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Screening and detection of extracellular cellulases (endo- and exo-glucanases) secreted by filamentous fungi isolated from soils using rapid tests with chromogenic dyes

Brigitte Sthepani Orozco Colonia and Aloísio Freitas Chagas Junior

Department of Agricultural Sciences and Technology, Federal University of Tocantins, Rua Badejós, Chácaras 69 e 72, Lote 07, Zona Rural, CEP 7740-2970 Gurupi, Tocantins, Brazil.

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The screening plate method is commonly used for previous detection of cellulases produced by micro-organisms with biotechnological potential. In this manuscript, the authors aim to evaluate the hydrolytic ability of different fungi isolated from soil for the production of cellulolytic enzymes for cellulose degradation and determining the enzymatic index (EI) in relation to the growth of fungal colony and halo. The fungi were grown in carboxymethyl cellulose medium (CMC 1% w/v) and Avicel medium (Cellulose microcrystalline 1% w/v) for the determination of endo-glucanases and exo-glucanases respectively at 28°C for 48 h. Four chromogenic dyes were used: Congo Red, Phenol Red, Trypan Blue and Gram's Iodine. Also, another screening method was compared using carboxymethyl cellulose medium (CMC 1% w/v) at 28°C for 96 h and exposed with Congo Red dye in buffer Tris HCl 0.1 M, pH 8.0. The results obtained allowed to find significant differences between the tested fungi, the growth time and chromogenic dyes. The strains with higher Enzymatic Index (EI) were JCO1, UFT1, UFT2 and UFT3 for endo-glucanases and JCO2, UFT1, UFT2 and UFT3 for exo-glucanases.

Key words: Cellulases, chromogenic dyes, filamentous fungi, endo-glucanase, exo-glucanase.

INTRODUCTION

In every year, millions of tonnes of waste of lignocelluloses formed by cell walls incorporated mainly of cellulose, being one of the compounds most abundant and hard-to-degrade in nature are generated (Sae-Lee and Boonmee, 2014); and are present in different sources such as agricultural, industrial, forestry and agro-industrial waste; becoming attractive feedstocks for the generation of different by-products (Gupta et al., 2012). Most of these residues are burned in the open, generating dioxins due to the combustion conditions and affecting the environment, human and animal health (CEC, 2014).

Cellulose is a polysaccharide constituted by crystalline structures comprised of chains of β(1–4)-D-glucose,

*Corresponding author. E-mail: bsorozco@uft.edu.br or chagasjraf@uft.edu.br. Tel: +556333113549.

recognized for its potential for energy generation and with recent studies for the production of second generation ethanol (Glass et al., 2013; Johnsen and Krause, 2014). However, to obtain these by-products, cellulose must be subjected to enzymatic degradation process with the help of a system that allows conversion into glucose units. Within enzymatic processes are included biological systems developed by different microorganisms such as bacterial and fungal species (Chakraborty and Mahajan, 2014). The cellulases produced by fungal species are formed by a cellulolytic complex of endo-β-1,4-gluca-nases (EC 3.2.1.4), exo-β-1,4-glucanases or cello-biohydrolases (EC 3.2.1.91) and β-glucosidases or cellobiases (EC 3.2.1.21) (Szakacs et al., 2010). The endo-glucanases are responsible for degrading cellulose chains internally, where at the same time, the cello-biohydrolases hydrolyze the reducing and non-reducing ends releasing cellobiose and finally these are hydrolyzed by glucosidases in glucose molecules (Gilbert, 2010; Glass et al., 2013). Therefore, the native fungal cellulases isolated from various sources such as soil and decaying wood are commercially important for their resistance to high temperature conditions, pH changes and by high levels of enzyme secretion (Juturu and Wu, 2014).

The screening method for the detection of extracellular enzymes using dyes developers or chromogenic such as Congo red, Phenol red, Trypan blue, Gram's iodine, Remazol brilliant blue, it has been commonly used for *in vitro* selection of polysaccharides degraders microorganisms and characterized as a simple, fast and cost-efficient technique (Yoon, et al., 2007; Kasana et al., 2008; Jo et al., 2011). Where, the cellulolytic activity is reflected by the appearance of clear halos that surround the colony and not degraded areas arise without exposure or color variation (Johnsen and Krause, 2014).

These previous tests applied in laboratory scale fermentations are important in the selection of indicator strains of cellulolytic activity, where the lignocellulosic waste are degraded and recovered by the same industries such as the production of bioethanol and paper pulping. This study aimed to evaluate the enzymatic capacity of different filamentous fungi isolated from soil for the production of extracellular cellulases using different chromogenic dyes (congo red, phenol red, trypan blue and gram's iodine). In this case, the enzymatic index calculated by colony growth in relation to enzymatic halo released by the endo-glucanases and exo-glucanases used as carbon sources: carboxymethyl cellulose (CMC) and cellulose microcrystalline (Avicel) respectively will be analyzed.

MATERIALS AND METHODS

Fungi strains

Eight fungi strains granted by the Microbiology Laboratory located in Biotechnology-Based Business Incubator of the Federal University of Tocantins, Gurupi Campus, Brazil were employed. Where, JCO1, JCO2, JCO3, JCO4 and JCO5 belong to the company JCO Fertilizers and were isolated from soils in Barreiras Municipality, Bahía, Brazil. The fungi UFT1, UFT2 and UFT3 were isolated from soils by Federal University of Tocantins, Gurupi Campus, in the town Lagõa da Confusão, Tocantins, Brazil. The strains were replicated and stored in PDA medium (Potato 200 g/L, dextrose 20 g/L, agar 15 g/L). The fungi grew at 28°C for 8 days and preserved at 4°C for 3 months.

Screening and evaluation of cellulases enzymatic activity

To determine production of Endo-1,4-β-D-glucanase, the strains were grown in CMC medium (Carboxymethyl Cellulose 1% w/v), adapted from Kasana et al. (2008), as sole carbon source (%w/v): 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 1% carboxymethylcellulose sodium salt, 0.05% peptone and 2% agar. For the productions of Exo-1,4-β-D-glucanase (Avicelase), the strains were grown in Avicel medium (Cellulose microcrystalline 1% w/v) composed of the same salts as the CMC medium (Jo et al., 2011). The plates were inoculated with a fungal mycelium disc of 1 cm diameter and incubated at 28°C for 48 h in darkness (Agustini et al., 2012).

Extracellular cellulases detection using indicator dyes

The enzymatic activity colorations using indicators were: Congo Red, Phenol Red, Trypan Blue and Gram's Iodine (Vetec, Synth, Vetec, Newprov). After the incubation period, the first plates were flooded with 10 mL Congo Red (0.1% w/v) solution. After 30 min, the solution was discarded. The crops were washed with 5 mL NaCl (0.5 M) solution for 10 min (Teather and Wood, 1982; Kim et al., 2000). The second and third sets of plates were flooded with Phenol Red and Trypan Blue respectively (Yoon et al., 2007) and it proceeded in the same way as with Congo red solution. The last set of plates was flooded with 10 mL Gram's Iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 min (Kasana, et al., 2008; Johnsen and Krause, 2014).

Comparison of the screening method using Congo Red

Also, it was compared and another screening method in plate described by Ruegger and Tauk-Tornisielo (2004) and proposed by Nogueira and Cavalcanti (1996) for the detection of Endo-1,4-β-D-glucanase was adapted: The strains were grown in carboxymethyl cellulose medium (CMC 1% w/v) as sole carbon source (%w/v): 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 1% carboxymethylcellulose sodium salt and 2% agar. The plates were inoculated with a fungal mycelium disc of 1 cm diameter and incubated at 28°C for 96 h in photoperiod. After that period, they were grown at 37°C for 16 h, 10 mL of Congo Red (0.1% w/v) in Tris-HCl buffer 0.1 M and pH 8.0. After 30 min, the solution was discarded. The crops were washed with 5 mL of NaCl (0.5 M) solution for 10 min (Kim et al., 2000).

Molecular identification of strains

After screening assays, the strains isolated from JCO Fertilizers JCO1, JCO2, JCO3, JCO4, JCO5 that are part of the fungi mix of Trichoplus product were identified through the sequencing of the ITS region, in the Biological Institute, São Paulo, Brazil. The isolates UFT1, UFT2 and UFT3 still were unidentified molecularly, therefore were compared with respect to fungi identified. DNA extraction was carried out according to the methodology of CTAB

Table 1. Molecular identification of fungi isolated from the Trichoplus product of JCO Fertilizer.

Isolated	Identified species	GenBank access	% Similarity index
JCO1	*T. asperelloides* GJS04-217	DQ381958	100
JCO2	*T. longibrachiatum* DAOM 167674	EU280099	100
JCO3	*T. harzianum* CIB T44	EU280077	100
JCO4	*T. harzianum* CIB T44	EU280077	100
JCO5	*T. asperelloides* GJS 04-217	DQ381958	100

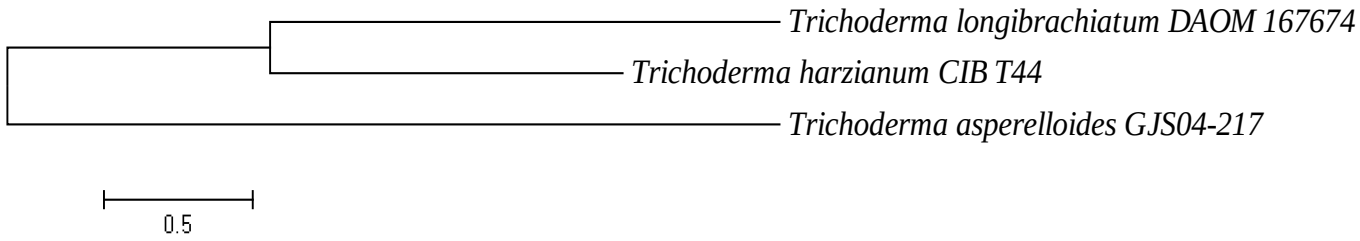

Figure 1. Phylogenetic tree analyzed using neighbor-joining method in the MEGA6 software.

(Cetyltrimethylammonium Bromide) described by Doyle and Doyle (1987). The polymerase chain reaction (PCR) for amplification of gene fragment encoding elongation factor (EF) was performed with the primer pair tef71F (5‟ – CAAAATGGGTAAGGAGGASAAGAC – 3‟) and tef997R (5‟ – CAGTACCGGCRGCRATRATSAG – 3‟) (product size of approximately 930 pb) (Shoukouhi and Bissett, 2008). The reactions were performed in thermal cycler PTC100 (MJ Research) according to the following schedule: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 60 s and final extension at 72°C for 4 min. The polymerase chain reaction (PCR) products were purified by precipitation with polyethylene glycol, according to the protocol described by Schmitz and Riesner (2006). The sequencing was conducted by chain termination method with the Big Dye 3.1 reagent (Applied Biosystems) in an automatic sequencer ABI3500 (Applied Biosystems). The sequences obtained were compared with sequences of voucher specimens deposited in the database of the ISTH International Subcommission on Trichoderma and Hypocrea Taxonomy). Identification of strains using molecular methods was carried out by sequences BLAST. The ITS sequences were retrieved from GenBank and the phylogenic trees were constructed using neighbor-joining method in the MEGA6 software (Tamura et al., 2013).

Data analysis

The enzymatic index was determined between the colony diameter and the enzymatic halo diameter (EI = Øh/Øc) (Herculano et al., 2011). The halo presence or clear area around the crop was the cellulase activity indicator evidenced by the enzymes secretion made by fungi through the culture medium (Sharma and Sumbali, 2014). The data was analyzed using IBM SPSS Statistics 19 by analysis of variance ANOVA and Tukey and Duncan multiple comparisons with a confidence level (p = 0.05).

The trials were conducted in triplicate and was performed in an experimental design for each of the dependent variables: Enzymatic Index (EI) of endo-1,4-β-D-glucanase and Exo-1,4-β-D-glucanase. Each design composed of three factors or independent variables: Strains, dyes and medium; with different levels or variations factor. In the case of 48 h of growth, the tests were performed in 8 × 4 × 1 factorial scheme per dependent variable. The second screening method was compared with respect to the average values obtained by the first method using CMC medium and Congo Red dye. The independent variables were: Strains, dyes, medium and time. Eight fungal strains, Congo Red dye with and without buffer, growth time 48 and 96 h, and two CMC medium described by authors with different methodologies were employed. The test was performed in 8 × 2 × 2 × 2 factorial scheme per dependent variable and in this case, for the detection of endo-1,4-β-D-glucanases.

RESULTS AND DISCUSSION

Five of the eight strains of filamentous fungi were identified molecularly (Table 1). The strains UFT1, UFT2, UFT3 were not yet sent for identification. The strains JCO1 and JCO5 were identified as the same species of *T. asperelloides* GJS04-217 and the strains JCO3 and JCO4 identified as *T. harzianum* CIB T44. Once identified molecularly, the three fungal species were analyzed using neighbor-joining method in the MEGA6 software (Figure 1). In the case of JCO1 and JCO5, the strains were in a separate group compared with the strains JCO2, JCO3 and JCO4. Therefore, T. longibrachiatum DAOM 167674 and T. harzianum CIB T44 showed a similar grouping. The results of screening methods were statistically analyzed by analysis of variance. The Fisher distribution test indicates a value of F-critical less than F-ratio for the CMC and Avicel medium (Table 2). Therefore, the Enzymatic Index Averages (EI) presents differences significant to the 1% of probability. In the second method with Congo Red (Figure 2), after 96 h of

Table 2. Analysis of variance of the experiment using the Fisher distribution test.

Source of variance	Sum of squares	Degrees of freedom (df)	Mean squares	F-ratio	F-critical
CMC ANOVA					
Between groups (Strains)	4.117	7	0.588	17.366**	2.849
Within groups (Dyes)	2.980	88	0.034		
Total	7.097	95			
AVICEL ANOVA					
Between groups (Strains)	7.511	7	1.073	38.321**	2.849
Within groups (Dyes)	2.464	88	0.028		
Total	9.974	95			

**Significant at 1% probability.

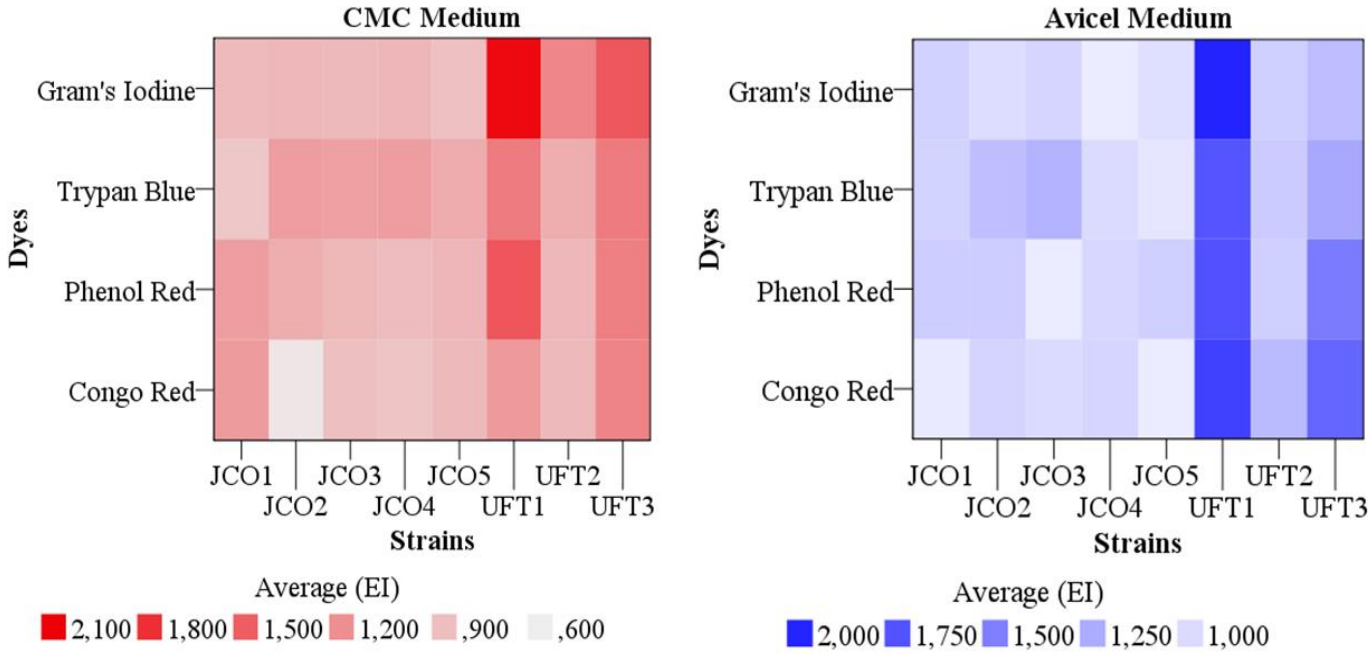

Figure 2. Statistical average group for the studied strains with different dyes in CMC and Avicel medium.*Second screening method for 96 h.

growth, some strains that were not evident development of halo at 48 h can be described as positive producers of Endo-glucanases as the strains JCO1, JCO3, JCO4 and JCO5. The enzymatic indices near 2.1 have better production of Endo-glucanases. In the case of Exo-glucanases, the fungi have a similar growth behavior with different chromogenic dyes. The enzymatic indices above 1.0 clearly represent enzyme secretion outside the colony and those with values below 1.0 defined the colony growth higher than the enzymatic halo.

Endo-glucanases production in CMC medium (Table 3) with 48 h of growth, the fungi strains revealed on Congo Red with more EI were UFT3, UFT2 and UFT1 with values of 1.269, 1.130 and 0.933, respectively. For the Trypan Blue dye, the best indices were in the strains UFT3, UFT1 and JCO2 with values of 1.327, 1.323 and 1.110, respectively. With Phenol Red dye, the best values were found in the strains UFT1, UFT3 and JCO1 with 1.450, 1.301 and 1.102, respectively. The growth evidenced in Gram's Iodine shows the highest rates to the strains UFT1, UFT3 and UFT2 with values of 2.024, 1.550 and 1.254, respectively. Comparing these with the second screening method with Congo Red at 96 h, the best indices were for the strains UFT1, UFT3 and JCO1 The similarities between the grouped strains can be explained once demonstrated by the molecular identification,

Table 3. Comparison of enzymatic index represented in CMC medium using ANOVA analysis of variance and post-hoc tests.

Strains	Congo Red	Trypan Blue	Phenol Red	Gram's Iodine	$\overline{TXEI}$	σ^2	$\hat{S}$	CV (%)	$S_{\bar{x}}$	HDS (p = 0.05)				
	$\overline{XEI}$									Tukey		Duncan		
JCO1	0.920	0.849	1.102	0.925	0.999	0.021	0.147	14.71	0.042	A		A		
JCO2	0.652	1.110	1.012	0.939	0.928	0.038	0.195	21.01	0.056	A		A		
JCO3	0.895	1.093	0.921	0.931	0.967	0.009	0.095	9.82	0.027	A		A		
JCO4	0.865	1.103	0.915	0.951	0.959	0.016	0.126	13.14	0.036	A		A		
JCO5	0.931	1.013	0.950	0.889	0.946	0.004	0.064	6.77	0.019	A		A		
UFT1	1.130	1.323	1.450	2.042	1.513	0.132	0.363	23.99	0.105		B		B	
UFT2	0.933	1.009	0.949	1.254	1.036	0.020	0.140	13.51	0.040	A		A		
UFT3	1.269	1.327	1.301	1.550	1.362	0.031	0.177	13.00	0.051		B			C
Significance										0.84	0.48	0.22	1.00	1.00

$\overline{XEI}$, Enzymatic index average; $\overline{TXEI}$, Enzymatic index total average; σ^2, Variance; $\hat{S}$, Standard deviation; CV, Coefficient of variation; $S_{\bar{x}}$, Standard error of mean; HDS, Homogeneous subsets; p, significance.

Table 4. Comparison of enzymatic index represented in Avicel medium using ANOVA analysis of variance and post-hoc tests.

Strains	Congo Red	Trypan Blue	Phenol Red	Gram's Iodine	$\overline{TXEI}$	σ^2	$\hat{S}$	CV (%)	$S_{\bar{x}}$	HDS (p = 0.05)					
	$\overline{XEI}$									Tukey			Duncan		
JCO1	0.923	1.042	1.074	1.045	1.021	0.017	0.132	12.93	0.038	A			A		
JCO2	1.038	1.152	1.076	0.989	1.064	0.007	0.083	7.80	0.024	A			A		
JCO3	0.997	1.215	0.903	1.030	1.037	0.034	0.185	17.84	0.053	A			A		
JCO4	1.030	0.998	1.021	0.906	0.989	0.004	0.067	6.77	0.019	A			A		
JCO5	0.906	0.935	1.061	0.977	0.970	0.007	0.085	8.76	0.025	A			A		
UFT1	1.847	1.733	1.756	2.006	1.835	0.084	0.289	15.75	0.084		B			B	
UFT2	1.181	1.085	1.057	1.057	1.095	0.014	0.117	10.68	0.034	A			A		
UFT3	1.647	1.275	1.522	1.162	1.401	0.056	0.238	16.99	0.069			C			C
Significance										0.60	1.00	1.00	0.11	1.00	1.00

$\overline{XEI}$, Enzymatic index average; $\overline{TXEI}$, Enzymatic index total average; σ^2, Variance; $\hat{S}$, Standard deviation; CV, Coefficient of variation; $S_{\bar{x}}$, Standard error of mean; HDS, Homogeneous subsets; p, significance.

where the strains JCO1, JCO2, JCO3, JCO4 and JCO5 belong to the same fungal genus. However, the enzymatic indices of unidentified isolates were higher (UFT1, UFT2 and UFT3) along with the species identified JCO1 and JCO2. In spite of that JCO1 is the same species that JCO5 (*T. asperelloides* GJS04-217) the JCO5 results were not significant. In the case of second screening method for 96 h of growth in CMC medium, the enzymatic halos presented values almost double in diameter compared with 48 h of growth. The highest values were of the strains JCO4, JCO2 and UFT2 with 71, 70 and 65 mm and with EI of 1.141, 0.971 and 1.283, respectively. However, the best enzymatic indices presented in this

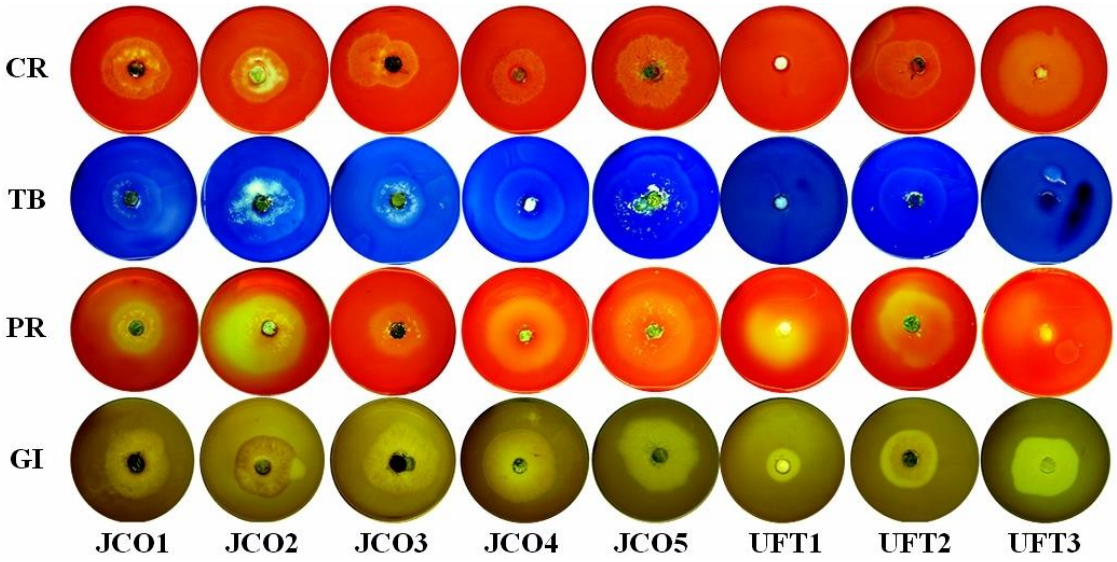

Figure 3. Evaluation of enzymatic activity in CMC medium (Carboxymethyl Cellulose 1% w/v) at 28ºC for 48 h using Congo Red (CR), Trypan Blue (TB), Phenol Red (PR) and Gram's Iodine (GI) as cellulase activity indicator.

method were evidenced by the strains UFT1, UFT3 and JCO1 with average values of 1.669, 1.440 and 1.373, respectively. Therefore, the previous results coincide with Tukey test; were, UFT1 and UFT3 belong to the group A and without significant differences between their values. Although, the Duncan test separate in different groups the strains UFT1, UFT3 and JCO1. Enzymatic indices data reported by Ruegger and Tauk-Tornisielo (2004) using the same screening method, where *Trichoderma harzianum* II presented EI of 1.0 and *Trichoderma longibrachiatum* did not provide growth of enzymatic halo, however, in the data found in this study, *T. longibrachiatum* DAOM 167674 (JCO2) and *T. harzianum* CIB T44 (JCO3 and JCO4) showed EI of 0.913, 1.272 and 1.185 of endo-glucanases respectively.

The screening performed in CMC medium (Figure 3) presented different color degradations. In Congo Red, the enzymatic halo change from red to opaque orange, the larger halos corresponded to strains UFT2, JCO5 and UFT3 with average values of 40, 39 and 38 mm, respectively. In Trypan Blue, the dark blue color change to light blue and the larger halos presented in UFT1, JCO2 and JCO5 with average diameters of 61, 57 and 56 mm, respectively. In Phenol Red, the red color was degraded by the enzyme production changing to yellow color, were the larger halos diameters were in the strains JCO5, JCO2 and UFT2 with average values of 57, 55 and 55 mm, respectively. In the case of Gram's Iodine, the medium presented a brown coloring initial and the enzyme secretion changed to beige, with higher average diameters in UFT3, JCO4 and JCO1 with values of 42, 38 and 37 mm. These strains despite having higher enzyme halos, the colony diameters were proportional. Therefore, were not necessarily the best to present a high enzymatic index.

The strains grown in Avicel medium (Figure 4) presented different colorations and enzymatic degradations compared with growths in CMC medium. In Congo Red, the red color changed to an orange color very similar to the original. The larger halos corresponded to strains JCO5, UFT2 and JCO4 with average diameters of 51, 48 and 46 mm, respectively. In Trypan Blue, the dark blue color changed to light blue color and the larger halos diameters in JCO5, JCO2 and UFT3 with average values of 45, 38 and 37 mm, respectively. In Phenol Red, JCO5, UFT3 and JCO4 presented the higher average diameters with values of 58, 49 and 48 mm, respectively. Were, the red color changed to yellow. Finally, the strains JCO5, UFT2 and JCO1 showed the best enzymatic diameters with average values of 52, 45 and 40 mm, respectively in Gram's Iodine dye. In the majority of the chromogenic indicators, the strain JCO5 presented the best enzymatic values. However, due to the proportional growth of the fungal colony, the best enzymatic indices corresponded to the strains UFT1, UFT3 and UFT2.

Conclusions

This study allowed us to identify the best strains for cellulases production such as Endo-glucanases and Exo-glucanases, the optimal growth conditions using two screening methods with two growth times and the chromogenic dyes most appropriated for growth halos measurement.

The eight fungal strains showed potential for production

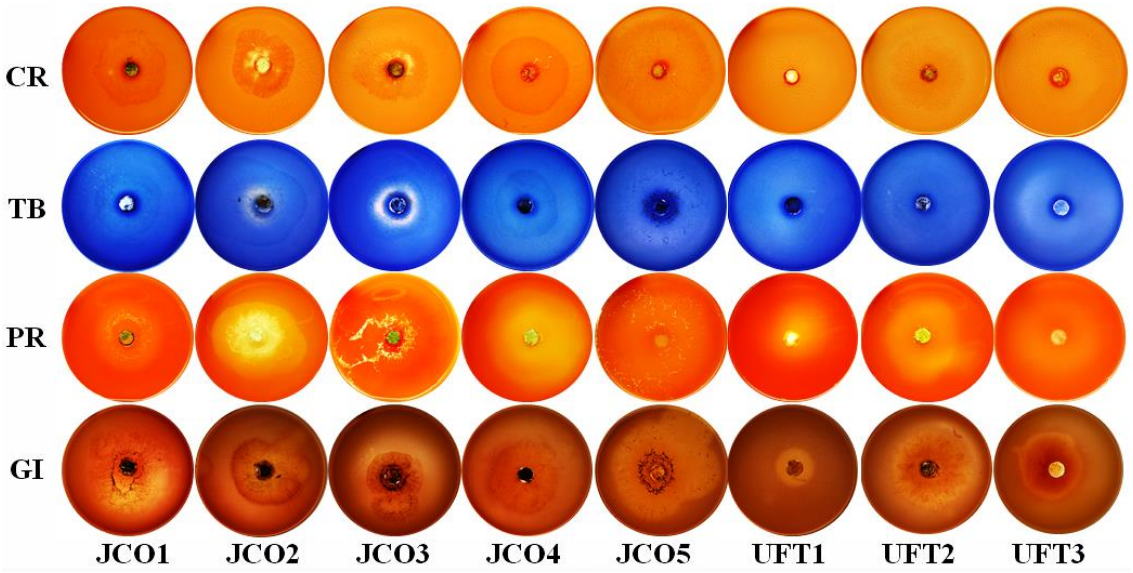

Figure 4. Evaluation of enzymatic activity in Avicel medium (Cellulose microcrystalline 1% w/v) at 28ºC for 48 h using Congo Red (CR), Trypan Blue (TB), Phenol Red (PR) and Gram's Iodine (GI) as cellulase activity indicator.

of cellulases. However, the best enzymatic indices of Endo-glucanases were presented by the strains UFT1, UFT2 and UFT3 with average values above 1.0. For the Exo-glucanases, the best indices were of the strains UFT1, UFT2 and UFT3 although the strains JCO1, JCO2 and JCO3 also had values above 1.0.

For screening methods, it was observed that the second screening method allowed verifying the results using double the time of growth enabling, the study of the production of enzymatic halos depending on the type of microorganism and its growth phase. However, the screening method for 48 h is ideal for rapid and effective detection of potential producers of cellulases.

In most cases, several researchers have used the dye Congo Red as chromogenic indicator for detection of cellulases (Jo et al., 2011; Sharma and Sumbali, 2014). However, in the present study, the best colorations were evidenced using Phenol Red in CMC medium with a best revelation of the contrast between the original color and the change caused by enzymatic degradation. Otherwise as occurred in Avicel medium, were the best views of contrast in the halos presented using Gram's Iodine also demonstrated by Kasana et al. (2008).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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