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Production of cellulases by solid state fermentation using natural and pretreated sugarcane bagasse with different fungi

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ABSTRACT

Lignocellulosic biomass is an important source of energy and it has been extensively studied in the production of second generation ethanol. For this purpose, the complex lignocellulosic matrix must be deconstructed by thermal or chemical pretreatment, followed by cellulose depolymerization using cellulolytic enzymes to release simple fermentable sugars. This process still has several techno-economical drawbacks, such as the cost of enzymes. Aiming to reduce the cost of theses enzymes, their production have been studied by solid-state fermentation (SSF) using low-cost agroindustrial residues as carbon source to the microbial growth. In this context, this work aimed at studying the operational conditions (temperature and moisture content) for the production of cellulases from the fungi *Penicillium* sp., *Rhizomucor* sp. and *Trichoderma koningii* INCQS 40331 (CFAM 422), utilizing natural sugarcane bagasse (NSB) and pretreated by acid-alkaline solution (AAB) and hydrogen peroxide (HPB) as substrates. The study was performed by using three-level factorial design (3^2) with central points. The results showed that the more suitable fungus for cellulases production was the *Trichoderma koningii (8.2 IU/g $_{substrate}$)*, followed by *Penicillum sp (1.7 IU/g $_{substrate}$)*. Moreover, the best results were obtained using NSB for all fungi. Statistical analyses showed that the temperature has a greater effect on the production of cellulases by the evaluated fungi.

1. Introduction

Cellulases are industrially important enzymes, which are widely used to bioethanol production from lignocellulosic biomass (Zhang and Sang, 2012; Behera and Ray, 2016). They are a cocktail of three types of complex enzymes namely, cellobiohydrolases (EC3.2.1.91), endoglucanases or CMCases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21), acting synergistically to convert the cellulose into glucose (Satyamurthy et al., 2016; Prasanna et al., 2016). The cellulases have a significant cost factor in the cellulosic ethanol production, mainly in countries where there is no industrial production of cellulases (Ellilä et al., 2017), which accounts for approximately 40% of the total cost (Behera and Ray, 2016).

Ellilä et al. (2017) reported that the carbon source could account for more than 50% of the total cost of producing enzyme cost whether pure glucose is used as carbon source. In this way, several strategies have been used to reduce the enzyme production cost, such as the solid-state fermentation (SSF), using lignocellulosic residues as carbon source and enzyme inducer (Cavka and Jonsson, 2014; Cunha et al., 2012; Jampala et al., 2017). These residues are abundant, renewable, cheap and readily procurable sources of nutrients for the growth of cellulases producing microorganisms (Saini et al., 2017). Many lignocellulosic residues, such as wheat bran, sugarcane bagasse, rice straw, wheat straw, waste paper, fruit pomace, corn cob and soy bran have been evaluated in the production of cellulases (Jampala et al., 2017; Irfan et al., 2017). Sugarcane bagasse may be an interesting substitute to expensive carbon sources, especially in Brazil, where it is readily available in large amounts at the sugarcane mills (Vasconcellos et al., 2015).

The biomass could be used in the raw or pretreated forms (Saini et al., 2017). The biomass pretreatment have been reported as a way to disrupt the recalcitrant structure of the lignocellulosic complex and to facilitate the accessibility of cellulose to the fungi attack (Rodríguez-Zúñiga et al., 2014). The choice of pretreatment has a large impact on

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fungal growth, because the pretreatment can generate toxic compounds potentially inhibitory for fungi. Vasconcellos et al. (2015) reported an improvement in the enzyme production by removal of phenolic compounds, known as potential inhibitors. Although there are several biomass pretreatments (e.g., biological, physical, chemical and physico-chemical pretreatments), the choice of one depends on the properties of each raw material, since different lignocellulosic materials have different physico-chemical characteristics. Among the different methods, chemical and thermochemical pretreatments are currently the most effective and include the most promising technologies for industrial applications (Alvira et al., 2010).

In addition to the cost factor, the substrate acts as an enzyme inducer, and its source may result in enzymatic cocktails with different catalytic potentials for the breakdown of cellulose (Pandey et al., 2016; Li et al., 2017). The use of lignocellulosic substrates, instead of other commercial inducer, can contribute to obtain specific pools of enzymes that may improve the yields of cellulose hydrolysis (Cunha et al., 2012).

In this context, this work aimed to evaluate the production of cellulases by filamentous fungi (*Penicillium* sp., *Rhizomucor* sp. and *Trichoderma koningii)* by solid-state fermentation using the solid fraction of the sugarcane bagasse as carbon source. Sugarcane bagasse was evaluated in its natural form and pretreated with acid-alkaline and with hydrogen peroxide solutions. The fungi were cultured according to a 3^2 factorial design to evaluate the best conditions of temperature (28, 33 and 38 °C) and moisture content (50%, 60% and 70%) totalizing 33 set of experiments for each fungus (11 set of experiments for each substrate, i.e., natural bagasse, pretreated with acid-alkaline solution and pretreated with hydrogen peroxide).

2. Material and methods

2.1. Preparation and analysis of substrates

Sugarcane bagasse was supplied by Destilaria Itaúnas S/A (DISA, Espírito Santo, Brazil). The material was dried at 55 °C until constant weight and milled to an average size of 6 – 20 mesh (2.36–0.85 mm) using a laboratory knife mill. This material is hereinafter named natural bagasse.

The sugarcane bagasse was pretreated with acid-alkaline solution as following: 10 g of bagasse were suspended in 200 mL of a 1% (v/v) sulfuric acid solution. The suspension was stirred at 100 °C for 40 min; the pretreated bagasse was filtered and washed with hot water until neutral pH. Thereafter, 200 mL of a 7% (w/v) sodium hydroxide solution were added to the washed acid-pretreated bagasse, and the suspension was kept under stirring at 100 °C for 40 min. After that, the pretreated bagasse was washed with hot water until neutral pH and with 50 mM citrate buffer pH 4.8. The solid residue was oven dried at 55 °C until constant weight.

The bagasse was also pretreated with hydrogen peroxide solution, as following: 15 g of bagasse were suspended in 600 mL of 7.3% (v/v) hydrogen peroxide solution at pH 11.5. The suspension was stirred at 25 °C for 60 min and the pretreated solid was recovered by filtration and washed until neutral pH. The solid residue was oven dried at 55 °C until constant weight. These procedures were adapted from Guilherme et al. (2015).

All substrates (natural bagasse and pretreated bagasse by acid-alkaline and hydrogen peroxide solutions) were submitted to chemical analyses. The ash and moisture content were performed according to an adaptation of the protocol described by Morais et al. (2010). The analysis of elemental composition, that corresponds to the percentage content in mass of the elements carbon (%C), hydrogen (%H), sulfur (% S), nitrogen (%N) and oxygen (O%), was performed in a Perkin Elmer CHNS/O 2400 Analyzer The hemicellulose and cellulose content were quantified according to protocol proposed by Morais et al. (2010). The extractives, non-structural components such as fatty acids, long chain alcohols, waxes, resins, phenolic compounds, terpenes, essential oils, sugars, salts, etc. (Rojas et al., 2014), were extracted in a Sohlet apparatus using toluene/ethanol (1:1) as solvents. The methodology was adapted by Morais et al. (2010). The Klason lignin content was quantified according to TAPPI T222 om-11 standard method (Technical Association of the Pulp and Paper Industry, 2011).

Table 1
Variables and levels used in the culture assays by SSF.

Variables	-1	0	+ 1
Culture temperature (T in Celsius degree)	28	33	38
Moisture content (MC in %)	50	60	70

Table 2
Chemical and elementar compositions of the untreated and pretreated bagasses.

	Natural sugarcane bagasse	Pretreated bagasse acid-alkaline	Pretreated bagasse hydrogen peroxide
Moisture (%)	5.37 ± 0.25	6.85 ± 2.07	0.28
Ash (%)	0.73 ± 0.02	2.13 ± 0.10	1.73 ± 0.10
Extractives (%)	13.96 ± 2.53	13.13 ± 1.12	10.64 ± 0.49
Lignin (%)	15.61 ± 3.72	8.23 ± 1.24	10.56 ± 0.29
Hemicellulose (%)	28.75 ± 2.99	2.71 ± 0.30	22.37 ± 0.97
Cellulose (%)	35.92 ± 0.25	75.01 ± 0.36	47.23 ± 0.78
%C	45.79	43.08	44.50
%H	6.23	5.97	6.01
%N	0.53	0.29	0.40
%S	1.08	0.99	1.01
%O	44.24	48.94	45.69

2.2. Enzymatic production

2.2.1. Microorganisms

Penicillium sp. and *Rhizomucor* sp. were isolated and supplied by the research group of the Department of Environment Engineering of the Federal University of Espírito Santo (Espírito Santo, ES, Brazil). *Trichoderma koningii* INCQS 40331 (CFAM 422) was supplied by Oswaldo Cruz Foundation (Rio de Janeiro, RJ, Brazil). The microorganism activation was carried out in agar plate containing 4.2% (w/v) potato dextrose agar at 28 °C for 5 days. After this period, spores were harvested by adding 10 mL of 0.1% Tween-80 (v/v).

2.2.2. Solid state fermentation (SSF)

The cultures were performed with the fungi *Penicillium* sp., *Rhizomucor* sp. and *Trichoderma koningii* using sugarcane bagasses (natural and pretreated with acid-alkaline and hydrogen peroxide solutions) as substrates. The assays were conducted in 250 mL Erlenmeyer flasks containing 5 g of substrate, mineral salt solution for 50%, 60% and 70% moisture (wet basis) and 2.0×10^7 spores/$g_{substrate}$. For the microorganisms *Penicillium* sp. and *Rhizomucor* sp. was utilized saline solution proposed by Mandels and Reese (1957), and for the microorganism *Trichoderma koningii* was used the saline solution proposed by Mandels & Weber (1969). The culture temperature for all assays was set to 28, 33 or 38 °C. The experiments were carried out in an incubator equipped with temperature control for 72 h (*T. koningii*) and 120 h (*Penicillium* sp and *Rhizomucor* sp). At the end of the fermentation, 50 mL of 2% (w/v) sodium chloride solution was added to the culture medium and stirred at 200 rpm for 1 h at 28 °C for enzyme extraction. After centrifugation (3500 rpm at 25 °C for 30 min), the supernatant was recovered and used to measure endoglucanase activity in the presence of carboxymethyl cellulose by the standard method proposed by Ghose (1987).

In order to evaluate the influence of the temperature and moisture content on the SSF, a 3^2 factorial design with two more central points was used (Table 1), totaling 33 experiments for each microorganism (11 for each bagasse). Statistical analyses of the data were performed in

Table 3
Endoglucanase activity of the crude broth after 72 h of SSF of different fungi using sugarcane bagasse as substrates, as follows: natural (NSB), acid-alkaline pretreatment (AAB) and hydrogen peroxide pretreatment (HPB).

Endoglucanase Activity (IU/$g_{substrate}$)											
	Variables		*Trichoderma koningii*			*Penicillium* sp.			*Rhizomucor* sp.		
Run	T (°C)	MC (%)	NSB	AAB	HPB	NSB	AAB	HPB	NSB	AAB	HPB
1	28	50	8.20	1.23	3.39	1.22	0.81	0.47	0.04	0.04	0.01
2	33	50	7.43*	0.86	1.50	0.26	0.08*	0.25	0.06	0.04	0.02
3	38	50	2.24	0.33	2.10	0.14	0.02	0.11	0.06	0.08	0.07
4	28	60	6.05	0.65*	3.19*	1.69	0.39	0.50	0.09	0.02	0.06
5	33	60	3.48	0.82	0.79	0.77	0.64	0.56	0.13	0.02	0.02
6	38	60	2.62	0.73	0.03	0.20	0.02	0.02	0.12	0.05	0.08
7	28	70	5.71	1.28	1.08	1.71	0.39	0.62	0.10	0.03	0.05
8	33	70	2.21	0.52	1.10	0.82	0.82	0.40	0.41	0.02	0.03
9	38	70	2.51	0.32	0.12	0.28	0.05	0.12	0.09	0.15	0.13
10	33	60	3.45	0.88	0.92	0.67	0.70	0.55	0.25	0.00	0.02
11	33	60	3.87	0.88	0.77	1.16	0.81	0.53	0.17	0.00	0.02

* outlier.

Table 4
Effect of temperature and moisture on the enzyme production by SSF of *T. koningii* and *Penicillium* using natural sugarcane bagasse (NSB) and bagasses pretreated with acid-alkaline solution (AAB) and hydrogen peroxide (HPB).

		T. koningii			*Penicillium sp.*		
Substrate	Factor	Effect	Std. Err.	p-value	Effect	Std. Err.	p-value
NSB	Mean	4.177	0.154	0.000	0.810	0.068	0.000
	(1) MC (%) (L)*	− 1.369	0.439	0.026	0.398	0.183	0.061
	MC (%) (Q)**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	(2) T (L)	− 4.196	0.391	0.000	− 1.334	0.183	0.000
	T (Q)	− 1.133	0.314	0.015	n.s.	n.s.	n.s.
	Interaction 1 L x 2 L	1.380	0.478	0.034	n.s.	n.s.	n.s.
AAB	Mean	0.840	0.041	0.000	0.434	0.045	0.000
	(1) MC (%) (L)*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	MC (%) (Q)**	0.258	0.088	0.026	n.s.	n.s.	n.s.
	(2) T (L)	− 0.882	0.113	0.000	− 0.498	0.115	0.003
	T (Q)	− 0.176	0.086	0.085	0.461	0.091	0.001
	Interaction 1 L x 2 L	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
HPB	Mean	1.291	0.148	0.000	0.357	0.033	0.000
	(1) MC (%) (L)*	− 1.563	0.376	0.006	n.s.	n.s.	n.s.
	MC (%) (Q)**	− 0.771	0.302	0.043	n.s.	n.s.	n.s.
	(2) T (L)	− 1.199	0.423	0.030	− 0.447	0.086	0.001
	T (Q)	n.s.	n.s.	n.s.	0.151	0.064	0.046
	Interaction 1 L x 2 L	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* (L) = linear factor.
** (Q) = quadratic factor, Std. Err. = standard error, n.s. = Not statistically significant.

$$\mathbf{A}_1 = 98.07 - 0.52\mathbf{MC} - 4.24\mathbf{T} + 0.05\mathbf{T}^2 + 0.01\mathbf{MCT} \quad R^2 = 0.97 \tag{1}$$

$$\mathbf{A}_2 = 6.50 - 0.00005\mathbf{MC}^2 - 0.26\mathbf{T} + 0.03\mathbf{T}^2 \quad R^2 = 0.91 \tag{2}$$

$$\mathbf{A}_3 = 37.18 - 1.00\mathbf{MC} + 0.01\mathbf{MC}^2 - 0.12\mathbf{T} \quad R^2 = 0.85 \tag{3}$$

$$\mathbf{A}_4 = 4.02 + 0.02\mathbf{MC} - 0.13\mathbf{T} \quad R^2 = 0.88 \tag{4}$$

$$\mathbf{A}_5 = -17.70 + 1.17\mathbf{T} - 0.02\mathbf{T}^2 \quad R^2 = 0.86 \tag{5}$$

$$\mathbf{A}_6 = -4.66 + 0.35\mathbf{T} - 0.01\mathbf{T}^2 \quad R^2 = 0.80 \tag{6}$$

Where: A_1 = Enzymatic activity (UI/$g_{substrate}$) using *T. koningii* and natural sugarcane bagasseA_2 = Enzymatic activity (UI/$g_{substrate}$) using *T. koningii* and pretreated bagasse with acid-alkaline solutionA_3 = Enzymatic activity (UI/$g_{substrate}$) using *T. koningii* and pretreated bagasse with hydrogen peroxideA_4 = Enzymatic activity (UI/$g_{substrate}$) using *Penicillium* sp. and natural sugarcane bagasseA_5 = Enzymatic activity (UI/$g_{substrate}$) using *Penicillium sp.* and pretreated bagasse with acid-alkaline solutionA_6 = Enzymatic activity (UI/$g_{substrate}$) using *Penicillium sp.* and pretreated bagasse with hydrogen peroxide

Statistic v. 13.0 software and the values were considered significant for p-values < 0.1.

3. Results and discussion

3.1. Chemical characterization of the substrates

The chemical and elementar compositions of the untreated and pretreated bagasses (using acid-alkaline and hydrogen peroxide pretreatments) are shown in Table 2.

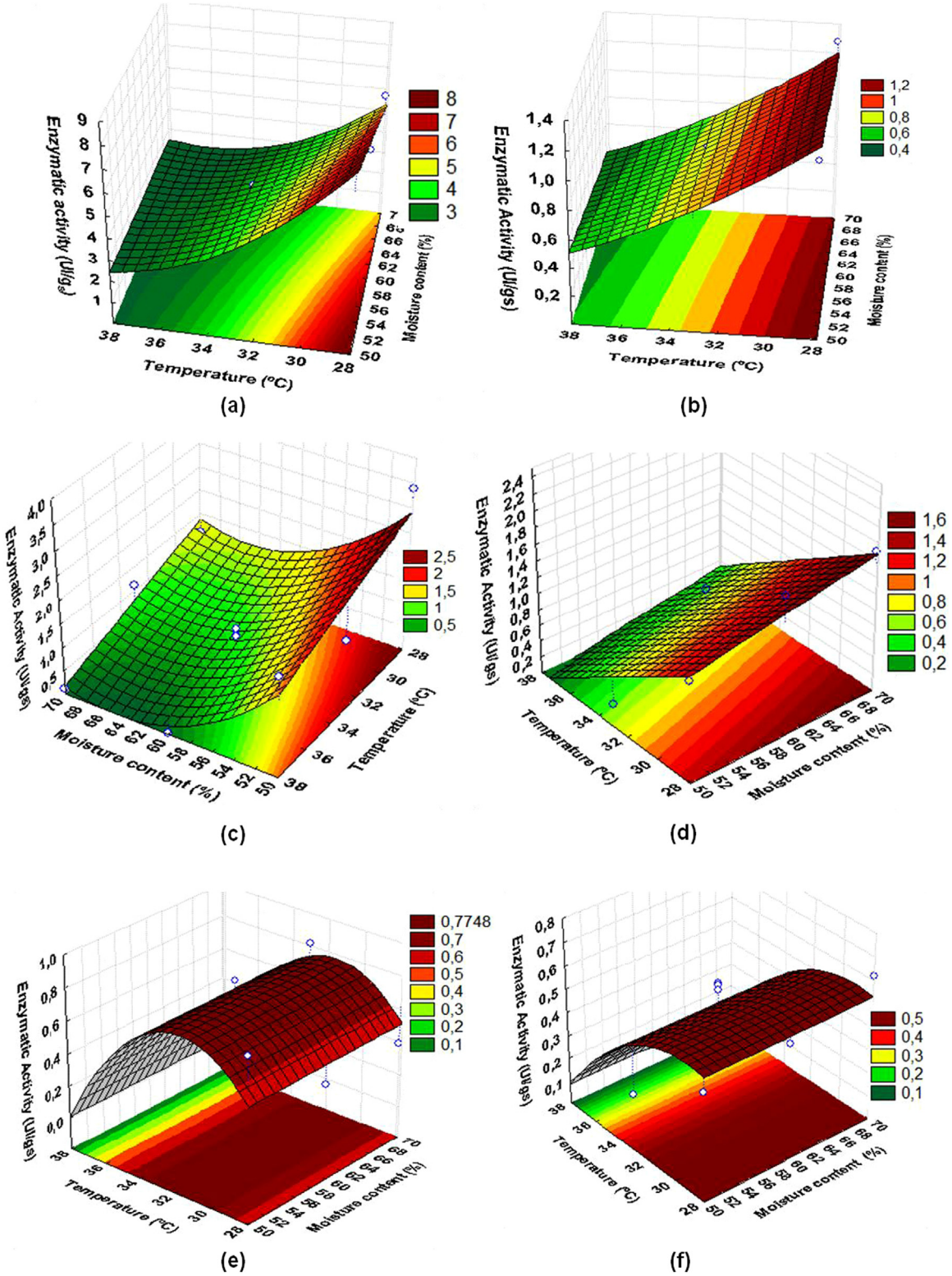

Fig. 1. Response surfaces for enzyme production by SSF of *Trichoderma koningii* using NSB (a), AAB (b) and HPB (c); and SSF of *Penicillium* sp. using NSB (d), AAB (e) and HPB (f).

The results showed that acid-alkaline pretreatment removed up to 47.3% and 90.6% of lignin and hemicellulose, respectively. However, when hydrogen peroxide was used in the pretreatment, only 32% and 22.3% of lignin and hemicellulose was removed, respectively. Similar findings were reported by Rezende et al. (2011), who used acid-alkaline (NaOH 2% or higher) pretreatment of the sugarcane bagasse. On the other hand, Guilherme et al. (2015) reported 55% removal of lignin from sugarcane bagasse using both acid-alkaline and hydrogen peroxide pretreatments. However, in this case values of hemicellulose removal were lower than achieved in this study. These results showed that acid-alkaline pretreatment is more efficient to delignification of the sugarcane bagasse, thus allowing the reduction of the lignocellulosic

residue recalcitrance. Lignin is considered to be the main contributor to biomass recalcitrance, although other factors also interfere, such as the content of hemicellulose and high content of acetyl groups in xylan chains (Carvalho et al., 2015; Chen et al., 2012).

3.2. Enzymatic production

The production of cellulases was performed with the fungi *Rhizomucor* sp., *Penicillium* sp and *Trichoderma koningii*. The fungi *Rhizomucor* sp. and *Penicillium* sp. were isolated from grease trap scum located in Vitória, ES, Brazil. The aim was to verify if these fungal strains of region are potential producer of cellulases. In the literature it is described that *Penicillium* species can be good producers of cellulases (Ritter et al., 2013; Behera and Ray, 2016), a fact not observed for *Rhizomucor*. *Trichoderma* is one of the most common producers of commercially available cellulases, then was used in this work to compare with the other microorganisms.

The enzymatic activities ($IU/g_{substrate}$) obtained in the SSF of *T. koningii, Penicillium sp.* and *Rhizomucor sp*. are presented in Table 3. The variables evaluated in the enzyme production were temperature (28, 33 and 38 °C) and moisture content (50%, 60% and 70%), using the natural sugarcane bagasse (NSB) and bagasses pretreated with acid-alkaline solution (AAB) and with hydrogen peroxide (HPB). In a review carried out by Paramjeet et al. (2018) it is possible to observe cultivation temperatures from 25 °C to 45 °C when using fungal strains.

It is noticeable the greater capability of the *T. koningii* of producing cellulases when compared to *Penicillum* sp. and *Rhizomucor* sp. The highest activity level (8.20 $IU/g_{substrate}$) was achieved at 28 °C and 50% moisture, using NSB as solid substrate. Liu and Yang (2007) studied the production of cellulases by *Trichoderma koningii* AS3.4262 using the waste from vinegar industry and obtained the best results after 84 h of incubation with optimal moisture content of 50% and 30 °C.

The pretreatment of the sugarcane bagasse did not result better assimilation by the fungi, resulting poor production of endoglucanases. Probably, the amorphous fraction of cellulose was removed during the pretreatment, which still present in the NSB was better assimilated by the fungi. According to Kumar et al. (2009) the pretreatment can remove the amorphous fraction of the cellulose, leaving the crystalline fraction. In the literature are found some works where it is verified that the amorphous cellulose was better inducer in the production of cellulases (Ahmed et al., 2009; Busto et al., 1996; Sanchez et al., 1999).

Besides, even washing the pretreated bagasse, residual degradation products, mainly phenolic compounds, could have inhibited the fungi growth. Phenolic compounds are derived from the hydrolysis of lignin during pretreatments and are toxic to the metabolism of many microorganisms (Lv et al., 2017). Van der Pol et al. (2016) verified that high concentrations of inhibitory by-products, such as organic acids and phenols, were produced during alkaline pretreatment. Bolado-Rodríguez et al. (2016) realized thermal, acid, alkaline and alkaline-peroxide pretreatment in wheat straw and sugarcane bagasse. They verified that all the pretreatments released formic and acetic acids and phenolic compounds, while 5-hydroxymetilfurfural (HMF) and furfural were generated only by acid pretreatment. The authors also verified that the alkaline peroxide pretreatment releases the highest formic acid concentrations and the value is higher for sugarcane bagasse.

The effects of temperature and moisture on the enzyme production were statistically analyzed and the results are presented in the Table 4, where are shown only values for statistically significant variables ($p < 0.1$). The SSF using *Rhizomucor* sp. resulted very low enzymatic activities; therefore the statistical analysis of these results was not performed. Statistical analysis of the data indicated that the residues were randomly and independently distributed, and followed a normal distribution, as well as all proposed models had good correlation coefficients (Eqs. 1 to 6). In this way, the adjusted models were appropriate to depict the data. For the experimental analysis, some planning trials were discarded: run 2 to *Trichoderma koningii* and NSB, run 4 to *T. koningii* whit AAB and HPB, run 2 to *Penicillium* sp. and NSB, because they are outlier detected from residue analysis.

The statistical analysis showed that moisture and temperature interfered in the enzyme production by *Trichoderma koningii*, using NSB, AAB and HPB as substrates, resulting in the Eqs. 1, 2 and 3, respectively. The effect of the temperature was higher in the enzyme production using NSB and AAB as substrates; however it was slightly lower using HPB. The response surfaces for the assays using the *T. koningii* microorganism with the substrates NSB, AAB, HPB, respectively, are shown in Figs. 1a, 1b, 1c. In these figures it is possible to observe the greater enzymatic activities are obtained for lower temperatures and lower moisture content (28 °C and 50%). For the *Penicillium* sp., statistical analysis showed that the moisture did not interfere in the enzyme production, only for the natural bagasse and its effect is less than the Temperature. One possible explanation would be a greater absorption of water by the structural components of the natural bagasse, which have been partially removed from the treated bagasse. Fig. 1d shows that when NSB was used, lower temperature (28 °C) and higher moisture content (70%) resulted in better enzyme production. When AAB was used, intermediate temperatures favored the enzyme production (Fig. 1e), in which the optimum temperature is 32 °C. In the assays using HPB, the optimum temperature was 29 °C (Figure 1f).

4. Conclusion

Among the fungi obtained in the region of the State of Espírito Santo, *Penicillium* sp. is the best producer of cellulolytic enzymes. The fungus *Rhizomucor* had insignificant production of cellulases. Although the *Penicillium* had better activity values, its production was lower when compared to *Trichoderma koningii*, which corroborates with the literature data. Regarding the substrate of the cultures, it was verified that sugarcane bagasse can be used as carbon source for the studied microorganisms and inducer in the production of cellulases. The best production results were obtained when bagasse was not pretreated. Among the operational variables studied, the temperature had a greater effect on the enzymatic production, resulting in better results when using temperatures between 28 and 30 °C.

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