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Screening, Characterization and Purification of Cellulase from Mango Mealybug *Drosicha stebbingi* Green (Homoptera: Margarodidae)

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MS and QAS developed basic idea and wrote the manuscript. RMM, MS, IR and QAS planned and performed the experiments. IR collected data. RMM and MUA critically reviewed the manuscript

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ABSTRACT

Arthropods have progressed with efficacious approaches to utilize plant cellulose for energy development and its study would be of a great importance in the field of bioenergy and integrated pest management. The study was aimed to evaluate cellulolytic activity which was detected in the gut of mango mealybug, (*Drosicha stebbingi*). Initially, cellulolytic activity was detected from crude proteins using substrate-agar plate assay and laterally confirmed by endoglucanase assay. Enzyme activity measured using the glucose standard curve was 725 U/ml after acetone precipitation. Thermal stability and optimum temperature were found to be 60°C for maximum activity. Likewise, enzyme was found more stable at 6.0 pH. Various mono and divalent cations were studied against the cellulolytic activity exhibited significant enhancing and inhibitory effect upon different concentrations. Effect of inhibitors (β-mercaptoethanol, EDTA) and various plant extracts on the enzyme activity were also tested. Cellulase was further purified and characterized through chromatographic techniques and electrophoresis. Gel filtration chromatography showed the presence of multiple forms of enzyme activities with different molecular weights. SDS-PAGE showed the presence of endoglucanase activities with molecular weight of 57 kDa.

INTRODUCTION

Successful and predominance distribution of plants are attributed by their cell wall made up of numerous cellulose polymers strengthened by van der Waals forces and hydrogen bonds. Cellulases is a complex enzyme which needs synergistic action of endo-β-1,4-glucanases (EGs), β-glucosidases (BGs) and cellobiohydrolases (CBHs) to hydrolyze the complex cellulose polymers into simple glucose (Lo *et al*., 2000). This mechanism of hydrolysis has great importance in the field of bioenergy to produce substitute fuel by utilizing cellulose (Kostylev and Wilson, 2012) as former techniques of hydrolyzing lignocellulose were not that cost effective. The limitations of previous techniques were related to inhibitory agents and enzyme stability (Kristensen *et al*., 2009). Recent decades back, it is believed that only fungi and bacteria have cellulolytic activity as a gut symbionts. Later, studies reported endogenous cellulase activity in wood feeding termites, *Nasutitermites takasagoensis* and *Reticulitermites speratus* and cockroach, *Panesthia cribrata* that do not possess the cellulolytic symbionts (Scrivener and Slaytor, 1994; Tokuda *et al*., 1997; Watanabe *et al*., 1997). Now endogenous cellulase has been reported from various insect orders Blattodea, Coleoptera, Diptera, Isoptera, lepidoptera, Orthoptera and Zygentoma (Pothula *et al*., 2019). Insect cellulases are categorized into four main glysosyl hydrolase families (GHFs) such as GHF1, GHF5, GHF9 and GHF45 (Tokuda, 2019). There are two main hypothesis about the origin of endogenous cellulase in the animal i.e. horizontal and vertical transfer where the genetic material may be inherited from non-animal organism and early metazoans, respectively. In insect, symbiont independent cellulose digestion is an inherited mechanism for plant cell wall digestion that would be a good source of unique cellulases (Watanabe and Tokuda, 2010; Calderón-Cortés *et al*., 2012). Furthermore, inhibition of such cellulases to control the insect pest have also got attention now (Tsvetkov and Yarullina, 2019).

The mealybug attained the position of being the most terrible agricultural insect pest of the tropics (Herren, 1981). Mango mealybug, *Drosicha stebbingi* Green (Homoptera: Margarodidae) is the most severe pests in

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indo-pak continent and is a rising risk to the mango fruit (Karar *et al*., 2006). Mealybug is only the reason for shocking the crop throughout its serious occurrence. It has a tendency to infest the diversity of the fruit and ornamental tree in addition with the mango for example plum (*Prunus domestica*), papaya (*Careica papaya*), peach (*Prunus persica*), and all citrus cultivars etc. in its peak infestation mango mealybug has been noted in different forest trees and huge amount of crawler and female approaching tree *Dalbergia sisso, Ficus religiosa, Populus abla* and *Bombyx ceiba* via stem (Neuenschwander, 2008). Primary cell wall of plant contains cellulose, hemicellulose and pectin, likewise plant sap also possess cellulose. Nymphs and adults are voracious feeder and suck cell sap from leaves and twigs. Mealybug feeding such high dietary cellulose content must have a cellulase enzyme either from both symbionts dependent, endogenous or from both origin to hydrolyze. Present study is aimed at (1) screening of both exogenous and endogenous cellulolytic activity of adults female mealybug and (2) further purification and characterization of cellulase which may act as a novel source for the applications in different industries.

MATERIALS AND METHODS

Insect

Female adult mealybugs were collected from infested mango orchard (locality: 25.447965°N, 68.573696°E) during April 2019. Specialized rubber scrappers were used for collection which can easily detach their bodies avoiding any injury. Collected bugs were stored at -10°C in deep freezer (Waves® 18ft^3, WDF318).

Crude enzyme extraction

White powder from dead mealybug was removed with distilled water and dried on filter paper. Appendages were removed in 0.9% physiological saline solution and left whole body were retained for enzyme analysis. Sodium acetate buffer of pH 7.0 was use to homogenize the mixture using mortar and pestle. Mixture was centrifuged at 10,000 × *g* for 20 minutes to separate supernatant. Ice-cold acetone (200ml) was added in filtered homogenate (50 ml) and left for protein precipitation at 4°C overnight. Precipitated protein was centrifuged at 10000 × *g* for 20 minutes. The pellets were air dried and dissolve in least quantity of sodium acetate buffer (pH 7.0) and stored at 4°C.

Screening of cellulase activity

Cellulolytic activity was screened using a modification of cellulase-congo red plate assay (Rehman *et al*., 2009). Pre-sterilized plates were prepared by adding 3% agar (5ml) and 2% carboxy methyl cellulose (CMC) (5ml). Further, 0.1M sodium phosphate buffer of pH 6.0 (5ml) was added in substrate- agar mixture. Made a well in center of each plate, poured 100µl of crude extract in it and kept for overnight at 37°C. The plates were then stained with 1% congo red solution and allowed to shake on digital shaker (BT302, Orbi-Shaker™) for 20 min and destained using 1M NaCl for 20 min to get clear circular zone of hydrolysis around wells.

Quantification of cellulase activity

Cellulolytic activity was measured by estimating reducing sugar released from substrate (CMC) using 3, 5-dinitrosallicylic acid (DNS) reagent. Reaction mixture was prepared adding 0.5ml CMC (2 % w/v) and 0.1 M phosphate buffer, pH 6.5 and incubated overnight at 4°C. 3 ml of DNS reagent was added to stop the reaction and then boiled at 100°C for 15 min in a water bath. Brown color was obtained of reducing sugar. Absorbance at 540 nm was measured using Unico® SQ2800 UV/VIS spectrophotometer and reducing sugar concentration was calculated by comparison with glucose standard curve.

Optimization for maximum cellulolytic activity

Optimization assay was performed at different pH, temperature and substrate concentrations. To determine maximum activity at different pH levels, 100 µl crude enzyme was mixed 0.5 ml of 2% of CMC and assay was performed at range of pH 4.8, 5.8, 6.0, 6.4, 6.6, 7.0, 7.4, 7.8 and 8.0. Likewise, for temperature optimization, assay was performed at temperatures 4°C, 25°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C. Furthermore, suitable substrate concentration was determined using 0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% CMC for maximum enzyme activity. Reducing sugar released from each reaction was assessed using DNS method.

Effect of mono and divalent cations on cellulolytic activity

Mono (Na+, K+) and divalent (Hg^{++}, Ca^{++}, Sr^{++}, Ni^{++}, Co^{++}, Mn^{++}, Cu^{++} and Pb^{++}) cations were individually test for their effect on cellulase enzyme. These cations are used in their salts form (NaCl, KCL, $HgCl_2$, $CaCl_2$, $SrCl_2$, $NiCl_2 6H_2O$, $CoCl_2.6H_2O$, $MnCl_2$, $CuCl_2.2H_2O$, and $PbSO_4$) at different concentrations of 0, 100, 200, 300, 400 and 500 ppm. DNS method was used to estimate the reducing sugars.

Effect of inhibitors on cellulolytic activity

Effect of inhibitors like ethylenediaminetetraacetate (EDTA) and β-mercaptoethanol was accessed on enzyme stability at different concentrations of 0, 100, 200, 300, 400 and 500 ppm. Here, enzyme was pre-incubated at 50°C for an hour and then residual enzyme activity was calculated using DNS method.

Purification of cellulase

Gel filtration

The gel filtration was performed to observe different isoforms of the cellulase. Initially, gel (Sephadex G-75) was swollen then poured in to the packed column. Concentrated crude enzyme sample was chromatographed through Sephadex G-75 suspension and equilibrated with 0.2M Sodium Phosphate buffer of pH 7.0. Crude sample (2ml) was loaded on the column and sodium phosphate buffer was used to elute the bounded proteins. Total 40 fraction (each 3ml) were collected (flow rate = 6 drops/minute). Cellulolytic activity and protein concentrations were determined from each fraction. Cellulolytic activity was measured at 540 nm, while protein concentration was measured at absorbance 280 nm with distilled was used as blank.

Anion exchange chromatography

Active fractions were pooled after gel filtration and was further purified by anion exchange chromatography DEAE-Sepharose cl-6b. Gel was equilibrated with 0.05M tris-Cl buffer, pH 7.2 at a flow rate of 20ml/hr. Pooled active fractions were loaded onto column at a flow rate of 2ml/hr. and, after absorption, 20 mM PBS (5ml) of pH 7.2 was used to wash the column at a flow rate 10 ml/hr to elute unbounded proteins. In the case of bounded proteins, stepwise sodium chloride elution (range 0.05-0.5M) was used for elution in 20mM PBS, pH 7.2 with flow rate 10ml/hr. Fraction with high cellulose activity were tested using the DNS method and their absorbance was detected at 280 nm. The active pooled fractions were stored and further used in SDS-PAGE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of active fractions (form anion-exchange) was performed for the detection of bands on gel. Once cellulase activity was collected into a single, fractioned sample was subjected to pre-casted polyacrylamide gels (Mini-PROTEAN®) and electrophoresis was conducted with running buffer (25mM Tris, 150mM glycine with 0.2% SDS) with constant electric power of 220V for 20 minutes at 4°C. 500 µl sample was loaded, prepared by adding 100 µl dye (0.01 g bromophenol blue in 1 ml H_2O) in 400 µl of enzyme sample. After electrophoresis, staining was done with coomassie brilliant blue-R250.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) to evaluate each treatment for their significant differences. Data about effect of cations and inhibitors were subjected to one-way ANOVA at $p < 0.05$ level. Significant means were compared by HSD tukey's tests. SPSS statistical package (SPSS 10, for windows, Chicago USA) was used to carry out all analyses.

RESULTS

Cellulase screening assay

Drosicha stebbingi crude enzyme sample was test for cellulolytic activity using Congo-red plate assay. Clear zone around the well (Fig. 1) against a red-stained background on agar plates was an indication of CMC degradation. Assay showed that cellulase activity is present in the crude enzyme sample.

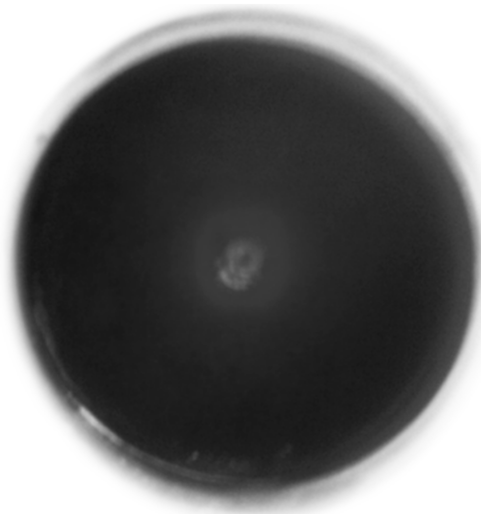

Fig. 1. Congo red plate assay of *Drosicha stebbingi* indication the presence of cellulase activity by formation of clear zone around the well at pH 5.8

Quantification of cellulase activity

Enzyme activity was measured using the glucose standard curve. Crude homogenate of *D. stebbingi* possessed activity of 420 U/ml while after acetone precipitation the cellulase activity was observed as 725 U/ml.

Effect of pH, temperature and CMC concentrations on enzyme activity

Figure 2 shows effect of pH, temperature and substate concentration on cellulase activity the enzyme activity. The peak cellulase activity was observed at pH 6.0 after which enzyme activity diminished pH range from 6.4 to 8.0. Likewise, reaction mixture showed highest activity of 2.64 U/ml at 60°C. Furthermore, maximum enzyme activity was observed with 2% of CMC.

Effect of mono and divalent cations on cellulase activity

Figure 3 shows effect of mono and divalent cations

on cellulase activity. The presence of strontium, nickle, Cobalt, potassium, copper and lead ions significantly decreased cellulase activity whereas little decrease was observed even in the presence of mercury. It seems that they are negatively correlated with cellulase activity. In the case of sodium and calcium cellulase activity was decreased initially at 100 ppm but later activity was increased than as it was at 100 ppm. The activity was increased in the presence of manganese ions. As its concentration increased from 0 to 500 ppm its activity increased two fold approx. which may suggest that manganese is acting as a co-factor to improve cellulolytic activity.

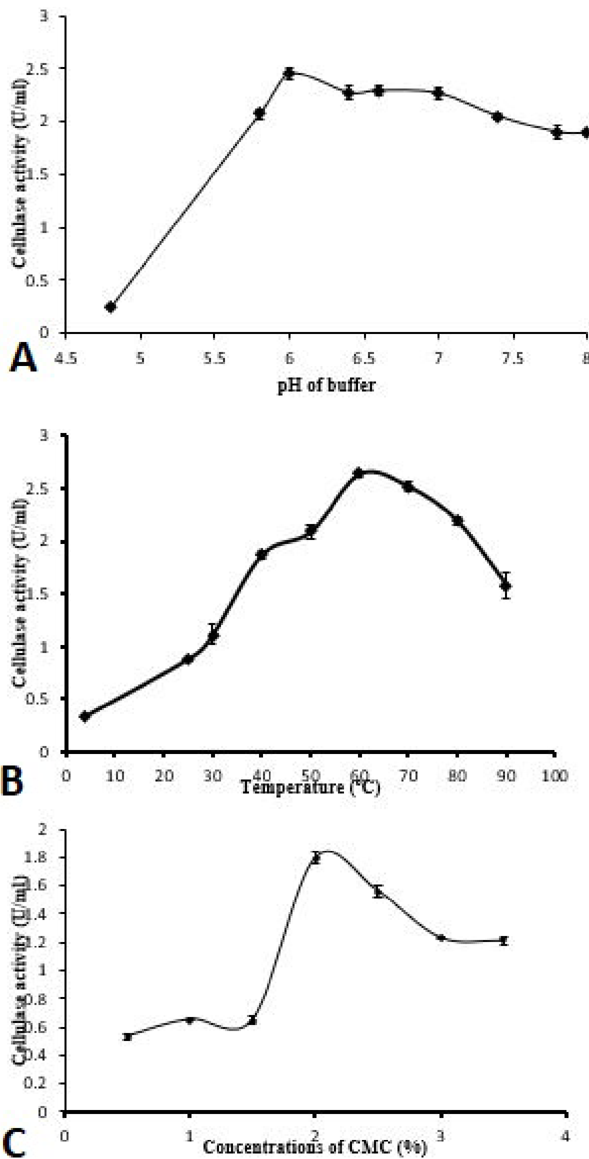

Fig. 2. Effect of pH (A), temperature (B) and substrate (C) on cellulase activity of *D. stebbingi.*

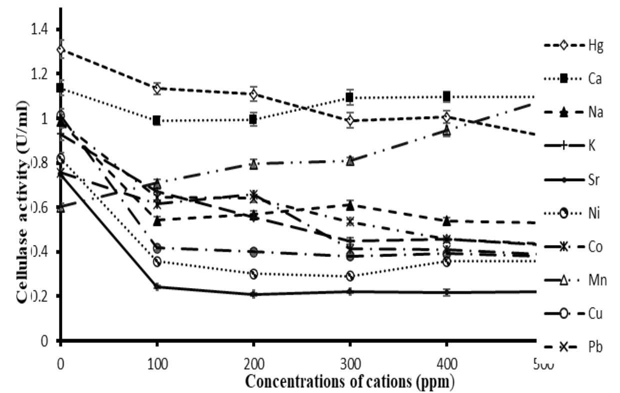

Fig. 3. Effect of mono and divalent cations on cellulase activity of *D. stebbingi.*

Effect of inhibitors on stability of cellulase activity

Stability of cellulase enzyme was assessed in the presence of EDTA and β-mercaptoethanol (Fig. 4). EDTA inhibited about 52.75% of cellulase activity at its maximum concentration (500 ppm). Likewise, cellulase activity was decreased up to 42.92% at 100mM concentration of β-mercaptoethanol in the reaction mixture.

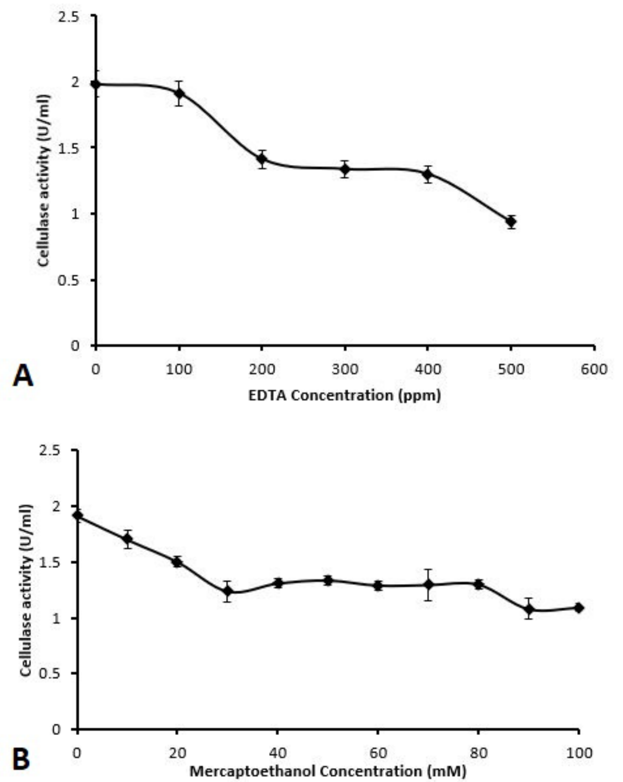

Fig. 4. Effect of EDTA (A) and β-mercaptoethanol (B) on cellulase activity of *D. stebbingi.*

Purification of cellulase enzyme

Figure 5 show fraction obtained after gel filtration, which were analyzed for the cellulolytic activity. Highest cellulase activity were found in the fractions no 16, 17, 18, 19, 20, 21, 26, 27, 28, 29, 30 and 31.

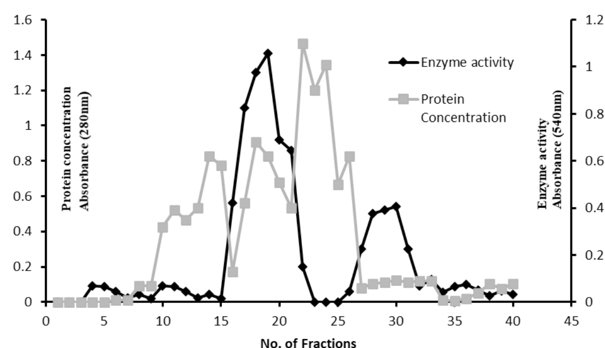

Fig. 5. Gel filtration for elution of cellulase on Sephadex G75 with flow rate 6 drops/minutes.

Concentrations of NaCl was used for stepwise solution of proteins form partially purified sample taken from gel filtration chromatography (Fig. 6). NaCl (0.05-0.5M) in 0.05M Tris-Cl buffer on pH 8.0 was used specifically for elution. Protein concentration was found high in the following fractions 18, 19, 20, 25, 26, 43, 44, 45, 46 and 48. Three fractions (number 19, 44 and 45) collected showed significant cellulase activity. These active fractions were further subjected to SDS-PAGE to calculate the molecular weight of protein.

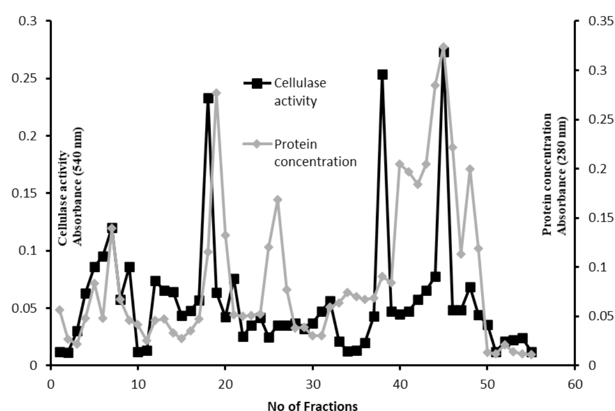

Fig. 6. Anion exchange chromatography for elution of cellulase active fractions on DEAE-Sepharose cl-6b with a flow rate of 10ml/h.

Molecular weight of enzyme was determined by polyacrylamide gel electrophoresis using protein ladder markers (LC 5625 Invitrogen) (Fig. 7).

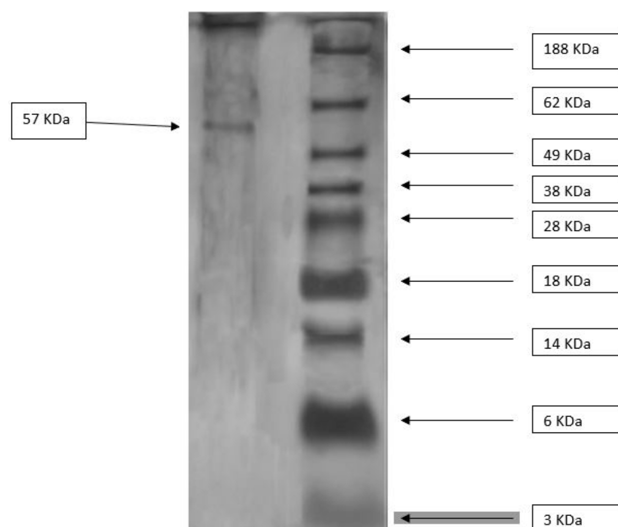

Fig. 7. SDS-PAGE of *Drosicha stebbingi* cellulase. The arrows on right indicate the molecular weight of markers while left side arrow indicates the molecular weight of cellulase enzyme.

DISCUSSION

In this study, we reported the characteristics of cellulase from *D. stebbingi* following the isolation, characterization and purification of enzyme. Cellulase is very significant hydrolytic enzyme. Several studies have been reported the properties of cellulase from wide range of insects including red flour beetle (*Tribolium castaneum*) (Willis *et al*., 2011), white spotted longhorn beetle (*Anoplophora malasiaca*) (Chang *et al*., 2012), stick insect (*Eurycantha calcarata*) (Shelomi *et al*., 2014), silver fish (*Ctenolepisma longicaudata*) (Pothula *et al*., 2019), and plant feed termite (*Zonocerus variegatus*) (Adelabu *et al*., 2019).

Maximum cellulase activity in *D. Stebbingi* was obtained using two step purification process. It is difficult to compare cellulase activity among reported studies because factors like substrate, pH, temperature and screening/ detection methods etc. can affect the enzyme activity (Fagbohunka *et al*., 2016). It would be interesting to make comparison of cellulase activity *D. stebbingi* with other insect having different mode of damage under the similar experimental condition.

Cellulase activity is dependent on pH and temperature of the reaction mixture. Previous study showed that cellulose present in the larval gut of yellow-

spotted longhorn beetle, *Psacothea hilaris* have maximum acitivity at pH 5.5 against CMC (Sugimura *et al*., 2003). Similarly, mulberry longicorn beetle, *Apriona germari* maximum activity of cellulase was observed at pH 6.0. These results coincided with our study, where *D. stebbingi* possessed maximum cellulase activity at pH 6.0 and slightly less peak at pH 6.5. But as pH went more alkaline, a significant decreased was observed in cellulase activity because the higher pH would lead to decline in cellulase activity (Li *et al*., 2013). pH value for maximum cellulase activity may vary from acidic to alkaline medium. This specifies that breakdown of internal bonds of cellulose and clipping of small units from the exposed chain ends are affective at slightly acidic pH. So, hydrolysis of cellulose into small reducing sugars (monosaccharides) is more efficient at slightly acidic or near at neutral pH (Prasetyo *et al*., 2010). The optimum temperature value was same (50°C) for maximum cellulase activity as to that obtained with cellulase mulberry longicorn beetle larvae *A. germari* (Lee *et al*., 2005). Likewise, in digestive tract of termite *Marcotermes mulleri*, 55°C temperature was observed (Fagbohunka *et al*., 2016). Many invertebrates and insects cellulases so far reported have maximum activity around 40-55°C with optimum pH from 5 – 6.8 (Xia *et al*., 2013; Mei *et al*., 2016). Temperature is an important part for cellulolytic activity to get bind on substrate and catalysis of reaction (Andreaus *et al*., 1999). Catalytic domain becomes deactivated beyond the temperature of maximum activity (Andreaus *et al*., 1999). Optimum temperature also reported to increase affinity of cellulase to the substrate for maximum activity (Baig, 2016).

Most of all tested cations like Sr^{2+}, Ni^{2+}, Co^{2+}, K^{+}, Cu^{2+} and Pb^{2+} inhibited the enzyme activity to various degree may be consequences of induction of alterations in the secondary and tertiary structure of enzyme or interaction of these cations with sulfhydryl group at catalytic site of cellulase (Yin *et al*., 2010). The cations that have strong affinity for ligands like phosphate, cysteinyl and histidyl may show the strong inhibition for the enzyme (Singh and Maret, 2017). In our study, all cation used were in the form of their chloride salts and Fagbohunka *et al*. (2015) described that chloride salts of Na^{+}, $NH4^{+}$, Mg^{2+}, Hg^{2+}, Zn^{2+}, Sn^{2+}, and Ba^{2+} generally inhibit the cellulase activity. Unlikely, manganese ions Mn^{2+} increased cellulase activity that suggest it might be behaving as a co-factor to enhance the cellulase activity.

Furthermore, cellulase performed very unstable in the presence of EDTA and β-mercaptoethanol. Study reported that β-mercaptoethanol slightly inhibit (14%) the cellulase activity (Fagbohunka *et al*., 2016) while EDTA was found strong inhibitor and significant decreased the cellulase activity (Goel *et al*., 2019) suggesting EDTA preformed as chelating agent for the co-factor that were essential for the cellulase activity.

Gel filtration (GF) and ion exchange chromatography (IEC) was used for digestive enzyme purification ever since alone or in integration with other techniques (Mubasshir *et al*., 2014). These techniques were adopted to cellulases from palm weevil *Rhynchophorus palmarum* (Rehman *et al*., 2009) and from mulberry longicorn beetle *A. germari* (Lee *et al*., 2005).

After purification of the crude proteins from *Drosicha stebbingi* through different chromatographic techniques (gel filtration and ion exchange chromatography), SDS-PAGE showed the molecular weights of cellulase was 57 kDa.

CONCLUSION

The finding of our research is a proof of the presence of a stable cellulase enzyme and thereby indicates the independent mode of cellulose digestion. Furthermore, as chloride salts are found strong inhibitor of Cellulase enzyme, therefore these cations or a combination of more than two would be used to design as a pesticide for that insect pest such that *D. stebbingi* and other insect pests may not be able to metabolize plant cell wall even if they consume. Resultant, the insect pests would be unable to hydrolyze the cellulose material and would lead to the death of an organism.

Statement of conflict of interest

The authors have declared no conflict of interest.

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