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Localization of cellulolytic system and *de novo* transcriptome analysis of whole larval gut of *Zophobas morio* (Coleoptera: Tenebrionidae) disclosed the existence of glycosyl hydrolase family-9

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The present study was aimed at localizing the cellulolytic system in the larval gut of *Zophobas morio*. Quantitative analysis revealed maximum cellulolytic activity in the midgut followed by foregut and hindgut. Additionally, examination of physicochemical characterization showed the highest endogenous cellulase activity of 2250.32 U/mg at 40°C and the enzyme was stable between pH 5 and 6. Distinct patterns of influence of divalent cations were also found. *De novo* transcriptome analysis of whole gut envisaged the prevalence of the endogenously transcribed cellulase gene. The assembled transcriptome identified the existence of an isoform of endoglucanase with significant resemblance to glycosyl hydrolase family-9 (GH9). Twenty-seven other members of GH family were also revealed in the study. Consequently, the existence of independent cellulolytic system with the larvae possessing its own cellulolytic gene belonging to the GH9 as revealed by the transcriptome makes them an optimum resource for bioenergy research.

Keywords: Cellulolytic system, GH9, Illumina, Transcriptome, *Zophobas morio*

Insects are considered to be the most fascinating creatures that have evolved to live in most habitats successfully surviving many environmental upheavals. Their success can be attributed partly to their ability to fly, colonise in new habitats and adaptations to subsist on huge range of foods. Of particular interest are those insect species that feed on plant biomass. The so called phytophagous and xylophagous insects are identified as the most effective lignocellulose digesters owing to their ability to efficiently degrade cellulose to produce glucose that can act as an energy source[1-3]. For instance, the higher-termites and scarabs can recycle up to 65% of the ingested cellulose[4,5]. Indeed, the discovery of the first endogenous cellulase in the termite *Reticulitermes speratus* shed light on the endogenous mode of cellulolysis and the perception that animals cannot produce their own cellulase and relied on endosymbionts fizzled out[6]. Following this, cellulases of endogenous nature were reported in different orders, including Blattaria, Coleoptera and Orthoptera[7-11]. The existence of numerous putative cellulase genes including four main glycosyl hydrolase families (GHF) such as GHF1, GHF5, GHF9 and GHF45 with glycosyl hydrolase family 9 (GH9) ubiquitously distributed in all the insect orders was also affirmed by the insect transcriptome sequencing data[12-14].

Zophobas morio is a species of darkling beetle which intensively feeds on porridge oat or bran of stored cereals that are a copious source of lignocellulose. This makes them an excellent model to explore the nature of cellulolysis. It is hypothesized in the present investigation that, does *Z. morio* grub possesses independent mode of cellulose digestion in the gut system? Accordingly, the study is aimed at (i) localizing cellulolysis in the larval gut of *Z. morio* and (ii) *De novo* transcriptome-based identification of endogenous cellulase transcripts in the whole larval gut of *Z. morio.* This is significant in supporting the prevalence of endogenous nature of cellulolysis in the gut of the insect which perhaps makes them an optimum candidate to prospect for the novel cellulolytic enzyme.

Materials and Methods

Collection and identification of insects

Zophobas morio is a species of darkling beetle whose larvae are generally identified as superworms. The grubs of *Z. morio* were maintained under laboratory conditions using the standard diet

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consisting bran of stored cereals. Further, they were identified using standard taxonomic keys.

Isolation of gut and extraction of cellulases

In order to ascertain the cellulolytic activity, all the processes of enzyme extraction were performed under sterile conditions. After wiping the collected larvae with ethanol, the whole gut, foregut, mid gut and hindgut were separately dissected out in 0.9% physiological saline solution containing 0.02% sodium azide (NaN_3) (Fig. 1). The gut contents and food particles were completely eliminated from the intestinal tract. About 100 mg of the dissected gut was extracted with equal volume of sodium acetate buffer (pH 5.5) using a mortar and pestle followed by centrifugation at 12000 x *g* for 20 min at 4°C. The collected supernatant was stored at -20°C and used for further assays.

Quantification of cellulase activity from different regions of larval gut of *Z. morio*

Gut extract prepared was mixed separately with equal volume of 1% carboxymethyl cellulose (CMC) in sodium acetate buffer, pH 5.5 and incubated for 1 h at 60°C. After that, equal volume of 3, 5-dinitrosalicylic acid (DNS) reagent was added to it. The reaction was stopped by incubating the mixture at 90°C for 10-15 min. The formation of dark brown colour indicated the amount of reducing sugars produced in these reactions and was measured with spectrophotometer at 540 nm[15].

Physicochemical characterization of cellulase activity

Effect of temperature on cellulase activity

Determination of thermal stability was done by pre-incubating the gut extract of the larvae at various temperatures ranging from 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 60 min at 60°C. The cellulase activity was then measured using DNS method.

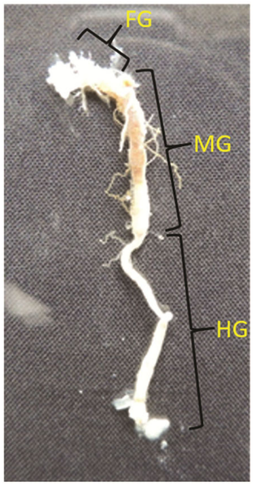


Fig. 1 — Larval gut of *Z. morio* showing FG-Foregut, MG-Midgut, HG- Hindgut

Effect of pH on cellulase activity

The optimum pH for cellulase activity was determined by mixing the gut extract of larvae with 1% CMC in buffers of different pH (4.0 - 8.0), followed by incubation at 60°C for 60 min. The cellulase activity was then measured using DNS method.

Effect of divalent cations on cellulase activity

To determine the effect of different divalent cations on cellulase activity, different cations including $CaCl_2$, $MgCl_2$ and $CuCl_2$ at different concentrations of 5, 10, 15, 20 mM were used by incubating with the gut extract. These were then incubated with 1% CMC assay buffer (0.1 M sodium acetate buffer, pH 5.5) and incubated at 60°C for 60 min. The resultant cellulase activity was then measured using DNS method.

Total RNA extraction and library construction

The whole gut of *Z. morio* was isolated in physiological buffer (12 mM NaCl, 1.3 mM Na_2PO_4, 1.2 mM KH_2PO_4). Following this, total RNA was extracted by Trizol Method. The quality of extracted RNA was checked on Qubit Flurometer (ThermoFisher #Q33238) using RNA HS assay kit (ThermoFisher #Q32851) following manufacturer's protocol. The purity of extraction and concentration of the RNA samples were measured on Nanodrop 1000. The RNA sample was then used to construct the sequencing library. Final libraries were quantified using Qubit 4.0 Fluorometer (ThermoFisher #Q33238) using DNA HS assay kit (ThermoFisher #Q32851) following manufacturer's protocol. The insert size of the library was also identified.

Illumina sequencing, *De novo* assembly and annotation

Competent libraries prepared were sequenced using Illumina NovaSeq 6000 system. Quality assessment of the raw Fastq reads of the sample was performed using FastQC v.0.11.9[16-18]. The reads were then *de novo* assembled using Trinity software. The Trinity assembled transcript FASTA file was subjected to the Diamond BLASTX v 2.0.6[19] against NCBI's non-redundant protein database (NRDB). The sequences that had blast hit were extracted and subjected to the

Evidential Gene tr2aacds pipeline[20] to generate a final assembly containing an NR unique sequence (uni-transcript) dataset. The okayset.mrna was subjected to the Diamond BLASTX v.2.0.6[19] against NCBI's non-redundant protein database (NRDB). Further, the assemblies generated in each step were subjected to the BUSCO v 2.0.6[20]. Quantitative assessment of completeness of the genome and quality assessment of the assembly contig fasta were carried out using Quast v5.0.2[21]. The final okayset.aa was taken for alignment against various databases using Diamond BLASTP v.2.0.6 and later subjected to Blast2GO for functional annotation[22]. The various steps involved in the *de novo* transcriptomics of whole gut of *Z. morio* were summarized (Fig. 2).

Identification of endoglucanase transcripts, prediction of functional domains, *in silico* modelling and molecular docking and identification of other members of glycosyl hydrolase family

A homology model was built based on the template with PDB ID 1KS8 using Modeller 10.1 by the satisfaction of spatial restraints[23]. The modelled structure was further validated using Protein Structure Validation Server. The model so generated was then used for molecular docking with the substrates CMC and microcrystalline cellulose using Autodock vina, PyRx interface[24]. The structures of the ligands were retrieved from PUBCHEM database. The energy of the ligands was minimized prior to docking studies using OpenBabel[25] in PyRx. In addition to this, the functional domains in the sequence of the transcript were identified using Conserved Domain search (NCBI)[26] and InterProScan[27]. The other members belonging to the glycosyl hydrolase family were also identified using CAZy database (Carbohydrate-Active enZYmes Database). This database describes the families of enzymes that are involved in degradation, modification and creation of glycosidic bonds and classifies into at least 108 different families[28].

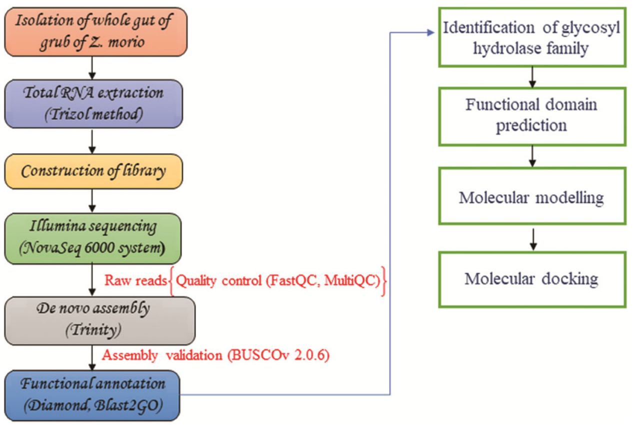


Fig. 2 — Various steps involved in *de novo* whole transcriptome analysis workflow

Statistical analysis

The experiments were conducted in triplicate, and the mean and standard error of the mean (X ± SE) were determined using the data (Sokal & Rohlf, 1973). The differences in mean data between the experimental groups were statistically analysed using student's t test.

Results

Quantitative analysis of cellulase activity in the different regions of the gut of *Z. morio* larvae

Using DNS method, the quantitative analysis of cellulase enzyme activity from different regions of the gut including foregut, midgut, hindgut of the larvae of *Z. morio* was performed. An activity of 1133.33 U/mg, 1668.00 U/mg and 1350.98 U/mg was shown by foregut, midgut and hindgut, respectively (Fig. 3).

Physicochemical characterization of cellulase activity from the whole gut crude extract of *Z. morio*

Effect of temperature on cellulase activity

Without the substrate, 100 µL of whole gut extract of *Z. morio* was pre-incubated at various temperatures in the range of 20 - 80°C. The cellulase activity was stable around 40°C to 60°C with highest activity of 2355 U/mg at 40°C (Fig. 4).

Effect of pH

At various pH ranging from 4.0 to 8.0, the effect of the pH on the activity of the cellulase from the whole gut extract was determined. The cellulase activity was

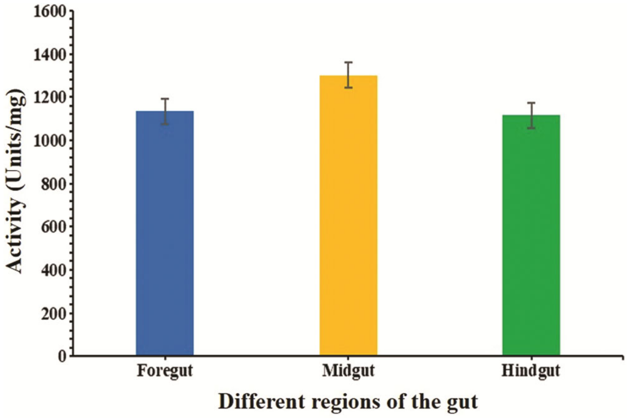


Fig. 3 — Cellulase activity in different regions of larval gut of *Z. morio*

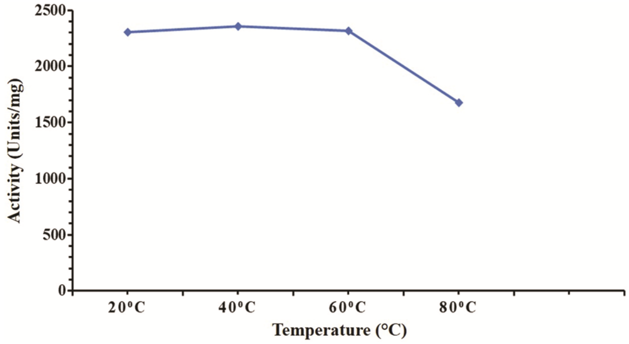


Fig. 4 — Thermal stability of cellulase activity from the whole larval gut extract of *Z. morio*

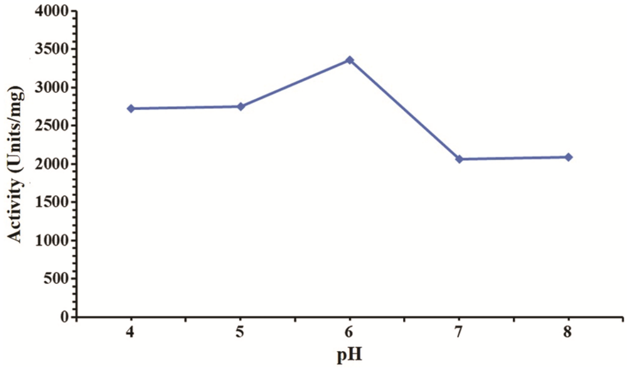


Fig. 5 — Optimal pH of cellulase activity from whole larval gut extract of *Z. morio*

stable in the optimum pH range between 5 and 6 (Fig. 5).

Effect of divalent cations

The effect of different divalent cations (Ca^{2+}, Mg^{2+} and Cu^{2+}) on cellulase activity of whole gut extract of *Z. morio* was analysed. The highest cellulase activity of 4029.65 U/mg was observed in 20 mM of Ca^{2+}, Mg^{2+} showed highest cellulase activity of 4422.15 U/mg for 15 mM concentration whereas Cu^{2+} showed highest activity at 5 mM concentration. The cellulase activity in the case of different concentrations of Cu^{2+} was less than that of the control (Fig. 6).

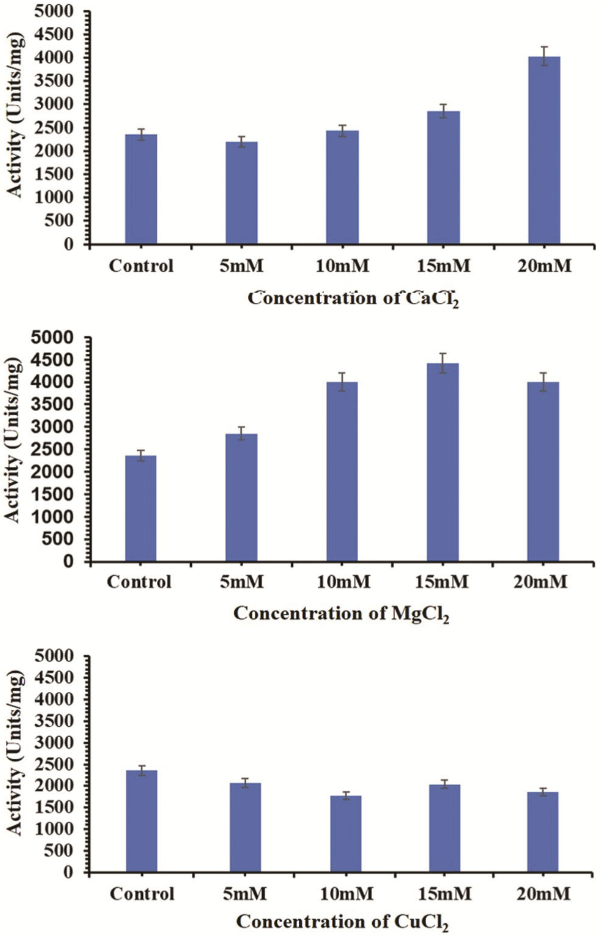


Fig. 6 — Effect of Ca^{2+}, Mg^{2+} and Cu^{2+} on the cellulase activity of whole larval gut extract from *Z. morio*

Table 1 — Blast hits and annotated sequences of whole gut

Database	Whole gut	
NCBI-nrdb	Blastx hits	43771
	Annotation	29652
SWISS PROT	Blastx hits	38851
	Annotation	31050
Tribolium castaneum protein sequence (GCA_000002335.3)	Blastx hits	39081
	Annotation	27195

***De novo* whole transcriptome assembly, identification of endoglucanase transcripts, *in silico* modelling, sequence alignment and docking**

RNA sample with RNA integrity number cut off greater than 6.4 was used to construct the sequencing library. The insert size of *Z. morio* whole gut was 276. Competent libraries of whole gut prepared were then sequenced using Illumina NovaSeq 6000 system that generated ~ 33.74 million raw reads. Data processing and *de novo* assembly produced a total of 1,14,739 contigs. The trinity assembled transcript FASTA file subjected to the Diamond BLASTX v 2.0.6 against NCBI's non-redundant protein database (NRDB) generated 43771 contigs. The final okayset.aa was taken for alignment against various databases using Diamond BLASTP v.2.0.6. The Blast2GO for protein annotation produced 29652 sequences. The details of BlastX hits and annotated sequences of whole gut are summarized (Table 1). Blast2GO software also annotated whole gut transcripts with GO keywords, KEGG pathways, and InterPro annotations (Table 2).

Of our particular interest are the endoglucanase transcript. One endoglucanase transcript with significant

Table 2 — Blast2GO software annotated whole gut transcript (ZM_WGGH9) with GO keywords and InterPro annotations

Transcript ID	GO IDs	GO names	InterPro IDs	InterPro GO IDs	InterPro GO names
ZM_WGGH9	P:GO:0005975; F:GO:0004553	P: carbohydrate metabolic process; F: hydrolase activity, hydrolyzing O-glycosyl compounds	IPR001701 (PFAM); PTHR22298:SF114 (PANTHER); PTHR22298 (PANTHER); IPR033126 (PROSITE_PATTERNS)	P:GO:0005975; F:GO:0004553	P:carbohydrate metabolic process; F:hydrolase activity, hydrolyzing O-glycosyl compounds

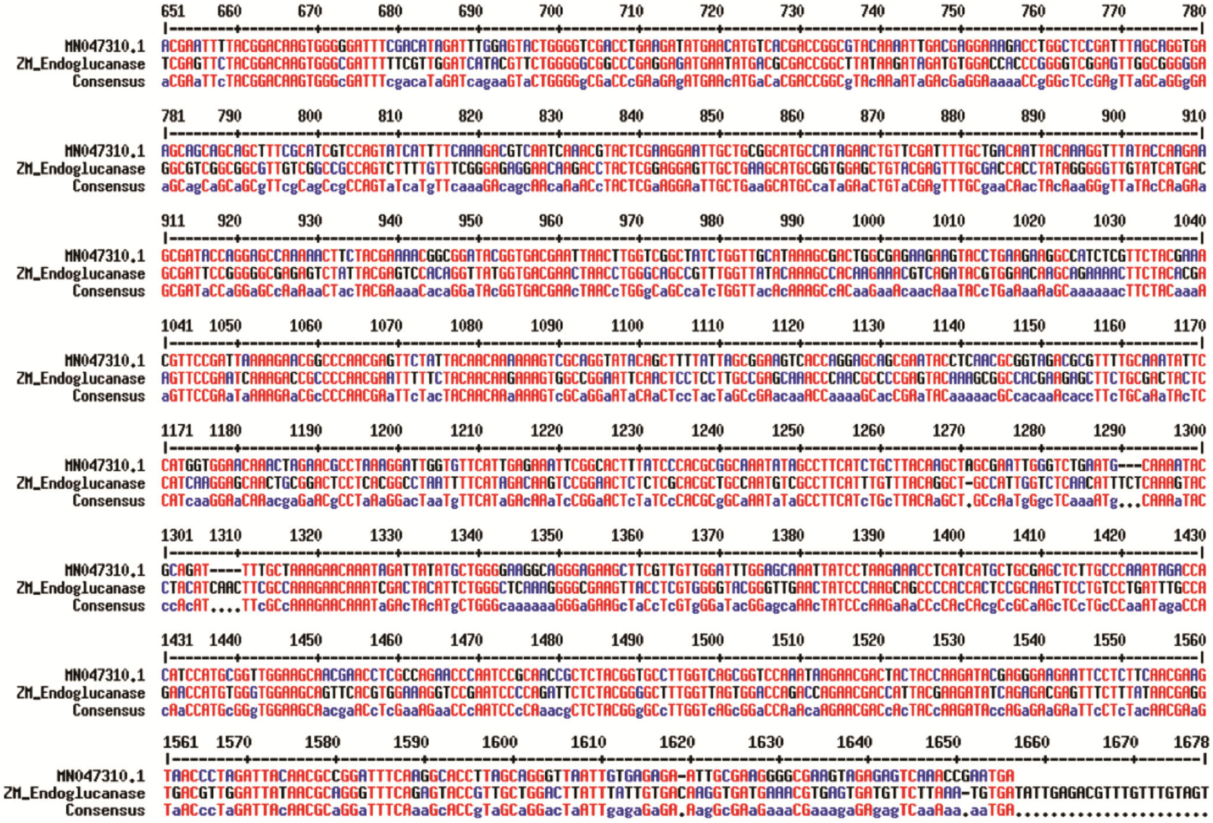


Fig. 7 — Pair wise sequence alignment of endoglucanase transcript from *Z. morio* and *O. rhinoceros* cellulase. Red regions denote the conserved sequences between the two

similarity to *Oryctes rhinoceros* cellulase (NCBI accession no. MN047310.1) was identified in the transcriptome of the whole larval gut of *Z. morio*. A pair wise sequence alignment was carried to find out the regions of similarity. The alignment of amino acid sequences of *O. rhinoceros* and *Z. morio* demonstrated significant similarity between the two (Fig. 7). Further CD search and InterProScan revealed its resemblance to GH9 family (Fig. 8). The structure for the same was further predicted using homology modelling with 1ks8: endoglucanase from termite, *Nasutitermes takasagoensis* (PDB ID) as template. The active site of the predicted structure was analysed using CASTP server. The structured model was then docked to the substrates CMC and microcrystalline cellulose using Autodock vina, PyRx interface (Fig. 9). The docking studies identified that the predicted structure possess affinity for both CMC and microcrystalline cellulose with a binding energy of -4.7 and -6.0, respectively.

Diverse members belonging to different families of glycosyl hydrolase were also found to be present as evidenced by the transcriptome data. The endo-β-1,4-glucanase identified in the current study belonging to GH9 family can act upon cellulose and efficiently degrade into similar forms including cellobiose or cellotriose. These substrates can be further acted upon by β-glucosidase (GH1) into glucose units. In addition to above members, 26 other members belonging to the GH family were also identified in the transcriptome of whole gut of *Z. morio* (Fig. 10). These results further substantiate the endogenous nature of cellulolytic system in *Z. morio*.

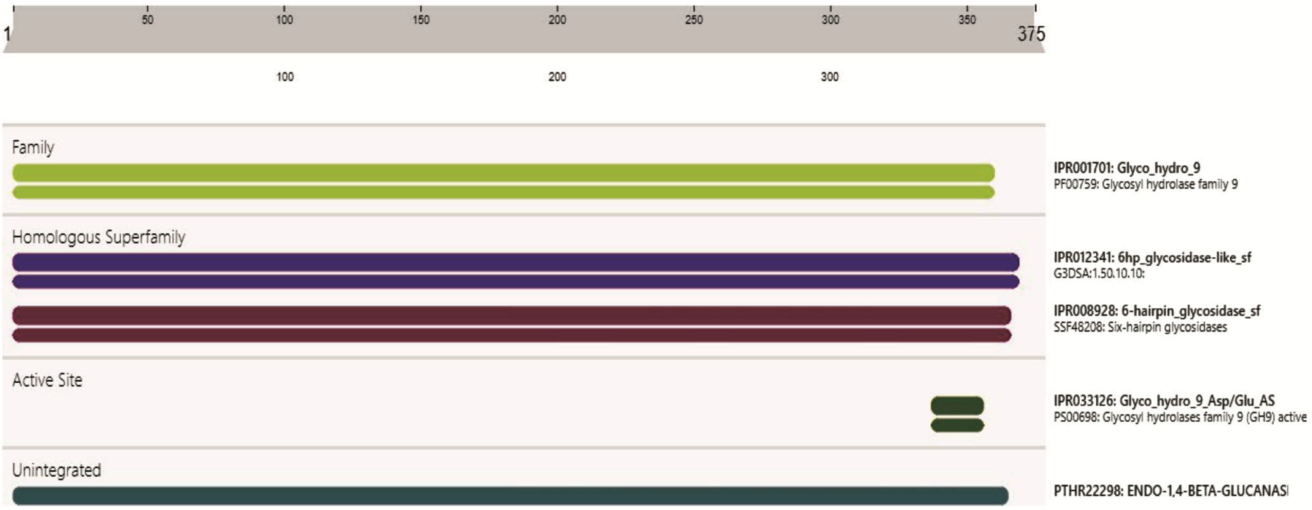


Fig. 8 — InterProScan revealing the presence of GH9 family domain

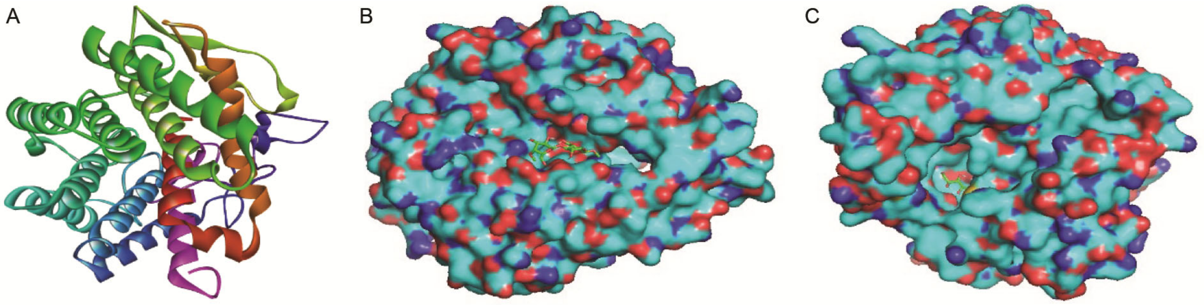
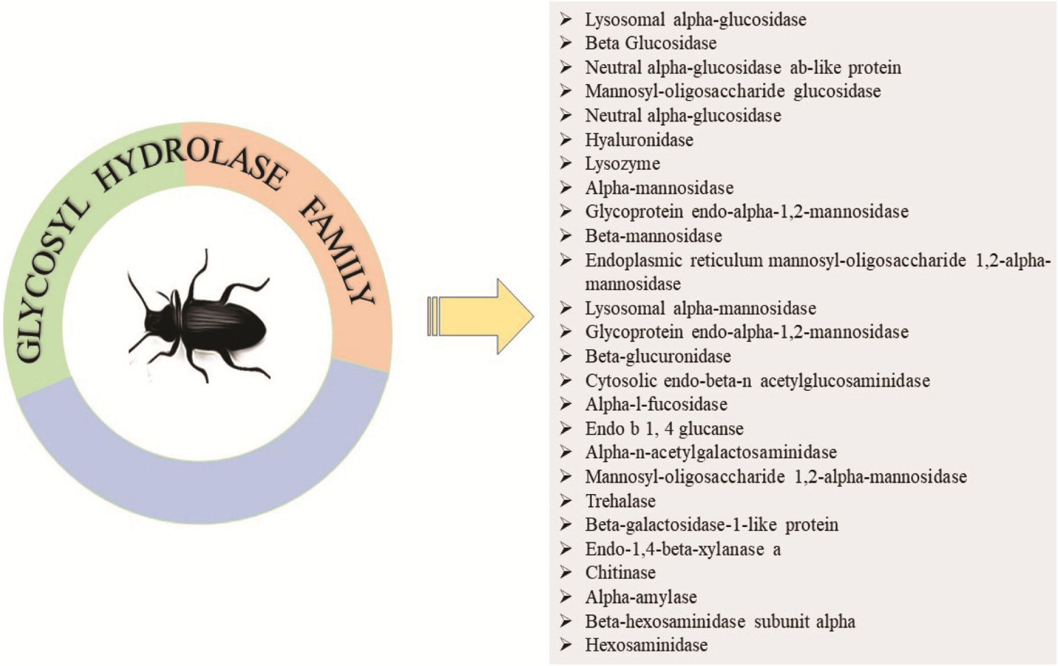


Fig. 9 — (A) Homology model of the endoglucanase transcript modelled using modeller 10.1; (B) CMC docked in the structure of endoglucanase transcript from *Z. Morio*; and (C) Microcrystalline cellulose docked in the structure of endoglucanase transcript from *Z. Morio*



- Lysosomal alpha-glucosidase
- Beta Glucosidase
- Neutral alpha-glucosidase ab-like protein
- Mannosyl-oligosaccharide glucosidase
- Neutral alpha-glucosidase
- Hyaluronidase
- Lysozyme
- Alpha-mannosidase
- Glycoprotein endo-alpha-1,2-mannosidase
- Beta-mannosidase
- Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase
- Lysosomal alpha-mannosidase
- Glycoprotein endo-alpha-1,2-mannosidase
- Beta-glucuronidase
- Cytosolic endo-beta-n acetylglucosaminidase
- Alpha-l-fucosidase
- Endo b 1, 4 glucanse
- Alpha-n-acetylgalactosaminidase
- Mannosyl-oligosaccharide 1,2-alpha-mannosidase
- Trehalase
- Beta-galactosidase-1-like protein
- Endo-1,4-beta-xylanase a
- Chitinase
- Alpha-amylase
- Beta-hexosaminidase subunit alpha
- Hexosaminidase

Fig. 10 — Diverse members of GH family identified from the transcriptome of whole gut of *Z. morio*

Discussion

Seclusion is not the convention of nature and no organism can exist alone. The diversified and intricate interactions between and within the species are the impetus that perpetuates the resilience of an ecosystem. From the perspective of biodiversity, herbivory in insects is contemplated to be the most intriguing phenomenon. Insects are endowed with the capability to digest the plant biomass[29,30]. Is this competence to digest plant biomass including cellulose, hemicellulose and pectin furnished by an independent cellulolytic system or are there some organisms (micro)[31] that lend a hand to accomplish the same?

Quantitative analysis of cellulase activity in various regions of the gut revealed maximum activity in the midgut (1300 U/mg) followed by foregut (1133.33 U/mg) and hindgut (1116.66 U/mg). Physicochemical characterization of cellulase activity from the whole gut crude extract of *Z. morio* demonstrated the highest activity of 2250.32 U/mg at 40°C and the activity was stable around 40°C to 60°C. Interestingly, higher stability of up to 55-60°C was also observed in the case of mulberry longhorn beetle, *Apriona germari*[32]. The cellulase activity was stable in the optimum pH range between 5 and 6. The effect of different divalent cations (Ca^{2+}, Mg^{2+} and Cu^{2+}) on cellulase activity of whole gut extract of *Z. morio* was also analysed. It was found that the highest cellulase activity of 4029.65 U/mg was recorded for Ca^{2+} at 20 mM concentration whereas Mg^{2+} showed the highest cellulase activity of 4422.15 U/mg at 15 mM concentration. Cu^{2+} showed highest activity at 5 mM concentration. These distinct patterns of influence of divalent cations were also found in the case of *Batocera horsfieldi*[33]. Remarkably, Ca^{2+} and Mg^{2+} augmented the activity in the case of the red palm weevil *Rhynchophorus ferrugineus*[34]. The competence exhibited by the termite, *Reticulitermes speratus* to conserve its ability to feed on wood even when the gut fauna was completely removed is contemplated to be the headway to the advancement of the endogenous nature of cellulose digestion in insects followed by the report of the existence of putative cellulase genes in about 30 insects belonging to different orders[1,7,13,32,35-41]. The studies on sequencing the transcriptome of five species of beetles and surveying openly accessible expressed sequence tags, 167 new beetle endogenous plant cell wall degrading enzymes belonging to eight diverse families of enzymes were described[42]. Among the members of the order Coleoptera that possess exorbitant diversity in size, morphology and behaviour are xylophagous or wood eating insects that utilizes cellulose as the sole carbon source. Even though the cellulase activity in Coleopteran larvae was ascribed to hindgut bacteria (*Oryctes nasicornis*) or to fungal enzymes (*Monochamus marmorator*), endo-b-1,4-glucanase belonging to the families GH9, GH45 and GH5 were found to be distributed in various families of Coleoptera[14]. Further, experimental evidences demonstrated the existence of endogenous cellulase gene in the digestive track of *Monochamus alternatus* with 14 cellulase enzymes[43]. On the contrary, not much studies are available in cellulolytic systems in other insect orders that contain species specialized to feed on cellulosic materials. *Z. morio* is a species of darkling beetle that feeds on lignocellulose-rich diet. *De novo* whole transcriptome analysis of whole gut of *Z. morio* identified the presence of GH9 family. Additionally, the sequence was found to be significantly similar to the cellulase transcript predicted from the *O. rhinoceros* transcriptome[43]. Interestingly, the amino acid sequences of *O. rhinoceros* cellulase showed significant similarity with termite cellulases[44]. The prevalence of other members of GH family was also revealed in the study. A total of 28 different members belonging to different GH family were also identified in the whole gut transcriptome of *Z. morio*. These group of carbohydrate active enzymes are crucial in sugar-based biofuel production.

A harmonious system comprising endoglucanases, exoglucanases and beta glucosidases bend efforts for the breaking down of this exceedingly complex lignocellulosic diet[45]. One of the crucial cellulolytic enzymes is endo-1,4-glucanase which hydrolyses internal β-1, 4 glycosidic bonds of amorphous cellulose microfibril chains. The activity of which is assessed by its potentiality to hydrolyze CMC, a frequently used substrate for evaluating cellulolytic activity due to its solubility in water which is attributed to the presence of methylated hydroxyl groups[9]. Over and above that, the gut fluids of insects manifest inordinate activity against CMC than microcrystalline cellulose[46]. Because two homologous sequences will have identical structures, homology models, also known as comparative models, are frequently constructed and employed in many biological applications to create credible structural models[47,48]. Homology modelling and molecular docking studies showed the cellulase gene identified possess affinity to both CMC and microcrystalline cellulose. Not surprisingly, a comprehensive investigation revealed that the gut fluids from 68 phytophagous insects spanning 8 different orders have a greater effect on CMC than crystalline cellulose[46].

The demand for sustained economic growth and a panacea to the energy crisis has made lignocellulose-based biofuels a prominent area of interest for academic and industrial communities around the globe[49]. Insects have developed incredibly efficient methods to harness energy from cellulose substrates and can be used in the field of bio ethanol production, bio fuels, pharmaceuticals etc[50,51]. This makes insects an optimum resource to prospect for novel cellulolytic enzymes. Endogenous cellulases have also been reported in different insect orders including Blattaria (termites and cockroaches), Coleoptera (beetles) and Orthoptera (crickets). Generally, insect cellulase genes have been uncovered using two main methods including sequence mining (bioinformatics approach) and functional assays (direct screening approach)[1,52]. The current study envisaged the prevalence of independent mode of cellulose digestion in the gut system of the grub of *Z. morio* in a comprehensive manner by utilising both the aforementioned approaches. It revealed the existence of GH9 family. The result validates further the prevalence of endogenous cellulolytic system in the gut of *Z. morio* larvae with strong evidence for the existence of its own cellulase. This makes them an excellent resource to prospect for novel cellulolytic enzymes that could be utilised for biofuel production. However, careful experimental investigation on the role of exogenous sources of cellulases are necessary to ascertain the comprehensive endogenous nature of cellulolytic system in the larvae of *Z. morio*.

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Conflicts of interest

All authors declare no conflict of interest.

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