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Cellulase from stored *Amorphophallus paeoniifolius* in clarification of apple juice

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Abstract

Crude enzyme fraction from stored corm of *Amorphophallus paeoniifolius* could clarify apple juices indicationg its potential use in fruit juice processing. The extract showed presence of cellulase activity which was confirmed by Congo red plate diffusion on incubating the plates for 24 hours at 50°C. Optimum temperature, pH, incubation time for cellulase activity was also determined by DNS method. Zymography studies and SDS gel electrophoresis confirmed that the molecular weight of the enzyme is 66 kDa.

Introduction

Plants usually contain 35–50% cellulose as per dry weight basis, making it the abundant organic compound in nature. Approximately $1.5x10^{12}$ tons of total annual biomass production is through photosynthesis (Klemm *et al*., 2002). Cellulose is a highly stable polymer consisting of β-1, 4-linked glucose units (Coughlan, 1992; Yin *et al*., 2010). Cellulases refer to cellulose-degrading enzymes with potential to convert cellulosic material into its subunit-glucose (Beguin and Aubert, 1994) but lignin, hemicelluloses and other back-up substances provide support against cellulolytic activities. Increasing demand for renewable energy sources has sparked growing interest in enzymes, capable of degrading cellulose to sugars that can then be used for the production of ethanol (Ekborg, 2007). Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars. 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 (Singh, 1999; Muthuvelayudham and Viruthagiri, 2006; Singh *et al*., 2007). Higher plant cellulases like from *Lantana camara* and *Cuscuta reflexa* are mostly involved in fruit ripening and senescence. Cellulases are used in the textile industry for cotton softening, denim finishing, in laundry, detergent market for colour care, cleaning, in the food industry for mashing, commercial food processing in coffee and it also performs hydrolysis of cellulose during drying of beans (Kirk *et al*., 2002; Cherry and Fidantsef, 2003; Karmakar and Ray, 2011). In the pulp and paper industries it has found application in deinking, drainage improvement and fibre modification. 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 (Sukumaran *et al*., 2005). High cost and low activity are the major impediments to the commercial use of cellulases (Yanase *et al*., 2010).

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 yet done in other countries (Misra *et al*., 2001; Singh and Wadhwa, 2014). It is a healthy low-fat food containing a good source of protein as well as starch. This aroid has not been explored much; the identification of cellulolytic enzymes in stored *Amorphophallus paeoniifolius* is unique work and can be commercially harnessed by the fruit juice industry which is among the largest agro-based industries worldwide. High juice yield is an important goal for juice production. Many modern processes for fruit and vegetable juice production employ enzymes as important processing aids to obtain higher yields

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and clarity (Yang *et al*., 2008).The existence of various carbohydrases in stored *Amorphophallus paeoniifolius* corm has been identified by our group. We also reported the presence of cellulase enzyme from peel of *Amorphophallus* (Wadhwa *et al*., 2014). This paper describes the isolation, characterization, confirmation and application of cellulolytic enzymes in clarification of apple juice from skinned *Amorphophallus* corm.

Materials and Methods

Preparation of corm homogenate as enzyme source

Amorphophallus paeoniifolius was purchased from local market at Ghaziabad. Corm was first peeled and cleaned with distilled water, the weighed corm was chopped into small pieces 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^{o}C in a refrigerated centrifuge to obtain a clear supernatant that was used as the enzyme source.

Carboxymethyl-cellulase (CMCase) and Filter-paperase (FPase) enzyme activity assay

CMCase and FPase activity was assayed using a modified method described by (Wood and Bhat, 1988), with some modifications. 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 (for CMCase) and Whatman no. 1 filter paper strip (1 x 3 cm, 25 mg) (for FPase) was added in a test tube and incubated at 50°C for 60 min. 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 (Miller, 1959). One ml 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 international unit of CMCase and FPase activity was expressed as 1 µ mole of glucose liberated from per ml enzyme per min under assay condition.

Protein assay

Protein concentrations were determined according to the dye binding method of (Bradford, 1976), using bovine serum albumin as standard.

Congo red plate diffusion assay

The evaluation of cellulase enzyme was identified employing the Congo red plate assay as described by (Teather and Wood, 1982). Agar plates were prepared containing 0.5% carboxy methyl cellulose incorporated into 1% w/v agar in milli Q water. The experimental steps were conducted in sterile condition in an incubator set at 50°C. The 50 µl enzyme sample was applied onto each agar well. Following a 24–48 hours 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 and Activity staining of cellulase

SDS-PAGE was performed on a slab gel containing 10% (w/v) polyacrylamide by the method of Laemmli (1970). The gel was stained with Coomassie brilliant blue (0.5% w/v) for 30 min and de-stained in 10% methanol and 5% acetic acid for a limited period of time.

The activity staining method was performed as described by Trudel and Asselin (1989) with some modifications. Carboxymethyl cellulose (0.5% and 1%) substrate was boiled and incorporated into the analytical 10% (w/v) polyacrylamide gel. The isolated 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 24 hours to renature the enzymes in gel. The polyacrylamide gels were stained for 30 min in Congo red and de-stained in 1 M NaCl for at least 30 min. Bands with lytic activity appeared as clear zones against bright red background.

Characterization of cellulase

Effect of temperature, pH and various metal ions on enzyme activity

The cellulolytic activity of the crude enzyme was measured at different temperatures (30 - 90°C), pH values (2 – 10) and with different metal ions (100 mM). The pH was adjusted using the following buffers (0.1 M) of citrate buffer (pH 2.0- 6.0), Tris (pH 7-10). The reaction mixture was pre-incubated for 15 min with all the mentioned temperature, pH and metal ions. After pre-incubation the activity of the enzyme was measured as per standard assay conditions.

Apple juice clarification by cellulases

Crude enzyme extract (30 units) was incubated with 5ml of freshly extracted apple juice, filtered through three layers of cheese cloth for 1 hour at 50°C. The contents of the tubes were stirred well in order to mix the enzyme with juice. The reaction was performed in water bath. Clarification of juice was

observed after 50 min. Test for starch and pectin was carried out to test the effectiveness of the enzyme treatment (results not shown).

Results and Discussion

Cellulase enzyme was isolated from corm of *Amorphophallus* and enzyme activity was estimated by the method of (Wood and Bhat, 1988). Optimum pH, temperatures 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 Figure 1(a). Thermal stability studies suggested that cellulase extracted from corm retained activity even at 90°C. CMCase activity was found to be optimum at pH 10 whereas FPase activity was found to be optimum at pH 5, as shown in Figure 1(b).

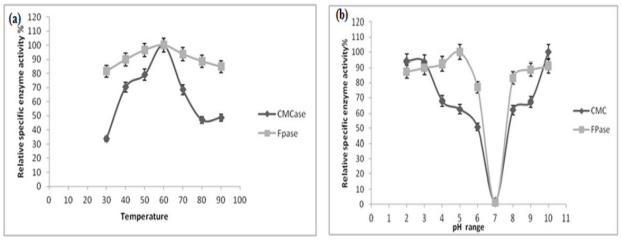

Figure 1. Effect of (a) temperature and (b) pH on enzyme activity CMCase and FPase. Maximum specific activity obtained for the enzymes was taken as 100% for the calculation of relative activity

CMCase retained its activity at both acidic and basic pH but interestingly at pH 7 both the CMCase and FPase activity decreased. Congo red plate diffusion assay confirmed the presence of cellulase activity in the isolated enzyme fraction and the clear zone were visualized under white light illumination. Control with distilled water shows no clear zone. Radial diffusion of enzyme into CMC incorporated gel produces hydrolysis zones, visualized by staining with congo red dye (results not shown).

Polyacrylamide gel electrophoresis and zymography studies where substrate CMC was polymerised in gel yielded bands with lytic activity after staining with Congo red as dark zones shown in Figure 2. The molecular weight of the enzyme is 66 kDa as determined by the zymographic studies. 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 and control without the metal ions was also taken. CMCase and FPase specific enzyme activity was calculated as shown in Figure 3.Control specific enzyme activity (without the addition of metal ions)

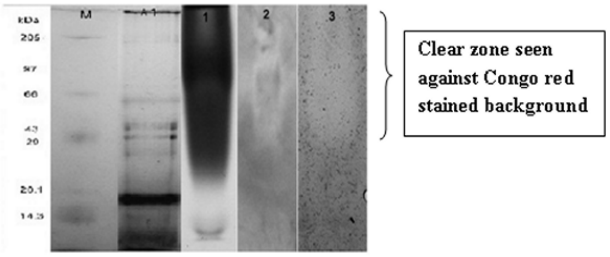

Figure 2. SDS PAGE, Native and Zymography of crude enzyme
M: Marker, lane A1 is SDS PAGE analysis of crude extract, lane 1 is the Native gel analysis of the crude extract, in lane 2 (substrate CMC 1%) and lane 3 (substrate CMC 0.5%) was polymerised in the native gel. Clear zones in lane 2 and 3 indicate that molecular weight of enzyme is around 66 kDa.

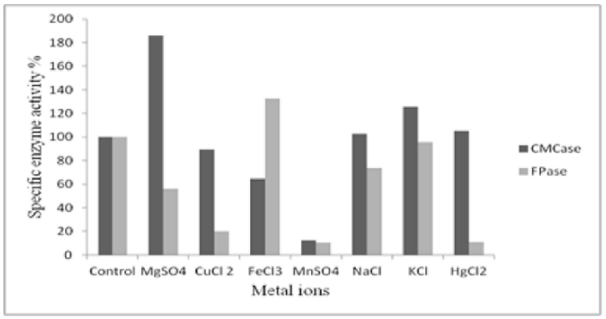

Figure 3. Effect of metal ions on cellulase specific enzyme activity

was taken as 100%. Mn^{2+} has strong inhibitory effect on both CMCase and FPase, whereas Mg^{2+} activated the CMCase activity maximum and Fe^{3+} strongly activated FPase activity.

Application of cellulase isolated from *Amorphophallus paeoniifolius* was checked for apple juice clarification. The enzyme extract, clarified freshly extracted apple juice. Freshly extracted and filtered apple juice was incubated with the enzyme extract in the ratio of 5:1 for 1hour at 50°C.Assay was done in triplicate and the clear juice was obtained after 1 hour of incubation with the enzyme as compared to the control tube which did not contain the extract.

Cellulases produced by stored *Amorphophallus* corms can degrade polysaccharide material of apple juice thus facilitating clarification. The use of cellulase from locally produced corms having good shelf life value can be favourable and economical for juice production. It is well known that for clarification of juices by cellulase, xylanases, pectinases are utilized (Vaillant *et al*., 1999). The availability of enzymes from plant species remains a best viable option which needs to be further explored. The stored corm extract showed the existence of cellulolytic activity with enhanced thermo stability and was stable under both acidic as well as alkaline conditions. This enzyme has potential to be exploited in the clarification of juices.

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