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PURIFICATION OF CELLULASES OF FILAMENTOUS FUNGI *Melanoporia* sp.

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ABSTRACT

Cellulases are the second largest class of industrial enzymes with potential for conversion of cellulosic biomass. Filamentous fungi are traditionally used for the production of cellulolytic enzymes. Melanoporia sp. is one of cellulase-producing fungi. Although fungal cellulase production is relevant, some purification steps might be important for its characterization. The results showed that cellulases of Melanoporia sp. were parcially purified by precipitation with ammonium sulfate (FPase and CMCase) and PEG 1500 (xylanase).

1. INTRODUCTION

Cellulases are the second largest class of industrial enzymes with growth in demand since 1995 for industrial applications. They offer potential for conversion of cellulosic biomass, which represents a larger source of the organic substrate (Patel et al. 2017). Filamentous fungi are traditionally used for the production of cellulolytic enzymes(Wilson 2011). The species *Trichoderma, Aspergillus* and *Penicillium* have been explored because they can produce a wide variety of cellulolytic enzymes (Panchapakesan & Shankar 2016). *Melanoporia sp.* is one of cellulase-producing fungi. This genus has the ability to hydrolise cellulose and hemicellulosic materials (Kim et al. 2003). Although fungal cellulase production is relevant, purification steps are important for its characterization. Thus, the objective of this study was to partially purify the cellulase produced by the filamentous fungi *Melanoporia* sp. CCT 7736 using coconut shell powder as a substrate for submerse fermentation.

2. MATERIALS AND METHODS

2.1. Fermentation Time

The spores were collected from the agar plate (stock culture) by the addition of 20 ml Tween 80 (2% v/v). A volume of 10% (v/v) of the spores suspension was transferred to a pre-inoculum medium with the following composition: $C_6H_{12}O_6$ (10 g/L), KH_2PO_4 (2 g/L), $(NH_4)_2SO_4$ (1.4 g/L), $MgSO_4.7H_2O$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.0016 g/L), $ZnSO_4.H_2O$ (0.0014 g/L), $CoCl_2.6H_2O$ (0.0037 g/L) and $CaCl_2$ (0.4 g/L). The strain as cultivated at 30 °C for 48 hours in an orbital shaker at 150 rpm. For the enzyme production, the carbon source was coconut shell powder (15 g/L), lactose (7.5 g/L), KH_2PO_4 (1 g/L), $FeSO_4.7H_2O$ (5 g/L) and Tween 80 (3% v/v). The enzyme production was

carried out at 30 °C for 96 hours (Oliveira et al. 2016).

2.2 Purification of enzymes

All purification procedures were performed using a 200 mM sodium acetate buffer (pH 5.5). Protein was measured by the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Step 1: ***Crude enzyme extract:*** The fermentation medium was mediated using Whatman no 1 filter paper to remove the coconut shell powder. Then the filtrate was centrifuged at 10.000 *g* for 10 min at 4 °C.

Step 2: ***Purification protocols:*** The crude extract was subjected to different purification methods: dialysis, precipitation with ammonium sulfate ($(NH_4)_2SO_4$) in three concentrations (0-30%, 30-60% and 60-90% w/v) and precipitation with non-ionic Polymer (PEG 1500 and PEG 4000).

2.3 Enzyme activity assay

The filter paper activity (FPase) was determined according to the Ghose methodology (Ghose 1987). An aliquot of 500 µL of sodium acetate buffer (200 mM and pH 5.5) was mixed with 250 µL of enzyme extract with an apropriate dilution in a glass tube containing Whatman No. 1 filter paper strips (1 × 6 cm). The mixture was incubated for 30min at 80 °C. The addition of DNS stopped the reaction. The concentration of reducing sugar was measured according to the DNS method (Miller 1959).

CMCase (endoglucanase) activity was determined by mixing in a glass tube 125 µL of enzyme extract and 125 µL of the activity solution consisting of buffer (sodium acetate 200 mM, pH 5.5) containing 2 % (w/v) of carboxymethylcellulose (Sigma). The mixture was incubated for 1h at 80 °C. The reaction was stopped by adding 250 µL of DNS reagent.

Xylanase activity was determined according to the Biely methodology (Biely et al. 1997): 125 µL of the enzyme, extract was mixed with 125 µL of the activity solution (sodium acetate 200 mM, pH 5.5 containing 1 % (*w/v*) of xylan from birch wood (Fluka). The mixture was incubated for 1h at 80 °C. The reaction was stopped by adding 250 µL of the DNS reagent.

All results were expressed as international enzyme unit (IU) per mL of enzyme (IU/mL). One IU is defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute at the assay conditions(Binod et al. 2007).

3. RESULTS AND DISCUSSION

The crude extract produced by *Melanoporia* sp. CCT 7736 was purified by different methods as shown in Table 1. The SDS-page of the recovered fractions is presented in Figure 1.

Table 1. Purification of cellulases from *Melanoporia* sp.

Sample fraction	FPase (FPU/mL)		CMCase		Xylanase	
	Specific Activity (UI/mgProt)	Purification Fold	Specific Activity (UI/mgProt)	Purification Fold	Specific Activity (UI/mgProt)	Purification Fold
Crude extract	0.20±0.03	1	0.81±0.06	1	0.61±0.13	1
Dialyzed	0.07±0.02	--	0.96±0.02	1.18	0.63±0.01	1.03
PEG 1500	0.166±0.01	--	43.21±0.00	53.34	1.51±0.11	2.47
PEG 4000	0.07±0.00	--	0.26±0.01	--	0.19±0.01	--
$(NH_4)_2SO_4$ 0-30%	0.02±0.00	--	Nd*	--	0.18±0.00	--
$(NH_4)_2SO_4$ 30-60%	7.41±1.06	37.05	112.79±0.05	139.24	Nd*	--
$(NH_4)_2SO_4$ 60-90%	7.84±2.07	39.20	109.88±2.30	135.65	Nd*	--

*Nd = not detected

The ammonium sulfate precipitation in the fraction 60-90% (w/v) proved to be the best method of purification for Fpase and CMCase. However, the xylanases were not purified with ammonium sulfate precipitation and the best method for them was the precipitation with PEG 1500 (table 1).

In SDS-PAGE of cellulase fractions, the precipitation with PEG 1500, PEG 4000 and ammonium sulfate precipitation in the fraction 60-90% (w/v) presented lesser loads of impurities (Figure 1).

For the characterization of the enzymes, new experiments are necessary for evaluating purification processes with combined methods. In addition, native electrophoresis would indicate the characteristics of these enzymes.

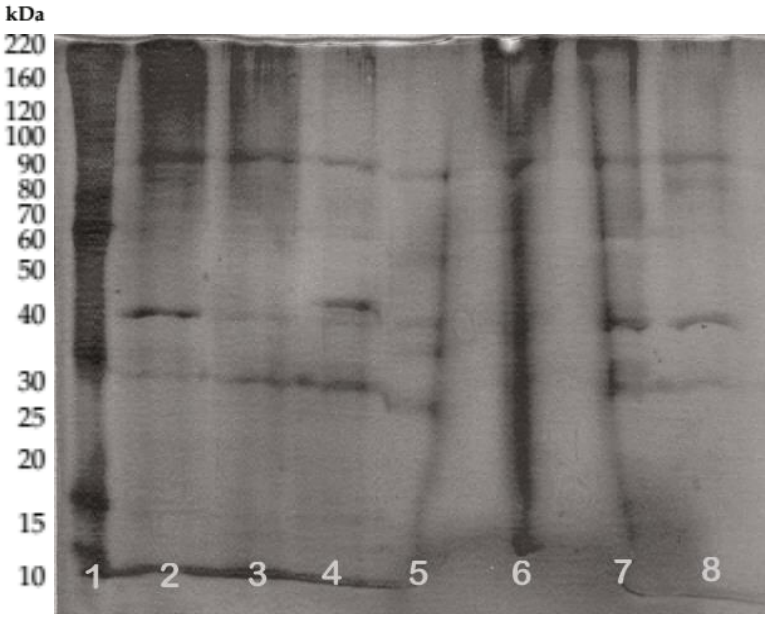

Figure 1 - SDS-PAGE of purified cellulase form *Melanoporia* sp. Lane 1: molecular weight marker, Lane 2: Crude extract, Lane 3: Dialyzed, Lane 4: PEG 1500, Lane 5: PEG 4000, Lane 6: 0-30% $(NH_4)_2SO_4$, Lane 7: 30-60% $(NH_4)_2SO_4$, Lane 8: 60-90% $(NH_4)_2SO_4$.

4. CONCLUSION

Cellulases of *Melanoporia* sp. were parcially purified by precipitation with ammonium sulfate (FPase and CMCase) and PEG 1500 (xylanase).

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