Ambiente & Água - An Interdisciplinary Journal of Applied Science
ISSN 1980-993X – doi:10.4136/1980-993X
www.ambi-agua.net
E-mail: ambi.agua@gmail.com

Evaluation of endoglucanase and β-glucosidase production by bacteria and yeasts isolated from an eucalyptus plantation in the cerrado of Minas Gerais

ARTICLES doi:10.4136/ambi-agua.2324

Received: 29 Aug. 2018; Accepted: 29 Apr. 2019

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ABSTRACT

The current global environmental and economic scenario is intrinsically related to the increase in fossil fuel consumption caused by technological development and world population growth. Thus, it is necessary to search for renewable sources of biofuel in an attempt to mitigate the effects of fossil fuels on the environment and the lack of these non-renewable fuels. The use of lignocellulosic biomass, an abundant and renewable resource in Brazilian regions, has contributed successfully to new research and technologies for second-generation ethanol production. The conversion of lignocellulosic biomass into fermentable sugars requires the use of cellulolytic enzymes produced by microorganisms found in the microbiota. This work evaluated the production of endoglucanase and β-glucosidase in a liquid medium containing carboxymethylcellulose by five microorganisms (bacteria and yeasts) from the bank of 348 isolates from eucalyptus soils in the Cerrado Mineiro. Microorganisms with the following enzymatic activity indexes were selected: IM1-74 (22), IM25-9 (5.33), IM32-90 (7.33), IM1-5 (10.33) and IM32-91 (5.44). The microorganisms with the highest enzymatic activity in the liquid medium were IM32-90 (endoglucanase = 0.214 U mL^{-1}) and IM32-91 (β-glucosidase = 0.067 U mL^{-1}).

Keywords: cellulase, enzymes, renewable sources.

Avaliação da produção de endoglucanase e β-glicosidase por bactérias e leveduras isoladas de plantações de eucalipto no cerrado de Minas Gerais

RESUMO

O atual cenário ambiental e econômico global tem uma relação intrínseca com o aumento do consumo de combustíveis fósseis causados pelo desenvolvimento de tecnologias e pelo crescimento da população mundial. Assim, é necessário procurar fontes renováveis de

biocombustíveis na tentativa de mitigar os efeitos dos combustíveis fósseis no meio ambiente e a falta desses combustíveis não renováveis. O uso de biomassa lignocelulósica, um recurso abundante e renovável nas regiões brasileiras, contribuiu com sucesso para novas pesquisas e tecnologias para a produção de etanol de segunda geração. A conversão da biomassa lignocelulósica em açúcares fermentáveis requer o uso de enzimas celulolíticas produzidas por micro-organismos encontrados na microbiota. Este trabalho avaliou a produção de endoglucanase e β-glicosidase em meio líquido contendo carboximetilcelulose por cinco micro-organismos bactérias e leveduras) do banco de 348 isolados de solos de eucalipto no Cerrado Mineiro. Foram selecionados micro-organismos com os seguintes índices de atividade enzimática: IM1-74 (22), IM25-9 (5.33), IM32-90 (7.33), IM1-5 (10.33) e IM32-91 (5.44). Os micro-organismos com maior atividade enzimática em meio líquido foram IM32-90 (endoglucanase = 0,214 U mL^{-1}) e IM32-91 (β-glicosidase = 0,067 U mL^{-1}).

Palavras-chave: celulase, enzimas, fontes renováveis.

1. INTRODUCTION

Global dependence on fossil fuels has caused unfavorable effects such as declining crude oil reserves, declining air quality and rising global temperatures. To promote sustainability and independence from fossil fuels, bioethanol is now considered to be a good substitute for a fossil fuel mixture or gasoline (Aditiya *et al.*, 2016; Toogood and Scrutton, 2018).

Lignocellulosic biomass represents a promising source of renewable energy, and can be used for the production of second-generation bioethanol (Squinca *et al.*, 2018). Currently, most of the lignocellulosic ethanol is produced by hydrolysis and fermentation. The enzymatic saccharification of plant biomass is the most efficient method for the breakdown of lignocellulose to sugars, although the cost of this operation is still one of the main bottlenecks in the commercialization of second-generation alcohol and has been reported as the most expensive point in the entire chain of bioethanol production, accounting for up to 40% of the total cost (Choudhary *et al.*, 2017; Cerda *et al.*, 2017). Decreasing the cost of cellulolytic enzymes remains one of the main goals of bioethanol research, as it can reduce the cost of these enzymes by up to 20% (Dodda *et al.*, 2018).

Cellulolytic enzymes include endo- and exoglucanases and beta-glucosidases that act synergistically and are necessary for the efficient hydrolysis of cellulose to soluble oligosaccharides (Lucarini *et al.*, 2017). The endoglucanases initiate hydrolysis by slicing the cellulose structure, uncovering reducing and non-reducing ends, whereas cellobiohydrolases (exoglucanases) act upon both ends to release cello-oligosaccharides and cellobiose units. Subsequently, β-glucosidases chop cellobiose to release glucose molecules. Further, during the hydrolysis reaction, the endoglucanases act on the amorphous region, which is the more soluble portion of the cellulose structure; meanwhile, cellobiohydrolases cleave β−1,4-glycosidic bonds from the chain ends (Srivastava *et al.*, 2018).

Cellulases have various kinds of industrial applications, including pulp and paper, laundry, food, animal feed, brewery and wine, textile, bioenergy industry (Idris *et al.*, 2017). Significant research has been developed focusing on the isolation, identification and optimization of these enzymes, thus leading to the marketing of cellulases. Commercially available cellulases are a formulation of endoglucanases, exoglucanases, and β-glucosidases (Obeng *et al.*; 2017).

The enzymatic process is pollution-free, economical and cost-effective (Li *et al.*, 2012). This gives a new horizon to finding cost-effective cellulose-producing microorganisms. Microorganisms such as aerobic bacteria, fungi, yeasts and actinomycetes produce cellulases that degrade cellulose by hydrolysis of β-1,4-glycosidic bonds of cellulose (Behera *et al.*, 2017).

The objective of this study was to evaluate the production of endoglucanase and β-

glucosidase by five microorganisms (bacteria and yeasts) isolated from the soil of a eucalyptus plantation in the cerrado region of the state of Minas Gerais, Brazil, with different periods of planting.

2. MATERIALS AND METHODS

For the evaluation of the production of endoglucanase and β-glucosidase, the 1-74, 25-9, 32-90, 1-5 and 32-91 microorganisms from the microorganisms bank of the Laboratory of Biotechnology and Genetics of the Department of Forest Engineering of the Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, Minas Gerais, Brazil, with previously calculated enzyme activity indices (EAI): EAI1-75 = 22, EAI25-9 = 5.33, EAI32-90 = 7.33, EAI1-5 = 10.33 and EAI32-91 = 5.44 were employed.

2.1. Culture medium and inoculum

To prepare the inoculants, the microorganisms assigned as described above were inoculated on a Petri plate with culture medium containing carboxymethylcellulose (CMC) as the main carbon source, according to the composition described in Table 1, and incubated in BOD (SL 200/334 SOLAB®) at 28°C for 96 hours.

Table 1. Composition of the culture medium used for initiation of inoculum growth.

Components	Amount (g)
$MgSO_4 . 7H_2O$	0.2
KH_2PO_4	0.4
K_2HPO_4	0.1
NaCl	0.1
Yeast extract	0.4
CMC	10
Agar	15
Distilled water	1000 mL

The culture medium was prepared from the mixture of the components described in Table 1. The pH was adjusted to 6.0, followed by sterilization in an autoclave at 121ºC for 15 min.

After growth was verified, the microorganism was scraped with the aid of a platinum handle and inoculated in 12 mL of culture medium with a composition similar to that mentioned above, but without agar, contained in a 15-mL Falcon tube. The tube was incubated in a SHAKER (SL 222 SOLAB®) at 28°C and 150 rpm for 96 h.

2.2. Culture of microorganisms for the production of endoglucanase and β-glucosidase

After 96 hours of growth, a 2-mL sample was withdrawn from the inoculum and the optical density was measured at 600 nm in a Lambda Bio PERKINELMER® spectrophotometer. The inoculum was then added to an Erlenmeyer flask containing 190 mL of the medium described above (without agar) and incubated in a SHAKER (SL 222 SOLAB®) at 28°C and 150 rpm for 240 hours. Two-mL aliquots were withdrawn at 24-hour intervals and centrifuged at 10,000 rpm for 10 min for evaluation of the production of endoglucanase and β-glucosidase.

2.3. Evaluation of endoglucanase production

Analysis of endo-1,4-β-glucanase activity was performed according the method modified from the International Union of Pure and Applied Chemistry (IUPAC) standard method, described by Ghose (1987). This method consists of hydrolysis of a solution of 1% CMC (Synth), followed by quantification of the soluble reducing sugars released over a given time

interval. The concentration of reducing sugars was determined according to the method described by Miller (1959) using 3,5-dinitrosalicylic acid (DNS) (Miller, 1959). The reaction was performed in Falcon tubes (15 mL) containing 1000 µL of 1% (w/v) CMC solution in 100 mmol.L-1 acetate buffer, pH 4.8 and 1000 µL of the enzyme extract. The mixture was incubated at 50°C, aliquots of 200 µL were withdrawn at 0-, 15- and 30-minute intervals, and 200 µL of DNS was added. A reaction without the presence of the enzyme extract (blank) was prepared by the addition of 100 µL DNS to 100 µL of distilled water. Samples and blank were heated in a boiling water bath (95 ± 1°C) for 5 minutes, followed by cooling in an ice-water bath (0 ± 1°C) and the addition of 2 mL and 1 mL of distilled water to the sample and the blank, respectively. The absorbance was measured at 540 nm using an analytical glucose curve at concentrations of 0 to 1.6 mg/mL in 0.2 mg mL^{-1} intervals as a reference (Figure 1).

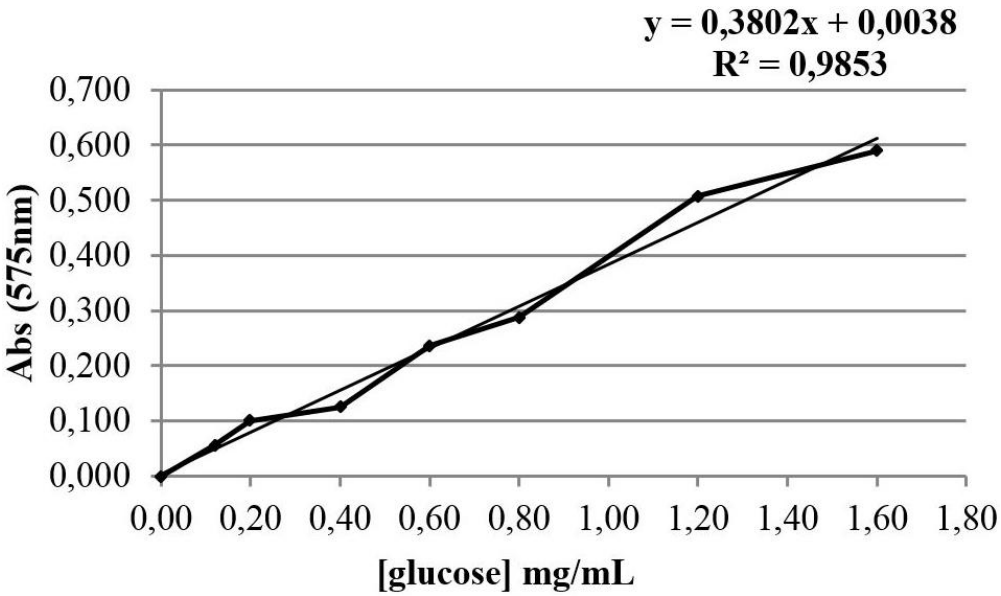

Figure 1. Analytical glucose curve.

One unit of endoglucanase activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars, expressed as glucose, per minute per unit volume of enzyme extract. Data were submitted to analysis of variance considering a level of significance (α) of 0.05 using software ORIGIN PRO 8.

2.4. Evaluation of β-glucosidase production

The assay of β-glucosidase activity was implemented using the method modified from the International Union of Pure and Applied Chemistry (IUPAC) standard method, described by Ghose (1987), which involves hydrolysis of a 1% solution of cellobiose in 100 mmol L^{-1} citrate buffer at pH 4.8 in the presence of the enzyme extract. The reaction medium was composed of 100 µL of the substrate solution and 100 µL of the enzyme extract in Eppendorf tubes. The tubes were incubated at 50°C in a water bath for 30 minutes, followed by boiling (95 ± 1°C) for 1 min and cooling in an ice bath. Aliquots of 15 µL were removed for quantification of glucose using the standard enzymatic procedure employing glucose oxidase/peroxidase (GOD-POD) (Lloyd and Whelan, 1969) with spectrophotometric measurement at 510 nm. For each enzymatic reaction, one was prepared without the presence of the enzyme extract (blank), following the same procedure described for the sample. One unit of β-glucosidase activity was defined as the amount of enzyme capable of releasing 1 µmol of glucose per minute of reaction, per unit volume (liter) of enzyme extract. Data were submitted to analysis of variance considering a level of significance (α) of 0.05 using software ORIGIN PRO 8.

3. RESULTS AND DISCUSSION

3.1. Profile of endoglucanase production

The five strains of microorganisms selected according to the enzyme activity index (EAI) were grown in medium containing CMC as the main source of carbon, and they were monitored for 240 h for evaluation of endoglucanase and β-glucosidase activities, as shown in Figures 2A, 2B, 3A and 3B.

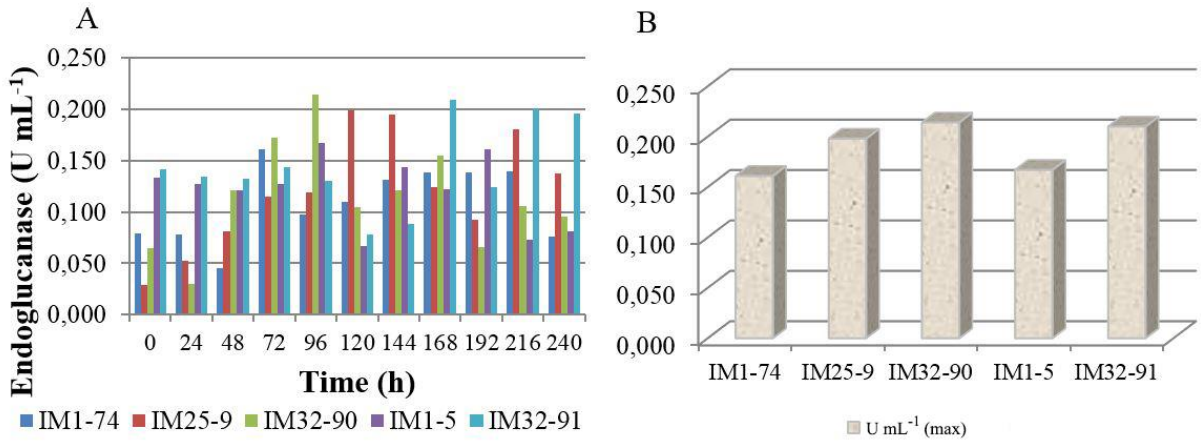

Figure 2. A: Punctual activities of endoglucanase from the five strains evaluated during 240 hours of culture. B: Maximum endoglucanase activity.

There is a relatively similar behavior for endoglucanase activities in all the strains (Figures 2A and 2B). The highest endoglucanase activities (CMCase) were observed at 96 hours of fermentation with the microorganism IM32-90 (0.214 U mL^{-1}), 120 hours with IM25-9 (0.198 U mL^{-1}) and 168 hours with IM32-91 (0.210 U mL^{-1}).

In general, the enzymatic activities of endoglucanases in this work were similar to those of other organisms already studied, as in Yang *et al.* (2011), who observed 0.24 U mL^{-1} in a study that demonstrated the presence of *Achromobacter xylosoxidans*, *Alcaligenes faecalis* and *Fusarium sporotrichioides*. A similar result was also obtained by Mohapatra *et al.* (2018) when evaluating cellulase production by *Aspergillus fumigatus* isolated from decomposing cellulosic waste, showing a higher endoglucanase activity (0.287 U mL^{-1}) obtained at 30°C, which shows that the cellulase activity is active in this temperature range. Carvalho (2013) obtained 0.164 U mL^{-1} by cultivating *Cryptococcus laurentii* in medium supplemented with 2% wheat bran.

The analysis of variance showed that there was no significant difference at 0.05 between the endoglucanase production of the five microorganisms evaluated (Table 2).

Table 2. Analysis of variance (ANOVA) of the endoglucanase production by the five micro-organisms evaluated.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	4	0.00947	0.00237	1.51983	0.24653
Error	15	0.02338	0.00156		
Total	19	0.03285			

One Way ANOVA
Overall ANOVA
Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are not significantly different.

3.2. Profile of β-glucosidase production

The production of β-glucosidase by the five selected strains was observed for 240 hours, as described in Figures 3A and 3B.

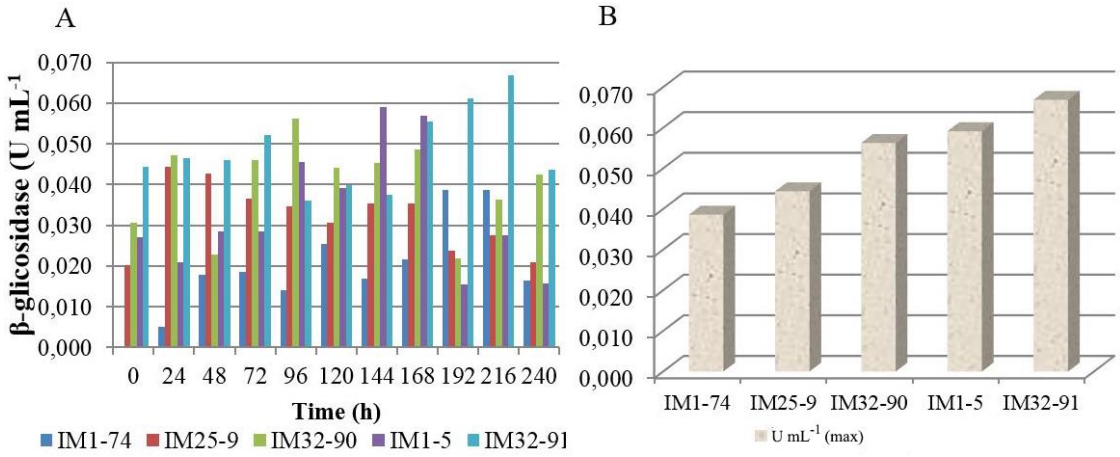

Figure 3. A: Punctual β-glucosidase activities of the five strains evaluated during 240 hours of culture. B: Maximum β-glucosidase activity.

The highest cellobiase activity (0.067 U mL^{-1}) was observed for the IM32-91 strain after 216 h of culture, followed by the IM1-5 strain (0.059 U mL^{-1}) at 144 h of culture. After 216 h of fermentation, the cellobiase activity of strain IM32-91 began to decrease. The IM1-74 strain with the highest enzymatic activity index (EAI = 22) had the lowest endoglucanase (0.161 U mL^{-1}) and β -glucosidase (0.039 U mL^{-1}) activities in liquid medium, as is shown in Table 3.

Table 3. Enzymatic activity indices expressed in solid medium and endoglucanase and β-glucosidase activity of the five strains evaluated in liquid medium.

Strain	EAI	Endoglucanase (U mL^{-1})	β-Glucosidase (U mL^{-1})
IM1-74	22.00	0.161	0.039
IM25-9	5.33	0.198	0.044
IM32-90	7.33	0.214	0.056
IM1-5	10.33	0.167	0.059
IM32-91	5.44	0.210	0.067

The analysis of variance showed that there was no significant difference at 0.05 between the β-glucosidase production of the five microorganisms evaluated (Table 4).

Table 4. Analysis of variance (ANOVA) of the β-glucosidase production by the five microorganisms evaluated.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	4	0.002	4.99157E-4	1.0913	0.41185
Error	10	0.00457	4.57398E-4		
Total	14	0.00657			

One Way ANOVA
Overall ANOVA
Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are not significantly different.

The IM32-91 strain, which has one of the lowest levels of enzymatic activity among the selected microorganisms (EAI = 5.44), was the best producer of β-glucosidase (0.067 U mL^{-1}) and the second best producer of endoglucanase (0.210 U mL^{-1}). The highest endoglucanase production was observed for strain IM32-90 (0.214 U mL^{-1}). The values of β-glucosidase production were very satisfactory compared to those found by Vyas and Chhabra (2017), who cultivated the yeast *Cystobasidium oligophagum* in a medium containing CMC as carbon source under the same conditions and obtained 0.031 U mL^{-1} after 72 hours of incubation. Priyanka *et al.* (2017) obtained 0.077 U mL^{-1} endoglucanase as the maximum value produced by the fungus *Paecilomyces variotii* when testing different concentrations of CMC as a carbon source. This value was about three times less than the maximum value obtained in this work for endoglucanase (0.214 U mL^{-1}).

CMC is considered to be a better substrate for cellulase production due to its soluble nature compared to other carbon sources, demonstrating its effectiveness in this work (Sohail *et al.*; 2016).

The production of cellulolytic enzymes can be increased by changes in culture medium conditions, such as supplementation of the culture medium and the use of recombinant microorganisms. Anusree *et al.* (2016) cultured recombinant *Corynebacterium glutamicum* bacteria with *Xanthomonas campestre* genes to evaluate the production of endoglucanase and *Saccharophagus degradans* for the production of β-glucosidase in medium supplemented with 20 $g.L^{-1}$ of CMC. They obtained 8 $\pm$ 0.4 U mL^{-1} of β-glucosidase and 4.5 $\pm$ 0.2 U mL^{-1} of endoglucanase. In medium supplemented with 20 $g.L^{-1}$ of cellobiose, they obtained 9.0 $\pm$ 0.5 U mL^{-1} of β-glucosidase and 2 $\pm$ 0.13 U mL^{-1} of endoglucanase. The strains evaluated in this work will be submitted for molecular identification.

The analysis of variance (ANOVA) with significance level 0.05 (Tables 2 and 4) showed that there was no significant difference in the production of endoglucanase and β-glucosidase by the five cellulase-producing microorganisms evaluated. These results indicate that any of the organisms evaluated can be used for the production of these enzymes, and their production processes may be optimized in future works.

4. CONCLUSION

The five strains evaluated were effective for cellulase production, presenting hydrolytic activities in carboxymethylcellulose and cellobiose, and proving that they are promising tools for the production of these enzymes. Further studies should be performed to increase cellulolytic capacity and enzyme extraction for commercial application.

5. ACKNOWLEDGMENTS

To Aperam Bioenergy LTDA, FAPEMIG, CNPq, CAPES and UFVJM for logistical and financial support to perform this work.

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