

PROPERTIES OF CELLULOLYTIC ENZYMES FROM PEEL OF *AMORPHOPHALLUS PAEONIIFOLIUS*

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Received: 24 Feb. 2014 Revised and Accepted: 10 Mar 2014

ABSTRACT

Objective: Peel of *Amorphophallus* is considered as waste. In this study we checked for the presence of cellulase enzyme in this waste and investigated its biochemical properties.

Methods: Presence of cellulase activity was confirmed by Congo red plate diffusion assay. The optimum temperature, pH, substrate concentration for both CMCase and FPase activity was also determined by DNS method. Effect of salts and metal ions were also investigated. Zymography studies confirmed the presence of cellulase enzyme.

Results: Optimum pH for CMCase was found to be 10.0 whereas pH 8 was optimum for FPase activity and optimum temperature was 60 °C. Hg²⁺ and Mn²⁺ has strong inhibitory effect on cellulase activity and molecular weight of cellulase is around 43 kDa. Cellulase from peel of *Amorphophallus* was also found to be thermostable at 90°C for 1 hour.

Conclusion: Thermostable and pH stable cellulase from agrowaste can be of potential use in textile, dyeing industry.

Keywords: Cellulase- Peel- FPase- CMCase- congo red- zymography

INTRODUCTION

Waste disposal of agro industries by-products and agricultural waste is a continuous serious problem, as they exert a detrimental impact on environment which needs to be addressed and managed. The fruits and vegetable waste are very rich in bioactive components, which are considered to have a beneficial effect on health. With increasing environmental awareness, efforts have been made to improve methods and ways of reusing fruits and vegetable wastes. The important purpose is the valorization of the bio components in by-products from fruit and vegetable industries. Agro industrial waste like fruits and vegetables skins are thrown in the garbage or utilized as feed or fertilizer.

These wastes are high value products and their recovery will be economically attractive. Carbohydrate-based agricultural products such as starchy tubers (Cassava, Sweet Potato and Cocoyam, Elephant foot yam) are important staple foods in the diet of people in most developing countries. Waste materials like peels resulting from the production; preparation and consumption of these starchy tubers creates disposal problem and potentially severe pollution problems resulting into a loss of valuable biomass and nutrients. Besides their pollution and hazardous aspects, in many cases, these peels might have potentials for recycling raw materials for conversion into useful products of higher value or even as raw material for other industries or for their use as food or feed after biological treatment [1].

The use of enzyme in the manufacture of various industrial products is wide spread [2]. Several agro-industrial by-products such as wheat bran, rice bran, molasses bran, barley bran, maize meal, soybean meal, potato peel and coconut oil cake have been screened as low-cost solid substrates for microbial production of enzymes to be used either in food applications or in other industrial sectors. Potato peel was reported to be an excellent substrate for the production of thermostable alpha-amylase; a starch hydrolyzing enzyme extensively used in different food industries, under solid-state controlled growth conditions and was successfully used in some applications [3,4]. Utilisation of these agro-industrial wastes provides alternative substrates and helps in solving pollution problems [5].

In India, most of the enzymes are imported at huge costs and there is a need for its commercial indigenous production to reduce the market price [6]. High cost and low activity are the major impediments to the commercial use of cellulases [7]. Cellulases are cellulose-degrading enzymes with a great potential to convert cellulosic material into its subunit-glucose [8]. Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research [9-11].

Higher plant Cellulases like *Lantana camara* and *Cuscuta reflexa* are mostly involved in fruit ripening and senescence. Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry, detergent market for color care, cleaning, and anti-deposition; in the food industry for mashing; and in the pulp and paper industries for deinking, drainage improvement, fiber modifications and for pharmaceutical applications. Cellulase is used for commercial food processing in coffee, and it also performs hydrolysis of cellulose during drying of beans. [12-14].

Utilization of *Amorphophallus paeoniifolius* peels for the production of cellulase enzyme can be an economical process. *Amorphophallus paeoniifolius* commonly known as Jimikand is an aroid which is mostly cultivated in Philippines, Malaysia, Indonesia and other South East Asian countries. In India, it is cultivated in Andhra Pradesh, West Bengal, Gujarat, Kerala, Tamil Nadu, Maharashtra, Uttar Pradesh, and Jharkhand. The net economic return is over 1 lakh rupees per ha. It has great export potential since its commercial cultivation is not in other countries [15, 16].

Some research on *Amorphophallus* corm has been carried out in our group. Earlier the identification of cellulolytic enzymes in stored *Amorphophallus paeoniifolius* corm has been done with potential of juice clarification from our group. [unpublished data] In our previously published paper we reported that peel contains a substantial amount of tannins and phenols, alkaloids and carbohydrates and has antioxidant potential. [17,18]. The present study summarizes most attempts of *Amorphophallus* peels utilization in food and non-food applications that include the extraction and verification of cellulase enzyme and its biochemical characterization.

MATERIALS AND METHODS

Material

Amorphophallus paeoniifolius was purchased from local market at Ghaziabad. Corm was first peeled and the peel of corm was cleaned with distilled water and dried on tissue paper. Peel was chopped into small pieces and packed in polyethylene bag. The chemicals used were of analytical grade.

Enzyme extraction and partial purification

Amorphophallus peel (10 g) was weighed and homogenized in ratio of 1:3 with 0.1 M Citrate buffer, pH 5.0. The homogenate was filtered through Cheese cloth and the filtrate was then centrifuged at 10,000 rpm 30 min at 4 °C in a refrigerated centrifuge to obtain a clear supernatant, then the supernatant was fractionated with solid (NH₄)₂SO₄.

Ammonium sulphate was added to supernatant to bring to 40-60% saturation was separated by centrifugation at 10,000 rpm for 20 min at 4 °C in a refrigerated centrifuge. Supernatant was discarded after centrifugation and the pellet was dissolved in 1 ml of 0.1 M Citrate buffer pH 5.0. The solution was then dialyzed against the same buffer at 4 °C for 48 h, with three time buffer changes. The dialyzed solution was collected and used as partially purified enzyme for all other analyses.

Enzyme assay

Carboxymethyl-cellulase (CMCase) activity: CMCase activity was assayed using a modified method described by Wood and Bhat with some modifications [19]. 0.1 mL of supernatant was added to 900 µl of 0.1 M Citrate buffer, pH 5.0 and 1.0 ml of 1%CMC was added in a test tube and incubated at 50°C for 60 minutes. The reaction was terminated by adding 3.0 mL of 3, 5-dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. [20]. One millilitre of Rochelle salt solution 40% was then added to stabilise the colour. The absorbance was recorded at 540 nm against the blank (without enzyme filtrate). One unit of CMCase activity was expressed as 1 µmole of glucose liberated from carboxy-methyl cellulose per minute.

Filter-paperease (FPase) activity: The activity of FPase was assayed according to the method explained by Wood and Bhat with some modifications. Briefly, the methods are similar to the CMCase assay method, but the substrate used was Whatman no. 1 filter paper strip (1 x 3 cm, 25 mg) soaked in 1 mL 0.1M citrate buffer (pH 5.0). The samples were incubated at 50°C for 60 minutes.

The reaction was terminated by adding 3.0 mL of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. One milliliter of 40 % Rochelle salt solution was then added. The absorbance was recorded at 540 nm against the blank. One unit of FPase activity was determined as 1 µmole of glucose liberated per ml enzyme per minute. Maximum specific activity obtained for the enzymes was taken as 100% for the calculation of relative activity.

Protein Assays

Protein concentrations were determined according to the dye binding method of Bradford using bovine serum albumin as standard [21].

Congo Red Plate diffusion Assay

The evaluation of crude endoglucanase enzyme was identified employing the congo red plate assay as described [22] Agar plates were prepared containing 0.5% carboxymethyl cellulose incorporated into 1% w/v agar in milliQ water. The experimental steps were conducted in sterile condition in an incubator set at 50 °C. The 50 µl crude enzyme peel sample was applied onto each agar well. Following a 24–48-h incubation period at 50 °C, the wells were washed off with distilled water and were stained with congo red solution for 30 min. The gels were soaked in 1 M NaCl until clear yellow zones were detected.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on a slab gel containing 10% (w/v) polyacrylamide by the method of Laemmli [23]. The gel was stained with Coomassie brilliant blue (0.5% w/v) for 30 min and destained in 10% methanol and 5% acetic acid for a limited period of time.

Activity staining of endoglucanase

The activity staining method was performed as described by [24] with some modifications. Carboxymethyl cellulose (0.5%) as substrate was boiled and incorporated into the analytical 10% (w/v) polyacrylamide gel. The isolated endoglucanase protein samples were run in the substrate incorporated gel at 100V constant voltage for 2-3 hours. After electrophoresis the gels were soaked in 0.1 M citrate buffer pH 5.0 for two 15 min changes for 24 hours to renature the enzymes in gel. The polyacrylamide gels were stained for 30 min in congo red and destained in 1 M NaCl for at least 30 min. Bands with lytic activity appeared as clear zones against bright red background.

Effect of temperature on enzyme activity and stability

The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30, 40, 50, 60, 70, 80, 90°C were studied. The reaction mixture was pre-incubated for 15 min with all the mentioned temperature, the activity of the enzyme was measured.

Effect of pH on enzyme activity

The cellulolytic activity of the crude enzyme was measured at different pH values (2 - 10). The pH was adjusted using the following buffers (0.1 M) of citrate buffer (pH 2.0- 6.0), Tris (pH 7-10) Reaction mixtures were incubated at 50°C for 60 min and the activity of the enzyme was measured as described previously.

Effect of various metal ions on Cellulolytic activity

The effects of metal ions [100 mM] were investigated by adding them to the reaction mixture and pre-incubated for 15 min at 50°C. The activity of the enzyme was measured as described earlier.

RESULTS AND DISCUSSION

Characterization of cellulase

Cellulase enzyme was isolated from peel of *Amorphophallus* and enzyme activity was estimated by the method of Wood and Bhat. Partial purification of crude enzyme was done by dialysis of 40-60% ammonium sulphate precipitated crude sample. Result was shown in Table 1. Optimum pH, temperature, substrate concentration were determined and investigation was carried out to determine the role of metal ions on enzyme activity.

Optimum temperature for CMCase and FPase both was found to be 60°C as shown in Fig.1. Thermal stability studies suggested that 52% activity of CMCase and 85.39% of FPase was retained even at 90 °C as shown in Fig. 2.

CMCase activity was found to be optimum at pH 10 whereas FPase activity was found to be optimum at pH 8, as shown in Fig. 2. Endoglucanase CMCase retained at both acidic and basic pH but interestingly at pH 7 CMCase activity decreased. In acidic conditions at pH 5.0, the CMCase retained its activity 92.87 % with respect to maximum CMCase activity at pH 10. The optimum activity obtained at respective pH was considered as 100% and graph represents relative activity. (Fig. 2)

The optimum substrate concentration was found to be 1% of CMC, 50 mg and Whatman filter paper 1 was found to be optimum for FPase (result not shown). Investigation was also carried out to determine the role of metal ions in the stabilization of cellulase enzymes. Enzyme was incubated with various metal ions at 50°C and the CMCase and FPase specific enzyme activity was calculated as shown in Fig 3. Hg²⁺ and Mn²⁺ has strong inhibitory effect on both CMCase and FPase. EDTA, Cu²⁺ and Fe²⁺ also have an inhibitory effect on CMCase but interestingly Fe³⁺ activated FPase activity and Mg²⁺ activated CMCase activity.

A specific enzyme activity was found maximum with Fe³⁺ in case of FPase where as Fe³⁺ was inhibiting the enzyme activity in CMCase. Zymography studies where substrate was polymerised in gel yielded bands with molecular weight of the enzyme is approx 43 kDa.

Endoglucanase activity was further validated by Congo red plate diffusion assay. Clear zones were visualized under white light

illumination as shown in Fig.4 where presence of clear yellow zones around the sample well confirmed the presence of CMCase activity. Polyacrylamide gel electrophoresis and lytic activity after staining with Congo red as dark zones is shown in Fig. 5. SDS PAGE analysis of crude extract was done in polyacrylamide gels in which the substrate CMC 1%, was polymerised in the native gel, and clear-clear zones in lane A1 and A2 indicate that molecular weight of enzyme is around 43 kDa.

Table 1: Partial purification of crude cellulase enzyme of *Amorphophallus peel*

Purification step	Total protein (mg)	Total activity units	Specific activity(U/mg)	Percentage yield	Purification factor
CMCase					
Crude extract	55	4016	73.02	100	1
40-60 %	0.31	124.08	130.61	3.09	1.79
FPase					
Crude extract	55	407	7.4	100	1
40-60 %	0.31	4.39	14.16	1.08	1.91

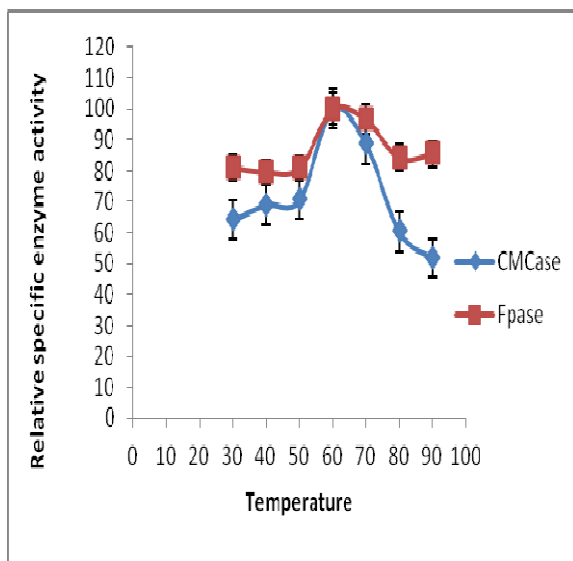


Fig. 1: Effect of temperature on enzyme activity CMCase & FPase

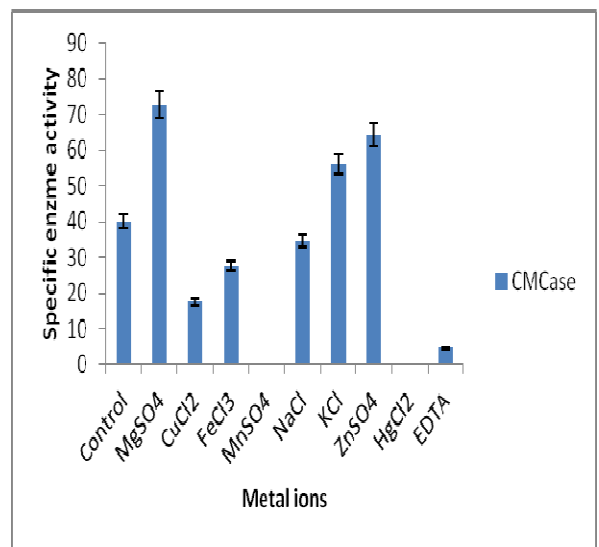


Fig. 3 (A): Effect of metal ions on enzyme activity A; CMCase

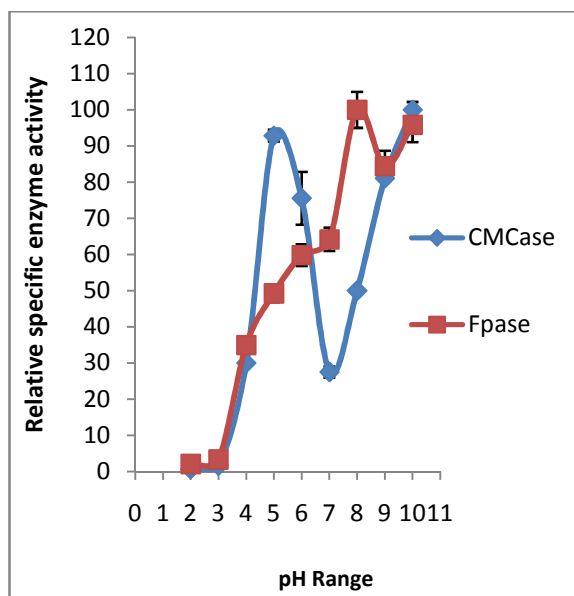


Fig. 2: Effect of pH on enzyme activity CMCase and FPase

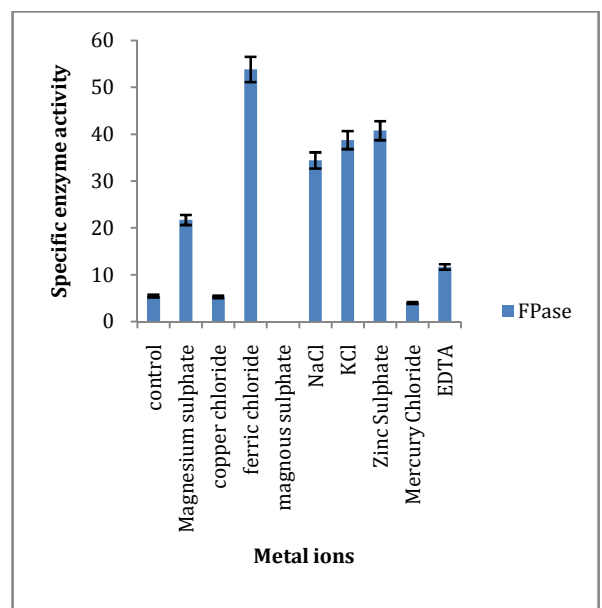


Fig. 3(B): Effect of metal ions on enzyme activity B; FPase

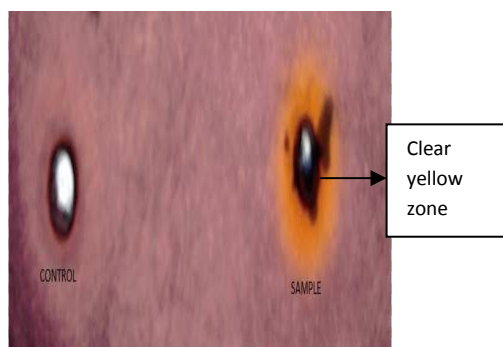


Fig. 4: Congo red plate diffusion assay

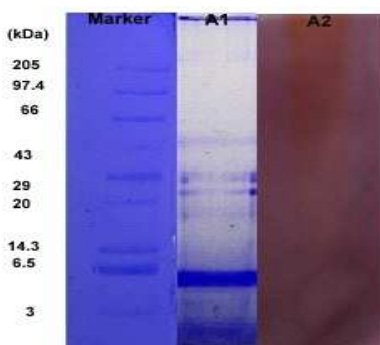


Fig. 5: SDS PAGE and Zymography of crude enzyme

CONCLUSION

Peel of *Amorphophallus* has great economic potential. Carbohydrases produced by waste peel of *Amorphophallus* can be further purified and utilized in industrial applications. The availability of enzymes from plant species also remains a best viable option which needs to be further explored. Production of enzymes by the agro waste can propose alternative paths for the reuse of agro-industrial waste, as well as adding economic value to these co-products. The cellulase enzyme from peel showed the existence of multiple forms, enhanced thermostability, and pH stability under acidic as well as under alkaline conditions. This enzyme has potential to meet the industrial needs.

ABBREVIATIONS

DNA; Di-nitro salicylic acid, FP; Whatman filterpaper No1, PAGE; Poly acrylamide gel electrophoresis

ACKNOWLEDGEMENT

We are thankful to Jaypee Institute of Information Technology University, Noida, India for providing the infrastructure facilities for this study.

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