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Purification and Partial Characterization of Cellulase Produced by *Aspergillus niger* Cultured on *Vitellaria paradoxa* shells

[1]Sulyman A. O, [2]Iyanda A. Y, [2]Opasola O. A, [3]Adedayo O, [1]Aladodo, R. A, [4]Balogun A, [1]Ajibola O. A, [1]Olukotun O. Z and [1]Hammed B. A

[1]*Department of Biochemistry, Kwara State University, Malete, Ilorin, Nigeria.*
[2]*Department of Environmental Management and Toxicology, Kwara State University, Malete, Ilorin, Nigeria.*
[3]*Department of Biosciences and Biotechnology (Microbiology unit), Kwara State University, Malete, Ilorin, Nigeria*
[4]*Department of Biological Science (Biochemistry Unit), Al-Hikmah University, Ilorin, Nigeria*

Abstract

This research investigated the purification and partial characterization of cellulase produced by *Aspergillus niger* cultured on *Vitellaria paradoxa* shells. Cellulase (endoglucanase) from *A. niger* was produced under optimum fermentation conditions at 35 °C, pH 4.7, *V. paradoxa,* 4 g/L, inoculum size of 10 mm and the fermentation media incubated for 120 hours. The crude endoglucanase obtained were partially purified by subjecting to ammonium sulphate precipitation, dialysis and gel filtration chromatography for further purification. The effect of temperature and pH on the activity of purified endoglucanase was determined. Cellulase was purified to 734.33 folds by Sephadex G-100 column chromatography with a specific activity and yield of 4.406 U/mg and 63.03% respectively. Fractions 4 and 7 contained the highest endoglucanase activity out of 18 fractions collected and the two fractions were pooled for further analysis. The activity of purified endoglucanase was optimum at a temperature of 40 °C and pH 5. Therefore, the purified endoglucanase produced may be explored in detergent industry.

Keywords: *Aspergillus niger*, endoglucanase, *Vitellaria paradoxa*, purification, partial characterization

Introduction

Cellulase (EC 3.2.1.4), an enzyme responsible for the hydrolysis of cellulose (the major component of plant framework) and related cello-oligosaccharide derivatives, can be produced from a wide range of agricultural wastes. Cellulase has a wide variety of industrial applications such as in textile (Gusakov *et al.*, 2000; Belghith *et al.*, 2001), detergents (Adrio and Demain, 2014; Shen *et al.*, 2017), pulp and paper (Lee *et al.*, 2017), and animal feed industries (Yuangklang *et al.*, 2017). The enzyme accounts for a significant share of the world enzyme market (Sukumaran *et al.*, 2005). Cellulases are usually produced by microorganisms when cultured on agricultural wastes (Shin *et al.*, 2000; Immanuel *et al.*, 2009) with fungi being the major cellulase-producing microbes (Shin *et al.*, 2000). A number of agricultural wastes have been reported as substrates for cellulase production.

**Corresponding author: +2348068486088*
Email address: abdulhakeem.sulyman@kwasu.edu.ng

Examples of such substrates include straws, corn cobs, wood wastes, peat, bagasse and waste paper (Acharya *et al*., 2008), but *Vitellaria paradoxa* shell has not been reported for cellulase production.

V. paradoxa is an indigenous plant that is widely distributed in nineteen countries across the African continent (Warra, 2011). Nigeria is the leading producer of shea, a product derived from *V. paradoxa*, accounting for about 60% of the total production in Africa (FAO, 2005). *V. paradoxa* tree grows in five states in Nigeria, including Niger, Kwara, Kebbi, Kaduna and Oyo (Shitu and Mohd-Nor, 2016). *V. paradoxa* nut is collected and processed mainly to shea butter by rural women across West Africa (Rousseau *et al*., 2015). Shea butter has witnessed an increasing international demand as an ingredient in cosmetic, pharmaceutical and edible products (Alander, 2004; Warra, 2011). This product is now attractive for markets across America, Asia and Europe (Lovett 2004). Shea butter is therefore, an economically important product to many rural communities in Africa (Garba *et al*., 2011). Despite the economic and nutritional importance of shea, a product derived from *V. paradoxa* plant, its processing presents major environmental and public health concern (Abagale *et al*., 2012). Shea nut processing to shea butter generates high amount of wastes, which include waste brown water, waste black sludge, and seed husks (Jibreel *et al*., 2013). The wastes are indiscriminately disposed, thereby becoming a menace to the environment and threat to the health of inhabitants (Ajayi, 2004; Sarkodie *et al*., 2016). Renewable resources such as agricultural wastes that pose threat to the environment can systematically be utilized to bring about resource productivity required to sustain human activity (Amin *et al*., 2014). The shells from *V. paradoxa* can alternatively be used as substrate for enzyme production (de Azeredo *et al*., 2007; Arvanitoyannis and Varzakas, 2008). Therefore, this research focuses on purification and partial characterization of cellulase from *A. niger* using *V. paradoxa* as substrate.

Materials and Methods

Substrate and chemicals

Agricultural waste, *Vitellaria paradoxa* shells were obtained from Ilesa Baruba, Baruten Local Government, Kwara State, Nigeria and used as carbon substrate. The substrate was air dried, pulverized to fine powder and stored in a polypropylene bag. All the chemicals used in this study were of analytical grade and were products of Sigma Chemical Co., USA.

Micro-organism, maintenance and inoculum development

Aspergillus niger was obtained from microbial culture bank, Department of Microbiology, Faculty of Natural Sciences, University of Ilorin, Ilorin, Nigeria. Spores of *A. niger* were grown and maintained on potato dextrose agar slants. It was then sub-cultured on fresh agar plate and incubated for 72 hours (Sulyman *et al*., 2018). This was maintained at 4 °C and used throughout the experiment.

Production and extraction of endoglucanase

Endoglucanase from *A. niger* was produced under optimum growth conditions. In order to achieve maximum cellulase production, *V. paradoxa* was inoculated for 120 hours at 35 °C, pH 4.7, concentration of *V. paradoxa,* 4 g/L and inoculum size of 10 mm. The fermentation media contained the following (per L): *V. paradoxa*, 4 g; peptone, 1 g; $(NH_4)_2SO_4$, 1.4 g; KH_2PO_4, 2 g; $CaCl_2$, 0.3 g; $MgSO_4.7H_20$, 0.3 g; Urea, 0.3 g. Flasks containing the media were shaken on an orbital shaker at 120 rpm for 5 days at 35 °C. After the stipulated fermentation period, the contents were filtered through muslin cloth and washed thrice with 0.05 M citrate buffer (pH 4.7). The filtrates were centrifuged at 15,000 × g (4 °C) for 5 minutes and supernatants were carefully collected. The supernatants collected were used as crude enzyme.

Determination of endoglucanase activity and protein concentration

Endoglucanase activity in supernatant was determined using spectrophotometry by following the method described by Ghose (1987). The reaction mixture contained 0.5 mL diluted crude enzyme with 0.5 mL of 1% carboxymethyl cellulose (CMC) as substrate in 0.05 M Na–citrate buffer (pH 4.7). The mixture was incubated at 50 °C and the reaction was stopped by the addition of 3 mL of 3, 5-dinitrosalicylic acid. The solution was transferred into boiling water for 5 min for colour development. It was then allowed to cool and 5 mL of distilled water was added. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by cellulase was determined by extrapolating the absorbance obtained from the glucose standard curve. The recorded activities were expressed as U/mL, while unit activity was defined as the amount of enzyme required to produce a unit increase in absorbance at specific wavelength (nm) per mL of reaction mixture. The protein concentrations in crude and purified enzyme extracts were determined by the method of Lowry *et al*. (1951), using bovine serum albumin as standard.

Partial purification of cellulase

The method described by De-Moraes *et al*. (1999) was adopted for the purification of cellulase (endoglucanase), with slight modification. Crude extract obtained from *Aspergillus niger* was centrifuged at 15,000 × g for 5 minutes at 4 °C to increase the clarity. Ammonium sulfate salts were added to the crude enzyme extract until it was 80% saturated and kept for 24 hours at 4°C. After centrifugation, the precipitated proteins were dissolved in 10 mL of 0.05 M citrate buffer (pH 4.7). The solution was dialyzed against 0.05 M citrate buffer for 24 hours with 4 regular changes of the buffer after every 6 h. Total proteins and endoglucanase activity of crude and dialyzed enzymes were determined. The dialyzed cellulase was applied to Sephadex G-100 column.

Sephadex G-100 column chromatography

The semi-purified cellulase was subjected to gel filtration chromatography using Sephadex G-100. Prior to the loading of column with Sephadex G-100 and semi-purified cellulase (endoglucanase), the column was washed with 20 mL of 0.05 M citrate buffer (pH 4.7). The calibrated column was packed to the height of 100 cm in a glass column with an internal diameter of 2.0 cm (Sharma *et al*., 2006). The semi-purified cellulase was introduced into the column and eluted with citrate buffer of pH 4.7. The flow rate was maintained at 0.2 mL/min. Eighteen fractions of 5 mL each were collected from Sephadex G-100 column. The endoglucanase activity and protein concentration were determined for each separate fraction.

Effects of temperature and pH on the activity of purified cellulase (endoglucanase)

The partially purified endoglucanase was incubated for 30 minutes with 1% CMC in 0.05 M citrate buffer (pH 4.7) at temperatures ranging from 10 to 60 °C and the endoglucanase activity was measured by following the method described by Ghose (1987). Also, the optimum pH of the purified cellulase was determined by incubating the enzyme for 30 minutes with 1% CMC in 0.05 M citrate buffer at pH values ranging from 2.0 to 12.0. The endoglucanase activity was assayed as described by Ghose (1987).

Statistical analysis

All data generated from this study were expressed as mean of three replicates (n=3). SPSS version 16 was used to analyze results and plot all the graphs. One-way analysis of variance with Dunnett's post hoc test was used for multiple comparisons. Values were considered statistically significant at 95% confidence level.

Results

Purification of endoglucanase

A brief purification summary of endoglucanase produced by *Aspergillus niger* cultured on *Vitellaria paradoxa* shells is presented in Table 1. The specific activity of cellulase was 0.006 U/mg for the crude cellulase, and there was an increase at each purification step. Although the yield was only about 63.03% after gel-filtration, 80-90% of the impurities were removed during the purification steps and the endoglucanase was purified with an increase in purification more than 734-fold. The elution profile of cellulase produced by *A. niger* on Sephadex G-100 is shown in Figure 1. Eighteen fractions were collected and the activity of cellulase as well as protein contents of each of the fractions was determined. The fractions (4 and 7) with highest peaks were pooled together and used for further analysis

Table 1: Purification summary of endoglucanase produced by *Aspergillus niger* cultured on *Vitellaria paradoxa* shells

Purification steps	Total volume (ml)	Endoglucanase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Percentage yield (%)
Crude enzyme	170	20.83±3.02	3537.70±5.12	0.006	1	100
$(NH_4)_2SO_4$	25	18.95±2.55	36.62±3.12	0.517	86.17	90.97
Dialysis	10	13.18±2.11	3.06±1.16	4.307	717.83	63.27
Sephadex G-100	5	13.13±1.98	2.98±1.02	4.406	734.33	63.03

Each value of endoglucanase activity and total protein is expressed as mean ± SEM of three different determinations

Effect of temperature and pH on the activity of purified endoglucanase

The partially purified endoglucanase was incubated at various temperatures ranging from 10 to 80 °C and the results obtained are presented in Figure 2. The activity of cellulase increased as temperature increased. The optimum temperature for the partially purified cellulase was found to be 40 °C after which the activity decreased sharply. Also, the effect of pH on the activity of purified cellulase was determined. Endoglucanase activity was greatly affected by change in pH. The optimum cellulase activity was observed at pH 5. The activity of cellulase was decreased more rapidly above or below the optimum pH value (Fig 3).

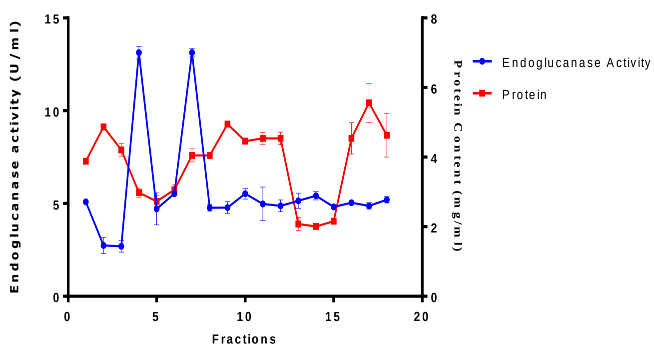

Figure 1: Elution profile of endoglucanase from *A. niger* purified on Sephadex G-100

Each value is expressed as mean ± SEM of three different determinations

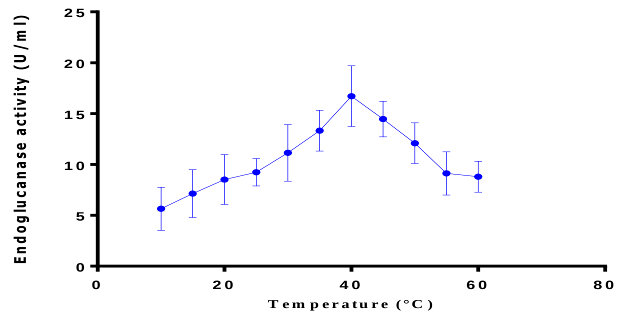

Figure 2: Effect of temperature on endoglucanase activity

Each value is expressed as mean ± SEM of three different determinations.

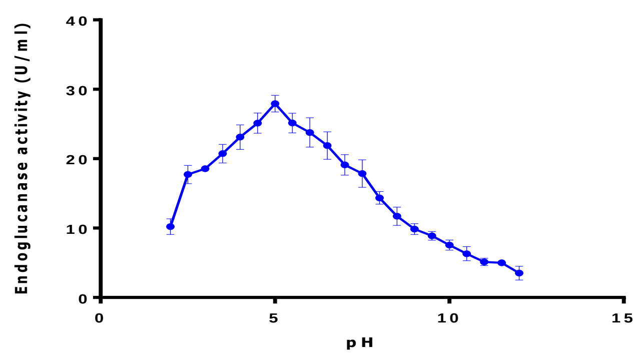

Figure 3: Effect of pH on endoglucanase activity

Each value is expressed as mean ± SEM of three different determinations.

Discussion

The growth of the *Aspergillus niger* in a fermentation medium containing *Vitellaria paradoxa* shell led to the synthesis of large amount of cellulase in the culture supernatants. The crude endoglucanase produced by *A. niger* cultured on *V. paradoxa* shell was purified through a multistep purification process. These multistep purification processes are ammonium sulphate precipitation, dialysis and gel filtration chromatography. The proteins were first salted out with ammonium sulphate at 80% saturation with a specific activity of 0.517 (U/mg). These were then subjected to Sephadex G-100 column chromatography and by gel filtration chromatography, the specific activity of endoglucanase increased to 4.406 (U/mg) and a reduction in the percentage yield from 100% to about 63%. This decrease in percentage yield might be due to denaturation of enzyme during the purification steps. Olama *et al.* (1993) reported a 99.8% loss of protein, and the specific activity was increased to about 22.8-fold when they purified cellulase from *Trichoderma viride* by DEAE-Sephadex A- 50 chromatography method followed by CM-Sephadex C-50. Also, Sultana (1997) observed 13.71 U/mg specific activities which were increased to about 32-fold in *Aspergillus sp.* by DEAE-cellulose chromatography. Po-Jui *et al.* (2004) reported that the specific activity was 38.22 U/ml and increased to about 9.04-fold from *Sinorhizobium fredee* by DEAE Sepharose anion-exchange column and followed by Phenyl-Sepharose column.

The decrease in cellulase activity above the optimum temperature may be due to denaturation of enzyme at a temperature higher than the optimum temperature. Macris *et al.* (1987) earlier reported optimum temperature 40 to 50 °C from *Neurospora crassa*. Also, Mishra (1988), Sandhya (1992), Cavazzoni and Manzoni (1994) and Wang (1999) reported optimum temperature of 50 °C for cellulase obtained from *A. aureoles* and *A. clavatus*, *T. viridiem*, *M. conica* and *T. reesei* QM 9414 respectively. From the results obtained in this study, it can be said that the partially purified endoglucanase was more active in relatively acidic environment than alkaline region. The decrease in endoglucanase activity above the optimum pH values might be due to destruction of active site as well as changes in secondary or tertiary structure of endoglucanase, a component of cellulase. Fungal cellulases with pH values between 4.5 to 6.0 have been reportedly obtained from *T. viridie* (Gupta and Gupta, 1979), *A. niger* and *A. terreus* (Goma *et al.*, 1982), *N. crassa* (Macris *et al.*, 1987), *A. aureolus* and *A. clavatus* (Mishra, 1988), *R. oryzae* (Amadioha, 1993) *V. diplasia* (Bhadauria *et al.*, 1997) and *T. reesei* QM 9414 (Wang, 1999).

Conclusion

This study revealed that *V. paradoxa,* an agrowaste, can serve as a carbon source for *A. niger* to produce relatively high amounts of cellulase enzymes and instead of being regarded as a waste can serve as a substrate for cellulase production. The endoglucanase produced was purified to 734-fold with a percentage yield of 63.03%. The purified endoglucanase has an optimum temperature and pH of 40 °C and 5 respectively. Therefore, the purified endoglucanase produced may be explored in detergent industry.

Conflict of interests

No conflict of interest was declared.

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