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Short Communication

The Influence of Carboxymethylcellulose Substrate on the Endoglucanase activity Produced by *Trichoderma atroviride* 102C1 and *Aspergillus awamori* IOC-3913 in Submerged Fermentation

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Abstract

Filamentous fungi are very important cellulose-degrading microorganisms in nature. The main cellulolytic fungi are *Aspergillus*, *Trichoderma*, *Penicillium*, *Acremonium*. Cellulases have played an important role in many industrial processes as fiber textile biobleaching, animal feeds, whitening fruit juices and wines. However, the main special attention has been devoted to the role of these enzymes in saccharification of lignocellulose biomass for biofuels production. Therefore, this study aimed to evaluate the cellulase production by mutant fungi *Trichoderma atroviride* 102C1 and *Aspergillus awamori* IOC-3913 and the effect of substrate on enzyme activity. *T. atroviride* 102C1 and *A. awamori* IOC-3913 produced endoglucanases, using sugarcane bagasse (SCB) and corn steep liquid (CSL) as raw-material. Endoglucanase activity was assayed using carboxymehtylcellulose (CMC) as substrate at low and medium viscosity (1.0 and 2.0% w/v), which has 400 and 1,100 of polymerization degree, respectively. The highest endoglucanase activity (2.63 $U.ml^{-1}$) was observed when *T. atroviride* and *A. awamori* (SCB 1.5% and CSL 0.3%) were grown together and the enzyme activity was detected in the presence of CMC low viscosity. To our knowledge, this is one of few studies reporting the effect of CMC viscosity on assays for CMCase activity.

ABBREVIATIONS

CMC: Carboxy Methyl Cellulose; LV-CMC: Low Viscosity Carboxy Methyl Cellulose; MV-CMC: Medium Viscosity Carboxy Methyl Cellulose; CSL: Corn Steep Liquor; SCB: Sugarcane Bagasse; CMCase: Carboxy Methylcellulase (endoglucanase)

INTRODUCTION

Biomass energy has an important role to play in the decrease of greenhouse gas emissions from fossil fuels [1,2], as well as creating new employment opportunities in rural areas [3]. Sugarcane bagasse is a carbon rich residue from sugar production [4]. The high yield and high content of cellulose and xylan makes this residue a competitive option for biofuels [5]. The use of a large amount (> 15%) of sugarcane bagasse for the production of bioethanol offers many advantages such as high concentration of sugars and ethanol and the reduction of costs [6]. Lignocellulosic materials are mainly composed of polymers

of cellulose, hemicellulose and lignin. This raw material used for bioethanol production can be obtained from agricultural residues and wood industries, or from crops specifically grown for this purpose [7,8]. Carboxymethylcellulose (CMC) is one of the most important cellulose by-products. It is a linear, long chain, water soluble, anionic polysaccharide derived from cellulose and used as a substrate to detect and quantify endoglucanase activity [9].

Cellulases secreted by filamentous fungi consist of three main components: endo-1,4-β-glucanase (EC 3.2.1.4), 1,4-β-D-cellobiohydrolase (EC 3.2.1.91), and 1,4-β-glucosidase (EC 3.2.1.21). These three enzymes act in synergy during the conversion of cellulose to glucose [10,11]. *Trichoderma reesei* is the most studied species of *Trichoderma* for cellulase production. Many traditional mutagenic strategies have been used to improve this characteristic. Nevertheless, these attempts have not been totally successful yet [12-15], because current enzyme production involves high costs and is still in early developmental stages [16]. *Aspergillus* species are also well known to be cellulase and xylanase producers and many species have been studied, including *Aspergillus terreus* [17], *Aspergillus niger* [18], *Aspergillus fischeri* [19], *Aspergillus niveus* and *Aspergillus ochraceus* [20]. *Aspergillus niger* and *Aspergillus oryzae* are the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients, and enzymes [21].

This research aimed to evaluate endoglucanase production and compare low and medium viscosity CMC from *Trichoderma atroviride* 102C1 and *Aspergillus awamori* IOC-3913. Three different fermentation conditions were studied: (i) *Trichoderma atroviride* 102C1, (ii) *Aspergillus awamori* IOC-3913 e (iii) co-culture with *Trichoderma atroviride* 102C1 and *Aspergillus awamori* IOC-3913.

MATERIALS AND METHODS

The *Aspergillus awamori* strain was obtained from the Fungal Culture Collection at Fundação Oswaldo Cruz (Fiocruz) in Rio de Janeiro, under the care of Dr. Maria Inez de Moura Sarquis. A wild strain, *Trichoderma atroviride* 676, was obtained from the culture collection of the CPqLMD (Centro de Pesquisa Leonidas and Maria Deane) of Fiocruz in Manaus, and was identified by Dr. Maria Inez de Moura Sarquis. The wild strain was exposed to mutagen agent 1.0% nitrosoguanidine for 12 min, which resulted in a new mutant strain *Trichoderma atroviride* NTG21. The new NTG21 mutant strain was exposed once again to 1.0% nitrosoguanidine for 15 min, from which a new mutant strain, 102C1, was studied. *T. atroviride* 102C1 showed increases in CMCase and xylanase activity of 154% and 340% respectively when compared to wild strain 676 [22]. *T. atroviride* 102C1 strain was selected for further investigation owing to its increased cellulase production, while *A. awamori* IOC-3913, a high beta-glucosidase producer was also studied.

Stock cultures were maintained on yeast extract-malt extract-agar [23] containing ($g.l^{-1}$): malt extract, 10; yeast extract, 4; glucose, 4 and agar, 15, after incubation at 28ºC for 15 days. Spore suspensions were prepared according to Hopwood et al. [24] after cultivation (28ºC/15 days) in the same medium and maintained as stock cultures in 20% (*v/v*) glycerol at -20ºC. Spore concentration was determined using a Neubauer counting chamber.

Enzyme production was carried out by submerged fermentation, in Erlenmeyer flasks with 1/5 of its volume of a culture medium based on the salt solution and urea described by Mandels and Weber [25], at pH 4.8, supplemented with different combinations of SCB and CSL. The composition of corn steep solids used in this work was determined according to the analysis by Laboratory of Analysis and Chemical Characterization on Pontifical Catholic University (PUC) in Rio de Janeiro, Brazil, coordinate by Dr Cristiane Maria de Mello Alves Portella. The content of chemical elements were nitrogen 7.3%, carbon 37.9%, hydrogen 6.1%, others elements 48.7%. The different combinations that generated five culture mediums are in Table 1. The condition SCB 2.5% (*w/v*) and CSL 0.7% (*w/v*) was done in triplicate (central point). Each Erlenmeyer flasks (250 ml), containing 50 ml of each medium, was inoculated with 50µl of a spore suspension (10^8 spores.ml^{-1}). Cells were incubated at 28ºC, under shaking conditions (200 rev. min^{-1}) for 3 days. To compare the CMCase production of the fungal strains, 3 fermentation conditions were assembled: (i) *T. atroviride* 102C1; (ii) *A. awamori* IOC-3913 and (iii) *T. atroviride* 102C1 + *A. awamori* IOC-3913 co-cultivated. The inoculum ratio of co-cultivated condition was 1:1. After 3 days of fermentation, the flasks were collected, and their contents were passed through a 0.45 µm filtration unit containing glass fiber membrane and collected for further analysis.

Endoglucanase (CMCase) activity was assayed by measuring the release of reducing sugars in a reaction mixture of 1.0 ml of the crude supernatant and 1.0 ml of carboxymethylcellulose (CMC) sodium salt (SIGMA-ALDRICH®) solution in 50mM sodium citrate buffer (pH 4.8) incubated at 50°C for 30 min. A comparison between 2 different types of CMC, at different viscosity levels, was investigated: 1.0 and 2.0% medium viscosity (MV-CMC), with a polymerization degree of 1.100 and molecular weight of 250 kDa, compared with 1.0 and 2.0% low viscosity (LV-CMC), with a polymerization degree of 400 and molecular weight of 90 kDa. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method [26]. One unit (U) of CMCase activity corresponded to 1 mol of glucose equivalents released per minute under the assay conditions [27]. All assays were performed in duplicates and results were expressed as average values. Variations between samples in the multiple assays were <10 %. The data were statistically analyzed by Experimental Design at the 0.10 probability level ($p < 0.10$)

RESULTS AND DISCUSSION

CMCase production by *Trichoderma atroviride* 102C1 and

Table 1: Media composition used in the different submerged fermentation conditions.

Experiment	SCB (% *w/v*)	CSL (% *w/v*)
1	1.5	0.3
2	3.5	0.3
3	1.5	1.1
4	3.5	1.1
5	2.5	0.7

Aspergillus awamori IOC-3913 (individually, Figures (1) and (2) and in co-culture (Figure 3), were evaluated by comparing the two types of CMC. The amounts of CMCase activity from the *Trichoderma atroviride* 102C1 strain ranged from 1.55 to 2.01 U.ml^{-1} (LV-CMC 1.0%) and 1.43 to 2.09 U.ml^{-1} (LV-CMC 2.0%). However, when MV-CMC was used as the substrate, at the same concentrations, the enzymatic activity was lower and ranged from 0.25 to 0.73 U.ml^{-1} (Figure 1), with $0.008 < p < 0.98$. The amounts of CMCase activity of *Aspergillus awamori* IOC-3913 ranged from 1.34 to 2.27 U.ml^{-1} (LV-CMC 1.0%) and 0.81 to 1.32 U.ml^{-1} (LV-CMC 2.0%). The same phenomenon, a decrease, was observed when the MV-CMC was used as the substrate (Table 3) and the enzymatic activity was less than 0.53 U.ml^{-1} (Figure 2), with $0.01 < p < 0.52$. The same effect was again observed in the co-culture (Figure 3), when the CMCase activity reached values between 1.93 to 2.63 U.ml^{-1} (LV-CMC 1.0%) and 1.20 to 1.97 U.ml^{-1} (LV-CMC 2.0%), with $0.002 < p < 0.68$. Enzymatic activity decreased when MV-CMC was used as substrate at 1.0% (range 0.51 to 0.74 U.ml^{-1}) and 2.0% (range 0.25 to 0.36 U.ml^{-1}). For *Trichoderma atroviride* and *Aspergillus awamori* strains, individually and in co-culture, the highest enzyme production (2.63 U.ml^{-1}) was seen in experiment 1, where SCB was 1.5% and CSS was 0.3%.

CMCase activity increased in all fermentation systems when LV-CMC was used as the enzyme substrate. In the first fermentation system (*Trichoderma atroviride* 102C1), CMCase activity increased 301% in comparison with MV-CMC. In the second system (*Aspergillus awamori* IOC-3913) the increase was 931%. When both fungi were grown together (third fermentation system), the increase was even greater, exceeding 950%. The high increase in enzymatic activity was in the presence of LV-CMC and can be explained by viscosity of the system. We observed in all cases that the viscosity of CMC substrate interferes significantly in enzymatic activity. This is the first report that quantifies the effect of CMC viscosity on endoglucanase activity assays and is important for bioprospecting and improving enzyme production. Another important aspect which must be considered is the degree of polymerization of the CMC substrate. All these factors influenced CMCase activity.

The enzymatic activity values showed that using LV-CMC results in higher activity than MV-CMC. Unfortunately in the literature, papers neglect to mention the viscosity of the CMC used in their CMCase assays. Castro et al [28], reported on endoglucanase activity (0.56 U.ml^{-1}) produced by *Trichoderma harzianum* using pretreated sugarcane bagasse after 4 days-fermentation and using MV-CMC. Wang et al [29], reported on endoglucanase production by *Trichoderma koningii* which was evaluated using different carbon sources with no mention of CMC viscosity. They reported high maximum activity (7.0 U.ml^{-1}) using cellulose as the carbon source. However, there was no specification about the viscosity of CMC used to detect endoglucanase activity. Mushtaq et al. [30], evaluated the endoglucanase production by *Aspergillus sydowii* from three different carbon sources (corn cobs, rice straw and sugarcane bagasse). The maximum activity (2.01 U.ml^{-1}) they obtained was after 4 days-fermentation, using corn cobs as carbon source. However, when sugarcane bagasse and rice straw were used as carbon source, the maximum endoglucanase activity was 1.87 and 1.99 U.ml^{-1}, respectively. The results demonstrate that the strains reported on here are similarly productive to previous studies.

CONCLUSION

Trichoderma atroviride 102C1 and *Aspergillus awamori* IOC-3913 were able to grow and produce good levels of CMCases using SCB and CSS as low-cost substrates and a mineral source. A maximum CMCase concentration of 1.97 U.ml^{-1} was observed from a culture medium containing 2.5% SCB and 0.7% CSS and

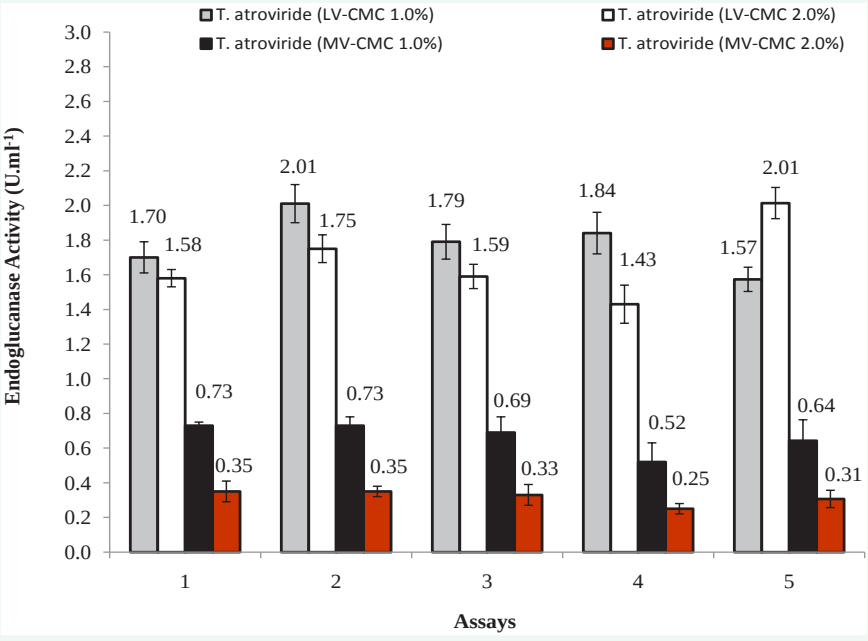

Figure 1 CMCase production by *T. atroviride* 102C1 on 5 different media composition and CMC low (LV-CMC) (grey and white column) and medium (MV-CMC) (black and red column) viscosity at 1.0 and 2.0% (*w/v*). The results are expressed as U.ml^{-1}.

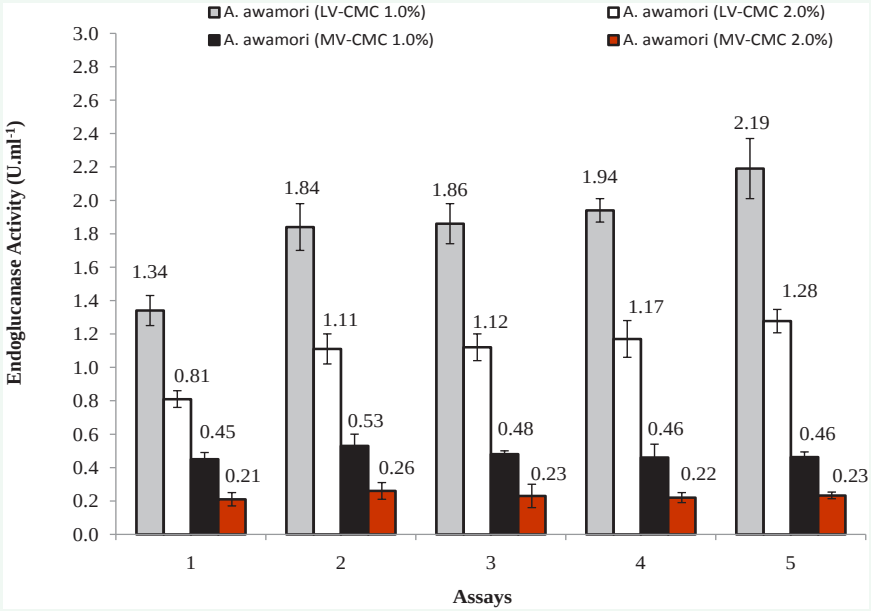

Figure 2 CMCase production by *A. awamori* IOC-3913 on 5 different media composition and CMC low (LV-CMC) (grey and white column) and medium (MV-CMC) (black and red column) viscosity at 1.0 and 2.0% (*w/v*). The results are expressed as U.ml^{-1}.

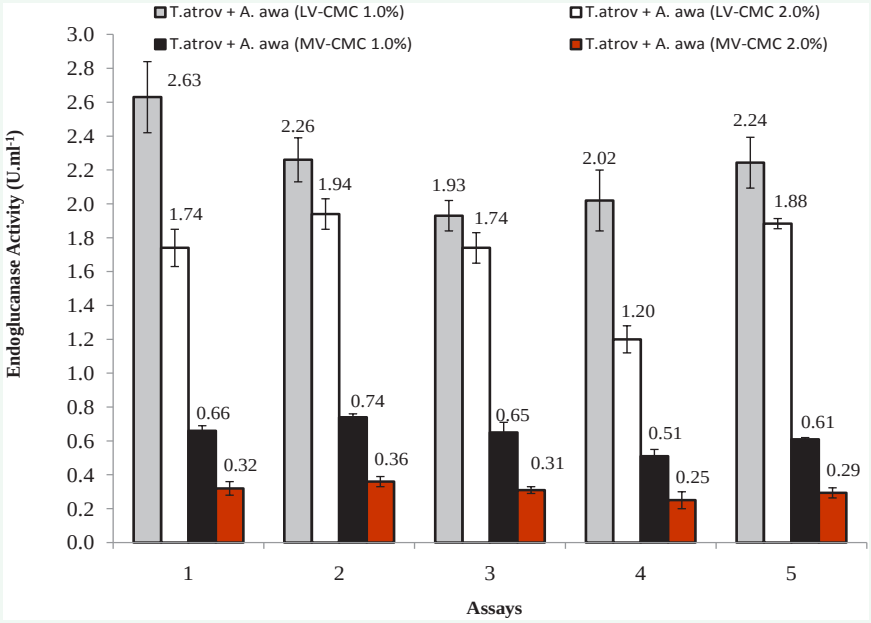

Figure 3 CMCase production by co-culture *T. atroviride* 102C1 and *A. awamori* IOC-3913 on 5 different media with CMC low (LV-CMC) (grey and white column) and medium (MV-CMC) (black and red column) viscosity at 1.0 and 2.0% (*w/v*). The results are expressed as U.ml^{-1}.

LV-CMC. The use of LV-CMC as the enzyme substrate was very promising when compared to MV-CMC. These findings suggest that a solution with lower polymerization degree increases the enzyme activities. The results herein demonstrate that the fungal strains studied are worthy of further investigation with biotechnological applications a possibility. The difference between using LV-CMC and MV-CMC is significant and it is clear that LV-CMC should be preferably used for endoglucanase activity assays.

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