulolytic activity (CA) with two methods (M1 and M2) of the three fungi (*Fusarium oxysporum*, *Aspergillus wentii*, and *Cladosporium* sp.) isolated from green beans of vanilla. The plate method (M1) was estimated by the diameter colony and enzymatic index (EI) and the quantitative assay method (M2) was evaluated by enzymatic (U/L) and specific activity (U/mg of protein). With both methods, conditions of pH 5, and 7 and carboxymethyl cellulose (CMC) concentrations of 0.1, 0.5 and 1%, (w/v) were used. The results obtained reveal that *Cladosporium* sp. had the highest degradative activity with EI = 1.16 (pH 5 and 0.1% CMC), enzymatic and specific activities with values of 17,548 U/L and 15,563.15 U/mg, respectively (pH 7 and 1.0% CMC). The cellulolytic activity of *F. oxysporum*, the values of EI (0.3) and enzymatic activity were higher at pH 5 (3094.2 U/L). While *A. wentii* presented minimal or no activity with the conditions mentioned with M1 and M2. Finally, both methods can be a quick and simple alternative to evaluate the degradative activity in fungi that persist in beans and may be associated with deterioration of vanilla. Also, the methods presented in this work can be used in future works with other fungi models isolated from vegetables.

INTRODUCTION

Vanilla is a major natural flavor widely used in many foods, beverages, pharmaceutics, cosmetics, tobacco and traditional crafts. It is obtained from beans of *Vanilla planifolia* Andrews, an orchid original from Mexico and Central America (De la Cruz-Medina et al., 2009). Although Mexico has lost its position as the major vanilla exporter, it continues being the center of origin and genetic diversity for this important orchid (Hernández-Hernández, 2011). One of the main reasons that limitits commercial production is the phytosanitary problem on the green vanilla beans that causes fruit putrefaction. This crop is susceptible to fungal phytopathogens such as *Phytophthora* spp., *Fusarium* spp., *Sclerotium* spp., *Calospora* spp. and *Cladosporium* spp. that cause serious damage (Bhai and Dhanesh, 2008; Qin et al., 2010; Khoyratty et al., 2015). Several fungi have been investigated for cellulase production such as: *Aspergillus niger* Tiegh. *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Cladosporium sphaerospermum* Penz., *Penicillium chrysogenum* Thom, *Microascus brevicaulis* S.P. Abbott, *Stachybotrys chartarum* (Ehrenb.) S. Hughes *Verticillium cycolsporum* and *Pseudothielavia*

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hamadae (Udagawa) X. Wei Wang and Houbraken– This cellulolytic activity is being used commercially due to yield high levels of extracellular cellulases (Pérez et al., 2002). Cellulolytic enzymes are synthesized by both macro and micro-fungi and are mostly well known as agents of decomposition of organic matter and in particularof cellulosic substrate (Srivastava et al., 2017). However, the fungal species capable of producing high levels of extracellular enzymes are normally involved in pathological processes in plants (Loginov and S˘ebela, 2016).

Fungi's survival on vanilla beans is due to their hydrolytic and/or oxidative enzymes production that degrade lignocellulose, the major component of biomass (Pinaria et al., 2015). Added to that, these micro-organisms use cellulose as source of nutrients by an enzymatic complex known as cellulase (Murashima et al., 2003; Johnsen and Krause, 2014). There are three types of enzymes: endo-1,4-β-D-glucanase (EC3.2.1.4), cellobiohydrolase (EC3.2.1.91) and β-D-glucosidase (EC3.2.1.21), that act synergistically to depolymerize cellulose. These enzymes use different mechanisms to hydrolize the glycosidic bonds of the cellulose fibers producing different size oligosaccharides and free sugars (Howard et al., 2013). The cellulolytic activity can be quantified by several methods, among the most used are the plate assays (agar plates with CMC as substrate), spectrophotometric method (3,5-dinitrosalicylic acid, DNS) (Johnsen and Krause, 2014) and quantitative measurements of cellulase production during cultivation under solid-state fermentation. These procedures are useful for confirming cellulase activity produced by microorganisms (Florencio et al., 2012). The aim of this study was to analyze the cellulolytic activit of the three fungus (*Fusarium oxysporum*, *A. wentii,* and *Cladosporium* sp.) from vanilla beans, *in vitro* conditions pH 5, and 7 and carboxymethyl cellulose (CMC) concentrations 0.1, 0.5 and 1%, (w/v).

MATERIALS AND METHODS

Vegetable material

Green vanilla beans with symptoms of basal rot were collected from various locations of the Totonacapan region in the states of Puebla and Veracruz, Mexico. The samples were stored in sterile polystyrene bags and brought to the laboratory.

Fungi isolation and identification

The infected fruits were then placed in sterile petri dishes with Potato Dextrose Agar (PDA) and were incubated at 28 ± 2°C. Mycelial were purified and transferred to PDA slants and pure cultures of the fungal phytopathogens were maintained for 120 h. They were examined for colony morphology as well as microscopic characteristics for organism identification. Microscopic analysis of fungi were related with information collected in the Mycobank (http:www.mycobank.org).

Total genomic DNA was extracted from fresh mycelium using Qiagen DNeasy Plant Mini Kit. The nuclear ribosomal internal transcribed spacers (ITS 1 and 2) and 5.8 s gene (ITS rDNA) were amplified using primers ITS1F or ITS5 and ITS4 according to Hoffman and Arnold (2010). Polymerase chain reaction (PCR) products were visualized using Midori green direct (Nippon Genetics), following electrophoresis on a 1% agarose gel, and positive amplicons were submitted to cleanup, and bidirectional Sanger sequencing. Sequences were assembled automatically following the Geneious 9.0.4 manual (Biomatters Ltd) (Ronquist et al., 2003). Consensus sequences were compared against the NCBI non-redundant database using BLAST to estimate taxonomic placement and submitted to GenBank. The morphological and molecular identifications of isolated microorganism were compared with the homology percentage of the sequences (98-100%) and their corresponding GeneBank accession number; *F. oxysporum* (KY305292), *Cladosporium* sp. (KY305290) and *A. wentii* (KY305291).

Screening of cellulase enzymes

The production of fungal cellulase has been found to depend on several factors including pH, T, incubation time and carbon sources (Ramanathan et al., 2010). In this studio the cellulolytic activity was analyzed by the in plate (M1) and the quantitative assay (M2) methods with several CMC concentrations (0.1, 0.5 and 1%, w/v) and pH (5 or 7). Two methods were used to compare the qualitative *(*M1) and quantitative (M2) results of cellulolytic activity from vanilla isolated fungi.

Method 1 - plate assays

Cellulose hydrolysis was determined by plate assays with CMC agar with 0.1% (w/v) yeast extract, ammonium sulphate (1 $g.L^{-1}$) as the nitrogen source on PBS buffer and pH 5, 7 (Figure 1A). Enzyme assay plates were inoculated by pitting with each fungy and incubated at 28°C for five days. Subsequently, the plates were stained with 2% (w/v) aqueous congo red and were left for 15 min. Excess stain was removed with distilled water, after the plates were flooded with 1 M NaCl to destain for 15 min (Muthukrishnan, 2017). The colonies and clear zone (diameter) were measured and analyzed with the following equation:

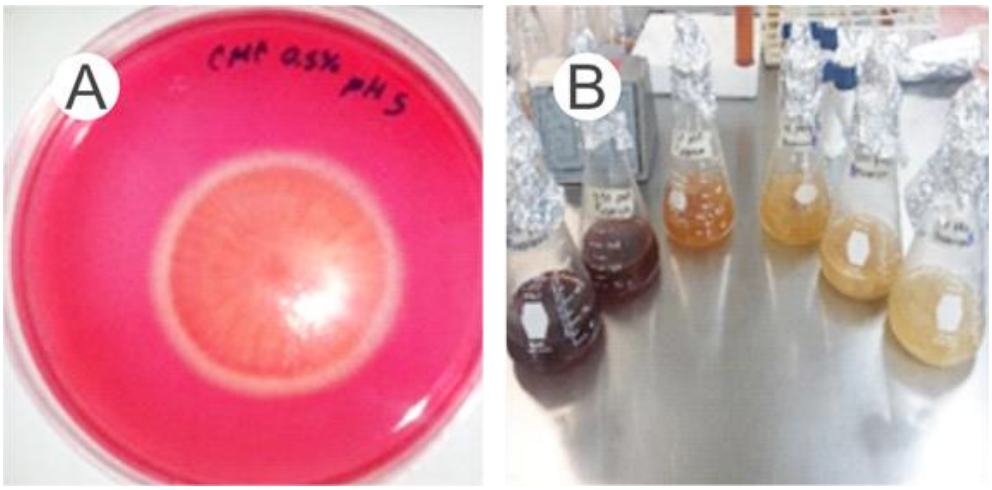

Figure 1. Pictures showing plate assay M1 (A) and quantitative assay M2 (B).

EI= (*dh/dc*) - 1

Where: EI, is the enzimatic index; *dh* is the diameter of hydrolysis; and *dc* is the diameter of colony.

Method 2 - quantitative assay

For the submerged fermentation process (Figure 1B) were inoculated selected fungi colonies (after five days growth) in natural potato broth was added 0.1% w/v of yeast extract, 0.1% m/v of $(NH4)_2SO_4$ 0.1% w/v and 0.5% w/v and 0.1, 0.5, 1% w/v of CMC. The media were sterilized at 15 psig for 15 min followed by pH adjustment to 5 or 7, with buffer Na_2HPO_4 and NaH_2PO_4 (0.1 M). The cultures were incubated at 37°C and shaken at 150 rpm for 15 d. Afterwards, the cultures were filtered through a 0.45 µm glass fiber filter and centrifuged at 3500 rpm by 15 min, these were used as the crude cellulases. The cellulase enzymatic activity was quantified using the procedure designed by Cianchetta et al. (2010) by measuring the amount of reducing sugar released from low viscosity CMC solution per minute using the glucose as the standard. The reaction mixture consisting of 0.5 mL CMC (w/v) in 0.5 mL of citrate buffer 0.1 M (pH 4.8) and 0.5 mL crude cellulose enzyme, this assay was realized under conditions of 50°C for 60 min. Finally, 1 mL of DNS (3,5-dinitrosalicylic acid, 3.5% w/w) was added and the final reaction was kept under conditions of T 95°C for 5 min.

The absorbance of samples was measured at 650 nm and the units of activity were expressed in terms of International Unit (U/mL), which are definined as the amount of enzyme that liberates 1 µmol/ minute/liter (U/L) of the reaction mixture.

Estimation of protein by Lowry's method

The specific enzymatic activity (U/mg of protein) was quantified with the Lowry method from liquid culture. A standard curve was prepared with Bovine Serum Albumin (BSA) at a concentration of 1 mg/mL and series of dilutions (0, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL) were made to duplicates with a final volume of 100 µL. The samples were transferred to the microplate, where 200 µL of Biuret reagent (0.5 mL of 1% cupric sulfate with 0.5 mL of 2% sodium potassium tartrate) and 50 mL of 2% sodium carbonate in 0.1 N NaOH were added. The mixture was incubated at room temperature for 10-15 min prior to the addition of 20 µL of Folin and Ciocalteu's (1 N) reagent. The absorbance of samples was measured at 650 nm (Cianchetta et al., 2010).

Experimental design and data analysis

The pH (5, 7) and CMC concentrations (0.1, 0.5 and 1%, w/v) were considered sources of variation, so that six treatments with the same number of replications were evaluated. A total of four replications with duplicate analysis were conducted. The data per treatment were analyzed using a model equivalent to a balanced complete random design (PROC ANOVA, SAS 2002). Comparison of means among treatments was performed with Tukey's test (SAS 2002). Statistical analyses were performed using Statistical Analysis System (SAS).

RESULTS AND DISCUSSION

The results of cellulase activity with the plate M1 showed

Table 1. Cellulase activity of fungi from vanilla beans, with different substrate concentrations and pH 5.0 assayed by the plate method.

Fungi	Substrate, CMC (%)	Clear zonediameter of (mm)	Enzymatic index (EI)
	0.1	45.50Aa	0.30 Ba
F. oxysporum	0.5	41.50 Bb	0.22 Bb
	1.0	40.50 Bb	0.17 BCb
	0.1	32.50 Ca	1.16 Aa
Cladosporium sp.	0.5	22.50 Db	0.96 Aa
	1.0	24.00 Db	1.00 Aa
	0.1	N/A	N/A
A. wentii	0.5	N/A	N/A
	1.0	N/A	N/A

The values are the average of four replicates. Different capital letters indicate significant differences ($p < 0.05$) between the values in the same column. Different lowercase letters indicate significant differences ($p < 0.05$) between values of substrate (concentration, CMC %). N/A: No activity.

Table 2. Cellulase activity of fungi from vanilla beans, with different substrate concentrations and pH 7.0 assayed by the plate method.

Fungi	Substrate (%)	Zone of clearance diameter (mm)	Enzymatic index (EI)
	0.1	43.06 Aa	0.22 BCc
F. oxysporum	0.5	40.18 Bc	0.13 BCb
	1.0	41.03 ABb	0.24 Ba
	0.1	24.0 Db	0.79 Aa
Cladosporium sp.	0.5	23.0 Db	0.71 Aa
	1.0	30.0 Ca	0.20 BCb
	0.1	N/A	N/A
A. wentii	0.5	15.3 Ea	0.34 Ba
	1.0	N/A	N/A

The values are the average of four replicates. Different capital letters indicate significant differences ($p < 0.05$) between the values in the same column. Different lowercase letters indicate significant differences ($p < 0.05$) between values of substrate (concentration, CMC %). N/A: No activity.

that all the assayed microorganisms were able to hydrolyze cellulose incorporated in media (Tables 1 and 2). Simmilarly, the EI of *Cladosporium* sp. and *F. oxysporum* revealed higher degradative activity ($p \pm 0.05$) than *A. wentii.* Khoyratty et al. (2015) demonstrated that these phytopathogens affect not only vanilla beans but also infect and damage stems, roots, leaves and shoots. Futhermore, Johnsen and Krause (2014) described that the high cellulolytic activity can be related by the necessity of the parasite to penetrate the host tissue by means of a process that is highly dependent on secreted enzymes. The primary functions of cellulolytic and hemicellulolytic enzymes are to hydrolyse the β1,4-glycosidic linkages present in the majority of plant structural polysaccharides, cellulose and hemicellulose respectively. Cellulases hydrolyze cellulose by synergistic action of their three enzymes such as β-1,4-endoglucanase, cellobiohydrolase and β-glucosidase, which cleaves internal β-1,4-glycosidic bonds and cellobiose is hydrolysed to glucose (Murashima et al., 2003; Deb Dutta et al., 2018).

In this work, pH was an important factor on the cellulolytic activity of *F. oxysporum* and *Cladosporium* sp., the values of the degradation zone and EI were higher at pH 5 (Table 1). These results are in agreement with those reported by Yuan et al. (2012) who obtained increased activity of hydrolase enzymes with pH near 5-6.

Rolling and Kerler (2001) reported that the genus *Aspergillus* sp. cause black and green rot at curing vanilla beans. Other reports confirm the production of hydrolytic enzymes including cellulases and pectinases of *A. niger* and *Aspergillus awamori* Nakaz species (Diaz et al., 2012; Desai and Iyer, 2016). However in this study, *A.*

Table 3. Cellulase activity fungi from vanilla beans, with different substrate concentrations and pH 5.0 assayed by the enzymatic method.

Fungi	Substrate (%)	Enzymatic activity (UI/L)	Specific enzymatic activity (UI/mg of protein)
	0.1	N/A	N/A
F. oxysporum	0.5	355.6Eb	866.67CDa
	1.0	590.21Ea	963.61CDa
	0.1	10653Cb	11553Bb
Cladosporium sp.	0.5	17548Aa	12747Bb
	1.0	15793Ba	15563Aa
	0.1	N/A	N/A
A. wentii	0.5	1255.6DEb	1701.1Ca
	1.0	2294Da	2223Ca

The values are the average of four replicates. Different capital letters indicate significant differences ($p < 0.05$) between the values in the same column. Different lowercase letters indicate significant differences ($p < 0.05$) between_values of substrate (concentration CMC %). N/A: No activity.

Table 4. Cellulase activity fungi from vanilla beans, with different substrate concentrations and pH 7.0 assayed by the enzymatic method.

Fungi	Substrate (%)	Enzymatic activity (UI/L)	Specific enzymatic activity (UI/mg of protein)
	0.1	393.92 Ca	1157.33 Cb
F. oxysporum	0.5	470.44 Ca	3094.20 Ca
	1.0	327.38 Ca	919.23 Cb
	0.1	13206.50 Aa	12320 ABa
Cladosporium sp.	0.5	10969.16 Ba	8452.3 Bb
	1.0	10977.48 Ba	15000 Aa
	0.1	77.85 Cb	286.91 Cb
A. wentii	0.5	122.77 Cb	10286 Ba
	1.0	749.08 Ca	N/A

The values are the average of four replicates. Different capital letters indicate significant differences ($p < 0.05$) between the values in the same column. Different lowercase letters indicate significant differences ($p < 0.05$) between values of substrate (concentration CMC %). N/A: No activity.

wentii did not present activity at pH 5 regardless of CMC concentration (Table 1), and a slight activity at pH 7 with 0.5% CMC (Table 2). This fungi did not exhibit clear zone with 0.1 and 1% CMC at any pH (Tables 1 and 2).

With regards to the concentration of 0.1% CMC, *F. oxysporum* and *Cladosporium* sp., presented values of EI 0.30 and 1.16, respectively (Table 1). This could be due to the fact that the plate method generates degradation zones more visible with lower concentrations of CMC (Samanta et al., 2011). According to Prasetyo et al. (2010) the molecular weight of CMC is other factor that can induce an increment in the production of enzymes to degrade the substrate to an available form for the microorganism.

Ramanathan et al. (2010) reported that *Fusarium* has the ability to produce cellulases at pH 7 when the medium is supplemented with different CMC concentrations. However, we found that *F. oxysporum* exhibited low hydrolysis (EI = 0.13-0.24) at pH 7 even though the size of colonies was greater (40-43 mm) (Table 2). The mycelial growth of *Fusarium oxysporum* f. sp. *Vanilla* causes rotting of the roots, stems, fruits and plant mortality (Adame-García et al., 2015; Khoyratty et al., 2015; Koyyappuratha et al ., 2015).

The maximum value of cellulolytic activity was of *Cladosporium* sp. (17548 U/L) at pH 7 and 0.5% CMC (w/v) (Table 4) whereas the lowest activity 77.85 U/L corresponds to *A. wentii* with pH 5 and 0.1% (w/v) (Table 3). Mushimiyimana and Tallapragada (2013) reported that *Cladosporium* sp. showed similar maximum activity

under submerged fermentation and 15 days of incubation. *A. wentii* presented maximum activities with pH 7 at 0.5 (1255 U/L) and 1.0 % (2294 U/L) of CMC, higher values than those obtained by Desai and Iyer (2016). While the obtained activity with *Cladosporium* sp. was higher than the reported by Gutiérrez-Ramírez et al. (2012); this difference is related to the experimental conditions (shaking, temperature) and the composition of the medium (potato dextrose broth, yeast extract and (NH4)2SO4 used in this study).

F. oxysporum presented significant differences with 0.1% CMC with higher activity at pH 5 (393.93 U/L); this result coincides with Ronquist et al. (2003) and Qin et al. (2010) who reported CMC enzymatic activity at broad ranging of pH from 4.2 to 10.3 with optimal value at pH 5.3, using 1 h of incubating at 50°C. The effect of pH on enzymatic activity coincides with Park et al. (2012) who observed that enzymatic activity is affected by the ionization of enzyme-substrate complex.

Tables 3 and 4 show the differences between the values of the enzymatic activity and specific activity of the different fungi under the different conditions of pH (5 and 7) and CMC (0.5, 1 and 1.5 w/v). The specific activities according to De Arriaga et al. (1979) are related to the higher concentration of proteins in the cellulase extracts and high affinity of these enzymes with the substrate (vanilla beans). *Cladosporium* sp. revealed differences ($p \leq 0.05$) in the enzymatic activity with maximum values of 1.0% of CMC and pH 5 or 7. These enzymatic activities may be related to the growth conditions of this fungus in vanilla beans, which have a pH of approximately 5 (Luna-Guevara et al., 2016). Abrha and Gashe (1992) and Yuan et al. (2012) considered that the cellulase complex of *Cladosporium* sp. presents activity with pH 4 to 8 with optimum activity value at pH 5. The same parameter contributed to the specific enzymatic activity of *F. oxysporum* with activities of 919 to 3094 U/mg of protein, in pH 5.0 (Table 3). While *A. wentii* enzymatic activity changed with relation to pH, with a maximum value of 10286 U/mg of protein and 0.5% CMC (Table 3), these results were higher than those achieved by Desai and Iyer (2016).

Conclusion

This study showed that fungi type and cellulose concentrations can influence on the production of extracellular enzymes of isolated fungi from vanilla beans. *Cladosporium* sp. and *Fusarium oxysporum* showed the higher enzymatic and specific enzymatic activities with 0.5 and 1% of CMC. The results obtained will be useful to identify and characterize the fungal extracellular enzymes involved in the pathogenesis of vanilla infections. Finally, both methods (plate and enzymatic) have the potential to be used to screen other fungi that affect to vanilla beans.

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