Purification and characterization of an endogenous cellulase from the digestive system of grub of banana pseudostem weevil *Odoiporus longicollis* (Olivier)

Sreeramulu Bhuvaragavan
University of Madras https://orcid.org/0000-0002-6358-116X
Thenozhiyil Reshma
University of Madras
Karuppiah Hilda
University of Madras
Ravichandran Balaji
University of Madras https://orcid.org/0000-0001-6023-6662
Mani Meenakumari
University of Madras
Narayanasamy Mathivanan
University of Madras
Sundaram Janarthanan (janas_09@yahoo.co.in)
University of Madras https://orcid.org/0000-0002-4987-6666

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Abstract

Insects have evolved with effectual strategies to utilise cellulose substrates for the energy source and can be used as an optimal resource in the field of bioenergy sector and insect-pest management by finding the novel cellulolytic enzymes from it. The present study was aimed at evaluating the endogenous cellulolytic system in the larval gut of banana pseudostem weevil *Odoiporus longicollis*. Initially, the cellulase activity was localized in the gut system in which the midgut showed highest activity of 2858 U $min^{-1}mg^{-1}$. The optimum temperature and thermal stability were found to be 60°C at the highest activity of 2712 U $min^{-1}mg^{-1}$. The enzyme was stable at a pH from 5 to 6. The effect of various divalent cations tested on the cellulase activity showed a differential enhancing and inhibitory activity upon varying concentrations. Purification of cellulase was carried out using anion exchange chromatography with the DEAE-Sepharose CL-6B matrix which was chosen according to the results of the batch assay. The total and specific activity of purified cellulase was 1166 U $min^{-1}mg^{-1}$ and 1190.46 U $min^{-1}mg^{-1}$, respectively, with 41.66 % recovery of activity. The molecular weight of the purified cellulase was determined as 47 kDa. The optimum temperature for the purified enzyme was 60°C with an activity of 1130 U $min^{-1}mg^{-1}$. The purified fraction had the highest activity between pH 5 and 6. Kinetic parameters of the purified cellulase enzyme were determined in which K_m and V_{max} values were 1.03 mg/ml and 343 U $min^{-1}mg^{-1}$, respectively. Mass spectrometry result identified the homology towards endoglucanase sequence belonging to the GHF5 family. The gut microbial cellulase activity showed no competence comparatively.

Introduction

The predominance and successful distribution of plants are credited by their rigid cell wall composed of abundant cellulose polymers strengthened by hydrogen bonds and van der Waals forces. This complex glycoside is hydrolysed into simple glucose by the action of cellulases such as endo-β-1,4-glucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases (BGs) among which EGs are common in nature (Lo et al., 2010). Interestingly, this hydrolysis mechanism has drawn attention in the bioenergy sector to produce alternate fuel by utilizing cellulose (Tomme et al., 1995) as of current cellulolytic technologies in hydrolysing lignocelluloses demands improvement, especially in cost management. The limitations of the former are related to the enzyme stability and inhibitory agents which opens up the search for novel enzymes (Kristensen et al., 2009). It was generally believed that only the bacteria and fungi possessed the cellulolytic enzymes especially as a gut symbiont in eukaryotes. This old-fashioned thought eventually faded after the first publications of purified endogenous cellulase from the wood-feeding cockroach *Panesthia cribrata* and the termites *Reticulitermes speratus* and *Nasutitermes takasagoensis* that do not harbour the cellulolytic symbiont (Scrivener and Slaytor, 1994; Tokuda et al., 1997; Watanabe et al., 1997). In recent times, endogenous cellulases from the insect orders Coleoptera, Isoptera, Blattodea, Orthoptera, Lepidoptera, Diptera and Zygentoma have been reported (Pothula et al., 2019; Willis et al., 2010a). The glycosyl hydrolase families (GHF) are a major group in which different types of cellulase (endogenous and exogenous) are categorised accordingly. Insect cellulases belong to the four main GHFs such as GHF1, GHF5, GHF9, and GHF45 (Calderon-Cortes et al., 2010; Tokuda, 2019). The origin of

endogenous cellulase in animals was explained with two hypotheses i.e. vertical and horizontal transfer where genes are inherited from the early metazoans and non-animal organisms, respectively. The symbiont independent cellulose digestion is an ancestral mechanism for plant cell wall (PCW) digestion in insects and this prospected them as an optimal source for novel cellulases (Calderón-Cortés et al., 2012; Martin, 1991; Scrivener et al., 1989; Watanabe and Tokuda, 2010). On the other hand, the uses of cellulases as potential candidates for inhibition to control the insect pests have also been in line of attention (Girard and Jouanin, 1999).

Banana stem weevil or banana pseudostem borer *Odoiporus longicollis* Olivier (Coleoptera: Curculionidae) which is an economically important pest of banana plantations feed the plant during flowering and bunch formation stages (Padmanaban et al., 2001). It was first recorded in Poonch and Rajouri district of the Jammu region as a major pest of banana plant (Musa spp.) which is grown in patches. They breed throughout the year and likely do not undergo winter rest (Azam et al., 2010). Grubs are voracious feeders and tunnels into the pseudostem and feed on the internal content. The banana pseudostem is composed of majorly cellulose, followed by hemicellulose and pectin (Ma, 2015). *O. longicollis* feeding such high dietary cellulose content must possess a cellulase enzyme either from endogenous, symbiont-dependent or from both the source to hydrolyse. In this context, the present study is aimed at i) localising the cellulolytic activity of both exogenous and endogenous sources from the gut of the grub of *O. longicollis* and ii) further purifying and characterising the enzyme which may act as a novel source for the applications in industry and other fields.

Materials And Methods

2.1. Insects

The fourth and fifth instar grubs were collected from the affected banana plants, in Pattambi, Palakkad, Kerala and suburban regions of Chennai, Tamil Nadu, India. The collected insect was identified using standard taxonomic keys.

2.2. Gut extracts

The collected larvae were wiped with ethanol and the head, whole gut, foregut, midgut and hindgut were separately dissected out in 0.9% physiological saline solution
(Fig. 1) and the gut contents including food particles were removed from the intestinal tract. For the assays and purification, the dissected tissues treated with saline containing sodium azide (NaN_3) and ampicillin (1 mg/ml) was used. The dissected gut was extracted using a mortar and pestle with an equal volume (w/v) of sodium acetate buffer (pH 5.5). The extract was centrifuged at 7000 x *g* for 20 minutes at 4°C. The supernatant was collected and stored at -20°C. The microbe-free gut extracts upon validated in the Petri plates were used for the assays.

2.3. Protein estimation

The protein concentrations of gut extracts and purified cellulase were determined using the method established by Lowry et al. (1951) using bovine serum albumin as a standard.

2.4. Isolation of gut bacteria

The whole gut extract was streaked onto the LB medium plates and incubated at 37°C for 24 h. The bacterial colonies were picked up, sub-cultured in LB medium broth, and re-streaked for the isolation of a single colony. The obtained colonies were used for the production of cellulase.

2.5. Cellulase-Congo red plate assay

The plates were prepared by adding the nutrient medium containing $NaNO_2$, K_2HPO_4, $MgSO_4$.7H2O, KCl, $FeSO_4$, along with carboxy methyl cellulose (CMC) (HiMedia). Wells were made in the plate and the extracts of head fluid, foregut, midgut, and hindgut were added on to the wells and kept for 24 h of incubation. Simultaneously, the isolated bacterial colonies were spotted on the cellulose plates and incubated at 37°C for 24 h. The plates were then stained with 1% Congo red for 20 min and destained with 1M NaCl for 15 min. The clear zone observed was measured using the scale (Miller, 1959).

2.6. Quantification of cellulase activity from isolated gut microbes and gut extract

The isolated gut bacterial colonies were inoculated in the nutrient broth and incubated for 24 h and 48 h at 37°C. Then, the produced cellulase in the nutrient broth was separated from the bacterial colonies by centrifuging at 3000 x *g* for 10 minutes. The supernatant was collected and the activity of produced cellulase was determined by 3,5-Dinitrosalicylic acid (DNS) method (Miller, 1959).

Gut extract and bacterial colonies produced cellulase were mixed separately with an equal volume of 1% CMC (dissolved in sodium acetate buffer, pH 5.5) and incubated for 1 h at 60°C. Then, an equal volume of DNS reagent was added to it. The reaction was stopped by incubating the mixture at 90°C for 10-15 min. The dark brown colour was obtained and the amount of reducing sugars produced in these reactions was measured with a spectrophotometer at 540 nm using the DNS method.

2.7. Physicochemical characterization of gut extract

2.7.1. Optimum reaction temperature

To determine optimal temperature, the whole gut extract of the larvae was incubated with 1% CMC in 0.1 M sodium acetate buffer (pH 5.5) at various temperatures ranging from 20°C to 80°C for 60 minutes using the DNS method (Miller, 1959).

2.7.2. Thermal stability

Thermal stability was determined by pre-incubating the gut extract in various temperatures (20°C to 80°C) for 10 min. Then, the pre-incubated samples were again incubated with 1% CMC in 0.1 M sodium acetate buffer (pH 5.5) for 1 h at 60°C. The activity was measured using the DNS method.

2.7.3. pH optima

To determine the optimal pH, the gut extract of the larvae was mixed with 1% CMC in buffers of different pH (4.0 - 8.0) at 60□C for 1 h and the activities of the solutions were measured. The buffers used include acetate (pH 4.0-6.0), phosphate (pH 7.0) and Tris-HCl (pH 8.0). The activity was measured using the DNS method.

2.7.4. Effect of divalent cations on cellulase activity

Divalent cations such as $CaCl_2$, $MgCl_2$, and $CuCl_2$ were individually applied at different concentrations of 5, 10, 15, 20 mM. These additives were dissolved with 1% CMC assay buffer (0.1M sodium acetate buffer, pH 5.5) at 60□C for 1 h. The amount of total reduced sugar was measured by DNS method.

2.8. Purification of cellulase

2.8.1. Batch assay

The binding ability of cellulase enzyme in the whole gut extract of *O. longicollis* was analysed with ion-exchange column matrices using batch assays to find a suitable matrix for the isolation of these molecules. The column matrices used were DEAE-Sepharose CL-6B and CM-cellulose. DEAE-Sepharose CL-6B (200 µl) was extensively washed with 20 mM phosphate-buffered saline (PBS) (pH 7.2) whereas CM-Cellulose was washed extensively using 20 mM sodium acetate buffer (pH 4.6). Then an equal volume of gut extract was applied and it was incubated at room temperature for about an hour. The residual was checked for cellulase activity by the DNS method to determine cellulase binding capacity of the column matrices used.

2.8.2. Anion exchange chromatography

Cellulase from the whole gut extract of *O. longicollis* was purified by anion exchange chromatography on DEAE-Sepharose CL-6B. A 13 × 1 cm diameter and 1 ml bed volume column of DEAE-Sepharose CL-6B was equilibrated with 20 mM PBS, pH 7.2 at a flow rate of 20 ml/ h. A total of 750 µl extract diluted to one fold with equilibration buffer was loaded onto the column at a flow rate of 1 ml/ h and, after adsorption, the column was washed using 5 ml of 20 mM PBS, pH 7.2 at a flow rate of 5 ml/ h to remove the unbound proteins. Bound proteins was eluted with step-wise sodium chloride elution (30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, and 100 mM) in 20 mM PBS, pH 7.2, at the flow rate of 20 ml/ h. Fractions containing cellulase were eluted in 50 mM NaCl and their absorbance was detected at 280 nm**.** The fractions were analyzed using Native-PAGE.

2.9. Molecular characterization of purified cellulase

2.9.1. Electrophoresis

The purified cellulase fraction was analyzed in both native-PAGE Tris/glycine (pH 8.3) buffer system (Maurer, 1971) and denatured PAGE (Laemmli, 1970). After electrophoresis, the gel was stained with coomassie brilliant blue-R250 and silver nitrate.

2.9.2. Zymogram

The zymogram was performed by following Schwarz et al. (1987) with minor modifications. Briefly, 12% SDS-PAGE with 0.5% CMC was used for zymogram. Samples were mixed with SDS sample buffer and kept at 4°C for 1 h for passive denaturation. Then the samples were loaded and run for electrophoresis in a refrigerator. After running, the gel was kept in 20% isopropanol (20 ml isopropanol in 80 ml PBS buffer) for 20 min. It was then washed with PBS for 6 times in 10 min intervals and incubated at 37°C in PBS overnight. The gel was stained with 0.1% Congo red dye at room temperature for 30 min and destained with 1M NaCl at room temperature for 15-20 min. Finally, 0.2% of acetic acid was added for better visualization of bands (Waeonukul et al., 2007).

2.10. Physicochemical characterization of the purified cellulase: Optimum temperature, thermostability, and pH optima

The methods for optimal temperature, thermal stability and pH optima for purified cellulase were carried out as mentioned for the crude gut extract.

2.11. Enzyme kinetics

The optimum substrate concentration for maximum enzyme activity was determined in terms of maximum reaction velocity (V_{max}) and Michaelis constant Km at which the reaction velocity is half-maximum (Cornish-Bowden, 2013; Laidler and Bunting, 1973; Michaelis and Menten, 1913).

For this, various concentrations of CMC in 0.1M sodium acetate buffer (pH 4.5) was incubated with purified enzyme preparation. The enzyme activity was measured at all concentrations. V_{max} and K_m were estimated graphically by plotting substrate concentration on X- axis against enzyme activity on the Y-axis. The accurate values of V_{max} and K_m were obtained from the doubled reciprocal plot (Line Weaver – Burk plot) (Lineweaver and Burk, 1934). The double reciprocal plot was obtained from Line Weaver – Burk equation, which states that $1/V_0 = K_m/V_{max}$ (1/[S]) +$1/V_{max}$. When $1/V_0$ plotted against (1/[S]) a straight line was obtained. This line had a slope of K_m/V_{max} an intercept of $1/V_{max}$ on the $1/V_0$ and an intercept of $1/K_m$ on the 1/[S] axis. Such a double reciprocal plot had the advantage of allowing a much more accurate determination of V_{max}.

2.12. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

The isolated enzyme was subjected to peptide mass fingerprinting and peptide sequencing using the standardized protocol for MALDI-TOF MS spectrometry (Wilm et al., 1996). The data obtained under the

mass spectrum were subjected to the database (NCBI non-redundant/Swiss-Prot) search using MASCOT (http://matrixscience.com) analysis. The calibrated peptide masses were searched with 100-500 ppm mass accuracy.

2.13. Multiple sequence alignment using ClustalW

The general purpose multiple sequence alignment program, ClustalW (Thompson et al., 1994) was used for sequence alignments of predicted peptide sequences from MASCOT search engine for the purified enzyme subjected to MALDI-TOF MS.

2.14. Statistical analysis

The data were subjected to One-way Analysis of Variance (ANOVA) to determine the significance of individual differences at $p < 0.05$ level. Significant means were compared by the Duncan's multiple range tests. All statistical analyses were carried out using SPSS statistical package (SPSS, Version 10.0 for Windows, SPSS Inc., Chicago, USA).

Results

3.1. Congo red plate assay

CMC degradation by different gut extracts such as the whole gut, head fluid, foregut, midgut and hindgut were tested in a CMC-agar plate with Congo red dye. CMC degradation was indicated by clear zone around the well (Fig. 2). The measurements of zone of activity were given in Table 1. CMC degradation by four isolates (I, II, III & IV) of gut bacteria were also tested for the production of cellulase. The obtained zone of activity was presented in Table 1.

Table 1 Analyses of cellulase activity (zone of activity) of gut extracts and gut bacterial isolates from the grub of *O. longicollis* by congo red plate assay.

S. No.	Gut extract[a]	Cellulase activity[b] (Zone of activity in mm)
1.	Whole gut extract	27±2
2.	Foregut	27±1
3.	Midgut	28
4.	Hindgut	22
5.	Head fluid	24
Gut bacterial isolates[c]		
1.	Isolate I	2
2.	Isolate II	3
3.	Isolate III	3
4.	Isolate IV	0

[a]100 µl of gut extracts that contained 578 µg, 550 µg, 570 µg, 600 µg, 580 µg of protein in extracts of whole gut, foregut, midgut, hindgut and head fluid, respectively. [b]Data based on median value of three separate determinations. [c]Bacterial isolates were spotted onto the plates. Sodium acetate buffer was used as a negative control.

3.2. Quantitative analysis of cellulase activity

The quantitative analysis of cellulase enzyme from head fluid, foregut, midgut, hindgut, and from the four isolates of gut bacteria (I, II, III, IV) were performed using the DNS method. The highest activity was shown by midgut followed by foregut, head fluid, and hindgut. The bacterial isolates I, II, III, IV had an activity in a range between 0 and 311 U $min^{-1}mg^{-1}$ for 24 h and decrease in activity was observed at 48 h of cellulase production (Table 2).

3.3. Physicochemical characterization of crude extract

3.3.1. The optimum reaction temperature and thermal stability

Gut extract with the substrate was tested in the range of 20-80°C. The reaction mixture showed the highest activity of 2800 U $min^{-1}mg^{-1}$ at 60°C which was determined as optimum temperature for further studies. The thermal stability of the cellulase was tested by the pre-incubation without the substrate in the same temperature range as above. The activity was stable around 40°C to 60°C with the highest activity of 2712 U $min^{-1}mg^{-1}$at 60°C
(Fig. 3).

3.3.2. Effect of pH

The effect of pH on the cellulase activity of the whole gut extract was determined at various pH ranges from pH 4.0 to 8.0. The cellulase activity was stable in the optimum pH range between 5 and 6 (Fig. 4).

3.3.3. Effect of divalent cations

The effect of different divalent cations (Ca^{2+}, Mg^{2+}, and Cu^{2+}) on cellulase activity of the whole gut extract was analysed and found to have varying ambiguous results. The highest cellulase activity of 5220 U $min^{-1}mg^{-1}$ was observed in 15 mM of Cu^{2+}. Mg^{2+} and Ca^{2+} showed highest activity of 3925 and 3050 U $min^{-1}mg^{-1}$, respectively for 10 mM concentration (Fig. 5).

Table 2 Quantitative analyses of cellulase activity of gut extracts and gut bacterial isolates from the grub of *O. longicollis* by DNS method.

S. No.	Gut extract	Cellulase activity[a,b] (U $min^{-1}mg^{-1}$)	
1.	Whole gut extract	2800	
2.	Foregut	2800	
3.	Midgut	2858	
4.	Hindgut	2479	
5.	Head fluid	2625	
Gut bacterial isolates	24 h	48 h	
1.	Isolate I	207	150
2.	Isolate II	311	300
3.	Isolate III	311	250
4.	Isolate IV	0	0

[a]One unit of cellulase activity is defined as the amount of enzyme that releases 1µmol of glucose min^{-1} at 60°C. [b]Data based on median value of three separate determinations.

3.4. Purification of cellulase from gut extract of O. longicollis

3.4.1. Batch assay

DEAE-Sepharose CL-6B and CM-cellulose were chosen as matrices for anion and cation exchange chromatography, respectively. The whole gut extract with cellulase activity of 2800 U $min^{-1}mg^{-1}$ was

made to adsorb with matrices to analyse the binding ability. The unbound, as well as stepwise eluted fractions (100 mM to 500 mM of NaCl) were checked for the cellulase activity. The unbound fraction from anion exchange matrix did not show any activity followed by eluted fractions that were observed with cellulase activity of 1460 and 550 U min^{-1} mg^{-1} in 100 mM and 200 mM and no activity in subsequent elution concentrations. The unbound fraction of cation exchange matrix showed cellulase activity of 2650 U min^{-1} mg^{-1} which was almost equal to the activity of the applied sample and eluted fractions did not show any activity in it (Table 3). Overall, it was inferred that cellulase had an effective binding ability with the DEAE-Sepharose CL-6B matrix and showed no binding to CM-cellulose.

3.4.2. Purification of cellulase using anion exchange chromatography

Based on the batch assay results, DEAE-Sepharose CL-6B was found to be an ideal matrix for the purification of cellulase and thereby anion exchange chromatography was adopted. Step-wise elution was carried out using various concentrations of NaCl. The sample volume of 1.5 ml (3.468 mg of protein) with cellulase activity of 2800 U $min^{-1}mg^{-1}$ was passed through the column. The unbound effluent was washed with 20 mM PBS, pH 7.2 till the baseline value was zero at 280 nm indicating that the matrix adsorbed all the activity from the gut extract. The bound protein was eluted at various NaCl concentrations ranging from 30 mM to 100 mM. Cellulase fraction was eluted at 50 mM NaCl concentration. Three out of four fractions collected showed significant cellulase activity of 1166 U min^{-1} mg^{-1}. A typical elution profile depicting the isolation of cellulase was shown in Fig. 6. As summarised in Table 4, this anion exchange chromatography method adopted for the purification of cellulase from the whole gut extract of the larva of *O. longicollis* resulted in about 41.66 % recovery of cellulase activity.

3.5. Molecular characterization of purified cellulase

3.5.1. Analysis of the purified cellulase in native-PAGE

The protein profiles of both gut extract and purified cellulase from the larvae of *O. longicollis* were analysed using discontinuous PAGE (8 %) under non-denaturing conditions (Fig. 7). The purified enzyme was visualized as a single protein band without any contamination in the polyacrylamide gel upon staining with coomassie brilliant blue (for gut extract) as well as silver nitrate (for the purified enzyme).

3.5.2. Zymogram

The cellulase zymogram analysis was performed for whole gut extract and eluted fractions from 50 mM NaCl concentration. The bands corresponding to enzyme activities were appeared and visualised using 0.1% Congo red dye (Fig. 8).

3.5.3. Analysis of the purified cellulase protein in SDS-PAGE

The protein profiles of both gut extract and purified cellulase from the larvae of *O. longicollis* were analyzed using discontinuous PAGE (12 %) under reducing conditions in the presence of SDS and β-mercaptoethanol (Fig. 9). A monomeric band without any polypeptide subunits appeared in the gel. The molecular weight of the band was corresponding approximately to 47 ± 2 kDa. Hence, the molecular weight of the purified cellulase was determined as 47 ± 2 kDa.

Table 3

Batch profile on the binding ability of column matrix to cellulase in gut extract.

Cellulase activity (U min^{-1} mg^{-1})						
Gut extract	2800					
Unadsorbed	100mM	200mM	300mM	400mM	500mM	
Incubation with DEAE SepharoseCL-6B	0	1460	550	0	0	0
Incubation with CM cellulose	2650	0	0	0	0	0

*Data based on median value of three separate determinations.

Table 4

Summary of purification of cellulase from the gut extract of *O. longicollis* by anion exchange chromatography DEAE Sepharose CL-6B[a].

Sample	Sample volume (ml)	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg of total protein)	Purification fold[c]	Recovery (%)[d]
Diluted crude gut extract (one fold)	1.5	3.468	800	230.68	1	100
Eluted fraction	3	0.280	333.33	1190.46	5.1	41.66

[a]Data represent mean values of three separate experiments. [b]One unit of cellulase activity is defined as the amount of enzyme that releases 1µmol of glucose min^{-1} at 60°C. [c]Purification fold = Specific activity of total isolated protein/Specific activity of the crude protein. [d]Recovery of activity (%) = (Total activity of isolated protein/Total activity of crude protein) x 100.

3.6. Physicochemical characterization of purified cellulase

3.6.1. The optimum reaction temperature and thermal stability

Purified cellulase with the substrate was tested in the range of 20 - 80°C. The reaction mixture showed the highest activity of 1166 U $min^{-1}mg^{-1}$ at 60°C which was determined as optimum temperature for cellulase activity. The thermal stability of the purified cellulase was tested by the pre-incubation without the substrate in the same temperature range as above. The activity was stable around 40°C to 60°C with the highest activity of 2800 U $min^{-1}mg^{-1}$ at 60°C (Fig. 10).

3.6.2. Effect of pH

The effect of the pH on the activity of purified cellulase was determined at various pH ranges from pH 4.0 to 8.0. The cellulase activity was stable in the optimum pH range between 5 and 6 (Fig. 11).

3.7. Enzyme kinetics

The effect of substrate concentration was studied by varying substrate concentrations from 0.1 - 3.0%. The eaction rate *versus* the substrate concentration curve was plotted to determine whether the enzyme follows the Michaelis-Menten kinetics and constants were determined from a Line Weaver-Burk plot. The V_{max} was obtained by plotting double reciprocal and Eadie-Hofstee plots. The K_m and V_{max} value of cellulase were 1.03 mg/ml and 343 U $min^{-1}mg^{-1}$, respectively (Fig. 12).

3.8. MALDI-TOF MS

The subjection of 47 kDa purified cellulase to MALDI-TOF MS analysis to find the relevant protein matching yielded the homology to the known cellulases. The MALDI-TOF MS fingerprints of the tryptic-digested purified cellulase with different m/*z* ratios in “lift” mode were obtained (Fig. 13). The acquired short peptide sequence “WLSGVDK” of a fragment with the *m*/*z* ratio of 804.3890, identified significant homology to endo-1, 6-beta-glucanase with 35 % of protein coverage (Fig 14A). The matched endoglucanase belonged to the cellulase family, glycosyl hydrolase 5 families (GHF5) which are also typically found in coleopterans. Hence, the various sequences of m/*z* 804.3890 were further extracted in all entries of search and aligned with coleopteran GHF5 cellulases which considerably showed the homology (Fig 14B).

Discussion

The cellulase that hydrolyses the CMC is denoted as endoglucanase as it prevents them from attacking the chain ends where it is substituted with carboxymethyl groups in place of open- ended sugar terminals. Such limitation is similar to the native cellulose present in the diet (Watanabe and Tokuda, 2001). Due to the presence of methylated hydroxyl groups in CMC facilitating water solubility, the CMC has been widely

used for the documentation of cellulase activity in assays (Willis et al., 2010a). The application of CMC in electrophoresis has its advantage over plate assays by visualizing the protein bands responsible for cellulolytic activity directly under partially denatured and low-temperature conditions (Schwarz et al., 1987). On the other hand, the crystalline part of the cellulose which is acted upon by cellobiohydrolase is usually found in hardwoods that by digestion produce the cellobiose (Yoshimura et al., 1996). The banana plant is known to have pesudostem and by the insect possessing mandibles that crush the stem into microparticles eliminates the assumption of crystalline cellulose presence and its testing in the study. The extensive study carried out with 68 phytophagous insects belonging to the 8 orders reported that gut fluids act more on CMC compared to the crystalline cellulose. Moreover, the coleopteran order displayed low levels of activity against the crystalline cellulose unlike with CMC implying the existence of phylogenetic relationships rather than the feeding habits (Oppert et al., 2010). The distribution of the cellulase and the invertebrate's diet seems to not correlate (Monk, 1976). Polysaccharide digestion by phytophagous insects is highly advantageous simply due to the increased yield of energy and nutrition from the available diet (Girard and Jouanin, 1999). The secretion of a cellulase completely by an insect is evolved independently in phyla. This gets reflected in the different types of cellulase in the divergent taxa (Liu et al., 2015). Observation of cellulase activities in insects that do not have symbiotic association; symbiont free sites like foregut and midgut; sterile hepatopancreas of gastropods altogether concluded that cellulases are secreted endogenously (Watanabe and Tokuda, 2001). The absence of cellulase gene in some important model insects argues about the critical interpretation of genome sequence and sampling artefacts (Davison and Blaxter, 2005).

To overcome this, the advent of pyrosequencing was used to unravel the presence of 167 plant cell wall degrading enzymes (PCWDE) belonging to eight different enzyme families in beetles (Pauchet et al., 2010). Willis et al. (2011) reported the first endoglucanase from an insect model organism *Tribolium castaneum*. The order coleoptera has been identified with the possession of endogenous cellulases (Martin, 1991). Over many endoglucanases were studied exclusively in beetles (Coleoptera) (Busconi et al., 2014; Calderón-Cortés et al., 2010; Lee et al., 2004, 2005; Wei et al., 2006; Xia et al., 2013). The cellulase characterized in this study showed higher activities in all the tested gut tissues with midgut being highest and hindgut being lower comparatively. The foregut and midgut are the major sites for the expression of large quantities of endogenous cellulase in the coleopteran species such as *Apriona germari*, *Phaedon cochleariae* and *Psacothea hilaris* (Wei et al., 2006). The anterior midgut structured naturally with infoldings to increase the surface area for cellulose digestion (Shelomi et al., 2014). Unlike hindgut where either bacteria and protozoan based cellulolysis occurs, the midgut majorly holds up one type of endo-β-1,4-glucanase (Tokuda et al., 1997, 1999). The cellulase expression was determined throughout the entire digestive tract of the cricket including hindgut in *Teleogryllus emma* (Kim et al., 2008). Endoglucanase expression was high in head fluids than the gut tissue in *T. castaneum* (Willis et al., 2011). In *Dissosteira carolina*, it is mainly localised in the foregut and midgut regions (Willis et al., 2010b). The highest cellulase activity for a Zygentoma was found to be in the head and foregut tissues (Pothula et al., 2019). In termites, it was seen with salivary glands and midgut (Tokuda et al., 1997). The foregut has 98% predominant activity in the wood-eating cockroach *P. cribrata* (Scrivener et al., 1989).

The highest crude cellulase activity in this study was observed to be 2858 U $min^{-1}mg^{-1}$ in the midgut. To date, in insect, the highest crude enzymatic activity ever reported in Zygentoma species was around 2500 (Units/g) proposing it as an insect model for high cellulase activity (Pothula et al., 2019). *O. longicollis* showing similar higher cellulase activity could also be envisioned as a model for it.

Only a mere number of bacteria were seemed to harbour the entire digestive tract of *O. longicollis*. The gut regions displaying cellulolytic activity in Orthoptera and Phasmotodea had low numbers of bacteria (Cazemier et al., 1997). The gut tissues used in this study for further assays were extracted with aseptic measures such as the use of antibiotic and sodium azide. No bacterial growth was observed in plates with these extracts. Aseptic conditions were maintained in the rearing of insects and removal of the gut to assure the elimination of cross- contamination of symbiont's cellulase in earlier studies. The activity was not affected even after the removal of bacteria (Lasker and Giese, 1956; Lo et al., 2010; Scrivener et al., 1989; Tokuda and Watanabe, 2007; Yokoe and Yasumasu, 1964). Attempts to isolate cellulose-degrading bacteria seemed to be unsuccessful or drawn mixed success in termites (O'Brein and Slaytor, 1982). The isolated gut bacteria's cellulase activity in the present study was extremely below par and negligible when compared to the gut extract activity implying a total differential pattern distinguishing endogenous from its counterpart. Nakashima et al. (2002) and Tokuda et al. (2007) in the wood-feeding termite have proven the existence of a dual cellulose-digesting system where both endogenous and symbiotic cellulase contributes. As in this study, the contribution of cellulolytic digestion from symbionts seemed to be trace as mentioned. A study on a range of digestive enzymes from a phytophagous beetle *P. cochleariae* encountered with the production of cellulases by microorganisms only in minor fractions suggesting that endogenous cellulase played a major role in PCW degradation (Girard and Jouanin, 1999). Due to the reason that hindgut is highly unlikely to produce the endogenous cellulase as it does not possess the secreting cells, we cannot be conclusive about the cellulolytic function from it (Chapman, 1998). With head fluid, foregut, midgut showing the enormous amount of cellulolytic activity in *O. longicollis*, we presume that a secretion out of enzyme to the hindgut from the upper digestive tract could be a reason that latter showing some activity. At times, the endoglucanases present in the salivary glands or upper digestive tract are presumed to be secreted in the digestive tract and reaching the hindgut where the latter shows the cellulase activity (Kim et al., 2008; Nakashima and Azuma, 2000).

Ion exchange chromatography (IEC) was used to purify cellulase ever since either solely or with a combination of other strategies (Eriksen and Goksøyr, 1977; Umezurike, 1979). Anion exchange chromatography (AEC) was adopted to purify EG from the mulberry longicorn beetle, *A. germari*, *T. castaneum* (Lee et al., 2004; Rehman et al., 2009) and glycosidases from palm weevil *Rhynchophorus palmarum* (Yapi et al., 2009, 2015). AEC was used for purifications of several other endoglucanases and glycosidases from insects, other invertebrates and even insect gut flagellates (Li et al., 2003, 2005; Marana et al., 2000; Scrivener and Slaytor, 1994; Willis et al., 2010b; Yang et al., 2004). The molecular weight of a purified cellulase in this study using AEC was determined as 47 kDa. The molecular weights of cellulase from Coleoptera were determined around 47 kDa, 51 kDa, 25 kDa, 55 kDa, 33 kDa, 29 kDa (Lee et al., 2004; Mei et al., 2016; Rehman et al., 2009; Sugimura et al., 2003; Wei et al., 2006; Willis et al.,

2011; Xia et al., 2013); of other insects around 47-55 kDa (Kim et al., 2008; Scrivener and Slaytor, 1994; Willis et al., 2010b) and in other invertebrates (nematodes and gastropods) around 44 to 50 kDa (Anzai et al., 1984, 1988; Li et al., 2005; Pothula et al., 2019; Smant et al., 1998). The specific activity of purified cellulase from *O. longicollis* is 1190.46 U/mg. On comparison with coleopterans and other insect order, the specific activities of EGs seemed around 12.11 U/mg, 992 U/mg, 812 U/mg, 928 U/mg, 1030.87 U/mg, and 1037 U/mg (Lee et al., 2004, 2005; Mei et al., 2016; Wei et al., 2006; Xia et al., 2013; Yang et al., 2004). The specific activity of multiple cellulases from other invertebrate were 7.60, 6.67, 6.45, 0.30 U/mg (Yin et al., 2011).

The influence of divalent cations on cellulase activity yielded differential patterns without the proportionality in terms of activity. Cellulase from the beetle *Batocera horsfieldi* has also shown similar kinds of results where ions and its concentration produced diverse effects on the activity (Mei et al., 2016) and in some cases, no influence occurred (Chararas et al., 1983). Although, cellulase activity from red palm weevil, *Rhynchophorus ferrugineus* was enhanced by the application of Ca^{2+} and Mg^{2+} which is quite similar to this study (Vatanparast et al., 2014). The optimum temperature and thermal stability of a cellulase from *O. longicollis* were 60°C with optimal pH between 5 and 6. Many insect and invertebrate cellulases so far reported have been stable around 40-50°C with optimal pH from 4 to 6.5 (Kim et al., 2008; Mei et al., 2016; Xia et al., 2013; Yin et al., 2011). But in some cases, high stability was observed up to 55-60°C (Lee et al., 2004, 2005; Wei et al., 2006) with at certain instances even after 24 h of incubation (Li et al., 2005) and in alkaline pH (Willis et al., 2011). The optimal pH 5-6 determined in this study is close to the gut luminal pH of *O. longicollis* such as 6.0-6.4 in the foregut, 6.8-6.9 in the midgut, and 6.1-6.4 in the hindgut (Singh and Prasad, 2010). These physiological pH values which are of the same in experimental conditions are necessary for the function of cellulase (Sugimura et al., 2003). With the favourable pH in hindgut of *O. longicollis*, it implies that the cellulase having its stability range between pH 5 and 6 could effectively exhibit activity in hindgut as well.

The K_m and V_{max} value of cellulase in *O. longicollis* were 1.03 mg/ml and 343 U/min/mg, respectively. The K_m and V_{max} values of cricket cellulase were 5.4 mg/ml and 3118.4 U/mg (Kim et al., 2008). The K_m and V_{max} values of EG1 from Australian wood cockroach *P. cribrata* are 9.4 mg/ml and 22.2 mg reducing sugar/min/mg protein, respectively. Correspondingly, the values for EG2 were 6.8 and 88.3l (Scrivener and Slaytor, 1994). The K_m values of two enzymes from a coleopteran insect were 2.55×10^{-3}M and 1.6×10^{-3}M, respectively (Chararas et al., 1983).

The partial peptide sequences obtained from the mass spectrometric analysis identified that the purified cellulase exhibited homology to endoglucanase belonging to the GHF5 family of cellulase. The first report of a GHF5 (a 47 kDa endo-β-1,4-glucanase) from arthropods was from a Coleopteran insect, *P. hilaris* (Sugimura et al., 2003). Endoglucanases belonging to the GHF5 have also been reported in coleopterans such as *A. germari* (47 kDa) and *Mesosa myops* (Liu et al., 2015; Wei et al., 2006). GHF5 in coleopterans lies in a separate clade where nematode and beetle cellulase resides in a way proposing that GHF5 could have diverged between prokaryotic and early eukaryotic cellulases (Calderón-Cortés et al., 2010; Kyndt et

al., 2008). Cellulase sequences of nematode and longicorn beetle show considerable similarity in phylogeny analysis (Lo et al., 2003). Insect GHF5 and GHF45 belong to very ancient gene families and specifically GHF5 is likely to be evolved from a common ancestor rather than through horizontal gene transfer (Watanabe and Tokuda, 2010).

Industrially used cellulolytic enzymes must be stable in bioreactors by withstanding heat and acidic conditions (Willis et al., 2010a). The physicochemical parameters of cellulase from *O. longicollis* infer that it is a highly stable enzyme. On the other hand, a study in assessing these digestive enzymes in coleopteran pests to assort them as potential inhibiting targets was also been in the line of interest (Girard and Jouanin, 1999). Apart from these commercial aspects, the role of endogenous cellulase in insects could also unearth new possible functions. The substitutions in cellulase sequence in deuterostomes might result in gaining new function such as like chitinases involving in innate immunity, no wonder that termites express the cellulases in many organs might open up the new hypothesis (Davison and Blaxter, 2005). The new findings of multifunctional cellulases in insects like xyloglucanolytic cellulase and a specific ancestral cellulase that lacks cellulolytic ability in almost all the insect orders necessitate the critical investigation with expansion of enzyme hypothesis (Shelomi et al., 2020). These divergent approaches to the insect cellulases indicate its multifaceted potentialities that hold a strong stand in research pursuits.

Conclusions

The finding of this study evident the presence of a stable endogenous cellulase belonging to the GHF5 family with predominant activity over an exogenous and thereby signifies the independent mode of cellulose digestion in the gut system of *O. longicollis.* Moreover, if characterised in the perspective of commerciality in the foreseeable future, this cellulase would possess potential in application prospects.

Declarations

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CRediT authorship contribution statement

Sreeramulu Bhuvaragavan: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Funding acquisition. **Thenozhiyil**

Reshma: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Karuppiah Hilda:** Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - review & editing. **Ravichandran Balaji:** Methodology, Software, Formal analysis, Data curation. **Mani Meenakumari:** Investigation, Data curation. **Narayanasamy Mathivanan:** Resources, Supervision. **Sundaram Janarthanan:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of interest

The authors declare no conflict of interests.

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Figures

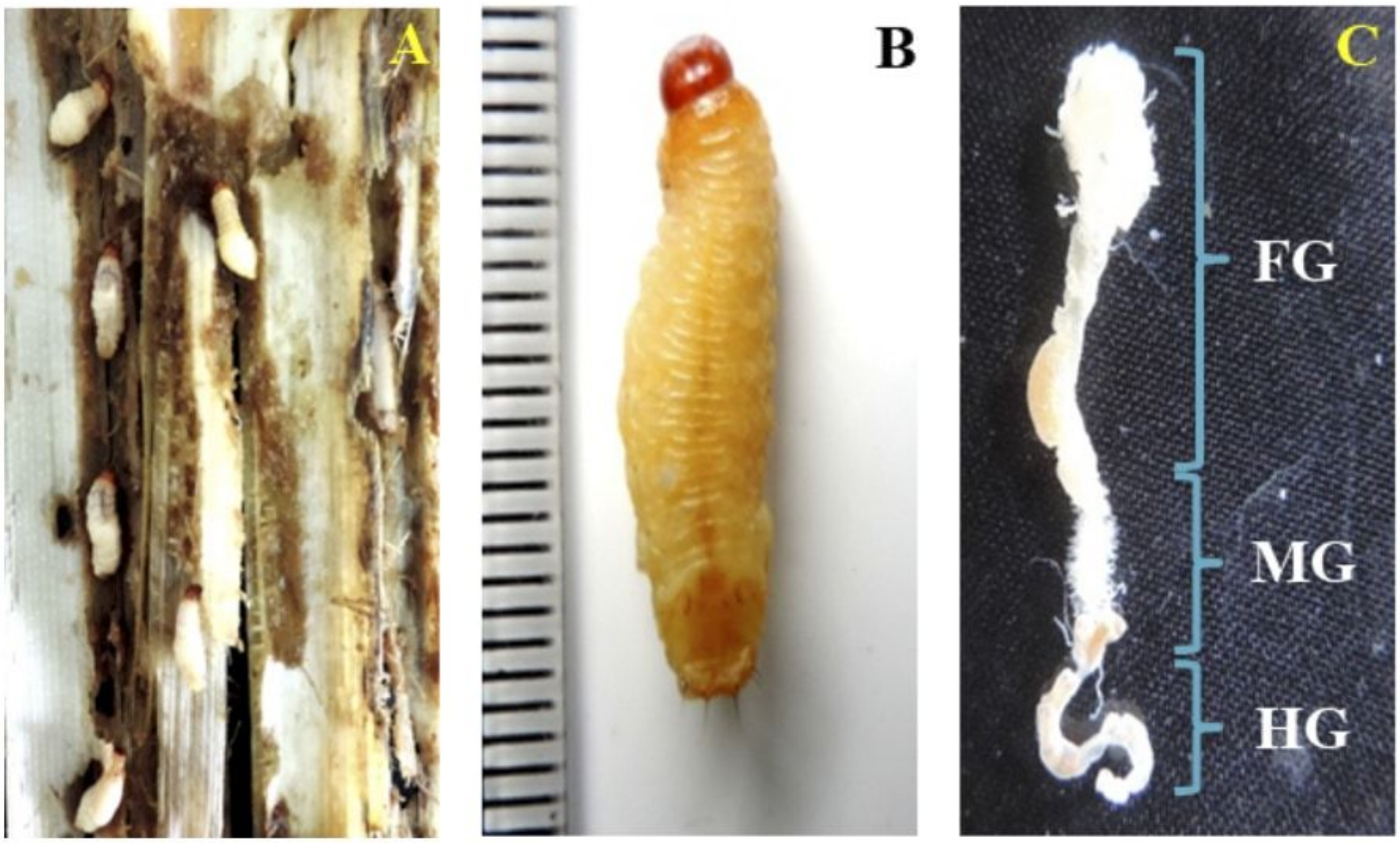


Figure 1

Collection and dissection of grub of O. longicollis. A, Grub infestation on the banana pesudostem. B, Grub and C, Alimentary canal (FG-Foregut, MG-Midgut and HG-Hindgut).

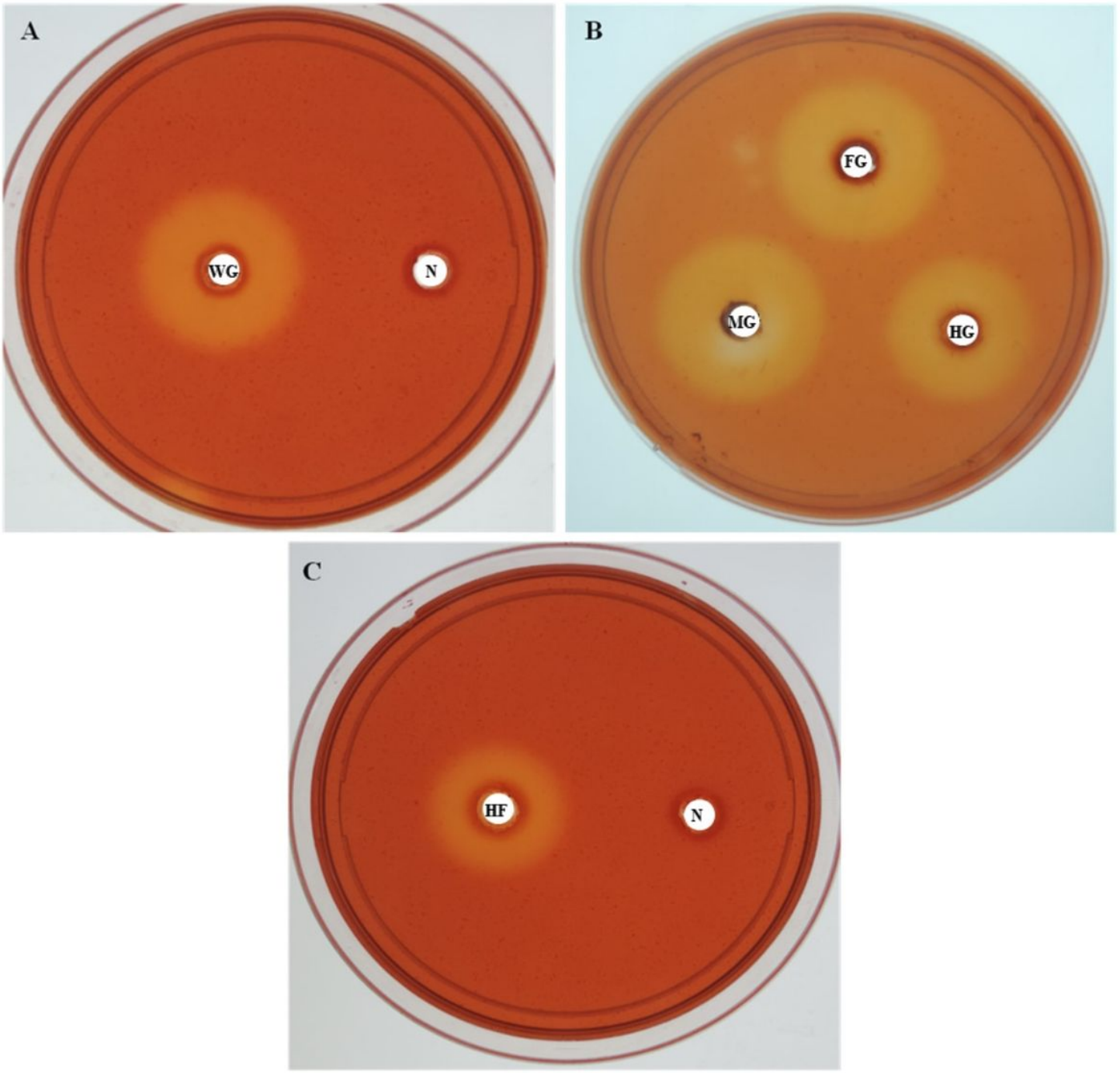


Figure 2

Congo red plate assay for zone of cellulase activity from the gut of O. longicollis grub. A, Zone of activity for the whole gut extract (WG). B, Zone of activity for the extracts of foregut (FG), midgut (MG) and hindgut (HG). C, Zone of activity for the extract of head fluid (HF) and N denotes negative control.

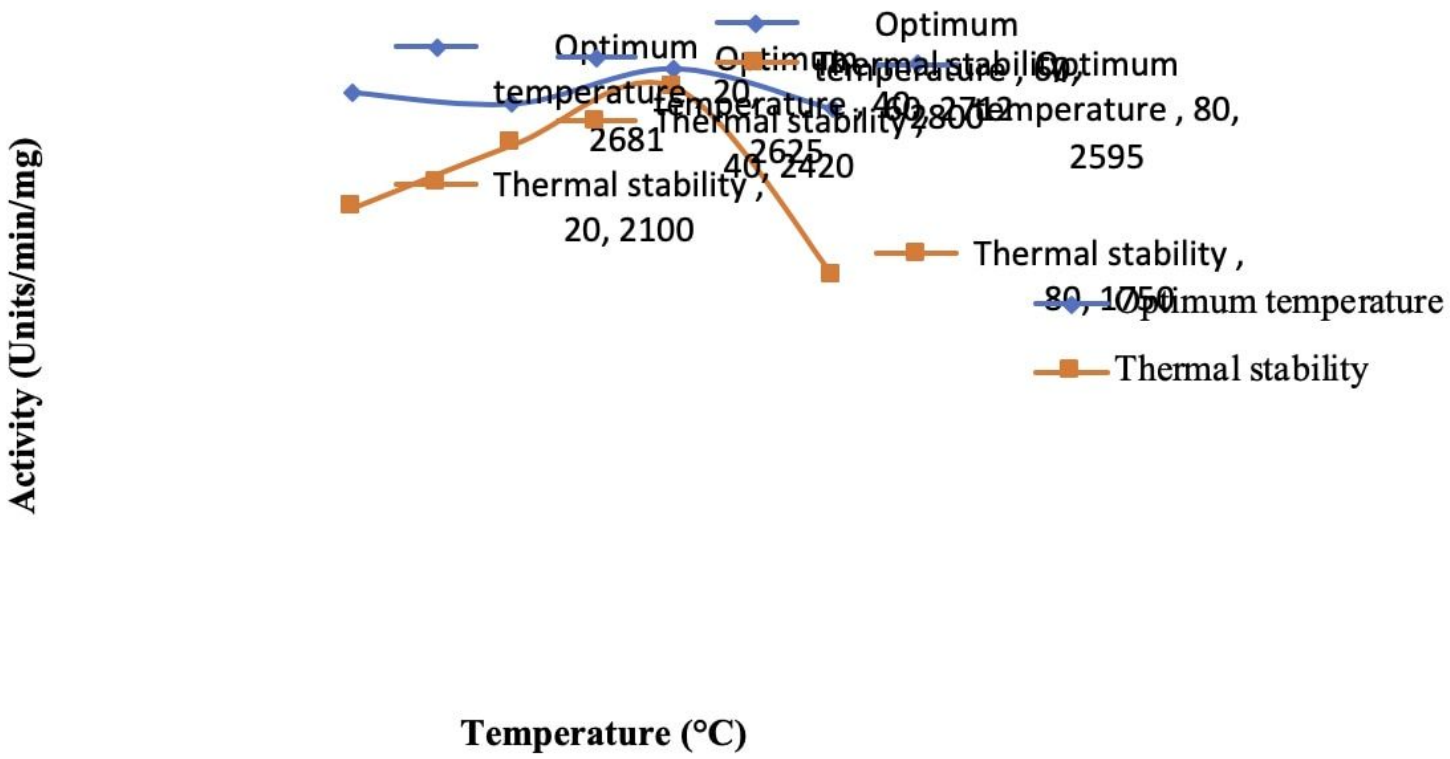


Figure 3

Optimum temperature and thermal stability of cellulase activity from whole gut extract of O. longicollis grub.

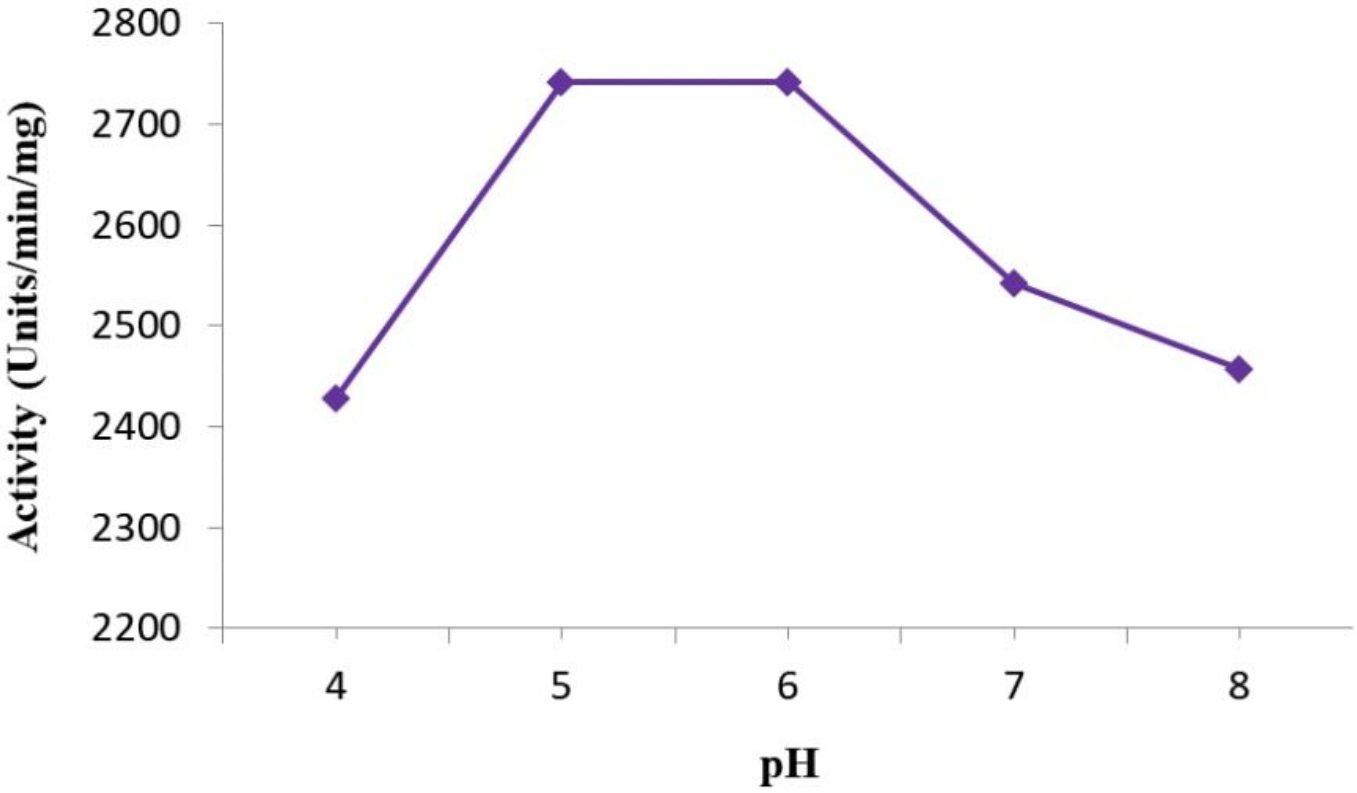
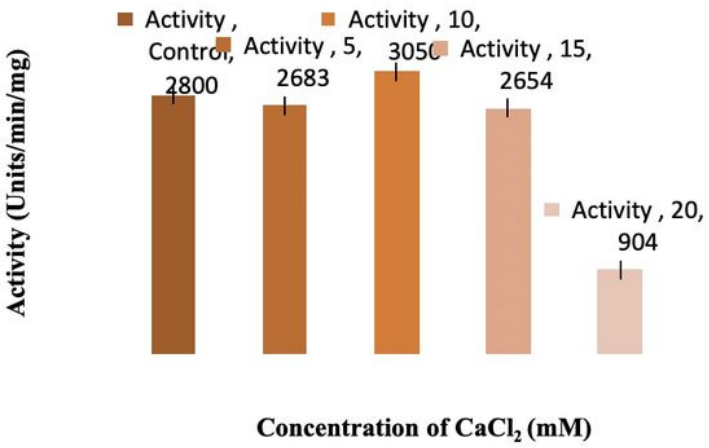
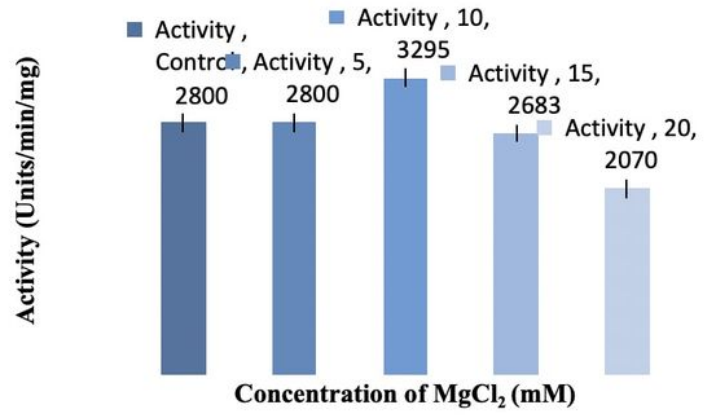


Figure 4

Optimal pH of cellulase activity from whole gut extract of O. longicollis grub.





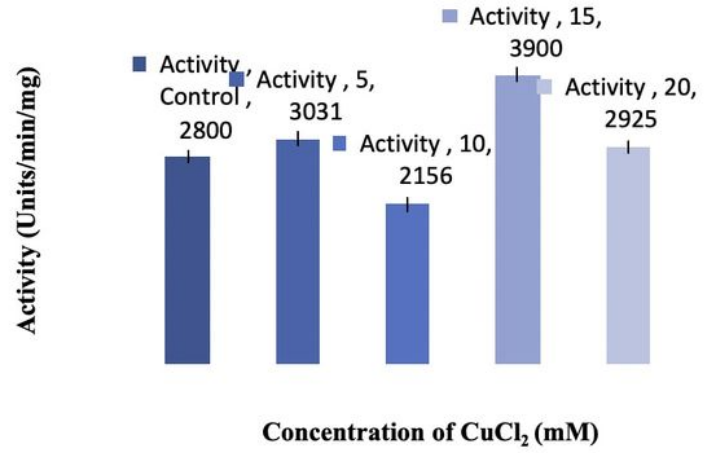


Figure 5

Effect of Ca2+, Mg2+ and Cu2+ on the cellulase activity of whole gut extract from O. longicollis grub.

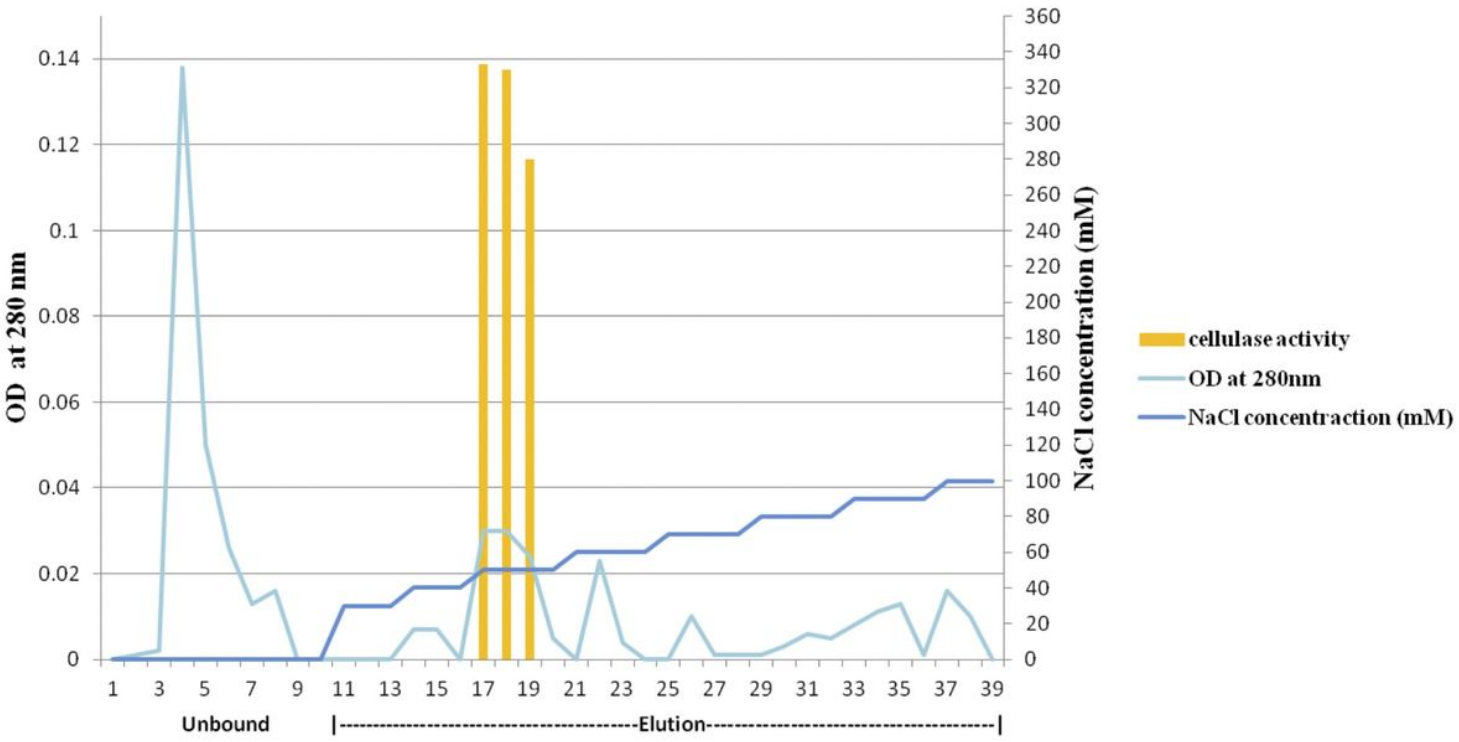


Figure 6

Elution profile of purified fractions of cellulase from the whole gut extract of O. longicollis by anion exchange chromatography using DEAE Sepharose CL-6B as column matrix.

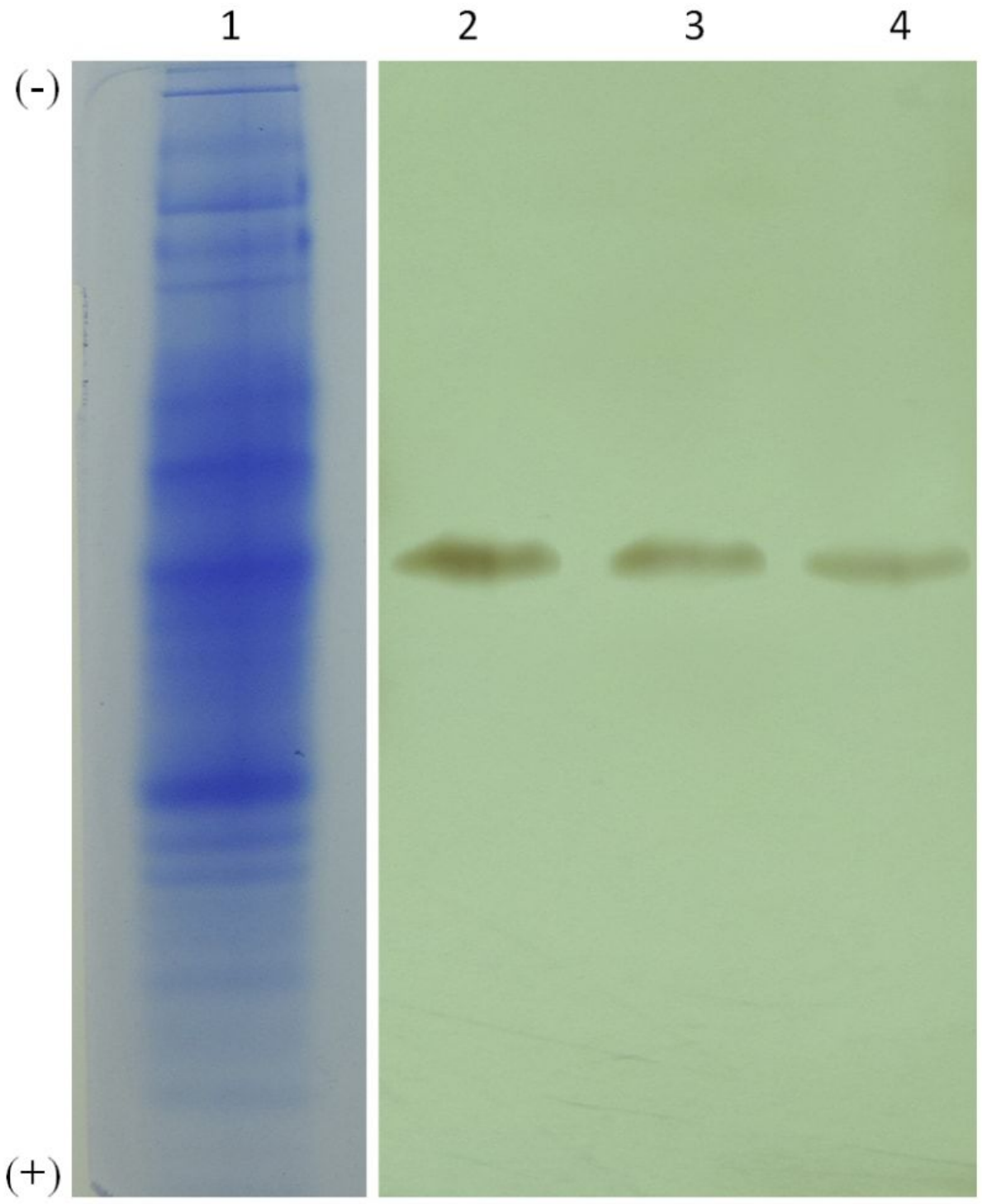


Figure 7

Native-PAGE (8 %) profile of the whole gut extract and purified cellulase enzyme from the grub of O. longicollis. Lane 1, Whole gut extract (28.9 µg) - CBB staining. Lane 2, Fraction 1 (50 mM NaCl) of purified cellulose (5 µg) - silver staining. Lane 3, Fraction 2 (50mM NaCl) of purified cellulase (4.8 µg) - silver staining and Lane 4, Fraction 3 (50mM NaCl) of purified cellulase (4.36 µg) - silver staining.

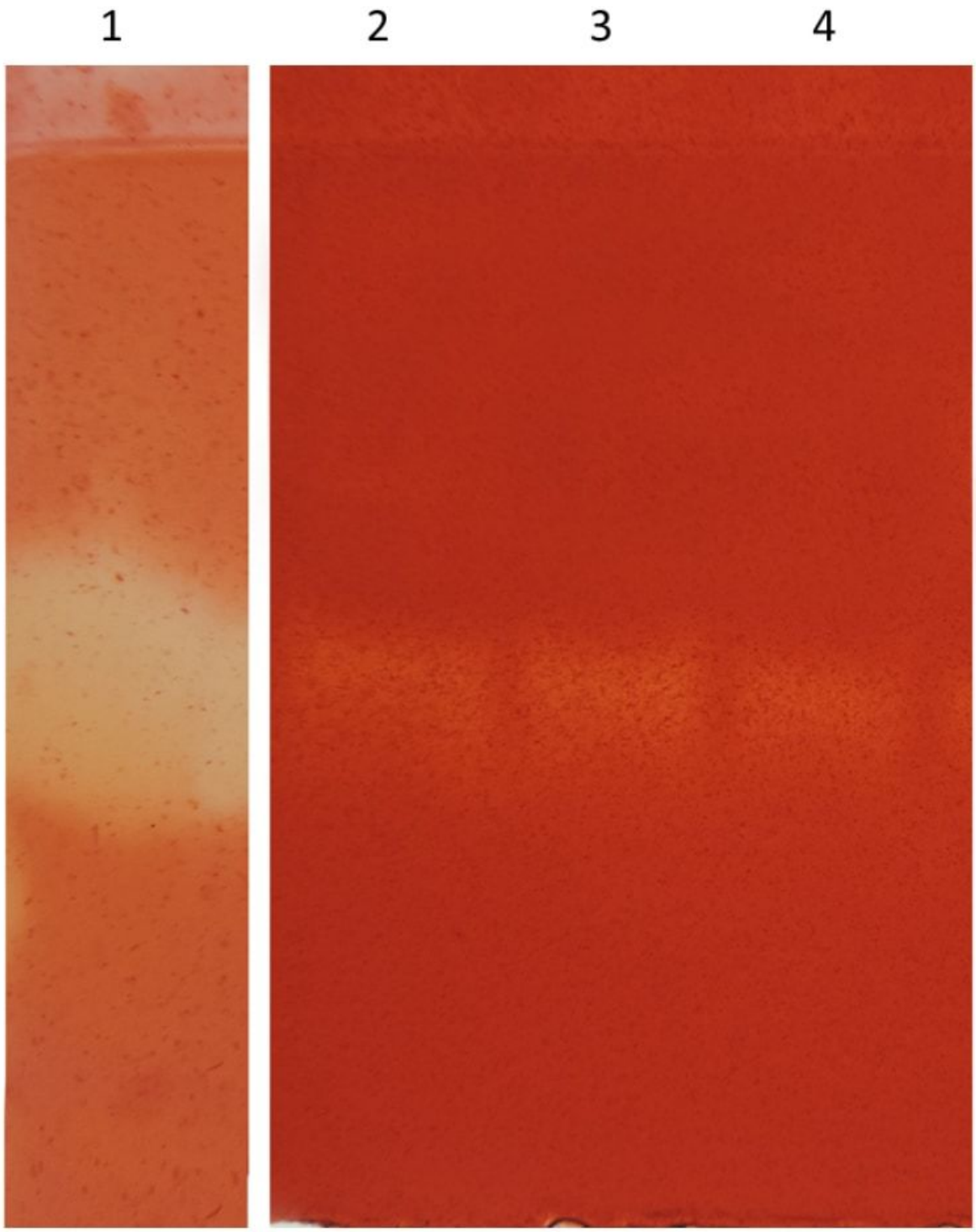


Figure 8

Zymogram (12% with 0.5% CMC) of whole gut extract and purified cellulase fractions from grub of O. longicollis with congo red staining. Lane 1, Whole gut extract (28.9 µg). Lane 2, Fraction 1 (50mM NaCl) of purified cellulase (5 µg). Lane 3, Fraction 2 (50mM NaCl) of purified cellulase (4.8 µg) and Lane 4, Fraction 3 (50mM NaCl) of purified cellulase (4.36 µg).

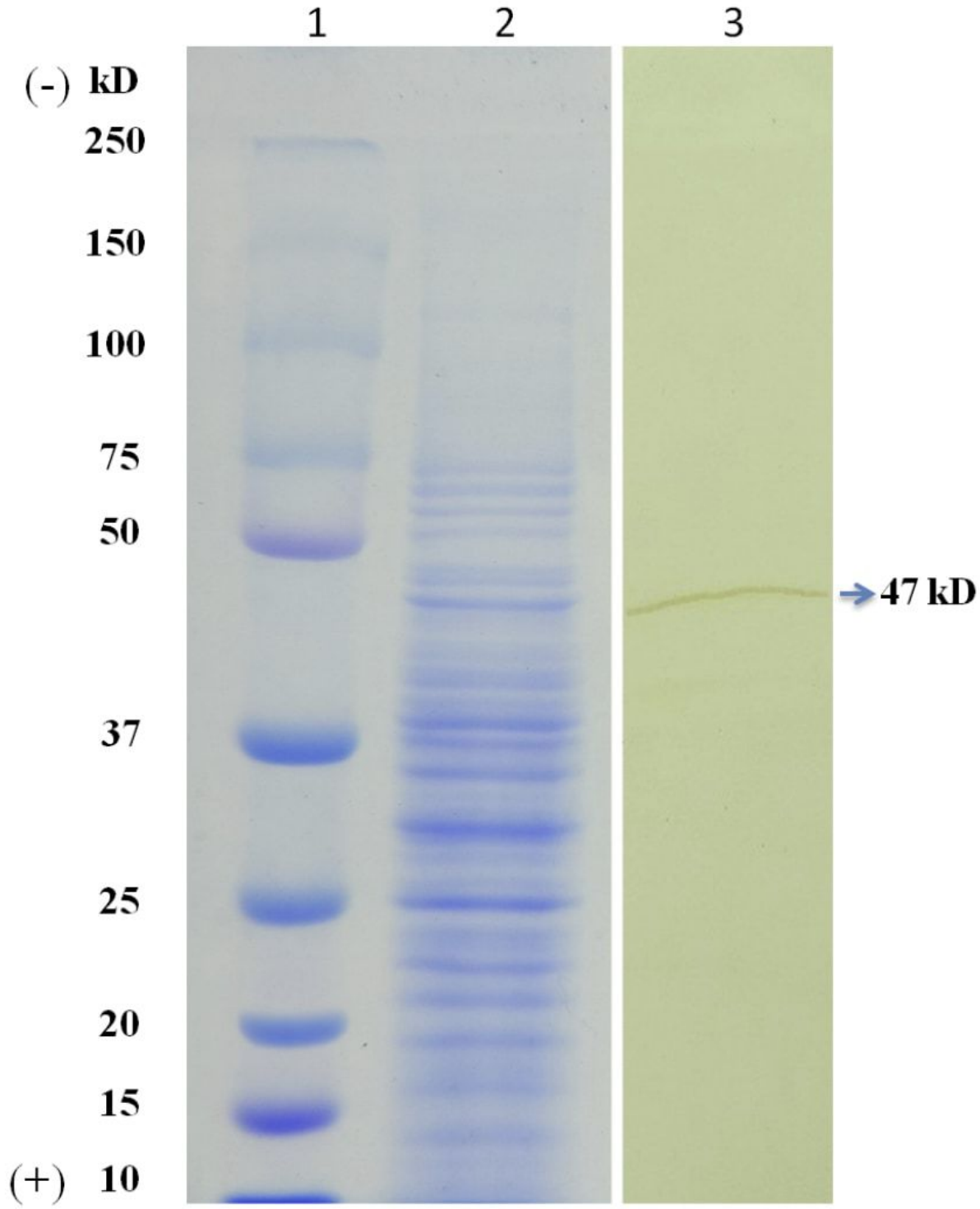


Figure 9

SDS-PAGE (12%) profile of the whole gut extract and purified cellulase enzyme from the grub of O. longicollis. Lane 1, Protein standard molecular weight (kDa) marker. Lane 2, Whole gut extract of O. longicollis (28.9 µg) - CBB staining and Lane 3, Purified cellulase enzyme (5 µg) - silver staining.

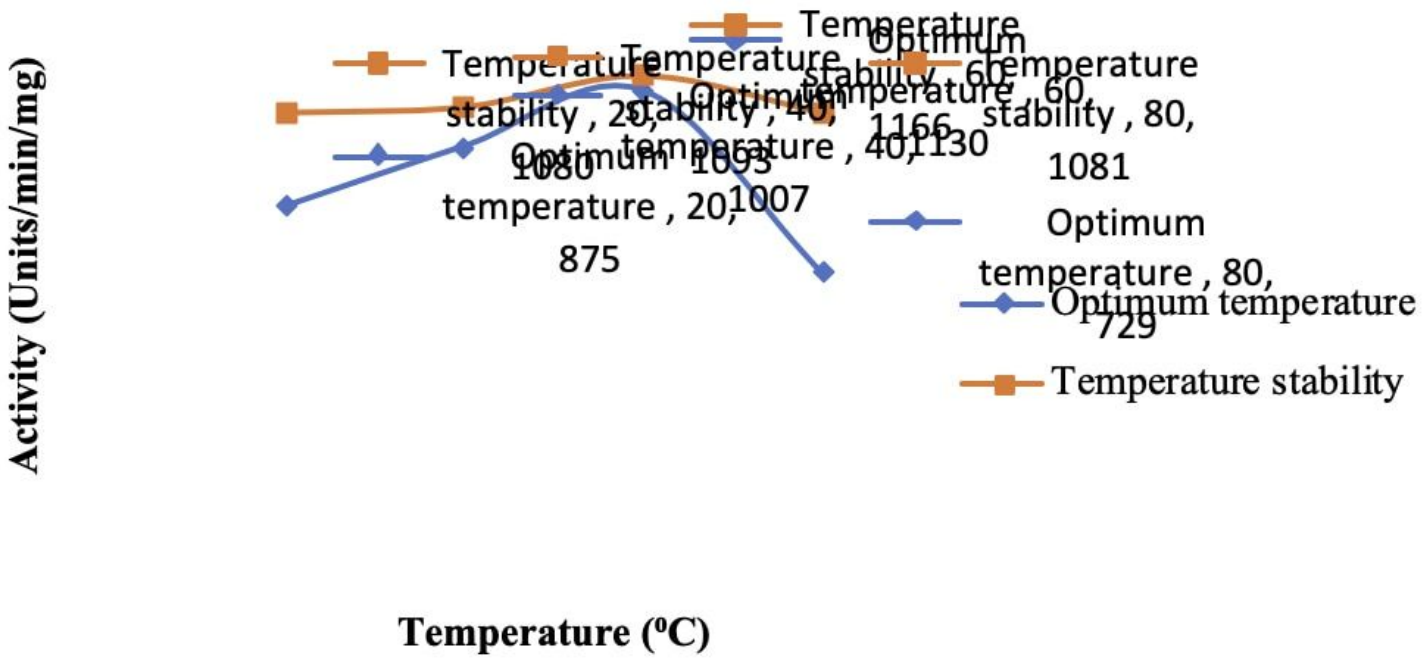


Figure 10

Optimum temperature and thermal stability of cellulase activity from purified cellulase of O. longicollis grub.

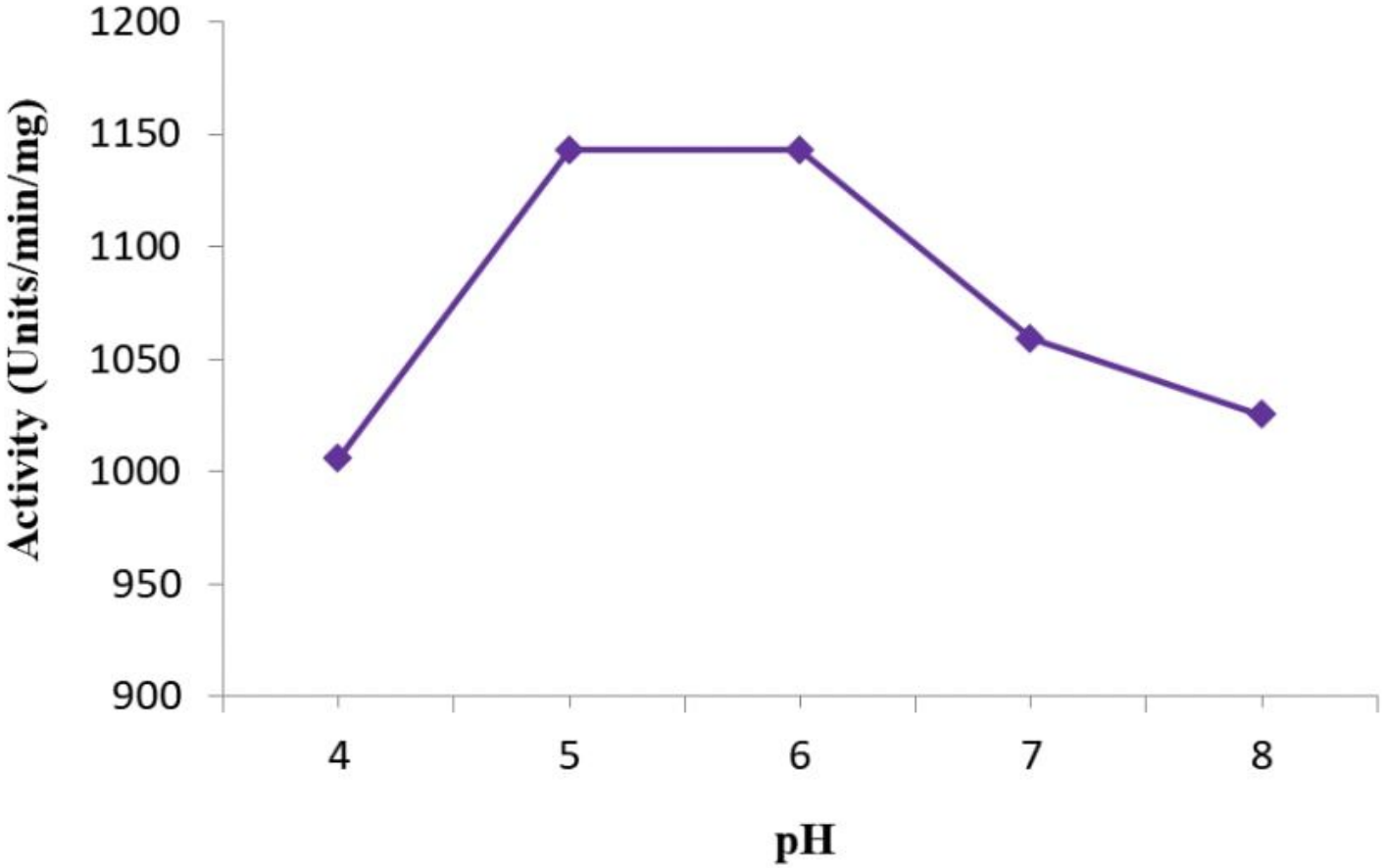


Figure 11

Optimal pH of cellulase activity from purified cellulase of O. longicollis grub.

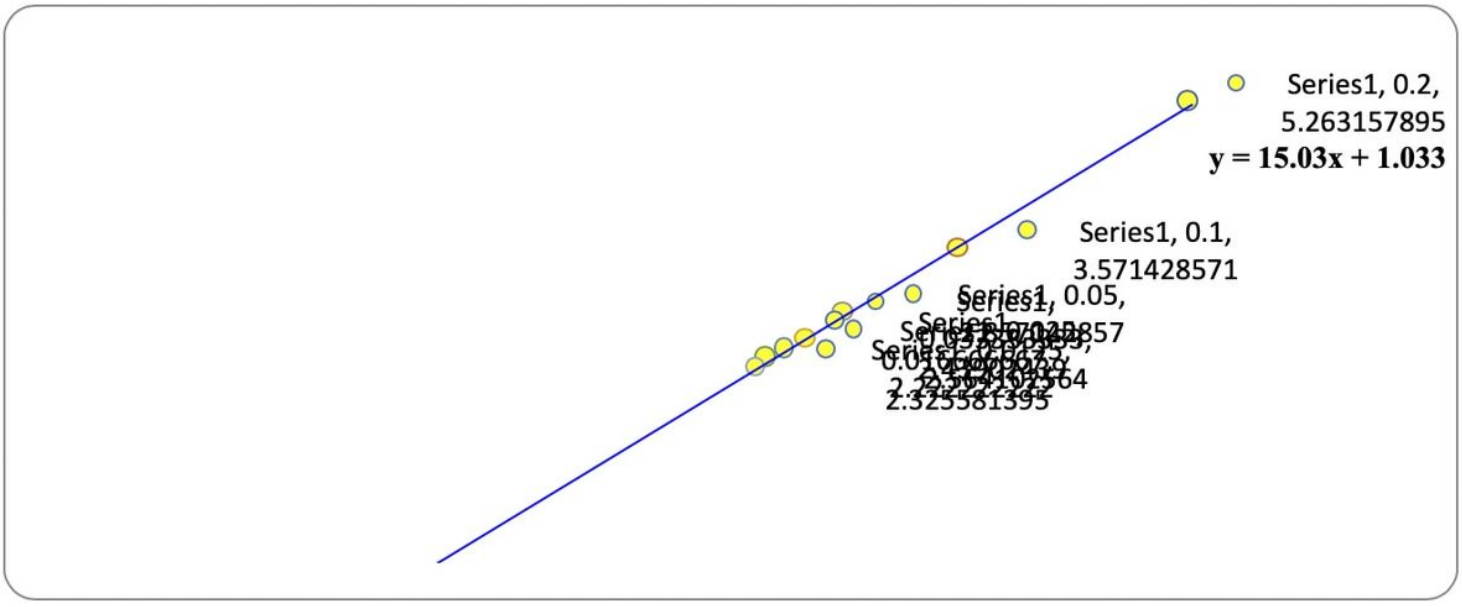


Figure 12

Enzyme kinetics of purified cellulase using Michaelis Menten kinetics and Line Weaver-Burk plot.

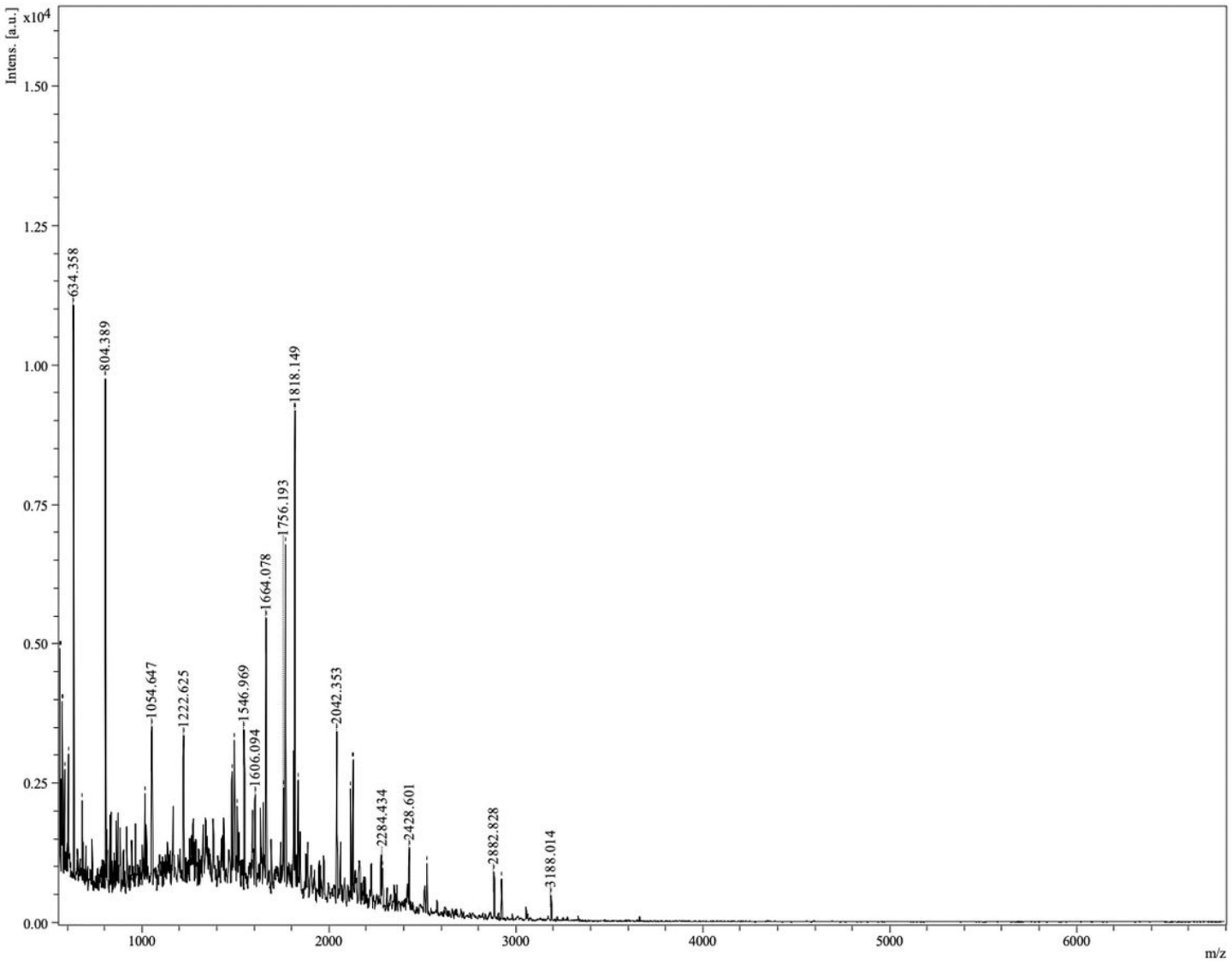


Figure 13

MALDI-TOF-MS fingerprint of purified cellulase from the grub of O. longicollis.

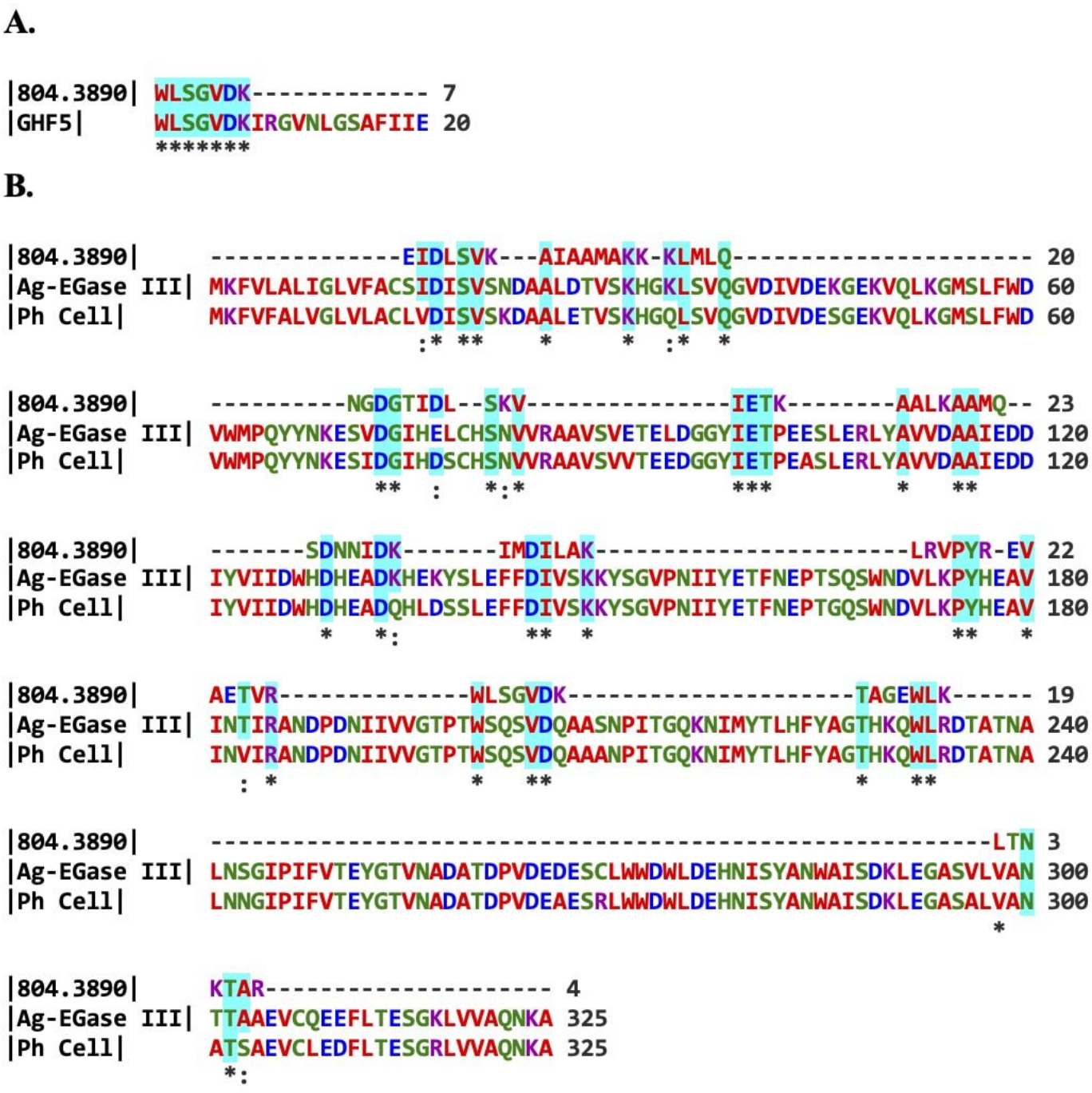


Figure 14

A. Sequence alignment of the amino acid sequences of GHF5 and short peptide sequence for m/z 804.3890 (WLSGVDK) obtained using mass spectrometry of purified cellulase. B. Sequence alignment of amino acid sequences of GHF5 from Coleopteran insects Apriona germari and Psacothea hilaris with short peptide sequences for m/z 804.3890 obtained using mass spectrometry of purified cellulase (* - Amino acid in that column was identical in all sequences, : - Conserved substitutions).