Minimum cocktail of cellulolytic multi-enzyme complexes obtained from white rot fungi via solid-state fermentation

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Abstract

The objective of this work was to optimize the total cellulase activity of the crude extract cocktails from five white rot fungi produced by solid-state fermentation, by means of the central composite design. The white rot fungi *Pleurotus ostreatus* PLO 06, *Pleurotus eryngii* PLE 04, *Trametes versicolor* TRAM 01, *Pycnosporus sanguineus* PYC 02 and *Phanerochaete chrysosporium* PC were tested. For optimization process aiming at the maximum value of total cellulase activity (FPase), the multi-enzyme cellulase complexes (crude extracts) of each fungus were mixed simultaneously in different proportions. There was increase in FPase activity for the cocktails formed by the extracts of the five fungi together, compared to the extracts of each fungus alone. The model presented the minimum cocktail of enzymes for maximum total cellulase activity, with 100.00 µL PYC; 100.00 µL PC; 100.00 µL PLO06; 100.00 µL PLE04 and 200 µL TRAM01. The maximum value found was of 304.86 U/L. The result of the cocktails was very relevant, showing that there is an enzymatic complementation in the extracts that should be further studied. Concentrated extract cocktails should also be evaluated for biomass saccharification.

Keywords Cellulase · Cocktail · Optimization · White rot fungi

Introduction

The cellulolytic enzyme complex is composed of endoglucanases, exoglucanases and β -glucosidases. The effect of this enzymatic complex is expressed by the synergistic action of these three different enzymes (Lee 1997; Kostylev and Wilson 2012; Lou et al. 2013). The cooperative form begins when endoglucanases disrupt the linear cellulose molecule, producing reducing and non-reducing ends that can be attacked by exoglucanases (Payne et al. 2011). Thus,

exoglucanases act by removing small cellulose chains and exposing more internal sites for endoglucanases binding. Activity of all cellulases, in particular CBHI, is inhibited by cellobiose. Therefore, the cleavage of cellobiose into glucose by β -glycosidase greatly reduces this inhibition, allowing cellulolytic activity continuity (Horn et al. 2012; Lou et al. 2013).

Different cellulolytic organisms have evolved to produce an arrangement of cellulases that, by means of different modes of action and substrate specificity, allow the exploration of very heterogeneous substrates (Duff and Murray 1996).

Enzymatic hydrolysis of cellulose from biomass sources requires multiple enzymatic activities for fermentable mono-saccharides generation (Sun and Cheng 2002). The minimum enzyme cocktail concept is the identification of the minimum number, minimum levels and optimum combination of enzymatic activities to achieve this requirement. Two main hypotheses lie behind this concept: (1) crude profile of cellulolytic multicomponents of enzyme preparations is not optimal for the degradation of pretreated lignocellulosic biomass cellulose; (2) it is possible to substitute a multi-enzyme crude preparation with designated combinations

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for the required minimum number of enzyme activities for biomass processing (Meyer et al. 2009).

Thus, it is necessary a projected production of cocktails, optimized for the minimum use of multicomponents and for maximum activity and/or degradation (Arias et al. 2016), within the concept of minimum cocktail of enzymes (Meyer et al. 2009).

Each fungus has a differentiated multienzymatic complex (Iandolo et al. 2011), which can be improved. This strategy for acquiring a more complete enzyme cocktail includes blending of enzyme extracts with complementing activities so as to obtain a more ample enzyme hydrolysis spectrum (Hu et al. 2011).

Therefore, the objective of this work was to optimize the total cellulase activity of the crude extract cocktails from five white rot fungi produced by solid-state fermentation, by means of the central composite design.

Materials and methods

Cellulase production by solid-state fermentation

For the solid-state fermentation, straw of forage sorghum BRS 655 (straw and stalk without grains), cultivar developed by Embrapa Corn and Sorghum, cultivated in the municipality of Sete Lagoas, was used. Sorghum was cut with 120 days of planting, sun dried and grinded. The ground material was stored in a dry place, away from light and moisture.

Sorghum straw was transformed into substrate, only with the addition of water until final moisture of 70%. Each 100 g of prepared substrate were packed in polypropylene bags with filter, closed with adhesive tape for later autoclaving. After sterilized at 121 °C for 90 min and cooled to room temperature, each polypropylene bag with substrate received two 2 cm diameter disks of the mycelium from each fungus, previously cultured in Petri dish with BDA for 7 days at 30 °C.

The white rot fungi *Pleurotus ostreatus* PLO 06, *Pleurotus eryngii* PLE 04, *Trametes versicolor* TRAM 01, *Pycnosporus sanguineus* PYC 02 and *Phanerochaete chrysosporium* PC, from collection of the Department of Microbiology/UFV, were tested.

The cultivation methodology was described by Silva (2001). For replication, the fungi were kept in test tubes, containing potato agar dextrose (BDA), sterilized at 121 °C for 15 min. After fungal growth for 7 days at 30 °C, tubes were cooled to 4 °C. Subsequently, fungi were replicated again in Petri dishes, for the removal of the inoculum in the form of 2 cm disks.

Bags were inoculated in laminar flow chamber and incubated in BOD at controlled temperature (28 °C) until complete colonization of the substrate. Monitoring of mycelial

growth was performed visually. After complete colonization, from 15 to 20 days for all fungi, cellulases enzyme activities were evaluated.

Enzyme complexes extraction

Five grams of each substrate were placed in 250 mL flasks containing 30 mL of sodium citrate buffer (50 mM, pH 4.8) and shaken at 150 rpm for 2 h at 5 °C.

Then, they were filtered in sieves, transferred to 2 mL microtubes and centrifuged at 12,000g at 5 °C for 5 min. Supernatants were collected and stored in a freezer at − 18 °C.

Enzyme assays

For the determination of total cellulase activity or FPase, the method described by Ghose (1986) was followed, with some modifications. Whatman # 1 filter paper strips of approximately 1 × 6 cm (50 mg), were added to the preincubated test tubes at 50 °C containing 1.5 mL sodium citrate buffer 50 mM pH 4.8 and 500 µL of the enzyme extract. After 1 h of incubation, hydrolysis was stopped with addition of 3 mL of (Dinitrosalicylic Acid) DNS, followed by quantification by this method (Miller 1956).

Solution absorbance was measured at 540 nm and the concentration of total reducing sugars was determined on the basis of a previously established glucose calibration curve. One cellulase total or FPase unit was defined as the amount of enzyme releasing 1 µmol of total reducing sugar equivalents in 1 min under the assay conditions.

Experimental design

For optimization process aiming at the maximum value of total cellulase activity (FPase), the multi-enzyme cellulase complexes (crude extracts) of each fungus were simultaneously mixed in different proportions.

Central composite design (CCD) was performed with five variables, corresponding to extracts of the following fungi: *Pleurotus ostreatus* PLO06, *Pleurotus eryngii* PLE04, *Trametes versicolor* TRAM01, *Pycnosporus sanguineus* PYC02 and *Phanerochaete chrysosporium* PC, And the levels studied were different volumes (µL) of crude extract of each fungus (Table 1), with their respective enzyme concentrations.

DCC matrix (Table 2) was generated by the Design-Expert 8.0 software for the five variables, with 49 trials.

Statistical analysis

In the central composite design (CCD), the results were analyzed statistically by means of analysis of variance and regression, considering the lack of fit, the significance of the

Table 1 Central compound design levels of factors for the optimization of the total cellulase activity of the enzymatic complexes from fungi *Pleurotus ostreatus* PLO06, *Pleurotus eryngii* PLE04, *Trametes versicolor* TRAM01, *Pycnosporus sanguineus* PYC02 and *Phanerochaete chrysosporium* PC

Factors	Levels (μL crude extract)				
	− 2.68	− 1	0	+ 1	+ 2.68
<i>Pleurotus ostreatus</i> PLO06	31.079	100	150	200	268.921
<i>Pleurotus eryngii</i> PLE04	31.079	100	150	200	268.921
<i>Trametes versicolor</i> TRAM01	31.079	100	150	200	268.921
<i>Pycnosporus sanguineus</i> PYC02	31.079	100	150	200	268.921
<i>Phanerochaete chrysosporium</i> PC	31.079	100	150	200	268.921

parameters and the regression coefficient. By means of the analysis of the response surface methodology, the best conditions for maximum total cellulase activity were determined in the experimental conditions intervals. With the use of the response surface methodology (RSM), the relations between the factors and the response can be better understood showing the behavior of each variable on the response studied, in this case, cellulolytic activity.

These graphs were generated by the Design-expert 8.0 program, based on the determined model, seeking the optimization of total cellulase activity. With the data obtained from numerical optimization, the response surface graphs of FPAse activity were generated by the volume variation between two (factors) extracts of the five fungi. The other factors were set in the values established by the numerical optimization. Thus, the variations of the two factors, within the minimum and maximum levels, generate a response surface seeking the maximum value in the response variable.

Results were analyzed by Design-Expert 8.0 software at a significance level of 5% ($P < 0.05$). For the planning of variable levels selection, the value of 5% ($P < 0.05$) for CCD was considered.

Results and discussion

Enzyme extracts were produced from the total colonization of the solid substrate (sorghum) by fungi, after 20 days of inoculation. Total cellulase activities of the white rot fungi are shown in Table 3.

In the optimization of total cellulase activity from cocktail of enzyme extracts (mixture of the crude extracts of the five fungi), the effects of the proportion of each of the

five fungal extracts simultaneously added were evaluated through the central composite design (DCC). Results of the enzymatic activities of FPA in the DCC can be visualized in Table 2.

In a direct evaluation, it can be verified that the mixture of the extracts led to the increase in the apparent total cellulase activity, as compared to the individual extracts of each fungus (Table 3). All values were higher in any proportion of the simultaneous mixing of the five fungi complexes. The maximum value found was 304.86 U/L. Probably this was due to the presence of several active isoenzymes in the extract of different cultures working synergistically, which was suggested by other researchers (Thurston 1994; Mansur et al. 2003; Horn et al. 2012; Lou et al. 2013).

In the regression evaluation of the data, the quadratic model was significant ($P < 0.05$). In the analysis of variance (Table 4) of the suggested quadratic model, there was no significant interaction between fungi complexes. However, all quadratic effects of the factors were significant. Only the linear component of the multienzymatic complex of *P. eryngii* fungus was not significant.

The coefficient of determination R^2 of 0.93, acceptable for biological experiments (Rodrigues and Iemma 2005), indicated that the percentage of variation explained by the model was 93%.

Table 4 (ANOVA) showed that the quadratic model adjusted for the process responses is satisfactory. Thus, predicted values from FPAse activity apparent were adjusted by the Eq. (1), taking into account only the significant factors:

$$\begin{aligned} \text{Activity} = & 165.60351 + 0.59361 \times \text{PYC02} + 0.19591 \\ & \times \text{PC} + 0.64172 \times \text{TRAM01} + 0.50591 \times \text{PLO06} \\ & + 1.06758\text{E} - 003 \times \text{PYC02}^2 + 8.26458\text{E} - 004 \\ & \times \text{PC}^2 + 8.75249\text{E} - 004 \times \text{TRAM01}^2 + 1.01738\text{E} - 003 \\ & \times \text{PLE04}^2 + 9.02472\text{E} - 004 \times \text{PLO06}^2 \end{aligned} \quad (1)$$

Surface response graphs for total cellulase activity are shown in Figs. 1 and 2. The model presented the minimum cocktail of enzymes for maximum total cellulase activity, with 100.00 μL PYC; 100.00 μL PC; 100.00 μL PLO06; 100.00 μL PLE04 and 200 μL TRAM01.

Other works show the feasibility of working with cocktails, by the increase in enzymatic activities or hydrolysis level of lignocellulosic biomass. Mayrink (2010), worked with the production of cellulosic cocktails by the mixture of crude extracts produced by *Trichoderma* sp. for

Table 2 Central compound design matrix for the optimization of total cellulase activity at different levels of crude extracts obtained from white rot fungi *Pleurotus ostreatus* PLO06, *Pleurotus eryngii* PLE04, *Trametes versicolor* TRAM01, *Pycnosporus sanguineus* PYC02 and *Phanerochaete chrysosporium* PC by solid-state fermentation of sorghum straw

Assays	PYC02	PC	TRAM01	PLE04	PLO06	Activity (U/mL)
1	100.00	200.00	100.00	100.00	100.00	271.45
2	200.00	100.00	100.00	100.00	100.00	276.37
3	100.00	100.00	100.00	100.00	100.00	288.59
4	200.00	200.00	100.00	100.00	100.00	292.54
5	100.00	200.00	200.00	100.00	100.00	293.94
6	200.00	100.00	200.00	100.00	100.00	284.47
7	100.00	100.00	200.00	100.00	100.00	298.65
8	200.00	200.00	200.00	100.00	100.00	295.12
9	100.00	200.00	100.00	200.00	100.00	286.05
10	200.00	150.00	100.00	200.00	100.00	275.74
11	100.00	150.00	100.00	200.00	100.00	302.58
12	200.00	31.08	100.00	200.00	100.00	284.62
13	100.00	268.92	200.00	200.00	100.00	295.38
14	200.00	150.00	200.00	200.00	100.00	285.38
15	100.00	150.00	200.00	200.00	100.00	304.86
16	200.00	150.00	200.00	200.00	100.00	289.83
17	100.00	150.00	100.00	100.00	200.00	258.8
18	200.00	150.00	100.00	100.00	200.00	260.46
19	100.00	150.00	100.00	100.00	200.00	273.34
20	200.00	150.00	100.00	100.00	200.00	261.32
21	100.00	150.00	200.00	100.00	200.00	277.05
22	200.00	150.00	200.00	100.00	200.00	263.8
23	100.00	150.00	200.00	100.00	200.00	278.89
24	200.00	150.00	200.00	100.00	200.00	271.84
25	100.00	150.00	100.00	200.00	200.00	251.95
26	200.00	150.00	100.00	200.00	200.00	244.71
27	100.00	200.00	100.00	200.00	200.00	271.44
28	200.00	100.00	100.00	200.00	200.00	266.06
29	100.00	100.00	200.00	200.00	200.00	267.43
30	200.00	200.00	200.00	200.00	200.00	270.48
31	100.00	200.00	200.00	200.00	200.00	276.59
32	200.00	100.00	200.00	200.00	200.00	277.8
33	31.08	100.00	150.00	150.00	150.00	292.05
34	268.92	200.00	150.00	150.00	150.00	260.46
35	150.00	200.00	150.00	150.00	150.00	269.05
36	150.00	150.00	150.00	150.00	150.00	276.64
37	150.00	150.00	31.08	150.00	150.00	258.65
38	150.00	31.08	268.92	150.00	150.00	288.42
39	150.00	268.92	150.00	31.08	150.00	276.56
40	150.00	150.00	150.00	268.92	150.00	274.53
41	150.00	150.00	150.00	150.00	31.08	298.1
42	150.00	150.00	150.00	150.00	268.92	249.74
43	150.00	150.00	150.00	150.00	150.00	258.98
44	150.00	150.00	150.00	150.00	150.00	269.37
45	150.00	150.00	150.00	150.00	150.00	265.09
46	150.00	150.00	150.00	150.00	150.00	259.59
47	150.00	150.00	150.00	150.00	150.00	263.34
48	150.00	150.00	150.00	150.00	150.00	262.65
49	150.00	150.00	150.00	150.00	150.00	270.79

Table 3 Enzymatic activities U/L of the enzyme complexes of white rot fungi *Pleurotus ostreatus* PLO06, *Pleurotus eryngii* PLE04, *Trametes versicolor* TRAM01, *Pycnosporus sanguineus* PYC02 and *Phanerochaete chrysosporium* PC obtained by solid-state fermentation of forage sorghum straw

Fungus	Total cellulase (FPase) (U/L)
<i>Pycnosporus sanguineus</i> PYC02	89.78 ± 4.98
<i>Pleurotus ostreatus</i> PLO06	94.05 ± 1.82
<i>Pleurotus eryngii</i> PLE04	83.70 ± 2.47
<i>Phanerochaete chrysosporium</i> PC	128.11 ± 9.82
<i>Trametes versicolor</i> TRAM01	144.88 ± 8.65

Table 4 ANOVA of the quadratic model of the total cellulase optimization of cocktails of extracts from white rot fungi, *Pleurotus ostreatus* PLO06, *P. eryngii* PLE04, *Trametes versicolor* TRAM01, *Pycnosporus sanguineus* PYC02 and *Phanerochaete chrysosporium* PC, obtained by solid-state fermentation of forage sorghum straw. $R^2 = 0.93$

Source	F	P > F	
Model	12.11	< 0.0001	Significant
A-PYC02	18.25	0.0002	n.s
B-PC	22.06	< 0.0001	n.s
C-TRAM01	34.61	< 0.0001	n.s
D-PLE04	1.931.10 ⁻⁴	0.9890	n.s
E-PLO06	136.13	< 0.0001	Significant
AB	0.19	0.6637	n.s
AC	0.11	0.7374	n.s
AD	0.61	0.4429	n.s
AE	0.28	0.5981	n.s
BC	2.95	0.0968	n.s
BD	0.43	0.5160	n.s
BE	0.025	0.8766	n.s
CD	0.012	0.9152	n.s
CE	0.57	0.4558	n.s
DE	1.50	0.2301	n.s
A ²	10.26	0.0034	Significant
B ²	6.15	0.0194	Significant
C ²	6.90	0.0138	Significant
D ²	9.32	0.0049	Significant
E ²	7.33	0.0114	Significant
Lack of fit	2.05	0.1900	n.s

n.s not significant

cellulose saccharification. This author produced the enzymatic cocktails C014 (T2T + T02), C015 (T02 + T08), C016 (T2T + T08) and C017 (T02 + T2T + T08). T2T, T02 and T08 extracts were obtained from the cultivation of

Trichoderma sp. in different carbon sources. In the evaluation of the enzymatic activities, the C15 and C16 cocktails presented the highest activities of endoglucanase and FPase, in comparison to the extracts. All cocktails showed high levels of cellobiase and β -glycosidase activities. In saccharification of AVICEL, cocktails were statistically similar to the extracts. The author concluded that there was an enzymatic cooperation in the cocktails. Probably, this allowed an increase in the enzymatic synergism, to improve the performance during the cellulose saccharification (Mayrink 2010).

Narain et al. (2010) used different mixtures of fungi extracts of the genus *Pleurotus* sp. for cell wall degradation of plants. They observed that hemicellulose presented the highest levels of degradation, influenced by the cocktails. There was a significant reduction of 13.6% in hemicellulose. The cocktail formed by the extracts of three fungi was the most effective in the degradation among all the other extracts used.

Visser et al. (2013) studied an enzyme cocktail produced by blending crude enzyme extracts from the fungi *C. cubensis* and *P. pinophilum*. Enzymes were blended at different concentrations of the two extracts to obtain preparations with different levels of cellulase and hemicellulase degrading enzymes, where synergy between the *C. cubensis* and *P. pinophilum* enzyme activities was evaluated. In relation to FPase activity, the 50:50 blend presented the highest levels of synergism for FPase. Synergy of the two crude enzyme extracts evaluated to activities of FPase attained nearly 80% for filter paper activity, in comparison to the theoretical activities expected based on simple mixing. Gottschalk et al. (2010) worked with blending enzyme extracts from *Trichoderma reesei* and *Aspergillus awamori* at different proportions. Those authors also found significant enzyme synergy, especially with relation to filter paper and carboxymethyl cellulase activities which for some blends exceeded 100%. Blending of crude enzyme extracts can result in enzyme synergy, resulting in enzyme activities greater than expected and therefore improved hydrolysis efficiencies (Gottschalk et al. 2010). However, enzyme synergy is extremely complex and may be dependent on a number of different factors (Kostylev and Wilson 2012).

Obtaining of preparations with high cellulolytic activity depends on the microorganism used and the enzyme production process. The ideal cellulolytic formulations can be obtained by the co-cultivation of superproductive strains of the main types of cellulases (Wen et al. 2004) and by the isolated production of the cellulases and subsequent mixing of the purified extracts, in pre-optimized proportions (Li et al. 2005).

Fig. 1 **a** Response surface of the total cellulase activity optimization at different amounts (μL) of fungal extracts PLO06 and PYC02. The other factors were fixed:: PC 200.00 μL , TRAM 200.00 μL and PLE04 200.00 μL . **b** Response surface of the total cellulase activity optimization at different amounts (μL) of fungal extracts PLO06 and TRAM01. The other factors were fixed:: PYC 100.00 μL , PC 200.00 μL and PLE04 200.00 μL . **c** Response surface of the total cellulase activity optimization at different amounts (μL) of fungal extracts PC and PYC02. The other factors were fixed:: TRAM

200.00 μL , PC 100.00 μL and PLO06 200.00 μL . **d** Response surface of the total cellulase activity optimization at different amounts (μL) of fungal extracts PLO06 and PLE04. The other factors were fixed:: PYC 100.00 μL , PC 200.00 μL and TRAM01 200.00 μL . **e** Response surface of the total cellulase activity optimization at different amounts (μL) of fungal extracts PYC02 and PLE04. The other factors were fixed:: PC 200.00 μL , TRAM01 200.00 μL and PLO06 100.00 μL

Use of cellulases mixtures from different microorganisms, or the use of cellulases and other enzymes in the hydrolysis of cellulosic materials has been widely studied (Sun and Cheng 2002), the addition of β -glycosidases in cellulase systems from *T. reesei* reach higher levels of saccharification. β -glycosidases hydrolyze cellobiose, which is an inhibitor of the cellulase enzymatic activity (Scheufele et al. 2012). Bussamra et al. (2015) produced an optimized cocktail for the hydrolysis of sugarcane bagasse and with an optimum blend of enzymes with higher saccharification levels compared to commercial enzymes.

According to Scheufele et al. (2012), as there is a deficiency of some specific enzymes produced by certain microorganisms, the use of enzymes of different origins results in a more balanced enzymatic system, with no limiting and detrimental factors to the process. Thus, use of crude extracts from different fungi has much potential to improve saccharification cocktails.

There was increase in total cellulase activity for the cocktails formed by the extracts of the five fungi together, compared to the extracts of each fungus alone. The result of the cocktails was very relevant, showing that there is an

Fig. 2 **a** Response surface of the total cellulase activity optimization at different amounts (μ L) of fungal extracts PC and PLE04. The other factors were fixed: PYC 100.00 μ L, TRAM 200.00 μ L and PLO06 100.00 μ L. **b** Response surface of the total cellulase activity optimization at different amounts (μ L) of fungal extracts PLO06 and PC. The other factors were fixed: PYC 100.00 μ L, TRAM 200.00 μ L and PLE04 200.00 μ L. **c** Response surface of the total cellulase activity optimization at different amounts (μ L) of fungal extracts TRAM01

and PYC02. The other factors were fixed: PC 200.00 μ L, PLE04 200.00 μ L and PLO06 100.00 μ L. **d** Response surface of the total cellulase activity optimization at different amounts (μ L) of fungal extracts TRAM01 and PLE04. The other factors were fixed: PYC 100.00 μ L, PC 200.00 μ L and PLO06 100.00 μ L. **e** Response surface of the total cellulase activity optimization at different amounts (μ L) of fungal extracts TRAM01 and PC. The other factors were fixed: PYC 100.00 μ L, PLE04 200.00 μ L and PLO06 200.00 μ L

enzymatic complementation in the extracts that should be further studied. Concentrated extract cocktails should also be evaluated for biomass saccharification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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