Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
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Universidade Estadual de Maringá
Brasil

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Acta Scientiarum. Biological Sciences, vol. 39, núm. 3, julio-septiembre, 2017, pp. 321-329
Universidade Estadual de Maringá
Maringá, Brasil

Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v39i3.32384

Production of cellulases by *Penicillium oxalicum* through solid state fermentation using agroindustrial substrates

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ABSTRACT. The purpose of this study was to define the factors that influence the production of cellulases by *Penicillium oxalicum*, a strain obtained from a leaf-cutting ant colony and identified based on the ITS gene. The experimental design included solid state fermentation using sugarcane bagasse and lignocellulosic sorghum as the lignocellulosic substrate. The variables were analyzed using a 2^{5-1} fractional factorial design, with three replicates on the central point. All the variables analyzed influenced the production of at least one of the three cellulose types analyzed. The highest values observed were: FPase 4.2 U g^{-1}, CMCase 9.2 U g^{-1} and Avicelase 8.4 U g^{-1} using lignocellulosic sorghum as the substrate. The best conditions for enzyme production were: incubation temperature at 40ºC, initial moisture of 60%, pH of 4.0 and a growth period of four days using lignocellulosic sorghum as the substrate.

Keywords: sugarcane bagasse; lignocellulosic sorghum; cellulolytic enzymes.

Produção de celulases por *Penicillium oxalicum* em fermentação em estado sólido usando diferentes substratos agroindustriais

RESUMO. O estudo teve como foco a determinação de fatores que influenciam a produção de celulases por uma cepa isolada de ninho de formigas cortadeiras e identificada por meio do gene ITS como *Penicillium oxalicum*. O processo produtivo foi Fermentação em Estado Sólido utilizando como substrato lignocelulósico bagaço de cana-de-açúcar e sorgo lignocelulósico. As variáveis foram analisadas através de um planejamento fatorial fracionário 2^{5-1}, com três repetições no ponto central. Todas as variáveis analisadas influenciaram a produção de pelo menos um dos três tipos de celulases analisados. As maiores atividades observadas foram: FPase 4,2 U g^{-1}; CMCases 9,2 U g^{-1} e avicelase 8,4 U g^{-1}, utilizando sorgo lignocelulósico como substrato. As melhores condições para produção foram: temperatura de incubação a 40ºC, umidade inicial 60%, pH 4,0, tempo de cultivo de quatro dias, utilizando como substrato sorgo lignocelulósico.

Palavras-chave: Bagaço de cana-de-açucar, sorgo lignocelulósico, enzimas celulolíticas.

Introduction

Lignocellulose is the most abundant organic compound in the biosphere; however, only a small amount produced in agriculture or forestry is used, the rest being considered waste, causing consequent deterioration of the environment (Sánchez, 2009). Much is being done to reduce losses of this resource and to diminish the resulting environmental degradation (Ballesteros, 2001) through the generation of a series of high-value products and by-products such as cellulolytic enzymes and cellulosic ethanol (Isikgor & Becer, 2015).

Despite the potential of cellulose, it is highly crystalline and compact (Aristidou & Penttila, 2000; Gray, Zhao, & Emptage, 2006; Pérez, Muñoz-Dorado, de la Rubia, & Martínez, 2002). Fortunately, some microorganisms, like *Penicillium oxalicum* are able to degrade this lignocellulosic material using hydrolytic and oxidative enzymes naturally produced by the microorganisms themselves (Xian, Wang, Yin, & Feng, 2016). These enzymes include cellulases, the main recruitable resource for the bioconversion of cellulosics to useful products, and usually the most costly part of the production process. In ethanol production, for example, the cost of cellulases is about 40% of the total cost of production.

Commercially, the production of cellulases is carried out on purified cellulose such as avicel and solka floc, which is quite expensive. Therefore, it is

possible to produce large quantities of cellulase using materials like lignocellulosic residues, which have much lower costs and would allow for large economically viable bioconversions (Chandra, Kalra, Sharma, Kumar, & Sangwan, 2010)

The strain *P. oxalicum* F-3380, was selected for identification and analysis in the present study because it showed the highest productivity among the isolates from leafcutter ant species' nests (*Acromyrmex balzani*) previously tested. These ants cultivate symbiotic fungus, prune, clean the garden, and take the substrate rejected by the fungus to a disposal site located outside the nest (Caldato, Andrade, Forti, Barbieri, & Lopes, 2010). The close relationship of lignocellulose with microorganisms that exist in the nest reinforces the idea that these organisms have a high ability to produce a series of catalytic enzymes and degrade polymers, cellulose, hemicellulose and lignin, existing on the substrate on which they grow.

The production of substrates for fermentation of these microorganisms and the consequent production of these lignocellulolytic enzymes are also a rational alternative for the use of agroindustrial lignocellulosic residues (Pandey, Soccol, Nigam, & Soccol, 2000). Solid State Fermentation (SSF) has emerged as a great resource for enzyme production by microorganisms; according to Hölker, Höfer, and Lenz (2004) the use of SSF has been shown to be particularly advantageous for the growth of filamentous fungi and the consequent production of enzymes, since it simulates their natural habitat and thus provides higher productivity when compared to the process of submerged fermentation. In addition, the enzymes produced by SSF are less susceptible to substrate inhibition problems and also have greater stability in temperature and pH variations.

However, it is known that the variables that affect the SSF process are numerous and need to be analyzed, because the success of the enzymatic production depends on the type of microorganism used and the setting of the most significant operating variables of the production process. Thus, based on the biotechnological potential of *P. oxalicum* F-3380 (Xian et al., 2016) with the evidence of high enzymatic activity in the leafcutter ant nests (Caldato et al. 2010), in the face of the abundance of residual lignocellulosic material available in nature (Sánchez, 2009), and based on the need to obtain ideal conditions for the SSF process using the strain, the study aims to define the most influential factors on cellulase production from *Penicillium oxalicum* F-3380, isolated from a leaf-cutting ant nest belonging to the species *Acromyrmex balzani*, using sugarcane bagasse SB and lignocellulosic sorghum LS as substrates.

Material and methods

Microorganism

The filamentous fungus F 3380 was obtained from an *Acromyrmex Balzani* colony (Hymenoptera: Formicidae), which is a leaf-cutting ant. The strain belongs to the microorganism culture collection: *Coleção de Culturas Carlos Rosa* at *Laboratório de Microbiologia Ambiental e Biotecnologia* from *Universidade Federal do Tocantins*. It was chosen due to present high cellulolytic activity according to previous research. The microorganism was revitalized in Petri dishes containing PDA for 7 days at 25° C and, with the addition of distilled water, spore suspensions were obtained.

Molecular identification

Genomic DNA was extracted from the strains following the protocol of Rosa, Vaz, Caligiorne, Campolina, and Rosa (2009). The ITS region was amplified and sequenced using the primers ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). Amplification was performed in a Mastercycler® nexus thermocycler (Eppendorf) using the GoTaq® DNA Polymerase kit (Promega Corp., Madison, WI, USA) in a final reaction volume of 25 µL containing 13.375 µL of ultrapure water; 2.5 µL of 25 mM $MgCl_2$; 1.0 µL of 10 mM dNTPs; 5.0 µL of 10 X buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl); 1.0 µL of the primer ITS1 (2 mM); 1.0 µL of the primer ITS4; 0.125 µL of 5 U of the enzyme Taq polymerase, 1.0 µL of DNA (50 ng μL^{-1}). For the amplification cycles, an initial denaturation was performed at 95° C for 2 minutes, followed by 39 denaturation cycles at 95° C for 1 minute; after that, the annealing of primers was performed at 52° C for 2 minutes and extension at 72° C for 2 minutes. A final extension was performed at 72° C for 10 minutes.

After amplification, agarose gel electrophoresis was executed at 1% (w/v) containing GelRed® (Biotium Inc. California, USA), and 1 X TBE buffer (2 mM EDTA, 1 M Tris-HCl, 0.1 M boric acid, pH 8.0 with the PCR product to see if amplification had occurred. It was visualized under ultraviolet light in a LPIX-EX imaging system (Loccus Biotechnology). The 1 Kb DNA Ladder (Promega Corp., Madison, WI, USA) was used as a molecular marker.

The amplified product was purified with a solution of Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-it®) per the manufacturer's instructions. After purification, the amplified

fragment was sequenced in both directions using the same PCR primers in an ABI 3500 XL automated sequencer (Life Technologies) according to the dideoxy or chain-termination method (Sanger, Nicklen, & Coulson, 1977) and using the BigDye Terminator kit v3.1 (Life Technologies). This step was carried out at the *Laboratório de Polimorfismo de DNA* of the *Universidade Federal do Pará* (UFPA).

After automatic sequencing, the nucleotide sequences of the microorganism were imported into the Geneious 6.1.8 program (Kearse et al., 2012) to be analyzed, aligned and edited. The consensus sequence, which was generated from the forward and reverse sequences, was manually corrected and aligned. This sequence was exported into a FASTA file extension (.fasta*/*. Fas) for further analysis, to search for and compare sequence identities in the GenBank database (www.ncbi.nlm.nih.gov/blast/) and sequences in the CBS Database (http://www.cbs.knaw.nl/Collections/).

Substrates (SB) (LS) and their pretreatment

Sugarcane bagasse (SB) was obtained from the company Bunge (Pedro Afonso, Tocantins, Brazil) and lignocellulosic sorghum (LS) was obtained from the company *EMBRAPA Milho e Sorgo* (Sete Lagoas, Minas Gerais, Brazil). SB and LS were passed through a sieve with a 20 mesh particle size and treated with 4% sodium hydroxide in an autoclave at 1.1 kg cm^{-2} of pressure for 15 minutes. After that, the pH was adjusted using phosphoric acid (pH 2 was maintained for 30 minutes) and the liquid portion was discarded; then, the solids were autoclaved with distilled water 1:1 (w/s) and dried at 65° C to a constant weight.

Enzyme production

All enzyme production was conducted in 250 mL Erlenmeyer flasks with each flask containing 5 g of substrate and the mineral salt solution described by Mandels and Weber (1969). The flasks were sterilized in an autoclave for 15 minutes, then cooled, and after that, inoculated with fungal spore suspension and incubated under constant stirring at 150 rpm. The variables were adjusted according to the experimental design. Enzyme extraction was done by adding citrate buffer (0.05 M, pH 4.8) followed by agitation for 1h, simple filtration and finally storage at -20° C for later analysis (Aguiar & Lucena, 2011).

Enzyme assay

Endoglucanase activity (CMCase) was determined according to the method described by Ghose (1987) using a reaction mixture containing 0.5 mL of the enzyme solution with 0.5 mL of 2% carboxymethylcellulose solution in citrate buffer (0.05M, pH 4.8), which was incubated at 50°C for 30 minutes. Exoglucanase (Avicelase) activity was determined according to that described for endoglucanase activity, but the incubation was carried out with 0.5 mL of 1% avicel suspension instead of carboxymethylcellulose. The FPase activity (filter paper activity) was determined by the Ghose (1987) method using 0.5 mL of the enzyme solution and a whatman n. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in 1.0 mL of 0.05 M citrate buffer at a pH of 4.8, with incubation at 50° C for 30 minutes.

The reducing sugar released was estimated using the DNS method described by Miller (1959). One unit (U) of enzyme activity in each case was defined by the amount of enzyme that produces one μmol of glucose from the substrate per minute of the reaction. The activities were expressed in U g^{-1} of dry substrate used in the process (U g^{-1} s).

Experimental design and Statistical Analysis

A 2^{5-1} fractional factorial design was performed with 16 observations and 3 replications in the central point, according to Rodrigues and Iemma (2005). The tested variables were: incubation temperature (TPT), moisture content (M), pH of the mixture (pH), incubation time (T), and inoculum concentration (IC); the responses were filter paper activity (FPase), endoglucanase activity (CMCase) and exoglucanase activity (Avicelase). All the results were analyzed using the software Statistica® 10.0 (Statsoft Inc., Tulsa, OK, USA), with a significance level of 95%.

Results and discussion

Fungal strain identification

This study molecularly identified the F33805 strain from the *Coleção de Culturas Carlos Rosa, Universidade Federal do Tocantins*. The sequence, which was generated using the ITS gene, shows 98% similarity with the strain *Penicillium oxalicum* (GenBank Accession LT558936.1) (Guevara-Suarez et al., 2016). For this reason, it was identified as *Penicillium oxalicum* and its identity was confirmed in the CBS Database http://www.westerdijkinstitute.nl/collections/), where it presented 99% similarity (IHEM Accession 2931).

Species from the genus *Penicillium* have been described as potential producers for commercial cellulases (Saini et al., 2015). Zhang et al. (2014) described fungi with cellulolytic activity, which were isolated from a subtropical and tropical forest in China and mostly from the genus *Penicillium*.

Difference between the evaluated substrates (SB) (LS)

The mean the of values obtained from the enzymatic activity with sorghum was higher than the ones using sugarcane bagasse (Figure 1). The activity using sugarcane bagasse varied between 3.39 and 0.32 U g^{-1} for FPase activity; 3.52 - 0.17 U g^{-1} for CMCases and 1.85 - 0.32 U g^{-1} for Avicelase activity. The cellulolytic activities obtained using LS varied between 4.2 and 2.2 U g^{-1} for FPase activity; 9.2 - 2.2 U g^{-1} for CMCases and 8.4 - 1.8 U g^{-1} for Avicelase activity.

Rocha, Barros, Fischer, Coutinho-Filho, and Cardoso (2013), upon analysis of different substrates' ability to induct the production of cellulolytic enzymes by *Aspergillus niger*, found that the highest values were obtained using sorghum, approximately 38 U g^{-1} compared to 5 U g^{-1} with sugarcane bagasse.

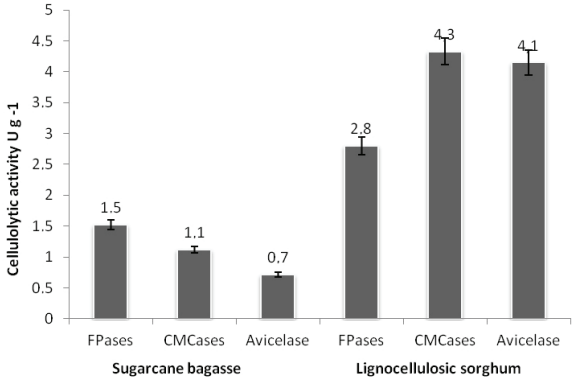

Figure 1. Mean of cellulolytic activity obtained using sugarcane bagasse and sweet sorghum as carbon sources.

Latifian, Hamidi-Esfahani, and Barzegar (2007) evaluated the effect of the following variables: initial pH, fermentation medium content and temperature, using rice straw as a substrate and a genetically modified strain of *Trichoderma reesei*; as a result, they obtained a maximum FPase activity of 1.1635 U g^{-1} under the following conditions: pH 5.0, medium containing 70% rice straw and incubation temperature was 30° C. However, Scheufele, Butzke, Marra, Hasan, and Fiorese (2012), in analyzing the effects of the following variables: time of hydrolysis, enzymatic dilution, concentration of Tween 80 and the solid:liquid ratio in SSF using *Trichoderma sp*, obtained a maximum enzymatic activity of 2.778 U g^{-1}. Basso, Gallo, and Basso (2010) used sugarcane bagasse as a substrate and obtained a maximum FPase activity of 2.3 U g^{-1} on the fifteenth day of the experiment. Singhania, Sukumaran, and Pandey (2007) obtained total cellulase activity of 0.17 U g^{-1} after 5 days of culture, using *T. reesei* RUT C30 in a wheat bran substrate. The current study observed greater values than those described above, with relatively less incubation time, which indicates that the filamentous fungus used has cellulolytic capacity and that sugarcane bagasse and sorghum are good substrates for SSF using this microorganism.

Fractional factorial design

The results of cellulolytic activity (FPases, CMCases, Avicelases) obtained from the 2^{5-1} fractional factorial design using sugarcane bagasse and Lignocellulosic sorghum as substrates are shown in Table 1.

Table 1. Fractional factorial design 2^{5-1} obtained during SSF using sugarcane bagasse and lignocellulosic sorghum as substrates.

Tests	Factors					Substrate Yield (U g^{-1})					
						SB			LS		
	TPT (X1)	M (X2)	pH (X3)	T (X4)	Ci (X5)	1⋆	2⋆	3⋆	1⋆	2⋆	3⋆
1	30	60	4	4	10^8	0.5	0.9	0.3	2.5	4.5	4.8
2	40	60	4	4	10^6	0.3	0.6	0.3	4.2	9.2	8.4
3	30	80	4	4	10^6	3.3	1.3	1.6	2.5	5.3	4.5
4	40	80	4	4	10^8	2.0	1.3	0.2	3.1	5.3	4.6
5	30	60	8	4	10^6	1.4	1.3	1.8	2.8	4.4	6.4
6	40	60	8	4	10^8	2.5	0.9	0.3	3.3	4.1	3.7
7	30	80	8	4	10^8	1.8	2.8	0.6	2.9	4.6	2.2
8	40	80	8	4	10^6	1.3	1.2	0.2	2.3	5.4	4.5
9	30	60	4	12	10^6	0.8	0.6	0.1	2.4	4.0	4.7
10	40	60	4	12	10^8	0.4	0.1	0.2	3.5	5.4	4.2
11	30	80	4	12	10^8	2.7	1.4	1.4	2.3	3.2	2.7
12	40	80	4	12	10^6	1.6	0.8	0.4	2.3	3.0	2.2
13	30	60	8	12	10^8	0.7	0.1	0.4	3.1	2.3	2.8
14	40	60	8	12	10^6	1.6	0.8	1.3	2.5	4.6	5.6
15	30	80	8	12	10^6	2.7	3.5	0.3	2.3	4.2	3.4
16	40	80	8	12	10^8	1.3	1.0	1.5	2.2	3.1	6.5
17	35	70	6	8	$5x10^7$	1.3	0.9	0.9	2.9	2.8	1.8
18	35	70	6	8	$5x10^7$	1.4	0.7	1.0	3.3	2.2	2.3
19	35	70	6	8	$5x10^7$	1.3	1.0	0.8	2.8	4.6	3.5

TPT - Incubation temperature (°C); M- initial moisture content (wt%); pH- initial pH of the mixture; T- incubation time; Ci - inoculum concentration; SB - sugarcane bagasse; LS - Lignocellulosic sorghum; 1⋆ - FPase; 2⋆ - CMCase; 3⋆ - Avicelase.

The data indicates that there was a large variation in cellulase activity in the different assays carried out; this variation increases the importance of the experimental design used in this study because it may contribute to achieving higher enzyme production by adjusting the variables involved in SSF.

Another way to confirm the importance of orienting experimental design to improve SSF conditions is to observe the values of enzymatic activity presented in the Central Point (CP) of the experiment (tests 17, 18 and 19). CP values were established, based mostly on the best results of similar cultivations performed in previous studies such as Kang, Park, Lee, Hong, and Kim (2004); Gao, et al. (2008) and Zhang and Sang (2012). From these values, ranges of the variables were expanded. CP tests resulted in an average activity of 1.1 U g^{-1} of SB and 2.9 U g^{-1} of LS; this shows that the experimental design performed promoted an average

increase in enzymatic activity of about 70% in sugarcane and 20% in sorghum, considering the highest values obtained in the experiment.

It is possible to observe in Table 1 that, for the assays using sugarcane bagasse as their substrate, the highest FPase activity (3.3 U g^{-1}) was in test 3, which was performed at a temperature of 30º C; initial moisture of 80%; pH of 4.0; over four days and an inoculum concentration of 10^6 spores mL^{-1}. In comparison, the highest CMCase activity (3.52 U g^{-1}) was in test 15, which was performed with an initial incubation temperature of 30º C; initial moisture of 80%; pH of 8.0; over twelve days and an inoculum concentration of 10^6 spores mL^{-1}. Also, the highest activity of avicelase (1.85 U g^{-1}) was seen in test 05, which was performed at an incubation temperature of 30º C; initial moisture of 60%; pH of 8.0; over four days and an inoculum concentration of 10^6 spores mL^{-1}.

In contrast, the results of fermentation using sorghum showed that the highest activities for FPase (4.2 U g^{-1}), CMCase (9.2 U g^{-1}) and Avicelase (8.4 U g^{-1}) were provided by combining test 2 levels, with a temperature of 40º C; 60% initial moisture; pH of 4.0; four days of incubation and an initial inoculum concentration of 10^8 spores mL^{-1}. Comparisons of cellulase activity produced by different fungal strains can be seen in Table 2.

Effect of the analyzed variables

Table 3 shows the significant effects of the factors and their interactions on FPase activity. Initial moisture influences the activity of FPase in both SB and LS substrates and this influence is positive for the SB substrate (1.07 U g^{-1}), which means that due to a rise in moisture from 60% to 80%, there was a gain in activity of 1.07 U g^{-1}. According to Martins, Kalil, and Costa (2008), low moisture reduces the solubility of the solid substrate, the swelling index and produces high superficial tension; this can explain the low yield presented in the tests with low moisture. However, the LS substrate's high values for initial moisture negatively affected FPase activity (-0.55 U g^{-1}), which means that due to the increase in initial moisture from 60% to 80%, there was a reduction of 0.55 U g^{-1} in enzymatic activity. According to Hölker et al. (2004), this fact can be related to fungus inhibition due to extrapolation of the water level.

The divergence of effects on the SB and LS substrates can be explained by natural and structural differences between the materials utilized, the chemical composition of the substrate itself and the distribution of its main components, such as lignin, hemicellulose and cellulose (Aguiar & Ferraz, 2011). According to Hölker et al. (2004), the appropriate level of moisture in solid state fermentation is variable, depending not only on the needs of the microorganism and the expression of desired metabolites, but also on the nature of the substrate used. This difference is a factor that may indicate an advantage for using sorghum as a substrate, in the sense that one problem with this type of enzyme production on a large scale is maintaining the same moisture levels even with a natural increase in temperature during SSF. Due to the fact that the studied microorganism is able to produce more in a sorghum substrate with low moisture, the maintenance of fermentation would be facilitated.

It was also observed (Table 3) that combinations of higher temperatures caused a reduction in enzymatic activity in SB (-0.39 U g-1), and increased production in SSF using LS. This enzymatic production capacity at high temperatures indicates that sorghum can maintain an ideal environment for fermentation by the filamentous fungus. This is a very promising result since heat removal is one of the greatest difficulties in SSF processes, due to the low thermal conductivity of the fermented material, a problem that increases for large-scale SSF (Durand, 2003). In the present study, it is noticeable that the sorghum substrate would be a better option than sugarcane bagasse for use on a large scale, due to its ease of temperature maintenance and considering the higher enzymatic activities obtained at high temperatures using the same fungal strain in fermentation.

The significant effects of the factors and their interactions on CMCase activity (Table 3) involve mainly M, TPT and pH; TPT alone presented a negative influence (-0.63 U g^{-1}) for SSF using SB. On the other hand, for SSF with the LS substrate, the T variable alone presented significance, though it was negative; when the time was increased from 4 to 12 days, CMCase activity was reduced to 1.8 U g^{-1}. The reduction or stabilization of the amount of enzymatic activity due to the increase in fermentation time may possibly be attributed to the exhaustion of nutrients or the accumulation of products that inhibit enzymatic synthesis or cell growth (Sanchez & Demain, 2008).

Biazus, Souza, Curvelo-Santana, and Tambourgi (2006), working with maize malt, observed that enzyme production, in principle, is slow, accelerating until it reaches its maximum value. Thenceforth, the concentration of products generated by the enzymes causes that part of them to become inhibited and their activity to be reduced to a constant value.

Table 2. Comparison of cellulase activities produced by cellulolytic fungi strains

Strains	Substrate	Cellulases U g^{-1}			References
		1⋆	2⋆	3⋆	
P. oxalicum	LS	4.2	9.2	8.4	Current study
P. oxalicum	SB	3.3	3.5	1.8	Current study
T. reesei	RB	1.2			Latifian et al. (2007)
Penicillium decumbens	SB	3.97			Long et al. (2009)
Trichoderma reesei	SB		5.4		Basso et al. (2010)
Aspergillus fumigatus	SB	0.14			Soni, Nazir, and Chadha (2010)
A. fumigatus	RB			1.7	Sherief, El-Tanash, and Atia (2010)
Aspergillus niger	SM and SB		2.2		Rodrigues-Zúñiga, Farinas, Bertucci Neto, Couri, and Crestana (2011)
Trichoderma sp	SB	2.7			Marra et al. (2015)

LS - Lignocellulosic sorghum; SB - Sugarcane bagasse; RB - Rice bran; SM - Soybean meal; 1⋆ - FPase; 2⋆ - CMCase; 3⋆ - Avicelase.

Table 3. Estimate of significant effects ($p < 0.05$) for FPase activity in SB and LS substrates.

	Substrate		Effect U g^{-1}	Standard Deviation	T (3)	P value	Lim. Conf. -95%	Lim. Conf. +95%
1⋆	SB	Mean/Interc.	1.59	0.06	24.34	0.000152	1.38	1.80
		M	1.07	0.13	7.52	0.004867	0.62	1.52
		M x pH	-0.82	0.13	-5.80	0.010200	-1.28	-0.37
		TPT x M	-0.73	0.13	-5.10	0.014564	-1.18	-0.27
		TPT x Ci	0.51	0.13	3.60	0.036673	0.06	0.97
		T	-0.39	0.13	-3.03	0.012697	-0.68	-0.10
		TPT x pH	0.37	0.13	2.87	0.016688	0.08	0.66
	LS	Mean/Interc.	2.80	0.07	39.77	0.000035	2.58	3.02
		M	-0.55	0.12	-3.58	0.037164	-104	-0.06
		TPT x pH	-0.53	0.12	-3.42	0.041791	-1.01	-0.04
		T	-0.38	0.12	-3.09	0.020262	-0.65	-0.09
		TPT x M	-0.35	0.12	-2.89	0.002505	-0.63	-0.07
		TPT	0.33	0.12	2.68	0.027853	0.05	0.60
2⋆	SB	Mean/Interc.	1.17	0.08	13.99	0.000790	0.91	1.44
		M	0.97	0.18	-3.46	0.040713	-1.21	-0.05
		TPT	-0.63	0.18	5.33	0.012924	0.39	156
		pH	0.58	0.18	2.94	0.012455	0.15	101
		T x Ci	-0.57	0.18	-2.89	0.013481	-1.00	-0.14
		TPT x M	-0.54	0.18	-2.76	0.017209	-0.97	-0.11
	LS	Mean/Interc.	4.26	0.26	39.77	0.000001	3.65	4.877
		T	-1.78	0.57	-3.58	0.016638	-3.11	-0.43
3⋆	SB	Mean/Interc.	0.77	0.05	15.96	0.000535	0.61	0.92
		TPT x T	0.57	0.10	5.43	0.012266	0.23	0.90
		M x pH	-0.49	0.10	-4.72	0.018011	-0.83	-0.16
		M x Ci	0.45	0.10	4.33	0.022676	0.12	0.78
		T x Ci	0.48	0.10	4.61	0.019194	0.15	0.82
	LS	No significant variables						

TPT - Incubation temperature (°C); M- Initial moisture content (wt%); pH- Initial pH of the mixture; T- Incubation time; Ci - Inoculum concentration; 1⋆ - FPase; 2⋆ - CMCase; 3⋆ - Avicelase.

Thus, the optimal incubation time for maximum enzyme production depends on the type of material, microorganism growth rate, carbon source and its standard production. For the production of Avicelases it is possible to observe that the variables were significant only when studied relating one factor to another, while for SSF using LS, no variable or interaction was significant with a 95% confidence interval.

The coefficient of determination (R-squared) values were observed at 0.97726 for FPase, 0.94624 for CMCase and 0.93391 for avicelase, which validates the use of equations 1, 2 and 3 and indicates that they can be used to predict enzymatic production tendencies. ANOVA analysis showed, through the F-test, that CMCase and Avicelase activities in LS were not significant. Thus, the models generated cannot be used.

$$FPase_{production} = 1.59 - 0.20X_1 + 0.54X_2 - 0.36X_1X_2 + 0.19X_1X_3 + 0.26X_1X_5 - 0.41X_2X_3$$

(Equation 1)

where: X_1 represents the incubation temperature; X_2 = is the initial moisture content; X_3 = is the initial pH of the mixture; X_4 = is the incubation time; X_5 = is the inoculum concentration.

$$CMCase_{production} = 1.17 - 0.31X_1 + 0.49X_2 + 0.29X_3 - 0.27X_1X_2 - 0.28X_4X_5$$

(Equation 2)

where: X_1 represents the incubation temperature; X_2 = is the initial moisture content; X_3 = is the initial pH of the mixture; X_4 = is the incubation time; X_5 = is the inoculum concentration.

$$Avicelase_{production} = 0.77 + 0.28X_1X_4 - 0.25X_2X_3 + 0.23X_2X_5 + 0.24X_4X_5$$ (Equation 3)

where: X_1 represents the incubation temperature; X_2 = is the initial moisture content; X_3 = is the initial pH of the mixture; X_4 = is the incubation time; X_5 = is the inoculum concentration.

Figure 2 displays the response surface generated from FPase production in SB. The best production region is in the extensions: Temperature (≤30 to 35° C), Moisture (70 to ≥80 %).

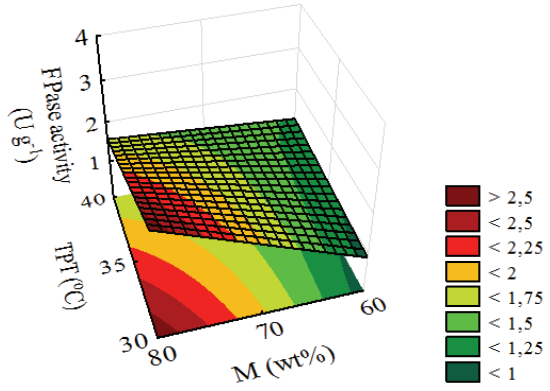

Figure 2. Response Surface of FPase activity obtained based on the SSF process using sugarcane bagasse as the substrate, considering Moisture (M) and Temperature (TPT) as factors.

In Figures 3 and 4 there is a region of best CMCase production in sugarcane bagasse in the following extensions: temperature (<30 to 35 °C), Moisture (75 to > 80 %), pH (4 to <6).

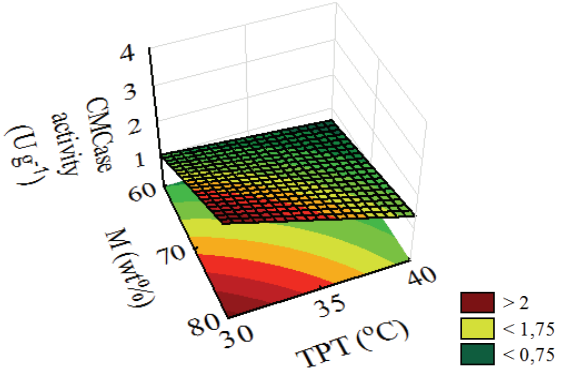

Figure 3. Response Surface of CMCase activity obtained based on the SSF process using sugarcane bagasse as the substrate, considering Moisture (M) and Temperature (TPT) as factors.

In Figures 5 and 6 it is possible to observe the existence of a region of best FPase production in sorghum. This region is in the following extensions: temperature (≥37 to ≥40° C), Moisture (≤60 to 70 %), pH (6 to <4).

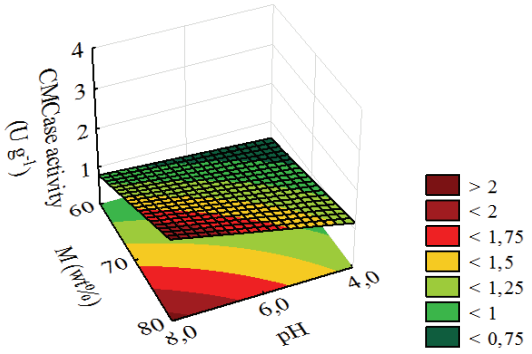

Figure 4. Response Surface of CMCase activity obtained based on the SSF process using sugarcane bagasse as the substrate, considering Moisture (U) and pH as factors.

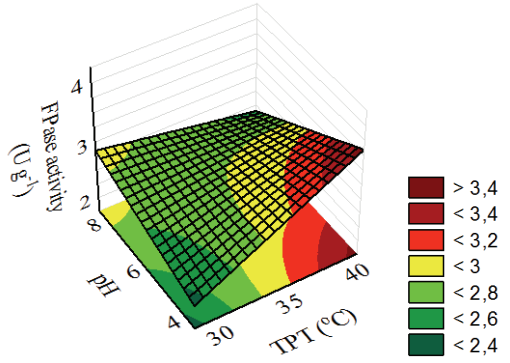

Figure 5. Response Surface of FPase activity obtained based on the SSF process using sorghum as the substrate, considering pH (pH) and Temperature (TPT) as factors

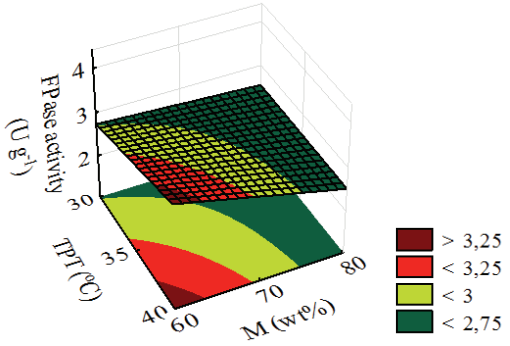

Figure 6. Response Surface of FPase activity obtained based on the SSF process using sorghum as the substrate, considering Moisture (M) and Temperature (TPT) as factors.

Conclusion

Cellulase production by Solid State Fermentation, using *Penicillium oxalicum* F 3380 and biomass LS and SB has shown to be promising due to high values of enzymatic activity; notably, the best performance was with LS. The huge fluctuation of enzymatic activity production in the tests performed and the differences in the influence of the variables in both substrates reinforce the importance of

detailed adjustments based on SSF parameters to obtain greater enzymatic production. The initial analysis in this study indicates the path for future optimization of SSF by *Penicillium oxalicum* using SB or LS as a substrate.

Acknowledgements

The authors thank Dr. Marcelo Mendes Pedroza and João Pedro Kappes Marques for their help with statistical analyses and the CAPES Foundation for the financial support through a doctoral scholarship.

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Received on June 21, 2016.
Accepted on April 10, 2017.