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Evaluation of different biological and chemical treatments in agroindustrial residues for the production of fungal glucanases and xylanases

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ABSTRACT

The production and use of fibrolytic enzyme complex in the hydrolysis of lignocellulosic materials is an important strategy for renewable bioenergy. The different carbon sources (residues) with or without some pretreatments (biological or chemical) were analyzed in order to increase the production of fibrolytic enzyme. Glycosyl hydrolases and xylanases were produced using *Trichoderma reesei* QM9414. The influence of some crude or pre-treated agro-industrial residues as a carbon source was previously investigated using shake-flask cultures. Subsequently, the selection of the best culture medium was studied under different pH and temperature conditions in stirred tank bioreactor. Fibrolytic activities reached a maximum of 0.85 ± 0.07 FPU mL^{-1} (total cellulase), 3.14 ± 0.01 CMC mL^{-1} (endoglucanase) and 1.25 ± 0.14 U mL^{-1} (exoglucanase) with the orange peel residue; and 93.08 ± 3.27 U mL^{-1} (xylanase) with sugarcane bagasse under alkali pretreatment. In the stirred tank bioreactor the cellulolytic activity increased to 1.76 ± 0.00 FPU mL^{-1}, about 2 times higher than in the shake-flasks or under studied conditions. The biosynthesis of the fibrolytic complex using agroindustrial residues supplemented was shown to reach a higher total cellulose production.

1. Introduction

The increased use of non-renewable energy sources over the decades has caused changes in the economy, society and the environment [1]. In this regard, serious environmental problems and future limitations of these sources indicate the need for environmentally friendly alternatives for energy sources. Thus, the technologies for production of alternative energy (bioethanol) in the world need to improve using agro-industrial residues in order to increase its production without the threat of competition with food production [1].

The lignocellulosic materials (LCM) are an alternative not fully utilized as feedstocks for bioenergy production. The main limitation of LCM use in bioprocesses is due to the recalcitrance characteristic due to the strong chemical bonds between cellulose, lignin and arabinoxylan in the vegetal cell wall making difficult its hydrolysis [2]. In this sense, low added value LCM residues can be used as rich sources of fermentable carbohydrates like sugarcane bagasse, orange peel, wheat bran, sugarcane bagasse, cotton cake residues, among others. These wastes have been studied as sugars feedstocks and fibrolytic enzymes production [3–5]. However, the challenge is the economic breakdown of these bonds in cellulose and hemicellulose to produce respectively glucose and xylose [3,6]. This process should be done by previous biological and/or chemical pretreatment and after an enzymatic hydrolysis [7]. The chemical pre-treatment is the most studied technique among various pre-treatment categories that was originally developed and therefore has extensively been used for delignification of cellulosic materials, and the most commonly used chemical pre-treatments include: acid and alkali based hydrolysis approaches [8]. However, the biological pretreatments are more effective, economical, eco-friendly and less health hazardous as compare to the physico-chemical or chemical-based pre-treatment approaches [8]. These enzymes are fibrolytic and their production can be done by fungal cultures with agroindustrial residues containing lignocellulosic compounds as inducers [4,5,7,9]. The cellulases are a complex of enzymes containing: (1) endoglucanases which cleave internal bonds in the amorphous regions of the cellulose chains, (2) exoglucanase which acts on the ends of the chains liberating cellobiose, and (3) β-glucosidases which hydrolyze cellobiose to glucose [7,10]. Agroindustrial residues can be pretreated for the production of xylanases [11], and xylanases can be used to produce fermentable sugars and fuels from xylan [12]. The complete cleavage of xylan is carried out by the synergistic action of β-xylanase and its accessory enzymes, including β-xylosidase, α-arabinofuranosidase, α-glucuronidase and acetyl xylan esterase [12,13]. The enzymatic hydrolysis of LCM into fermentable sugars followed by fermentation is a promising

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route for the conversion of cellulosic materials into ethanol [7,9] but the cost of these enzymes for this purpose is high. Therefore, the development of an economical and efficient bioprocess to produce these enzymes is necessary, and the knowledge of the more convenient low cost inducers is strategic. In the present work bioprocess were investigated for fibrolytic enzymes production using agroindustrial residues and pretreatment. In addition, the physicochemical conditions of *Trichoderma reesei* QM9414 culture were optimized to increase the fibrolytic enzymes production.

2. Materials and methods

2.1. Reagents and substrates

The reagents used were: 0.2 mol L^{-1} of acetate-citric acid solution (pH 5.6), 0.2 mol L^{-1} of citrate-phosphate (pH 3–7), 0.2 mol L^{-1} of phosphate (pH 7–8); 1,4-β-glucanase –(AB Enzymes – ROHAMENT® CL) (E.C. 3.2.1.4); filter paper Whatman n°1, Carboxymethylcellulose (Sigma-ALDRICH), Xylan (Sigma-ALDRICH), Avicel pH – 101 (Sigma-ALDRICH); 3,5-Dinitrosalicylic acid (Sigma-ALDRICH) were used for enzymes determination. The agroindustrial residues were obtained from different regions of Brazil: Sugarcane bagasse (São Joaquim Distillery – Cândido Mota – SP – Brazil); Orange peel (Citrovita Agroind. Catanduva – SP – Brazil); Cotton pulp (Louis Dreyfus Commodities – Paraguaçu Paulista – SP – Brazil); Wheat bran (Moinho Nacional – Assis – SP – Brazil) and Broken rice (Canda Cooperative – Assis – SP – Brazil). All agroindustrial residues were washed with running water and dried at 28 °C in laboratory stove (Quimis – Q317M-23). After drying, the residue was triturate (250–450 micrometers) in mill (Cienlab – CE-430/SM).

2.2. Microorganisms

The microorganisms used in the biological pre-treatment were *Rhizopus oligosporus* var. *oligosporus* and *Rhodotorula mucilaginosa* (fibrolytic yeast). *Trichoderma reesei* QM9414 was used as a model for the production of fibrolytic enzymes. The fungi were grown in petri dishes (30 mL of Potato Dextrose Agar medium – 5% – w v^{-1}) for 7 days and 28 °C. These plates were used to prepare of inoculum for the liquid cultures.

2.3. Culture and production media

The lignocellulosic residues used in enzymatic production are in Table 1 and the summary of the enzymatic production is in Graphical abstract (GA). The carbon sources used were divided in: crude residues (sugarcane bagasse, orange peel, rice grits, cotton cake) or residues pretreated by biological or chemical methods. *T. reesei* QM 9414 culture was performed by culture medium formulated by these residues mixed or not with sucrose and lactose, and salts adapted from Silva [5].

2.4. Biological pretreatment

The residue wheat bran and the sugarcane bagasse (Table 1) were pretreated by different fungi, and these substrates were used in the production of enzyme complexes. The wheat bran was pretreated using the fungus *Rhizopus oligosporus var. oliporus* due to its amylolytic activity [14]. The pretreatment consisted of fungal culture by the inoculation of 1.10^8 spores of *R. oligosporus* mL^{-1} in 250 mL Erlenmeyer flasks containing 10 g of wheat bran and 25 mL of nutrient solution (60% (m v^{-1}) humidity): 2.5% (m v^{-1}) of $(NH_4)_2SO_4$; 1.25% (m v^{-1}) of KH_2PO_4; 1.25% (m v^{-1}) of urea; for 5 days at 30 °C in solid state fermentation (SSF), according to Freitas et al. [14]. The sugarcane bagasse was pretreated with *Rhodotorula mucilaginosa* by aseptic inoculation of 10^6 yeast cells mL^{-1} in 100 mL culture medium – 5% (m v^{-1}) of sugarcane bagasse and nutrient solution: 0.2% (m v^{-1}) of yeast extract; 0.13% (m v^{-1}) of $(NH_4)_3PO_4$; 0.1% (m v^{-1}) of $(NH_4)_2SO_4$; 0.031% (m v^{-1}) of K_2SO_4; 0.0028% (m v^{-1}) of $ZnSO_4$; 0.0012% (m v^{-1}) of $MnSO_4$; 0.024% (m v^{-1}) of $MgSO_4$; in submerge fermentation (SmF) at 28 °C, pH 5.5 and 180 rpm, for 120 h. The residues of these cultures were dried at 50 °C for 48 h (Table 1).

Table 1
Some pretreatments and inducers mixtures for enzymatic production in a *Trichoderma reesei* QM9414 culture in submerged fermentation.

Code	*Carbon source*	*Complement*[a]	*Pretreatment*[b]
SB	Sugarcane bagasse	X	X
SB + Lac	Sugarcane bagasse	1% lactose	X
SB_Yeast	Sugarcane bagasse	X	*Rhodotorula mucilaginosa culture*
SB_NaOH	Sugarcane bagasse	X	*NaOH-6%/121 °C/ 45 min*[d]
SB_NaOH + Suc	Sugarcane bagasse	1% sucrose	*NaOH-6%/121 °C/ 45 min*
SB_acid	Sugarcane bagasse	X	*0.1% Sulfuric acid/ 60 °C/12 h*[e]
SB_acid + Suc	Sugarcane bagasse	1% sucrose	*0.1% Sulfuric acid/ 60 °C/12 h*
WheB_olig	Wheat bran	X	*Rhizopus oligosporus*
OP	Orange peel	X	X
OP + Lac	Orange peel	1% lactose	X
OP + Sac	Orange peel	1% sucrose	X
OP + SB[c]	Orange peel + Sugarcane bagasse	X	X
OP +SB + Lac	Orange peel + Sugarcane bagasse	1% lactose	X
BRice	Broken rice	X	X
BRice + Suc	Broken rice	1% sucrose	X
CB	Cotton bagasse	X	X

[a] 1% – lactose or sucrose (m v^{-1}).
[b] Pre-treatments made with fermentation of the respective microorganisms.
[c] Orange peel and sugarcane bagasse (1:1–m m^{-1}).
[d] Method adapted from Silva et al. [5].
[e] Method adapted from Yang et al. [15].

2.5. Chemical pretreatment

The sugarcane bagasse (SB) was chemically pretreated 6% (m v^{-1}) NaOH at 121 °C (1 atm) in 1:2 (m v^{-1}) of bagasse (250–450 micrometers) for 45 min in autoclave, according to Vásquer et al. (2007). Subsequently, the pretreated substrate was washed with deionized water to adjust the pH (6.0) in pH meter, centrifuged (FANEM 206 BL) for 20 min at 3000 rpm, dried at 50 °C for 48 h. The acid pretreatment was performed according to Yang [15], in two steps: First one was the acid hydrolysis: low temperature (60 °C) in 1:9 (m v^{-1}) of bagasse (250–450 micrometers) and sulfuric acid 0.1% (v v^{-1}). The mixture was placed in a 2 L fermenter (BioFlo/CelliGen 115) at 60 °C for 12 h at 200 rpm. Subsequently, the pretreated substrate was filtered and washed with deionized water to adjust the pH (6.0). The second step consisted in a second hydrolysis of the bagasse obtained from the first step by the adjustment of the pH 6.0 with 2 M NaOH and its autoclavation at 121 °C (1 atm) for 45 min. In the next step, the substrate was diluted in the ratio 1:12 with distilled water and centrifuged for 20 min at 3000 rpm, dried at 50 °C for 48 h (Table 1).

2.6. Shake flask cultures

The fermentation and production of cellulolytic and xylanolytic enzymes by *Trichoderma reesei* QM 9414 was adapted from Silva [5]: Inoculum was obtained by the aseptic transfer of 10 mm diameter circles of the fungus culture to flasks (250 mL) containing 80 mL of culture medium formulated by: 3% (m v^{-1}) of the residues as carbon source and the nutrient solution: 0.11% (m v^{-1}) of K_2HPO_4; 0.1% (m v^{-1}) of $(NH_4)_2SO_4$; 0.0017% (m v^{-1}) of $MgSO_4{\cdot}7H_2O$; 0.0028% (m v^{-1}) of $ZnSO_4{\cdot}7H_2O$; 0.1% (m v^{-1}) of yeast extract; 0.1% (m v^{-1}) of

Table 2
Temperature and pH of *T.reesei* QM9414 culture for enzymatic production by Central Composite Design.

Experiments[a]	Variables in coded units		Variables in original units	
	Temperature (°C) − X_1	pH X_2	Temperature (°C)	pH
1	−1	−1	26.5	3.8
2	−1	+1	26.5	5.2
3	+1	−1	33.5	3.8
4	+1	+1	33.5	5.2
5	−1.41	0	25.0	4.5
6	+1.41	0	35.0	4.5
7	0	−1.41	30.0	3.5
8	0	+1.41	30.0	5.5
9	0	0	30.0	4.5
10	0	0	30.0	4.5
11	0	0	30.0	4.5

[a] Selected carbon source in the previous stage of the work.

$NH_4H_2PO_4$; 0.06% (m v^{-1}) of KCl. For some treatments the medium composition was supplemented by 1% (m v^{-1}) of sucrose or lactose (Table 1). The formulated media were autoclaved at 121 °C for 15 min and then placed in an incubator (Tecnal – TE-421) at 180 rpm (28 °C and pH 4.5). Samples of the extracts produced through the cultivation were removed aseptically in the 3rd, 5th, 7th, 10th and 15th days for measurements of the enzyme activities.

2.7. Determination of the pH and the temperature: surface-response methodology − central composite design (CCD)

The best substrates were selected in the previous stage for enzymatic production (fermentation in shaker). The production of enzyme using *T. reesei* QM9414 was performed by central composite design (CCD) and response surface methodology, in different pH and temperatures (independent variables) and enzymatic activities (dependent variables) (Table 2), using the best time and substrate obtained in the previous step.

2.8. Stirred tank bioreactor cultures (2 L laboratory-scale bioreactors)

The quantification of fibrolytic enzymes production by *T. reesei* QM9414 in 2 L fermenter (BioFlo/CelliGen 115) was performed, in the best conditions obtained in the previous tests (30 g L^{-1} bagasse, 28 °C and pH 4.5). 1.8 L of the culture medium was inoculated with 10% (v v^{-1}) of the inoculum from the pre-culture. The total culture period was 15 days and equipped with an automatic monitoring system and control facilities for agitation, pH, aeration, temperature, antifoam. Temperature was maintained at 28 °C and the agitation at 250 rpm. The dissolved oxygen (DO) concentration was monitored as a function of time and the aeration rate was adjusted to 1.7 L min^{-1}. The pH was set to 4.5 ± 0.2 and was controlled by the automatic addition of 2 M HCl or 2 M NaOH. The foam was controlled by an automatic addition of antifoaming agent. The enzymatic activity was quantified in 3rd, 5th, 7th, 10th and 15th days of fermentation.

2.9. Characterization of cellulases: influence of temperature, pH and thermostability

The influence of temperature and pH of cellulolytic complex was evaluated for total cellulose activity [16]. These parameters were determined by enzymatic activity in different temperatures (30–80 °C) in 0.2 mol L^{-1} of acetate-acid acetic buffer (pH 5.6) and pH (3–10) in different reaction buffers (0.2 mol L^{-1} of citrate-phosphate for pH 3.0–7.0; 0.2 mol L^{-1} of phosphate for pH 7.0–8.0; 0.2 mol L^{-1} of Tris-HCl for pH 8.0–9.0; 0.2 mol L^{-1} of glycine-NaOH for pH 9.0–10.0) at 50 °C in 60 min of reaction. The thermostability of the enzyme extract was determined by the previous steps of the enzyme incubation in the temperature stability tests for 1 h in the absence of substrate. The residual cellulolytic activity was measured at different time periods (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 min).

2.10. Analytical measurements

The Glycosyl hydrolases were evaluated for total cellulase (filter paper activity – FPase) as well as endoglucanase (CMCase) which were determined as described by Ghose [17]. The avicelase activity (exoglucanase) was done by the technique of Tomme [18]. Xylanase activity was determined by the method described by Gomes [19]. Reducing sugar was measured by the dinitrosalicylic acid method [20]. Tests for enzymatic activity were conducted in triplicate, and data were submitted to analysis of variance (ANOVA), and the means were compared by Tukey test, using the program GraphPad Instat – Version 3.05 (Rutgers University). The analyses of the experimental matrices were made through the program Statistica – Version 7.0. The treatments were analyzed statistically and considered significant at $p < 0.05$.

3. Results and discussion

3.1. Influence of biomass in the production of the enzyme complex by T. reesei QM9414 in shake-flask

In a first step, experiments were designed to evaluate the effects of different carbon source in the production of fibrolytic enzymes by the fungus *T. reesei* QM9414 in submerge fermentation (SmF). Different agroindustrial residues were evaluated as carbon source for fibrolytic enzyme production (Table 1). The compilation of the data showed the best substrate. The orange peel (*OP* + *Suc* or *OP*) were the best substrates for cellulolytic complex production. For xylanases production, the best substrate was the sugarcane bagasse pretreated with NaOH (*SB_NaOH*) without supplementation (Table 3).

The profile of the total cellulase for the different carbon sources and different days of fermentation (SF) is shown in Fig. 1a. The carbon source in the culture medium had a significant influence on the cellulolytic complex production. The best substrate was the orange peel (*OP* + *Suc*) and 1% sucrose (m v^{-1}) with values of 0.85 ± 0.07 PU mL^{-1} or 25.61 ± 2.29 FPU g^{-1} of substrate in the 10th day of fermentation. This value was significantly higher than others ($p < 0.05$) (Table 3 and Fig. 1a). Comparing the 7th to 10th day there was a significant ($p < 0.05$) increase (37.3%) in the cellulolytic activities.

The orange peel supplemented with sucrose (*OP* + *Suc*) also presented the best results of endoglucanase with 3.14 ± 0.01 CMC mL^{-1} and 104.69 ± 0.33 CMC g^{-1} of substrate (10th day of fermentation), followed by the same substrate without supplementation (*OP*) and supplemented with lactose, respectively, 2.89 ± 0.02 CMC mL^{-1} (10th day of fermentation) and 2.20 ± 0.04 CMC mL^{-1} (7th day of fermentation), both statistically different ($p < 0.05$) (Table 3 and Fig. 1b).

The best carbon source for the exoglucanase production was the orange peel without supplementation (*OP*), 1.25 ± 0.14 U mL^{-1} or 37.79 ± 4.41 U g^{-1} of substrate (7th day of culture) (Table 3 and Fig. 1c). The orange peel with addition of sucrose (*OP* + *Suc*), considered the best substrate in the total cellulase and endoglucanase production, was 38% inferior in this activity than OP, probably due to the inhibition of sucrose in the fungal metabolism of exoglucanase production. The other substrates showed lower values, such as 0.14 ± 0.01 U mL^{-1} for sugarcane bagasse treated with NaOH and 0.54 ± 0.09 U mL^{-1} for sugarcane bagasse under alkaline treatment (both on the 15th day of culture). The level of exoglucanase obtained with orange peel is in the range (1.22 and 2.77 U mL^{-1}) reported by Beoliel [21] for partially pretreated sugarcane bagasse (SCB) with the microorganisms *Trichoderma harzianum* L04 and *T. reesei* Rut C30.

The best result for the production of xylanases were sugarcane

Table 3
Influence of different carbon sources on the production of total cellulase (FPase), endoglucanase (CMCase), exoglucanase (Avicelase) and xylanase by *T. reesei* QM9414 in fermentation on flasks. The optimum cultivation time (hours) are indicated.

Sourcecarbon[a]	Total Cellulase (FPU mL^{-1}– time)[b]	Endoglucanase (CMC mL^{-1} – time)[b]	Exoglucanase (U mL^{-1} – time)[b]	Xylanase (U mL^{-1} – time)[b]
SB	0.11 ± 0.00–168 h	1.01 ± 0.18–168 h	0.44 ± 0.09–240 h	21.11 ± 2.05–168 h
SB + Lac	0.06 ± 0.01–168 h	1.12 ± 0.11–168 h	0.45 ± 0.20–240 h	23.74 ± 1.03 − 168 h
SB_Yeast	0.09 ± 0.01–360 h	1.70 ± 0.01–360 h	0.52 ± 0.04–240 h	73.60 ± 6.66–360 h
SB_NaOH	0.04 ± 0.00–360 h	0.40 ± 0.00–120 h	0.54 ± 0.09–360 h	93.08 ± 3.27–360 h
SB_NaOH + Suc	0.02 ± 0.00–72 h	0.40 ± 0.00–168 h	0.14 ± 0.01–360 h	56.45 ± 0.33–240 h
SB_acid	0.15 ± 0.03 − 360 h	1.24 ± 0.01–360 h	0.86 ± 0.01–240 h	4.76 ± 0.09–360 h
SB_acid + Suc	0.12 ± 0.00–168 h	1.53 ± 0.00–360 h	0.15 ± 0.01–168 h	5.09 ± 0.07–240 h
WheB_olig	0.06 ± 0.00–168 h	1.17 ± 0.05–360 h	0.52 ± 0.01–168 h	52.39 ± 7.37–240 h
OP	0.57 ± 0.05–240 h	2.89 ± 0.20–240 h	1.25 ± 0.14–168 h	47.08 ± 2.38–240 h
OP + Lac	0.20 ± 0.03–240 h	2.20 ± 0.04–168 h	0.52 ± 0.05–240 h	34.53 ± 2.77–240 h
OP + Suc	0.85 ± 0.07–240 h	3.14 ± 0.10–240 h	0.79 ± 0.06–240 h	44.06 ± 0.93–240 h
OP + SB[c]	0.15 ± 0.01–240 h	1.78 ± 0.09–240 h	0.06 ± 0.00–240 h	37.08 ± 2.01 − 168 h
OP +SB + Lac	0.20 ± 0.07–240 h	1.92 ± 0.17–240 h	0.50 ± 0.03–240 h	36.30 ± 3.23–168 h
BRice	0.17 ± 0.01–120 h	0.53 ± 0.03–120 h	0.13 ± 0.01–72 h	0.61 ± 0.02–120 h
BRice + Suc	0.18 ± 0.00–240 h	1.22 ± 0.00–72 h	0.26 ± 0.00–240 h	1.37 ± 0.00–120 h
CB	0.03 ± 0.01–168 h	0.42 ± 0.03–168 h	0.04 ± 0.04–240 h	22.23 ± 2.73–168 h

[a] Substrates described in the Table 1.
[b] Enzymatic activity according to the previously described techniques.
[c] Orange peel + Sugarcane bagasse (1:1–m m^{-1}). 3 days – 72 h; 5 days – 120 h; 7 days – 168 h; 10 days – 240 h; 15 days – 360 h.

bagasse and alkali pre-treatment (*SB_NaOH*) with 93.08 ± 3.27 U mL^{-1} or 2792.41 ± 98.22 U g^{-1} of substrate. This result was significantly higher ($p < 0.05$) than the other cultures which used the sugarcane bagasse (pre-treated or not) (Table 3 and Fig. 1d). The carbon source is an important variable for the xylanase production since lignocellulosic materials are good substrates for the

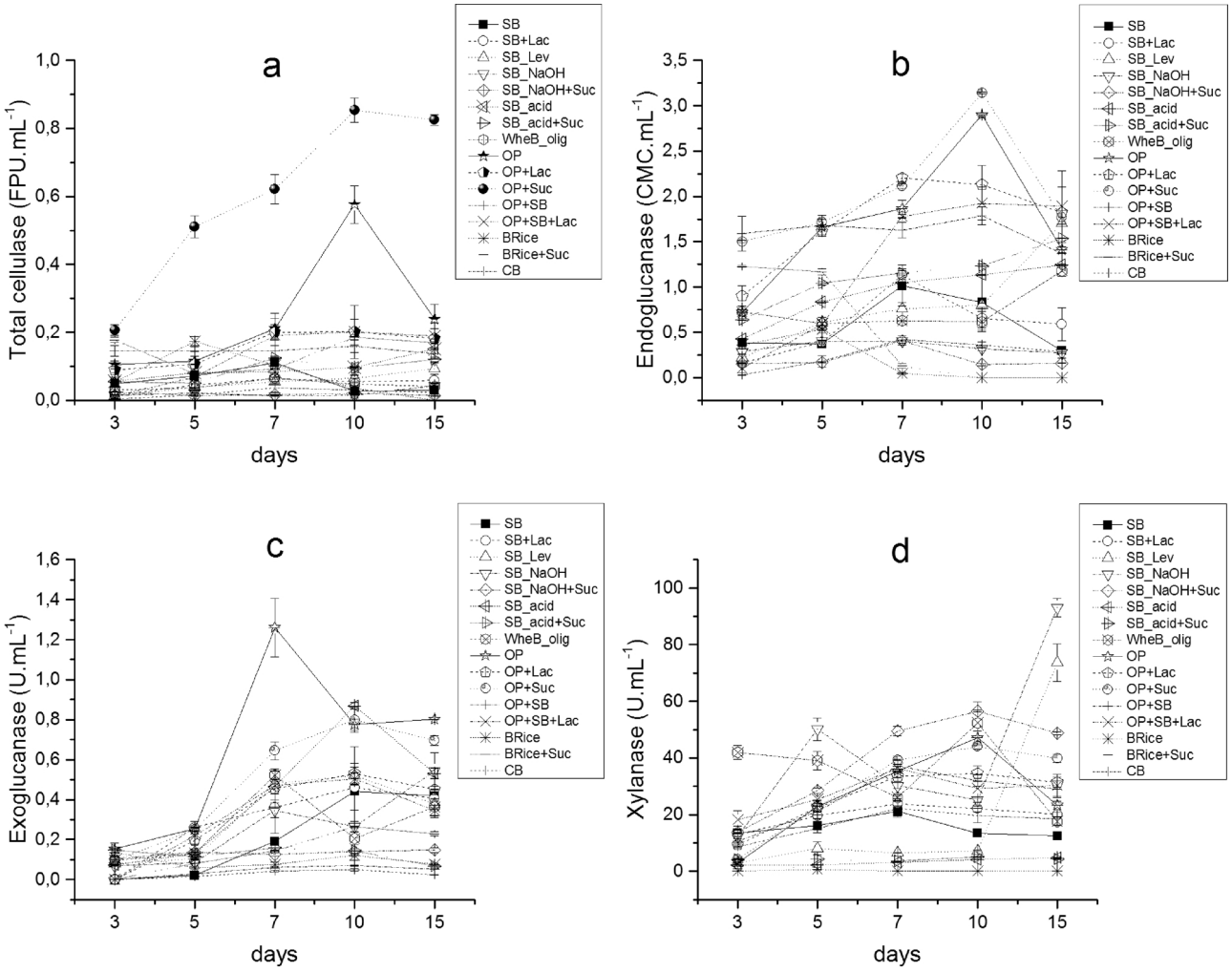

Fig. 1. Effect of different carbon sources on the production of fibrolytic enzymes: a) Total cellulase (FPU mL^{-1}); b) Endoglucanase (CMC mL^{-1}); c) Exoglucanase (U mL^{-1}); d) Xylanase (U mL^{-1}).

Table 4
Central Composite Design of pH and temperature of *T. reesei* QM9414 submerged culture for the fibrolytic enzymes production.

Expe	Variables in coded units		Response							
	Temp (°C) X_1	pH X_2	Total Cellulase (FPU mL^{-1})		Endoglucanase (CMC mL^{-})		Exoglucanase (U mL^{-})		Xylanase (U mL^{-})	
			Expe[a]	Pre[b]	Expe	Pre	Expe	Pre	Expe	Pre
1	−1	−1	0.68	0.70	2.57	2.79	1.42	1.29	33.29	35.65
2	−1	+1	0.66	0.69	1.72	1.84	1.16	1.16	34.10	36.84
3	+1	−1	0.68	0.66	2.05	1.93	0.93	0.99	37.09	35.64
4	+1	+1	0.49	0.48	2.05	2.16	0.91	0.86	25.84	24.77
5	−1.41	0	0.61	0.57	1.90	1.70	1.36	1.43	34.95	31.65
6	+1.41	0	0.37	0.39	1.27	1.31	1.02	1.00	20.98	23.02
7	0	−1.41	0.84	0.84	2.96	3.07	0.99	1.03	42.44	42.08
8	0	+1.41	0.72	0.71	2.57	2.55	0.84	0.85	36.07	35.17
9	0	0	0.86	0.87	3.37	3.24	0.77	0.82	45.15	45.19
10	0	0	0.86	0.87	3.32	3.24	0.87	0.82	45.02	45.19
11	0	0	0.89	0.87	3.27	3.24	0.84	0.82	45.44	45.19

[a] Enzymatic activities obtained during the experiments in 10 days.
[b] Predicted enzymatic activities in accordance with the significant parameters.

production of xylanolytic enzymes [22]. Michelin [11] obtained 750 total U of xylanase with *Aspergillus terricola* and corncob (CCs) particles. Delabona [3] produced xylanases with *Aspergillus fumigatus* using SSF culture and obtained the following activities for different substrate: wheat bran (1055.62 U g^{-1}), a 1:1 (m m^{-1}) mixture of sugarcane bagasse and wheat bran (821.52 U g^{-1}), soybean (484.21 U g^{-1}) and a 1:1 (m m^{-1}) mixture of orange peel and wheat bran (437 U g^{-1}). In other work with submerged fermentation in flasks and the strain *T. harzianum* (P49P11), Delabona [23] reported 36.96 U mL^{-1} (96 h) for steam pretreated bagasse, and only 15.02 U mL^{-1} in 72 h, when they used pretreated bagasse with steam followed by delignification and sucrose supplementation.

Sucrose supplementation demonstrated a significant gain for cellulase production. As shown for total cellulase, the value was high by 48.1% based in the culture without this sugar (*OP*) ($p < 0.05$) and 4.24 times higher than the culture with lactose (*OP* + *Lac*) (Table 3 and Fig. 1a). The residues mixture which was composed by raw sugarcane bagasse and orange peel, supplemented with lactose showed a 27% higher activity (*OP* + *SB* + *Lac* = 0.201 ± 0.07 FPU mL^{-1}) than these residues without lactose. However, xylanases production values were not higher, the supplementation of this substrate with sucrose presented only 56.45 ± 0.33 U mL^{-1} (*SB_NaOH* + *Suc*), while the acid pretreatments showed a decrease of almost 95% in the xylanase productions (Table 3).

Delabona [23] evaluated different carbon sources and their combinations on the production of xylanolytic and cellulolytic enzymes in submerged fermentation with *T. harzianum* P49P11. The best result were 0.50 FPU mL^{-1} (bagasse pretreated with steam followed by delignification with NaOH), 0.78 FPU mL^{-1} (crude sugarcane bagasse), and 0.85 FPU mL^{-1} (1% w w^{-1} of sucrose in the medium with sugarcane bagasse). This same level of total cellulases was obtained in the present work but with orange peel and sucrose in a culture of *T. reesei* QM9414. Ortiz [4] exhibited only 5.11 ± 0.33 FPU g^{-1} in culture of *Trichoderma reesei* CBS836.91 in SSF and wheat bran. The presence of sucrose in the culture medium was important for these works and for Silva [5] working with the same fungus *T. reesei* QM9414, in 7 days (168 h) and submerged fermentation (SmF) using orange peel as sole carbon source. The authors only obtained a maximum of 0.15 FPU mL^{-1} (28 °C and pH 4.5).

In the protocols involving acid pretreatments of sugarcane bagasse, the highest values were 0.15 ± 0.03 FPU mL^{-1} (*SB_acid*) and 1.53 ± 0.00 CMC mL^{-1} (*SB_acid* + *Suc*), demonstrating a significant ($p < 0.05$) increase in the enzyme production when compared to the crude substrate (*SB* = 0.11 ± 0.00 FPU mL^{-1}; *SB_acid* + *Suc* = 1.01 ± 0.18 CMC mL^{-1}). However, the alkaline pretreatment was not effective, because the substrate showed a maximum value of 0.04 ± 0.00 FPU mL^{-1} and 0.40 ± 0.00 CMC mL^{-1} (*SB_NaOH*). A similar result was obtained for the biological pre-treatment (*SB_Yeast*) once the FPAse activity was 16% lesser than in the crude sugarcane bagasse. The lower xylanase activities observed in the cultures grown using acid pretreated sugarcane bagasse were probably due to the reduction of the hemicellulose concentration after this pretreatment. Canilha [24] studied the sugarcane bagasse pretreatment with dilute sulfuric acid, and reported a decrease in the hemicellulose concentration after the bagasse pretreatment and this reason explains why this substrate is not convenient for xylanase production. Benoliel [21], using *T. harzianum* L04 strain in submerged culture with sugarcane bagasse partially delignified (cellulignin) as carbon source reported only 6.51 U mL^{-1} of xylanase activities. The other substrates presented inexpressive results (Table 3 and Fig. 1d). The pretreatment is an essential step to disarray the layers of lignocellulose prior to enzymatic hydrolysis [7,9]. Sharma et al. [7] affirms that various pretreatments using microorganisms of lignocellulose are gaining popularity due to its financial and environmental benefits. However, careful selection of the suitable microbial consortium for efficient pretreatment of biomass is a critical step. According to the same authors, the co-culture of bacteria and/or fungi in consolidated bioprocessing is highly beneficial in the breakdown of complex biopolymers due to their high enzyme activity.

In addition, there was a decrease in all fibrolytic activity at the end of the cultures. For example, for the substrate *OP* + *Suc* there was a decrease of approximately 5% of total cellulase activity from the 10th to the 15th day of culture (Fig. 1a). This fact may be explained by the possible presence of protease activity during this period, as noted in the literature [5,25].

3.2. Central composite design (CCD) of fibrolytic production: temperature and pH

Based on the results obtained, the fungal culture was performed with the culture medium formulated with the orange peel with sucrose in a central composite design (CCD) (Table 4 and Fig. 2).

The regression equations obtained after the analysis of variance (ANOVA) expressed the level for each fibrolytic activity as a function of independent variables. The final response equations that represent a suitable model for this enzyme production are given in Eqs. (1)–(4). These equations describe the activity predicted as a function of the coded variables in a new parametrization model. They contain only statistically significant terms: X_1 and X_2 are temperature (°C) and pH, respectively:

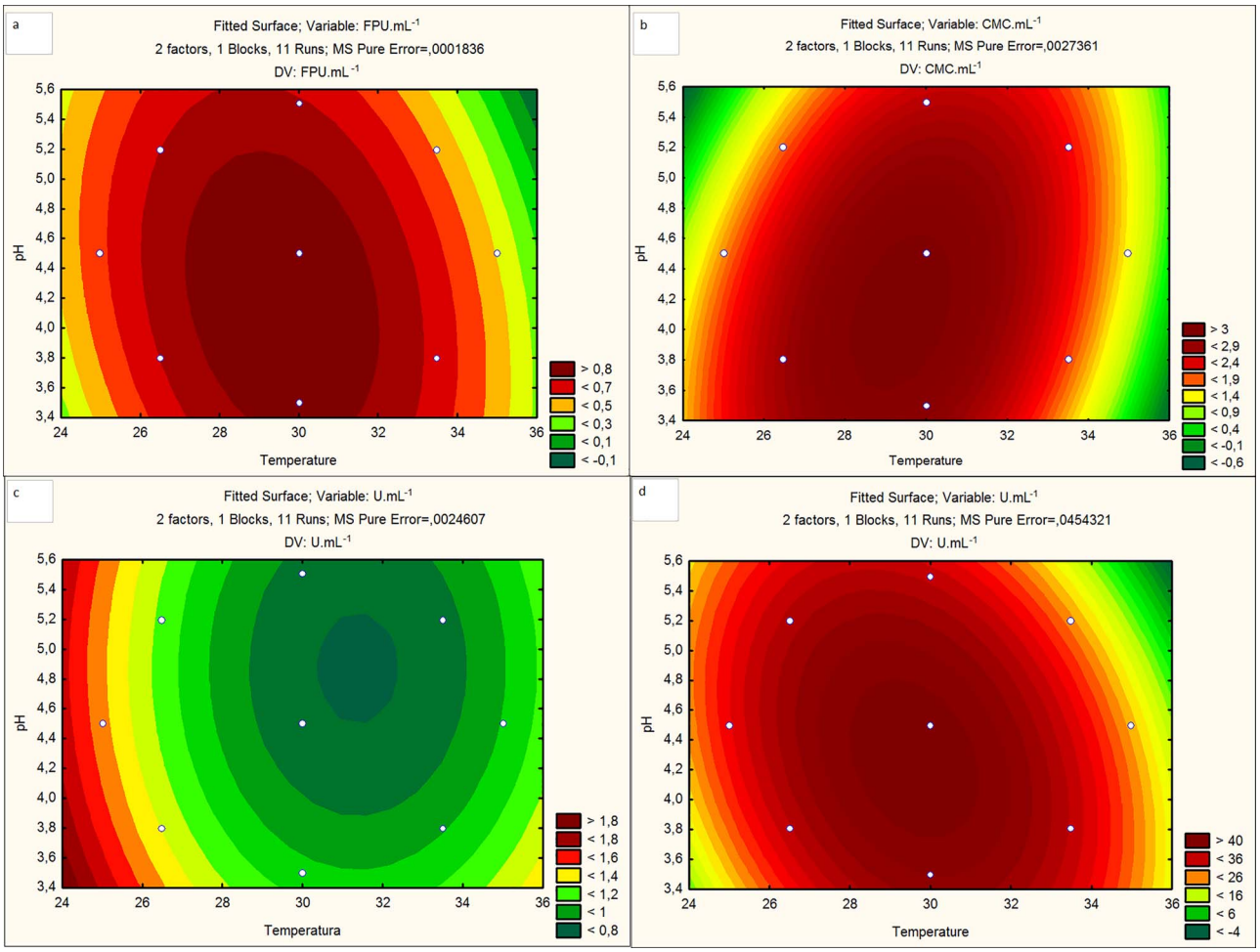

Fig. 2. The response surface graphs of the pH and temperature for enzyme production using orange peel supplemented with sucrose: a) Total cellulase (FPU mL^{-1}); b) Endoglucanase (CMC mL^{-1}); c) Exoglucanase (U mL^{-1}); d) Xylanase (U mL^{-1}).

$$\textit{Total cellulase}\ (\text{FPU mL}^{-1}) = -16.7028 + 0.9984X_1 - 0.0156X_1^2 + 1.3425X_2 - 0.0964X_2^2 - 0.0180X_1X_2,\ R = 0.986 \quad (1)$$

$$\textit{Endoglucanase}\ (\text{CMC mL}^{-1}) = -59.1959 + 3.8573X_1 - 0.0714X_1^2 - 0.6021X_2^2 + 0.0864X_1X_2,\ R = 0.981 \quad (2)$$

$$\textit{Exoglucanase}\ (\text{U mL}^{-1}) = 22.39286 - 1.09734X_1 + 0.01572X_1^2 - 1.89109X_2 + 0.11815X_2^2,\ R = 0.956 \quad (3)$$

$$\textit{Xilanase}\ (\text{U mL}^{-1}) = -855.163 + 47.525X_1 - 0.714X_1^2 + 92.529X_2 - 6.562X_2^2 - 1.231X_1X_2;\ R = 0.949 \quad (4)$$

The total cellulase values obtained in the experiments of CCD (2^2) range from 0.37 FPU mL^{-1} (assay 6–35 °C and pH 4.5) to 0.89 FPU mL^{-1} (assay 11–30 °C and pH 4.5) in 11 assays performed (Table 4). The temperature and pH of the culture were significant ($p < 0.1$) and the contour graphs are presented in Fig. 2a. A high interaction between these two parameters was observed, according to the elliptical profile observed. The highest total cellulase for the variables studied was obtained at 28–31 °C and pH 4.2–4.6. For the endoglucanase production, the minimum value obtained was 1.27 CMC mL^{-1} (35 °C and pH 4.5) and the maximum was 3.37 CMC mL^{-1} (30 °C and pH 4.5). However, for this enzyme the pH culture was not significant ($p > 0.1$) (Fig. 2b). The avicelase or exoglucanase activity did not reach the optimum range for the independent variables (Table 4). The minimum value was 0.77 U mL^{-1} (30 °C and pH 4.5) and the maximum was 1.36 U mL^{-1} (25° and pH 4.5), the graph indicated a lower pH (pH < 4.0) and low temperature (< 25 °C) range for the maximum enzyme production (Fig. 2c).

Xylanolytic enzymes demonstrated a similar range of pH and temperature (Fig. 2d). The minimum and maximum values were respectively 20.98 U mL^{-1} and 45.44 U mL^{-1} at 35 °C and 30 °C, in pH 4.5. The best result of the fungi culture for xylanases production was 28–31 °C and pH 4.2–4.6. The experimental and predicted results can be considered satisfactory (28 °C and pH 4.5) for all enzymatic activities analyzed (Table 5).

3.3. Stirred tank bioreactor (Fermenter – 2 L)

The fungi culture in a stirred tank bioreactor (Fermenter – 2 L) was performed using the selected conditions in the flask cultures in shaker: orange peel with sucrose supplementation as carbon source, 15 days of submerged culture of *T. reesei* QM9414 at 28 °C and pH 4.5. The highest total cellulase was 1.76 ± 0.00 FPU mL^{-1} or 58.66 ± 0.07 FPU g^{-1} of substrate (15th day). This result was two times superior than that obtained in a previous culture in shaker (Fig. 3a) ($p < 0.05$). The endoglucanase activity was 4.28 ± 0.09 CMC mL^{-1} or 142.94 ± 3.07 CMC g^{-1} of substrate (1.5 times higher – Fig. 3b). However, the production of exoglucanase (0.79 ± 0.06 U mL^{-1}) and xylanases (44.06 ± 0.93 U mL^{-1} in shaker were slightly higher than in the stirred tank bioreactor, respectively (0.55 ± 0.00 U mL^{-1} and 36.85 ± 9.21 U mL^{-1}– Fig. 3c and d) ($p < 0.05$).

These results demonstrated a better efficiency for enzymatic production in stirred tank bioreactor, probably due to the better control of physicochemical conditions. In this sense, the controlled pH appeared

Table 5
Predicted values for the central composite design.

	Predicted values					Experimental value
	Predicted	*-90,% Conf.*	*+90,% Conf.*	*-90,% Pred.*	*+90,% Pred.*	
FPU mL^{-1}[a]	0.8500	0.8289	0.8711	0.8051	0.8948	0.8534
CMC mL^{-1}[b]	3.0425	2.9729	3.1120	2.8747	3.2103	3.105
U mL^{-1}[c]	0.9790	0.9018	1.0562	0.8148	1.1432	0.905
U mL^{-1}[d]	44.061	43.729	44.393	43.356	44.766	44.09

[a] Total cellulase (FPU mL^{1}) – R = 0.98617; Adj = 0.97234.
[b] Endoglucanase (CMC mL^{-1}) – R = 0.96776; Adj = 0.94626.
[c] Exoglucanase (U mL^{-1}) – R = 0.92558; Adj = 0.87596.
[d] Xylanase (U mL^{-1}) – R = 0.94909; Adj = 0.89817.

to be more favorable for enzyme production, as observed previously in bioreactor culture [26]. Cunha et al. [27] working with both shake-flask and stirred tank bioreactor, with a wild type of *Aspergillus niger* A12 and pretreated sugarcane bagasse, obtained the highest endoglucanase activity of 1.72 CMC mL^{-1} in shake-flask cultivations and 1.59 CMC mL^{-1} in stirred tank bioreactor, and the maximum of xylanase in stirred tank bioreactors (4.21 U mL^{-1}) using pretreated bagasse. Ahamed & Vermette [28] studied the cellulases production through the co-culture of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA in a 3 L stirred tank bioreactor with operating volume of 1 L, reported of mixed culture experiments exhibited a highly significant increase in the production of volumetric enzyme activity (98.4 U/L/h), filter paper activity (7.1 U mL^{-1}), carboxymethyl cellulase activity (4.7 CMC mL^{-1}), soluble proteins (2.1 mg mL^{-1}). The control condition obtained by the bioreactor was essential to increase the cellulolytic complex production, with a better homogeneity of the substrate [29]. The results proved that the fungi culture fermented with lignocellulosic residues in the stirred tank bioreactor improved the production of cellulolytic enzymes and should be considered during the development of the bioprocess engineering.

3.4. Effect of temperature, pH and thermostability analysis

The fibrolytic complex produced in stirred tank bioreactor (2 L) by *T. reesei* QM 9414 and using *OP* + *Suc* was evaluated in thermostability. The cellulolytic activity provide a reduction of 19% when incubated at higher temperatures (1.86 ± 0.04 FPU mL^{-1} −60 °C; 1.50 ± 0.08 FPU mL^{-1} −65 °C) ($p < 0.05$) (Fig. 4a). The enzyme obtained in the culture was also evaluated in acid stability, the optimal result was pH 5.5 (1.78 ± 0.05 FPU mL^{-1}). In that case, there was a reduction of

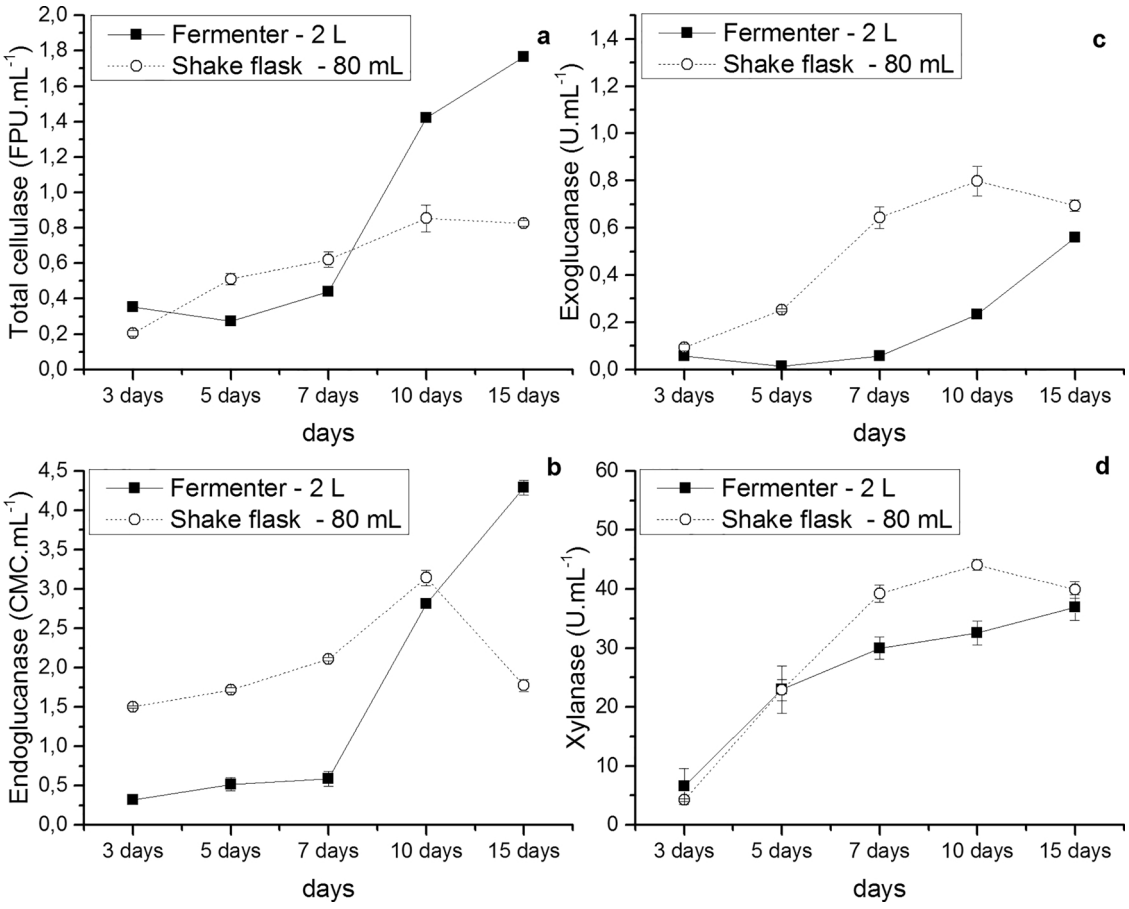

Fig. 3. Fibrolytic enzymes assay fibrolytic enzymes production by orange peel supplemented with 1% (m v^{-1}) of sucrose, fermented by *T.reesei* QM9414 in a fermenter (2 L) and in shaker using Erlenmeyer of 80 mL for 15 days at 28 °C in pH 4.5: a) Total cellulases (FPU mL^{-1}; b) Endoglucanase (CMC mL^{-1}); c) Exoglucanase (U mL^{-1}); d) Xylanases (U mL^{-1}).

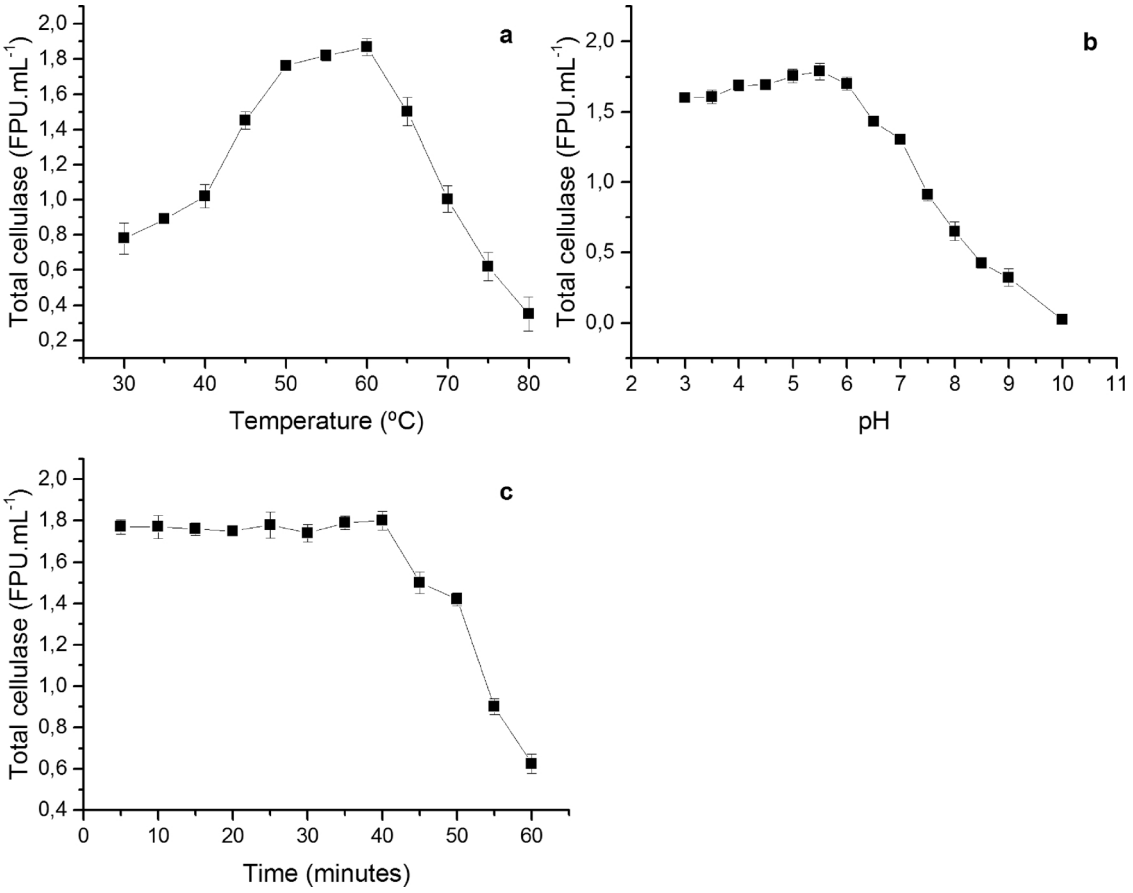

Fig. 4. Enzyme stability tests: a) Effect of temperature on total cellulase (FPU.mL^{-1}) in pH 5.6.; b) Effect of pH on total cellulase activity at 50 °C; c) Residual cellulase activity in 60 min of incubation at 60 °C.

Table 6
Comparison between the production of fungal fibrolytic enzymes by different authors.

Authors	Microorganism	Substrate	Total Cellulase (FPU mL^{-1})		Endoglucanase (CMC mL^{-1})		Exoglucanase (U mL^{-1})		Xylanase (U mL^{-1})	
			shake flask	*tank bioreactor*	*shake flask*	*tank bioreactor*	*shake flask*	*tank bioreactor*	*shake flask*	*tank bioreactor*
This work	*T. reesei* QM9414	Table 1	0.85	1.76	3.14	4.28	1.25	0.55	93.08	36.85
Benoliel [21]	*T. harzianum* L04 strain and *T. reesei* Rut C30	Partially pretreated sugarcane bagasse					1.22 and 2.77		6.51	
Delabona [3]	*A. fumigatus*	Wheat bran, sugarcane bagasse, soybean, orange peel							1055.62[a]	
Delabona [23]	*T. harzianum* P49P11	Steam pre-treated bagasse	0.85						36.96	
Silva [5]	*T. reesei* QM9414	Orange peel	0.15							
Cunha [27]	*A. niger* A12	Pre-treated sugarcane bagasse			1.72	1.59				4.21
Michelin [11]	*Aspergillus terricola*	Corncob (CCs) particles							750 total U	
Ahamed [28]	*T. reesei* RUT-C30 and *A. niger* LMA	Cellulose substrate into soluble sugars		7.1		4.7				

[a] Delabona [3] measured the enzymatic activity in U g^{1}, in this work a xylanase activity of 2792.41 ± 98.22 U g^{1} of substrate.

only 5% when the pH decreased to 4.5 (1.69 ± 0.02 FPU mL^{-1}) ($p > 0.05$) (Fig. 4b). The enzyme complex obtained in this work was able to retain 100% of its activity at 60 °C for 40 min (1.80 ± 0.04 FPU/ mL^{-1}) and had a 21% reduction in its activity when incubated for 55 min (0.9 ± 0.03 FPU mL^{-1}) (Fig. 4c).

The thermal stability is one of the major obstacles to the use of cellulases in industrial process, being responsible for increasing costs and reducing the process efficiency. In addition, lignocellulose hydrolysis at relatively high temperatures (above 50 °C) is an optimum condition for many fungal cellulases, and it may offer a potential advantage and potentially faster hydrolysis rates [30]. In Table 6 the comparative between different enzymatic activities of several authors is related.

4. Conclusions

The biosynthesis of fibrolytic complex using orange peel supplemented with sucrose and *Trichoderma reesei* QM9414 was shown to

reach a higher total cellulose production. Sucrose supplementation in the formulated medium also increased the enzymes production in other substrates. The acid pre-treatment of sugarcane bagasse favored the production of the cellulolytic enzymes and alkaline pre-treatment made easier the xylanases production in *T. reesei* QM 9414 culture. The specific temperature and pH determined by Central Composite Design in the fungal culture significantly increased the enzymes production. The importance of the mixture of some residues with specific sugars for cellulases production or alkaline pretreatment of the fiber for xylanases production in a fungal culture were confirmed in this work to reach good levels of fibrolytic enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2018.02.008.

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