ENDOGLUCANASE PRODUCTION BY ENDOPHYTIC FUNGI ISOLATED FROM *Vitis labrusca* L. WITH PEANUT HULL AND SAWDUST AS SUBSTRATES

PRODUÇÃO DE ENDOGLUCANASE POR FUNGOS ENDOFÍTICOS ISOLADOS DE Vitis labrusca L. UTILIZANDO CASCA DE AMENDOIM E SERRAGEM COMO SUBSTRATOS

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ABSTRACT: Endoglucanases are enzymes widely employed in different industrial fields, albeit with high production costs. Studies on new microbial sources and low-cost substrates are highly relevant, including those on agro-industrial. Current analysis evaluates peanut hull (PH) and sawdust (SD) as substrates for submerged cultures of 14 endophytic fungi isolated from grapevine (*Vitis labrusca* L.) cultivars Bordô and Concord. Endophytes were grown on a carboxymethylcellulose (CMC) medium and the cup plate assay showed that eight strains (belonging to genera *Cochliobolus*, *Diaporthe*, *Fusarium* and *Phoma*) had positive results: enzymatic halos ranged from 10.8±0.02 to 15.5±0.07 mm in diameter. *Diaporthe* sp. strains (GenBank accession codes KM362392, KM362368 and KM362378) and *Fusarium culmorum* KM362384 were highlighted as the most promising sources. Further, PH and SD as substrates for the fermentation of these fungi were evaluated by the cup plate assay and endoglucanase activity assay. Highest halo diameters were obtained for *Diaporthe* sp. KM362392: 16.1±0.01 mm (CMC), 14.5±0.01 mm (PH) and 14.7±0.03 mm (SD). The fungus also presented the highest levels of endoglucanase activity: analysis of variance revealed that CMC (3.52±0.98 μmol/min), PH (2.93±0.23 μmol/min) and SD (3.26±0.38 μmol/min) were similarly efficient as substrates. Results deepen knowledge on *V. labrusca* endophytes that may be endoglucanase sources, even though further optimizations in submerged cultures with PH and SD should be undertaken to increase the enzymatic production from these wastes.

KEYWORDS: Cellulase. Microbial enzymes. Endophytes. Agro-industrial wastes. Submerged fermentation.

INTRODUCTION

Endophytic fungi colonize intra- or inter-cellular spaces of healthy plant tissues (FELBER et al., 2016; STONE et al., 2000) without causing any apparent damage; they are ubiquitous organisms found inside all plants (RODRIGUEZ et al., 2009). During their long coexistence process with host plants, these fungi developed important characteristics to maintain a stable symbiosis. Since cellulases hydrolyze the plant cell wall during colonization by microbial endophytes (DUTTA et al., 2014), there is a great interest in endophytic sources of cellulase for industrial application. In fact, the search of microbial enzymes has already detected several endophytic sources of hydrolytic enzymes, as recently reported by Fouda et al. (2015), Orlandelli et al. (2015, 2017a) and Ribeiro et al. (2018).

Cellulolytic enzymes form a complex of three groups that catalyze cellulose hydrolysis to glucose: (1) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91); (2) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4); (3) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21). Exoglucanases act on the reducing or non-reducing ends of cellulose polysaccharide chains and release glucose or cellobiose as major products.

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Endoglucanases cut randomly at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides, whereas β-glucosidases hydrolyze soluble cellodextrin and cellobiose to glucose (LYND et al., 2002).

Dollar-wise, cellulases represent the third largest industrial enzyme worldwide. However, if, in the future, ethanol from lignocellulosic biomass through the enzymatic route becomes a major transportation fuel, these enzymes may occupy the largest volume of industrial enzymes (SINGHANIA et al., 2013). In particular, endoglucanases have a great potential application in textile and detergent industries, paper recycling, juice extraction, animal feed additives and renewable energy (LIN et al., 2016). The main disadvantage is that cellulases have high production costs. However, industrial wastes may replace the cellulose as substrate, obtaining low-cost enzymes.

Large amount of wastes is annually generated by food, agricultural and forestry industries, causing a serious disposal issue. For instance, about 222 millions/m^3 of waste from the lumber industry are generated worldwide and the peanut production exceeds 13 million tons/year (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2014), mostly discarded as waste.

Fungal cellulase researches mainly focus on *Fusarium solani*, *Penicillium pinophilium*, *Sporotrichum pulverulentum*, *Trichoderma reesei* and *Trichoderma viride* strains (McKELVEY; MURPHY, 2011). Only scanty data in the literature report endophytes *Alternaria*, *Bipolaris*, *Cochliobolus*, *Diaporthe*, *Fusarium* and *Phoma* as cellulase sources (BEZERRA et al., 2015; CHRISTAKOPOULOS et al., 1995; LUMYONG et al., 2002; PENG; CHENG, 2007). The biosynthesis of microbial products is affected by culture medium composition and cultivation conditions (ELISASHVILI, 2012). Further studies under different culture conditions are necessary to provide an in-depth knowledge on endophytic strains that may be enzymatic sources. This study aimed to compare the use of carboxymethylcellulose, peanut hull and sawdust as substrates for the endoglucanase production by *Vitis labrusca* L. endophytes.

MATERIAL AND METHODS

Endophytic fungi

Fourteen fungal strains of genera *Alternaria*, *Bipolaris*, *Cochliobolus*, *Diaporthe*, *Fusarium* and *Phoma* (Table 1) were used. Fungi were isolated from healthy leaves of Bordô and Concord cultivars of *V. labrusca* (FELBER et al., 2016) and belong to the fungal culture collection of the Laboratório de Biotecnologia Microbiana, Universidade Estadual de Maringá, Maringá PR Brazil. Molecular identification was based on the sequencing of ITS1-5.8S-ITS2 region of rDNA. Sequences were deposited in the GenBank database (FELBER et al., 2016).

Agro-industrial wastes

Peanut hull (PH), obtained from local vendors in Maringá PR Brazil, in the form of food and beverage production wastes, was blended and sieved to obtain 1-mm particles. Sawdust (SD) was obtained from the local lumber industry and preserved in its original size.

Submerged fermentation

All endophytes were previously grown in Petri dishes with potato dextrose agar medium (SMITH; ONIONS, 1983), at 28±2ºC, for seven days. Three 5-mm mycelial plugs of each fungus were transferred to 125-mL Erlenmeyer flasks containing 50 mL of Manachini's solution (MANACHINI et al., 1987), comprising 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$, 0.9 g/L $Na_2HPO_4.2H_2O$, 1 g/L yeast extract, and volume completed to 1 L with distilled water. The following substrates (0.5% w/v) were added to the medium: carboxymethylcellulose (CMC) (Sigma-Aldrich, St. Louis, MO, USA) was used for the initial screening of cellulase-positive endophytes; PH or SD was used for comparison to CMC. Negative control consisted of liquid medium incubated without fungal inoculation. Flasks were incubated in triplicate, at 28±2ºC, for 168 h under stationary condition and filtered with sterile gauze to separate the fungal mycelia. Cell-free supernatants were used as crude enzymatic extracts.

Cup plate assay

Crude enzymatic extracts were inoculated (50 μL) on Petri dishes containing CMC-agar medium (18 g/L agar, 10 g/L CMC, 0.1 M Na^+ acetate buffer, pH 5.0) with the surface perforated for cup plates (6-mm diameter). Positive control consisted of commercial cellulase from *Aspergillus niger* (powder, off-white, ~0.8 U/mg) purchased from Sigma-Aldrich (St. Louis, MO, USA). The experiment was performed in triplicate and dishes were incubated at 28±2ºC for 24h. The dishes were flooded with 0.1% Congo red solution (in 0.1 M Tris buffer pH 8.0) for 30 min and de-stained with 0.5 NaCl solution (in 0.1 M Tris buffer pH 8.0) for 15 min. Cellulase activity was evaluated by the

development of colorless halos on a red background and measured in millimeters.

Endoglucanase activity assay

A modified version of the carboxymethylcellulase assay for endo-β-1,4-glucanase described by Ghose (1987) was used. The 0.5 mL-aliquots of crude enzymatic extracts were pipetted into test tubes triplicates containing 0.5 mL of CMC solution (1% w/v) in sodium citrate buffer (50 mM; pH 4.8). After 15 and 30 min of incubation at 40ºC, 1 mL of 3,5-dinitrosalicylic acid (DNS) (MILLER et al., 1959) was added. Tubes remained at 100ºC for 5 min and immediately transferred to a cold-water bath. The volume of each tube was completed to 3 mL with distilled water.

Blank tubes in each assay contained crude enzymatic extracts and CMC solution (1% w/v), which were immediately mixed to DNS (corresponding to time zero). Absorbance was measured in a Libra S60PC spectrophotometer (Biochrom, Cambourne, CBE, UK) at 540 nm. One unit of endoglucanase was the amount of enzyme required to liberate 1 µmol/min/mL glucose.

Statistical analyses

To compare the best culture medium for the enzymatic production by each fungus and the best fungal producer by submerged fermentation in each culture medium, results of cup plate and endoglucanase activity assays were analyzed by ANOVA (analysis of variance) and means were compared by Tukey test ($p<0.05$) with SISVAR 5.3 (FERREIRA, 2011).

RESULTS AND DISCUSSION

Screening of cellulase-positive endophytes

Cellulose is the most abundant renewable carbon source on the Earth's crust. In nature, microorganisms degrade cellulose by cellulase production. In fact, enzymes from filamentous fungi are the most efficient cellulose-degrading tools (GUTIÉRREZ-ROJAS et al., 2015). Consequently, fungal sources of enzymes were initially screened by the cup plate assay, where halo formations revealed that the inducing substrate was hydrolyzed by microbial cellulases. Table 1 shows that eight fungal endophytes (belonging to genera *Cochliobolus*, *Diaporthe*, *Fusarium* and *Phoma*) produced cellulolytic enzymes when grown on CMC medium. *Alternaria* and *Bipolaris* strains did not have positive results. Since fungal metabolic activity is affected by nutritional and physical parameters, the *V. labrusca* endophytes should be further evaluated according to their capacity for the production of cellulase with different cultivation condition.

Table 1. Screening of cellulase-positive endophytes using carboxymethyl cellulose (CMC) as carbon source for submerged fermentation. Results of cup plate assay are given as mean±standard deviation.

Endophytic fungi/ Controls	Host plants	Halos (mm)
Diaporthe sp. KM362368	B	15.5±0.07[b]
Fusarium culmorum KM362384	C	15.1±0.04[b]
Diaporthe sp. KM362392	C	15.0±0.05[b]
Diaporthe sp. KM362378	B	13.5±0.02[c]
Phoma sp. KM362379	B	12.4±0.02[d]
Cochliobolus sp. KM362367	B	11.9±0.06[de]
Diaporthe sp. KM362382	B	11.1±0.03[ef]
Phoma sp. KM362373	B	10.8±0.02[f]
Bipolaris sp. KM362374	B	00.0±0.00[g]
Fusarium sp. KM362390	C	00.0±0.00[g]
Bipolaris sp. KM362380	B	00.0±0.00[g]
Alternaria sp. KM362386	C	00.0±0.00[g]
Alternaria alternata KM362389	C	00.0±0.00[g]
Cochliobolus sp. KM362388	C	00.0±0.00[g]
Positive control	-	25.8±0.02[a]
Negative control	-	00.0±0.00[g]

Means of triplicates followed by different letters are significantly different by Tukey test ($p<0.05$). Host plants: Bordô (B) or Concord (C) cultivars of *Vitis labrusca*. Positive control: cellulase from *Aspergillus niger* (powder, off-white, ~0.8 U/mg; Sigma-Aldrich) diluted (1 mg/mL) in distilled water. Negative control: liquid medium incubated without fungal inoculation.

ANOVA showed statistically significant differences among the enzymatic halos, ranging between 10.8 and 15.5 mm in diameter; the highest rates were obtained for *Diaporthe* sp. KM362368 (15.5±0.07 mm), *Fusarium culmorum* KM362384 (15.1±0.04 mm), *Diaporthe* sp. KM362392 (15.0±0.05 mm) and *Diaporthe* sp. KM362378 (13.5±0.02 mm). Therefore, enzymatic production of the fungal strains was subsequently evaluated using agro-industrial wastes as substrates.

Comparison of the influence of different substrates on enzymatic activity

Cellulases may be produced by microorganisms on cheap lignocellulosic substrates (SRIDEVI et al., 2015). SD contains approximately 48% cellulose, 17% hemicellulose and 12% lignin (SRIDEVI et al., 2015), while PH contains about 39%, 23% and 21% cellulose, hemicellulose and lignin, respectively (CHUWECH; RAKARIYATHAM, 2014). In Brazil, the above-mentioned substrates are agro-industrial wastes and represent low-cost raw materials, which may be used to produce value-added compounds such as enzymes.

After the initial screening, CMC and agro-industrial wastes (PH and SD) were compared by the cup plate and endoglucanase activity assays (Table 2). Enzymes used for producing plant-based ethanol (i.e., bioethanol) have gained more importance in recent years, including endoglucanases (YENNAMALLI et al., 2013). Therefore, among the cellulases, it was chosen to be quantified by the endoglucanase activity assay. Table 2 shows that three *Diaporthe* (= *Phomopsis*) endophytes were the best sources of endoglucanase. A recent study has also highlighted *Diaporthe anacardii* PL01 (with halos measuring 15.02 mm and 1.60 µmol/min of endoglucanase activity) and *Diaporthe* sp. PL67 (12.89 mm and 1.23 µmol/min) as the best enzymatic sources (RIBEIRO et al., 2018). The genus occurs as plant pathogens, endophytes or saprobes (GOMES et al., 2013) and plays an important role in decomposition in plant senescence (CARROLL; PETRINI, 1983), related to their lignocellulolytic activity (JORDAAN et al., 2006).

Table 2. Effect of different carbon sources on enzymatic activity of endophytic fungi, evaluated by cup plate (halos in mm) and endoglucanase activity assays (µmol/min). Results are given as means of triplicates±standard deviation.

Fungi/ Controls	Inducer substrates		
	CMC	**PH**	**SD**
	Halo degradation (mm)		
Diaporthe sp. KM362392	16.1 ± 0.01^{Ab}	14.5 ± 0.01^{Bb}	14.7 ± 0.03^{Bb}
Diaporthe sp. KM362368	15.7 ± 0.03^{Ab}	14.1 ± 0.06^{Bb}	12.0 ± 0.03^{Cc}
Diaporthe sp. KM362378	14.3 ± 0.01^{Ac}	00.0 ± 0.00^{Bc}	00.0 ± 0.00^{Bd}
F. culmorum KM362384	13.7 ± 0.08^{Ac}	00.0 ± 0.00^{Bc}	00.0 ± 0.00^{Bd}
Positive control	25.7 ± 0.06^{a}	25.7 ± 0.06^{a}	25.7 ± 0.06^{a}
Negative control	00.0 ± 0.00^{d}	00.0 ± 0.00^{c}	00.0 ± 0.00^{d}
	Endoglucanase activity (µmol/min)		
Diaporthe sp. KM362392	3.52 ± 0.98^{Aa}	2.93 ± 0.23^{Aa}	3.26 ± 0.38^{Aa}
Diaporthe sp. KM362368	3.14 ± 0.09^{Aab}	1.57 ± 0.15^{Bb}	1.15 ± 0.02^{Cb}
Diaporthe sp. KM362378	2.65 ± 0.24^{Aa}	0.63 ± 0.15^{Bb}	0.57 ± 0.07^{Bb}
F. culmorum KM362384	2.05 ± 0.24^{Aa}	0.63 ± 0.12^{Bb}	0.73 ± 0.10^{Bb}

Means followed by different lower-case letters (columns) or upper-case letters (rows) are significantly different by Tukey test ($p<0.05$). Inducer substrates: CMC (carboxymethylcellulose), PH (peanut hull) and SD (sawdust). Positive control: cellulase from *Aspergillus niger* (powder, off-white, ~0.8 U/mg; Sigma-Aldrich) diluted (1 mg/mL) in distilled water. Negative control: liquid medium incubated without fungal inoculation.

ANOVA showed that CMC, PH and SD were similarly efficient for *Diaporthe* sp. KM362392, with results varying between 3.52±0.98 and 2.93±0.23 µmol/min. On the other hand, *Diaporthe* strains showed the highest activities when grown on CMC (CMC > PH ≥ SD): 3.14±0.09 and 2.65±0.24 µmol/min, respectively for *Diaporthe* sp. KM362368 and KM362378. The fungal

biosynthesis is a strain-dependent process and may foreground the differences in compounds secreted by closely related species cultivated under the same conditions (DIAMANTOPOULOU et al., 2014), as observed in our study for these *Diaporthe* strains. In corroboration, marked differences were already reported for other metabolic products obtained for endophytes from this genus (ORLANDELLI et al., 2016, 2017b). CMC was also the most suitable substrate for the endoglucanase activity of *F. culmorum*, where rate (2.05±0.24 µmol/min) was statistically higher than that for other substrates.

PH is scantily employed for cellulase production. Results highlighted *Diaporthe* sp. KM362392 (2.93±0.23 µmol/min) and *Diaporthe* sp. KM362368 (1.57±0.15 µmol/min) with the highest endoglucanase activity when PH was used as substrate. When grown under submerged fermentation with PH, *A. niger* and *Humicola insolens* had enzymatic production of 0.54 U/mL (MOHITE; MAGAR, 2010) and 1.0 U/L (BORKAR; THAKRE, 2014), respectively.

In the case of SD, the four endophytes showed differences in enzymatic activities, with means ranging between 0.57±0.07 and 3.26±0.38 µmol/min. Highest rate was observed for *Diaporthe* sp. KM362392. These enzymatic activities are superior to that (approximately 0.07 IU/mL) obtained for the submerged cultivation of *Aspergillus flavus* in a bioreactor with alkaline pretreated SD (OJUMU et al., 2003) and for the enzymatic hydrolysis of alkaline pretreated SD by *A. niger* (approximately 0.18 U/mL) (ACHARYA et al., 2008).

CONCLUSIONS

Some *V. labrusca* endophytes may produce endoglucanase from agro-industrial wastes, with *Diaporthe* isolates as promising enzymatic sources. *Diaporthe* sp. KM362392 produced statistically similar amount of endoglucanase with carboxymethylcellulose, peanut hull or sawdust.

Current investigation contributes towards the reuse of agro-industrial wastes abundantly generated in Brazil. Further investigations should be undertaken to increase the enzymatic production by using the wastes tested.

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RESUMO: Endoglucanases são enzimas amplamente empregadas em diferentes setores industriais; embora sua produção apresente custos elevados. Estudos sobre novas fontes microbianas e substratos mais baratos são de grande importância, incluindo os resíduos agroindustriais. Nesse estudo, casca de amendoim (CA) e serragem (SE) foram testadas como substratos para o cultivo submerso de 14 fungos endofíticos isolados das cultivares Bordô e Concord de videira (*Vitis labrusca* L.) Os endófitos foram crescidos em meio contendo carboximetilcelulose (CMC) e o ensaio *cup plate* mostrou resultados positivos para oito fungos (pertencentes aos gêneros *Cochliobolus*, *Diaporthe*, *Fusarium* and *Phoma*); os halos enzimáticos variaram entre 10,8±0,02 e 15,5±0,07 mm de diâmetro. Linhagens de *Diaporthe* sp. (códigos de acesso no GenBank KM362392, KM362368 e KM362378) e *Fusarium culmorum* KM362384 se destacaram como produtores mais promissores. Então, o uso de CA e SE como substratos para a fermentação desses fungos foi avaliado pelo ensaio *cup plate* e pela quantificação da atividade de endoglucanase. Os maiores halos enzimáticos foram obtidos para *Diaporthe* sp. KM362392: 16,1±0,01 mm (CMC), 14,5±0,01 mm (CA) e 14,7±0,03 mm (SE). Esse fungo também apresentou os maiores níveis de endoglucanase: a análise de variância revelou que CMC (3,52±0,98 µmol/min), CA (2,93±0,23 µmol/min) e SE (3,26±0,38 µmol/min) foram substratos similarmente eficientes. Esses resultados expandem o conhecimento sobre endófitos de *V. labrusca* que são fontes de endoglucanases; futuras otimizações quanto ao cultivo submerso com CA e SE podem ser utilizadas para aumentar a produção enzimática a partir do uso desses resíduos.

PALAVRAS-CHAVE: Celulase. Enzimas microbianas. Endófitos. Resíduos agroindustriais. Fermentação submersa.

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