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Purification and characterization of cellulase from May beetle (*Phyllophaga errans*) gut

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Abstract. *Ezima EN, Adeqbesan BO, Osonuqa IO, Adefuye AO, Adepoju AR, Bello TH, Olalekan SO. 2024. Purification and characterization of cellulase from May beetle (*Phyllophaga errans*) gut. Asian J Trop Biotechnol 21: 52-58.* Cellulose constitutes a significant portion of plants' primary and secondary cell walls. Insects, traditionally believed to lack the ability to digest cellulose, have evolved distinctive strategies for cellulose degradation. May beetle (*Phyllophaga errans* LeConte, 1859) is herbivorous insects that feed on numerous plant species, including vegetables, maize plants, and other fresh food crops, causing a lot of damage to farm products. The ability of these beetles to thrive on plant diet may be due to the presence of highly effective cellulose digesting enzyme (cellulase) in their gut which aids the breaking down of cellulose into glucose. Therefore, this study focused on the extraction and purification of cellulase from the *P. errans* gut sourced from a farm in Ijebu-Ode, Ogun State, Nigeria. Following the dissection and homogenization of the beetle's guts, cellulase isolation and purification were carried out utilizing techniques such as ammonium sulfate precipitation, ion-exchange chromatography on CM-Sephadex G-200 and gel filtration using Sephacryl S-200 gel. The resulting pure cellulase from the gut of the *P. errans* exhibited a purification fold of 43.71 and a yield of 21.61% with a specific activity of 70.38 units/mg of protein. Characterization revealed the enzyme's native molecular weight of 24.6 kDa, Km (0.67 mM), and Vmax (192.31 mg/mL/min), with optimal activity observed at pH 9 and 70°C. The cellulase from the *P. errans* gut showcased distinctive characteristics that, if properly harnessed, could pave the way for practical applications in various industries, particularly in developing an efficient pesticide for pest management and environmental conservation.

Keywords: Cellulase, gut, May beetle, pest control, *Phyllophaga errans*, purification

INTRODUCTION

Polysaccharides are major constituents of cell walls and serve as energy reserves within plant and animal cells. In plants, starch acts as the primary energy reserve, while animals store energy in the form of glycogen. Phytophagous insects disrupt plant cell walls to access the storage polymers in cell content. This disruption can result from mastication, but it more commonly involves the action of digestive enzymes (Rehman et al. 2009; Barcoto and Rodrigues 2022). Cellulose, a linear polymer of β-1,4-linked D-glucose units, is the most abundant biopolymer on Earth and is a key structural component in plant cell walls, providing rigidity and strength to plant cells (Li 2021; Hemati et al. 2022). Efficient biodegradation of cellulose requires the coordinated effort of a sophisticated enzymatic system called cellulases due to its resistant characteristics (Chatterjee et al. 2015; Jayasekara and Ratnayake 2019). These enzymes catalyze the breakdown of β-1,4-glycosidic linkages in cellulose, converting it into smaller sugar units that can be further utilized in various applications.

Cellulases have received significant attention due to their potential applications in industries such as biofuel production, textile manufacturing, food processing, and waste management (Atousa et al. 2017; Sreeramulu et al. 2023). The demand for sustainable and eco-friendly processes has driven the search for novel and efficient cellulase sources. Although cellulases are produced by a variety of microbes such as bacteria, fungi, and protozoa, as well as in herbivorous invertebrates such as arthropods, nematodes, and mollusks (Barzkar and Sohail 2020), recent research has highlighted the potential of insect-derived cellulases as promising alternatives (Fagbohunka et al. 2017; Soeka and Ilyas 2020). The nutritional significance of cellulase for leaf-feeder organisms was previously deemed negligible due to its resistance to digestion in most animal species. The discovery of GH9 cellulases in facultative leaf-consuming grasshoppers (Ademolu and Idowu 2011) and GH45 cellulases, as well as GH11 xylanases in herbivorous beetles (Pauchet and Heckel 2013), implied that the function of cellulases in other plant-eating insects requires reassessment.

Presently, the existence of cellulases in invertebrates and insects has been documented across various insect orders such as Isoptera, Blattodea, Coleoptera, Orthoptera, Diptera, and Lepidoptera (Chatterjee et al. 2015; Tokuda 2019; Uddin et al. 2021). The presence of cellulase has also been documented in the intestinal fluids of many insect species of different orders (Imran et al. 2016; Fagbohunka et al. 2017; Soeka and Ilyas 2020). It is commonly believed

that the cellulase activity found in these insects is due to symbiotic gut microorganisms. Herbivorous insects, particularly, depend on their gut microbes to break down plant materials like cellulose and lignin (Li 2021; Rajeswari et al. 2021). Cellulases of microbial origin play a vital role in the bioconversion of cellulose, acting as phytopathogens that produce a range of enzymes (Zhang et al. 2016). Insects host many microbes in their gut and thus serve as a reservoir of microbial diversity (Rajeswari et al. 2021; Hemati et al. 2022). Cellulolytic enzymes have been well-studied in wood-feeding insects, which depend on endogenous or symbiotic microbial enzymes to break down lignocellulose into assimilative sugars or nutrients (Afzal et al. 2019; Hemati et al. 2022). However, the role of cellulases in monophagous leaf-feeding (phytivorous) insects is less explored.

May beetle belong to a large family called scarabs (Scarabaeidae). Several Scarabaeidae beetles have been identified as capable of degrading cellulose; these include *Protaetia brevitarsis* Lewis, 1879 larvae (Wang et al. 2022a), *Trypoxylus dichotomus* Linnaeus, 1771 (Wang et al. 2022b), *Gastrophysa viridula* De Geer, 1775 beetle (Busch et al. 2018). Adult May beetle (*Phyllophaga errans* LeConte, 1859) is leaf feeders, feeding on foliage of trees and shrubs, causing significant damage, especially in large numbers. The ability of these beetles to thrive on this diet may be due to the presence of a highly effective cellulose-digesting enzyme (cellulase) in their gut, which helps break cellulose into glucose that is readily absorbed and used as their energy source. Therefore, understanding the nature and characteristics of *P. errans* cellulolytic enzymes will help design effective pesticides to control the beetle and contribute to understanding insect-derived cellulases.

MATERIALS AND METHODS

Materials

The reagents and chemicals used in this work were analytical, sourced from BDH Chemicals Limited (Poole, England), Sigma Aldrich Chemicals Company (St. Louis. Mo. USA), Pharmacia Fine Chemical (Uppsala, Sweden), Eastman Kodak (Rochester, N. USA) and Pierce Chemical Company (Rockie, Illinois USA)

Methods

Collection of beetles and sample preparation

Phyllophaga errans samples were collected nightly from maize and vegetable farms in Ijebu-Ode, Ogun State, Nigeria. They were stored in a well-ventilated container and taken to the laboratory. The beetles were washed in normal saline and stored at 4^{o}C until use. Before dissection and excision of the beetle's gut, the beetles were thawed and rinsed in normal saline. All the collected gut samples were weighed (25.6 g) and homogenized in 3 volumes of 0.1 M acetate buffer pH 5.5. The resulting homogeneous solution was centrifuged at 4,000 rpm for 15 minutes, and the supernatant obtained was used as the crude enzyme. The beetles were identified at the Zoology Department of Olabisi Onabanjo University, Ago-Iwoye, Ogun State.

Enzyme assay

The assessment of cellulase activity was conducted using an enzymatic assay method initially described by Afzal et al. (2019) with minor adjustments. This process involves quantifying the quantity of reducing sugar liberated from the reaction between the enzyme and substrate (CMC) using the DNSA (3, 5-dinitrosalicylic acid) technique. A standardized glucose curve was employed to determine the quantity of glucose liberated. The assay mixture comprised 0.5 mL of the substrate (CMC) solution (1%, w/v in 0.1 M acetate buffer of pH 5.5) mixed with 0.1 mL of the enzyme sample. Subsequently, the mixture was incubated for 30 minutes at 37^{o}C with gentle agitation. Following the incubation period, 0.4 mL of DNSA reagent was introduced to the mixture and incubated in a boiling water bath for 10 minutes, then cooled in an ice bath before adding 3 mL of distilled water to dilute the solution. The absorbance at 540 nm was measured after the incubation. Therefore, to ensure reliability, all experiments were repeated thrice. Cellulase activity was defined as the quantity of enzyme necessary to release 1 mg of glucose under the specified assay parameters.

Estimation of protein concentration

The Bradford (1976) method was used to determine the enzyme's protein concentration using Bovine Serum Albumin (BSA) as a standard.

Purification cellulase

Ammonium sulfate precipitation

The crude enzyme obtained from the gut of *P. errans* was brought to 70% ammonium sulfate saturation. The calculated amount of solid ammonium sulfate was added to the crude enzyme mixture and stirred gently until well dissolved. The mixture was refrigerated for 12 hours and then centrifuged at 6,000 rpm for 30 minutes; the precipitate was suspended in an aliquot amount of 0.1 M acetate buffer of pH 5.5 and stored at 4^{o}C for further use. The enzyme precipitate was dialyzed against several changes of 0.1 M acetate buffer pH 5.5 to remove the ammonium sulfate salt for the ion exchange chromatography.

Ion exchange chromatography on CM-Sephadex G-200

The salt-free enzyme obtained after dialysis from the previous step was administered onto a chromatographic column (2.5x40 cm) containing CM-Sephadex G-200 resin, equilibrated with 0.1 M acetate buffer at pH 5.5. The enzyme was separated using the same buffer with a flow rate of 30 mLh^{-1}. Next, 5 mL portions were collected, and the enzyme's protein and cellulase activity were assessed. The fractions exhibiting high activity levels were combined and concentrated in 50% glycerol.

Gel filtration on Sephacryl S-200

The recovered enzyme solution from glycerol concentration was then applied to the Sephacryl S-200 column already equilibrated with 0.1M acetate buffer of pH 5.5. The column was washed with the same buffer at a flow

rate of 30 mLh^{-1}. Next, 5 mL fractions were collected, and the enzyme's protein concentration and cellulase activity were determined. The fractions were pooled where high activity was observed.

SDS-PAGE

The purity and molecular weight of the enzyme were ascertained on SDS-PAGE (Laemmli 1970) with resolving and stacking gel compositions of 12 and 5%, respectively. The molecular weight of the enzyme was calculated with the help of a protein ladder marker, 10-220 kDa. Next, 30 µm of sample buffer was added to 90 µml of active *P. errans* gut cellulase fraction from Sephacryl S-200, and the mixture was boiled for 5 minutes to denature the enzyme. The same procedure was performed on the protein marker, ammonium precipitate, and crude enzyme. After gel polymerization, denatured enzymes and protein markers were stacked at 100 Volts, and the process was run in a separating gel at 150 Volts for 2 hours in a Bio-Rad electrophoresis. The electrophoresis was stopped when the dye fronts were approximately 1 cm from the end of the separation gel. The gel was then fixed for one hour in a 10% (w/v) acetic acid solution and 40% (w/v) methanol. It was then stained overnight with shaking to ensure uniform mixing and Coomassie brilliant blue R-250 adsorption onto the protein bands. A solution of 5% methanol and 7.5% glacial acetic acid in distilled water was then used to destain the gel completely.

Effect of pH on Phyllophaga errans *gut cellulase*

The impact of pH on enzyme activity was investigated by assessing enzyme activity within the pH range of 4 to 11 at a temperature of 37ºC, employing diverse buffers with varying pH levels: citrate (pH 3-5), phosphate (pH 6-8), and borate (pH 9-11). The cellulase activity was assayed as previously described.

Effect of temperature on Phyllophaga errans *gut cellulase*

Cellulase activity was evaluated within a temperature range of 20 to 100ºC at pH 5. The assay mixture was first incubated at the respective temperature for 10 min before the commencement of the reaction by introducing 500 µL of the purified enzyme that had also been equilibrated at the same temperature. The assessment of cellulase activity followed the standard procedure as previously outlined.

The study of the kinetic properties of Phyllophaga errans *gut cellulase*

The kinetic parameters, Km and Vmax, were determined for the purified *P. errans* gut cellulase using 1% (w/v) CMC as substrate. The concentration was varied from a final concentration of 1 to 10 mg/mL CMC and the assay was conducted in triplicate measurements. The kinetic parameters were determined from the Lineweaver-Burk plot of the reciprocal of the initial velocity (1/V) against the reciprocal of the substrate concentrations [1/S].

RESULTS AND DISCUSSION

The investigation presented in this study offers valuable insights regarding the purification and characterization process of cellulase extracted from the gut of *P. errans*. The outcomes of the purification techniques are briefly outlined in Table 1. The cellulase obtained from the gut of the *P. errans* exhibited a specific activity of 70.38 U/mg of protein with a yield of 21.61% and was purified to homogeneity. The results of the purification processes are shown in Figures 1 and 2, which illustrate the use of ion-exchange chromatography on Sephadex G-200 and Sephacryl S-200. Verification of the enzyme's purity was conducted through SDS-PAGE, displaying a single band for the purified enzyme as depicted in Figure 3. The molecular weight was determined to be 24.6 kDa, also using SDS-PAGE as shown in Figure 4.

Table 1. Summary of the results of the purification procedures of cellulase from the gut of *Phyllophaga errans*

Procedures	Volume (mL)	Activity (units/mL)	Protein (mg/mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude enzyme	30.0	1.14	0.709	21.27	34.20	1.61	100	1.00
70% Ammonium sulfate $(NH_4)_2SO_4$	10.5	3.38	1.750	18.36	35.46	1.93	103.7	1.20
Ion-exchange Chromatography CM-Sephadex G-200	55.0	0.09	0.025	1.38	4.29	3.12	12.54	1.94
Gel Filtration Sephacryl S-200	35.0	0.21	0.003	0.11	7.39	70.38	21.61	43.71

Values for activity and protein are the mean of the triplicate assay for *Phyllophaga errans* gut cellulase activity and protein concentration. Cellulase activity was defined as the amount of enzyme needed to release one milligram of glucose under the specified assay conditions

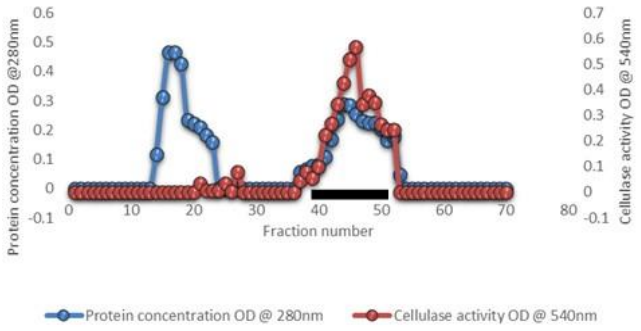

Figure 1. Sephadex G-200 elution profile of the ion-exchange chromatography of cellulase from the gut of *Phyllophaga errans*. Fractions with very high cellulase activity (40-50) were pooled for subsequent analysis

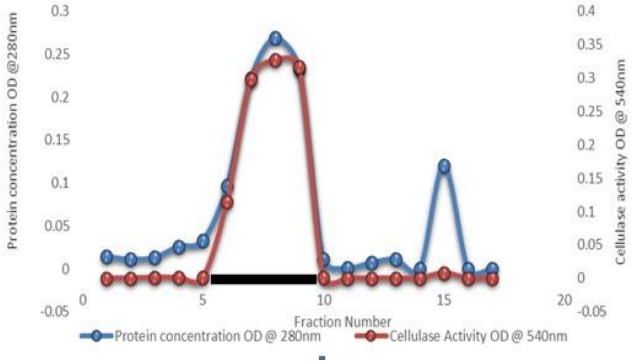

Figure 2. Sephacryl S-200 elution profile of the cellulase ion-exchange chromatography from the *Phyllophaga errans*' gut. Fractions with very high cellulase activity (5-11) were pooled for subsequent analysis

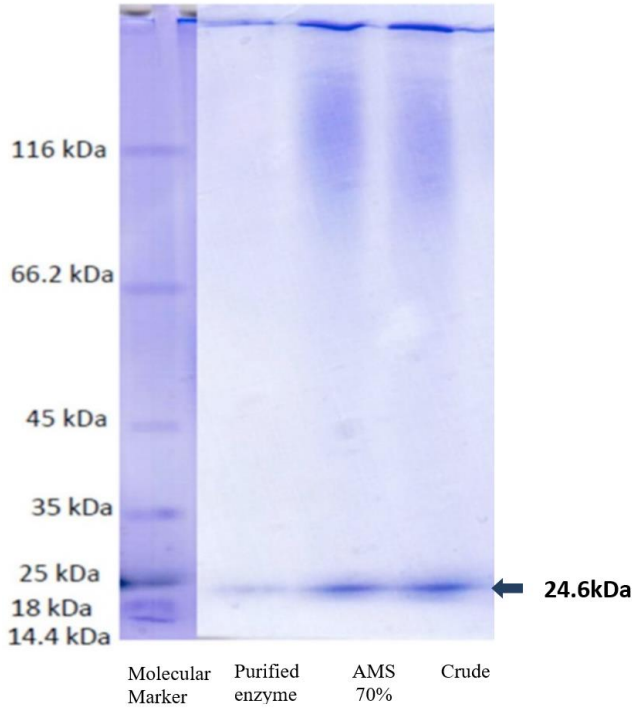

Figure 3. SDS-Polyacrylamide slab gel electrophoresis of *Phyllophaga errans*' gut cellulase (Lane 1 protein marker, Lane 2 purified enzyme, Lane 3 ammonium sulfate precipitate, and Lane 4 crude enzyme)

The specific activity (70.38 U/mg) obtained in this study surpassed the findings from a previous study on cellulase sourced from worker termites *Amitermes eveuncifer* Silvestri, 1901, which recorded a specific activity of 0.25 U/mg (Ezima et al. 2014). In a separate study, Haloi et al. (2012) reported a lower specific activity of 0.687 U/mg for Grasshopper *Hieroglyphus banian* Fabricius, 1798 (Orthoptera: Acrididae), while Fagbohunka et al. (2017) gave an account of 5.04 U/mg for soldier termite *A. eveuncifer* cellulase. Furthermore, Pachauri et al. (2020) observed a specific activity of 30 U/mg for cellulase sourced from a novel isolate of fungi *Trichoderma longibrachiatum* Rifai, while Goswami et al. (2022) reported a specific activity of 31.4 U/mg for bacteria *Novosphingobium* sp. Cm1. In contrast, Islam and Roy (2018) and Sreeramulu et al. (2023) reported significantly higher specific activities of 2655 U/mg and 2858 U/mg, respectively, for cellulase-producing bacteria found in molasses and larvae of the banana pseudostem weevil, *Odoiporus longicollis* G.A.K.Marshall, 1930 (Coleoptera: Curculionidae).

Various literature sources have reported different molecular weights for cellulases from various microbes and insects, ranging from 35 to 184 kDa (Atousa et al. 2017; Banerjee et al. 2020; Malik and Javed 2021). A recent study by Fouda et al. (2024) revealed a molecular weight of 436 kDa for Thermotolerant *Bacillus subtilis* F3. So far, reports of cellulase with low molecular weight are few. The molecular weight of *P. errans* cellulase aligns closely with the findings of Rahman et al. (2014), who identified an enzyme called AkEG21, from the common sea hare *Aplysia kurodai* Baba, 1937 with a molecular weight of 21 kDa. Furthermore, Listyaningrum et al. (2018) documented a molecular weight of 18 kDa for *Bacillus* strains isolated from carrageenan solid waste. It has been noted that cellulases with low molecular weights exhibit thermal stability and offer advantages in diverse applications such as heterogeneous expression and protein-engineering studies. According to Cowan and Fernandez-Lafuente (2011), enzymes with lower molecular weights are more amenable to modification, facilitating processes like immobilization or genetic manipulation and ultimately enhancing enzyme specificity.

Environmental conditions such as pH and temperatures have great effects on enzyme performance and stability; the characterization of the optimal pH and temperature of *P. errans* gut cellulase revealed optimal activities at pH 9 and 70°C respectively (Figures 4 and 5), indicating a moderately thermostable enzyme that works well in alkaline medium. The enzyme possessed a unique thermostability, with activity stability over a broad range of

temperatures, from 50°C to 75°C. Similar temperature optima have been reported (Padilha et al. 2015; Maswati 2022). Other thermostable cellulases have been reported from different sources with optimum temperatures between 50°C to 60°C (Atousa et al. 2017; Kim and Ku 2018; Shyaula et al. 2023; Sreeramulu et al. 2023). Lower optimum temperatures of 30-45°C were also reported (El-Sersy et al. 2010; Rahman et al. 2014; Banerjee et al. 2020; Fagbohunka et al. 2021). However, Zhang et al. (2016) reported an optimum temperature as low as 28°C for cellulase from *Pseudomonas mendocina.*

Many documented insects and microbial cellulases have neutral pH optima (Lee et al. 2008; Fagbohunka et al. 2017; Kim and Ku 2018), few acidic optima (from 3 to 5) had also been reported (Rahman et al. 2014; Atousa et al. 2017; Shyaula et al. 2023). The optimum pH of 9 obtained for the *P. errans* gut cellulase is one of the highest so far reported. Listyaningrum et al. (2018) reported an optimum pH of 8 for *Bacillus* strains isolated from the Carrageenan solid waste. The alkaline nature of the *P. errans* cellulase could be of great importance for the solubilization of plant biomass and efficient cellulose degradation in the insect, as suggested by Wang et al. (2022a). Our study also revealed that although cellulase enzymes from *P. errans*' gut can work well in an alkaline medium (8-9) higher pH values have a detrimental effect on the enzyme.

The cellulase enzyme from the gut of the *P. errans* exhibited promising characteristics for various industrial applications, especially due to its optimal activity within the temperature range of 50-70°C (Figure 5). The enzyme shows high efficacy in environments with elevated pH levels and temperatures, making it particularly suitable for applications in textile treatments, paper and pulp processing, and specific stages of biofuel production and other industrial processes requiring enzymatic activity in harsh environments.

The insights into an enzyme's catalytic efficiency and substrate affinity are mostly provided by the Kinetic parameters of the enzyme, specifically the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m). Understanding these parameters is crucial to monitoring enzyme behavior and optimizing its performance in various applications. The K_m and V_{max} values estimated for cellulase from the *P. errans* gut were 0.67 mg/mL and 192.31 units/mL/min (Figure 6). Though the K_m and V_{max} values documented for cellulases vary between different species, the K_m for the *P. errans* cellulase is lower than that documented by Sreeramulu et al. (2023) with K_m and V_{max} of 1.03 mg/mL and 343 units/mL/min for the gut cellulase of the larvae of banana pseudostem weevil, *O. longicollis*, and Afzal et al. (2019) reported K_m and V_{max} of 2.24 mg/mL and 454.05 µg/mL/min for cellulase isolated from *Bacillus licheniformis* HI-08 from the hindgut of wood-feeding termite. Likewise, Shyaula et al. (2023) reported K_m and V_{max} values were 1.8 mg/mL and 10.92 µg/mL/min for cellulase of *B. licheniformis* PANG L isolated from Himalayan soil, while Pachauri et al. (2020) gave an account of K_m and V_{max} values for cellulase isolated from *T. longibrachiatum* of 0.121 mg/mL and 0.421 µmol/min.

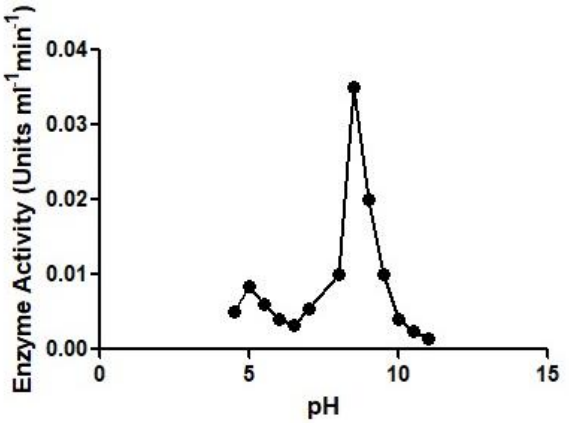

Figure 4. Effect of pH on cellulase activity from *Phyllophaga errans*. Enzyme activity was assayed within the pH range of 4 to 11. The values shown represent the average of triplicate experiments

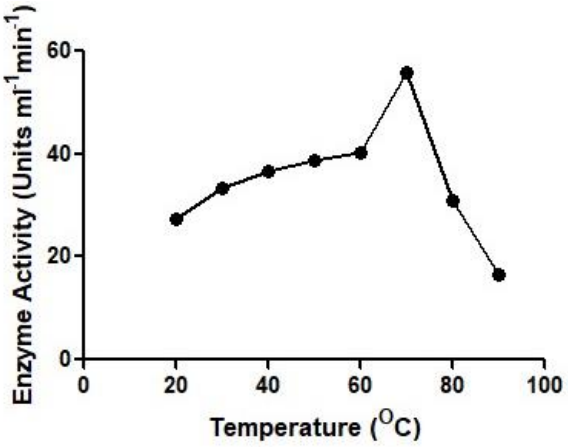

Figure 5. Effect of temperature of *Phyllophaga errans* cellulase. The cellulase activity was evaluated at temperatures between 20 and 100ºC at pH 5. The values shown represent the average of triplicate experiments

The variations in K_m values reflect the different enzymatic efficiencies and substrate affinities of cellulases from various species, highlighting the diverse adaptations in utilizing cellulose as an energy source. Since adult *P. errans* feed mainly on the foliage of trees and shrubs high in cellulose content, the beetle requires an effective cellulase for effective nutrition. Therefore, the Km of 0.67 mg/mL showed that cellulase from the *P. errans*' gut has an effective cellulase with a high affinity for cellulose.

Insects from diverse taxonomic orders have been discovered to exude β-glucosidase and endo-β-1,4-glucanas enzymes that exhibit significant attraction towards cellulose and cellobiose, respectively, thereby emphasizing their cellulolytic potential (Watanabe and Tokuda 2010; Shelomi et al. 2014; Linton 2020). The cellulose in the gut of the *P. errans* is presumed to be Indigenous owing to its strong affinity towards substrates, optimal temperature conditions, and capacity to operate efficiently under high pH environments.

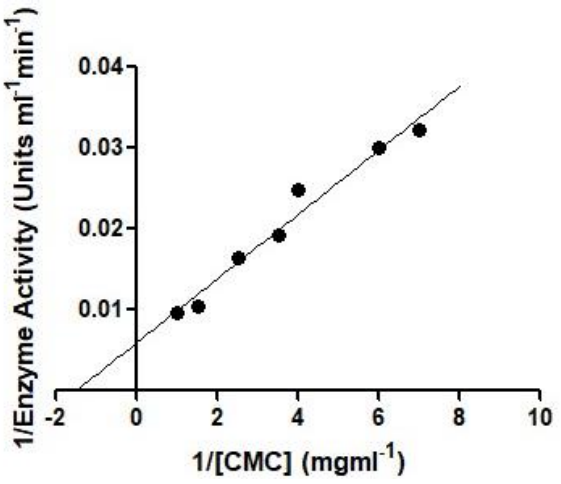

Figure 6. Lineweaver-Burk plot of the purified cellulase from the gut of *Phyllophaga errans*. The concentration of the substrate (CMC) was varied between 1 to 10 mg/mL, and the plot of the reciprocal of initial velocity (1/V) against the reciprocal of the substrate concentrations [1/S] was used to estimate the values of the enzyme Km and Vmax

In conclusion, the cellulase extracted and identified from *P. errans* guts was effectively purified and characterized. The enzyme demonstrates features that establish it as a viable option for various industrial processes, especially for waste treatment and the production of biofuels that demand strong enzyme efficacy in harsh conditions. Furthermore, the efficient cellulolytic properties of cellulases obtained from the gut of *P. errans* could be harnessed for developing pesticides aimed at managing beetle populations in farming settings, where they present significant risks. This objective can be accomplished through targeted approaches to either suppress or hinder the activity of cellulases, disrupting their digestion and depleting their source of nourishment. It is strongly advised that additional research be carried out, particularly focusing on optimizing the extraction and purification processes through techniques like enzyme stabilization, enhanced purification methods, or the application of advanced biotechnologies such as genetic engineering, which substantially increases the enzyme yield. Also, further work is recommended to confirm the cellulase's origin from the *P. errans*'s gut, whether it is truly endogenous or from the symbiotic microbes. Again, more research is advised to validate the cellulase source derived from the *P. errans*'s digestive system, determining whether it is endogenous or originating from the symbiotic microorganisms residing in the gut.

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