Biomacromol. J., Vol. 4, No. 1, 7-15, July 2018.

Biomacromolecular Journal
www.bmmj.org

A Novel Cellulase from the Earthworm *Allolobophora Chlorotica*

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(Received 8 September 2018, Accepted 27 September 2018)

ABSTRACT

Cellulase is one of the most commercial and applicable enzymes which is produced chiefly by fungi, bacteria, protozoa and metazoa that catalyze cellulolysis, the decomposition of cellulose and of some related polysaccharides. Recently, the demand for this enzyme has been increased mostly for the various industrial purposes. It has been assumed that the milieu of earthworm's intestine is the final destination of digestive enzymes such as cellulase. For the first time, this study is aimed to shed a light on the characteristics of cellulase activity in the coelomic fluid and body extract of the earthworm *Allolobophora chlorotica*. Practically, following the preparation of homogenate and coelomic body in cold-ice and enzyme activities at different conditions were investigated and based on results, the optimum pH and temperature for cellulase activity were pH 9 and 50 °C, respectively for the two sources. Given the results, the earthworm contains a novel cellulolytic enzyme which is more stable at alkaline pH. A side from optimal pH and temperature, the addition of metal salts, including $CaCl_2$, NaCl, $MgCl_2$, KCl increase cellulase activity, although, $HgCl_2$ and EDTA have inhibitory effects on the enzyme. Due to common properties of cellulase from coelomic and body extracts it can be concluded that coelomic cellulase contents can be secreted from digestive tracts to facilitate the digestion of the food. Furthermore, based on results, the novel cellulase is active at alkaline pH and moderately thermostable which can be used in biotechnological industries after purification.

Keywords: Earthworm, *Allolobophora chlorotica*, Cellulase, Coelomic fluid, Body extract

INTRODUCTION

By the aid of cellulase in their digestive tract, earthworms have a key role in the decomposition of soil organic materials and other wastes from anthropogenic activities like agricultural wastes.

Proteins extracted from earthworms indicate cytolytic, agglutinating, proteolytic, hemolytic and mitogenic activities [1]. In addition, the extract obtained from the tissue of earthworm has been used to treat various kinds of ailments since the source of valuable compounds is known to be present in earthworm tissues including proteins, peptides, enzymes and physiologically-active substances [1].

Moreover, cellulase is an enzyme capable of hydrolyzing the glucosidic bonds. Three main types of enzymatic activities of the cellulase have been reported:
1-endoglucanase or 1,4-beta-D-glucan-4-glucaohydrolases (EC:3.2.1.4),
2-exoglucanase or 1,4-beta-D-glucan-4-glucancellobio-hydrolases (EC:3.2.1.91),
3- beta-glucosidase or beta-glucosid glucohydrolases (EC: 3.2.1.21) [1].

This enzyme was initially developed for the food industry and animal feed. Due to a variety of applications, cellulase is currently the third largest industrial enzyme worldwide [2] as it can be used in texture, fruit juices and paper industries and in animal feed to improve digestibility as well. It must be noted that a major shortcoming in the fossil fuels and the arising need to find an alternative source of renewable energy has led to an interest in the bioconversion of lignocellulosic biomass using cellulases [3].

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Till date, several types of research on enzymes from earth worms have been carried out. In 1998, it has been demonstrated that dried powder from earthworm show protease, chitinase, amylase and cellulase activities [4]. Furthermore, in 1999 some researchers have reported the glycolytic and cellulolytic activities in the intestine of the many species of earthworms *viz. Pontoscolex corethrurus*, *Millsonia anomala*, *Horrnogaster elisae*, *Hyperiodrilus africarius*, *Polypheretima eloizgata*, *Dichogaster terrae nigrae*. Although some earthworm species have shown poor enzyme activities, they have been equipped with the enzymes to digest the required amounts of plant tissues. Moreover, they have proved that different parts of the intestine show unequal expression of these enzymes [5]. Meanwhile, it has been indicated that the changes in the cellulase activity in the intestine of two species of earthworms *Lampito mauritii and Eudrilus eugeniae* in the presence of substrates dependent on nutrition, type of food and physiological condition of each species [6]. After that, a gene encoding cellulase called phhEG from the intestine of *Pheretima hilgendorfi* has been identified. Based on the aforementioned research, the intestine of earthworm expresses an endogenous cellulase to digest food [7].

In recent decades, the demand for this enzyme has been increased. A major problem for industrial application of cellulase is the cost production and low yield of this enzyme [8]. As cellulases are rather costly enzymes, therefore a significant reduction in cost will be of great importance for their commercial use in biorefineries [9].

The aim of this study was to evaluate the characteristics of cellulase activity in the coelomic fluid and body extract from earthworm *Allolobophora chlorotica* to introduce a possible novel cellulose-degrading enzyme. Here, it is worthy to be noted that, for the first time, we have compared the activities of cellulase from both coelomic fluid and body extract of the earthworm *Allolobophora chlorotica* at different conditions. The earthworm *Allolobophora chlorotica* is one of the commonest earthworm exists in mull soils of pH above 4.5 level [10].
In the present study, cellulase activity was measured spectrophotometrically and subsequently different parameters include optimal pH and temperature, deactivation constants, enzyme half-life and also the effects of different inhibitors and activators on the activity of the enzyme are calculated.

MATERIALS AND METHODS

The earthworm samples were collected from greenfield of Shahrekord University campus and then transferred to the animal biology laboratory where they were kept at room temperature for several days to adapt. To commence the tests, following washing the earthworms were added to the certain amounts of phosphate buffer (50 mM, pH 7) in a beaker on the ice bath. The coelomic fluid was extracted using a electric shock by means of 9v battery. Furthermore, to obtain the body extract, worms were gently crushed into the pieces and homogetaed by electric homogenizer.
Then, the mixture was centrifuged at 600 rpm for 5 min at 4 °C and the resulted supernatant was collected in the sterile microtubes. Finally, the supernatants and coelomic fluids were stored at -20 °C until use.

To assay cellulase activity, the supernatant was added to 50 mM_phoshphate buffer in the presence of carboxy methyl cellulose (CMC) as a substrate for a short time.Cellulase activity was measured according to method as reported in previous studies using 5,3-dinitrosalicylic acid (DNS) [11]. DNS mixture was prepared as follows: 0.35 g of DNS, 0.66 g of NaOH, 10.2 g of Rochelle salt, 0.28 g of sodium metabisulfite ($Na_2S_2O_5$) were dissolved in 50 ml of double-distilled water. It is worthy to be noted that for any experiment the value of absorbance at 450 nm was converted to concentration using standard glucose and the activity was reported in the terms of μmol min^{-1} per assay volume. Considering the total protein concentration for each assay, cellulase activity was calculated as specific activity (μmol min^{-1} mg^{-1}). All experiments repeated in triplicate and the average values were used and then the plots were drown by Excel 2010 software. All of the materials were purchased from Merck chemical company representative in Iran.

Effect of pH on the Cellulase Activity

To investigate the effect of pH on the cellulase activity, the following buffer systems were used: citric acid monohydrate (pH 4 and 5), sodium hydrogen diphosphate (pH 6 and 7), Tris (pH 8 and 9), glycine-sodium hydroxide (pH 9.8 and 10.6) with a concentration of 50 mM. The reaction mixture was also prepared by the addition of

1100 ul of each of the above-mentioned buffers to 400 ul of body extract/coelomic fluid (0.5 mg ml^{-1}) and 500 µl of 2% CMC, then, the mixture were_incubated at room temperature for 80 min. Afterwards, 1000 ul of the DNS was added and the tubes were gently placed in a boiling water bath at 60 °C. Following heat incubation, samples were centrifuged and yellow upper phases collected and finally the absorbances were measured at 540 nm spectrophotometerically. Thereafter, the absorbance values were converted to concentration using a standard curve of glucose to determine the values of enzyme activities [12].

Effect of Temperature on the Cellulase Activity

For this, the temperature ranging from 10-70 °C with an interval of 10 °C were used. After the preparation of the assay mixture with the above-mentioned conditions, the experiment was carried out according to earlier report [12].

Determination of pH Stability of Cellulase

The pH stability of the cellulase was assessed for both body extract and coelomic fluid with a pH ranging from 4-10.6 as follows: body extract/coelomic fluid was added to a solution of each of the buffer systems (citric acid monohydrate pH 4; Tris pH 9 and glycine pH 10.6) and then incubated at room temperature for 80 min. Followingly, cellulase activities for these samples were measured at an optimum pH of 9. For this purpose, a mixture of body extract or coelomic fluid was prepared with each of the mentioned buffer and pH stability was measured with an interval of 20 min for 80 min at room temperature. Finally, after centrifugation at 6000 rpm for 5 min the upper phase was removed and its absorbance was read at 540 nm [13].

Thermal Deactivation and Determination of the Half-life of Cellulase

Thermal deactivation of the cellulase was separately measured within the temperature ranges of 30-70 °C with an interval of 10 °C for 80 min for both of the body extract and coelomic fluid as following: A 4-ml mixture containing each body extract or coelomic fluid was kept at any given temperature for 80 min. then, For every 20-min interval, a volume of the heat-treated body extract or coelomic fluid was cooled on ice for 30 min and the activity of cellulase was determined at room temperature and optimum pH and finally the absorbance measured at 540 nm. A mixture of CMC and DNS was used as the control sample. The control sample was not heated and directly cooled on ice. Eventually, the half-life of the cellulase was calculated for each of body extract and coelomic fluid at any given temperature as reported in the earlier study [12]. To draw the thermal deactivation plot, enzyme activity at any time (A) was divided by enzyme at zero time (A_0) to calculate A/A_0 ratio, and then the ratios were drawn against time.

Effects of Metal Salts and EDTA on the Cellulase Activity

The effects of the metal salts include KCl, $MgCl_2$, $HgCl_2$, NaCl, $FeCl_3$, $CaCl_2$ and EDTA on the cellulase activity were studied with the following conditions: three concentrations (1, 5 and 10 mM) of any of the salts were used in a reaction mixture containing Tris (pH 9), body extract or coelomic fluid and 2% CMC and incubated for 80 min at room temperature. Subsequently, after the addition of DNS and heat treatment, the heated mixture was centrifuged at 6000 rpm for 5 min, and then after removing the yellowish upper layer the absorbance of the upper phase, measured at 540 nm. The activity of each sample has been shown compared with the control sample which lacks any of the aforementioned salts.

RESULTS

Effects of pH on the Cellulase Activity

The results of the effects of pH on the enzyme activity for coelomic fluid and extract body indicated that the cellulase activity is decreased at lower pH (acidic) while it is increased in higher pH (alkaline) and reaches maximum at pH 9 (Fig. 1). Thus, pH 9 was regarded as the optimum level for cellulase from both body extract and coelomic fluid.

Determination of pH Stability of Cellulase

Data in Fig. 2 shows the effects of various pH values (4, 9 and 10.6) on cellulase stability A: from body extract and B: from coelomic fluid of the earthworm. The slope of the plot of pH stability indicates that at optimal pH level (pH 9), cellulose is highly stabile which means that the earthworm

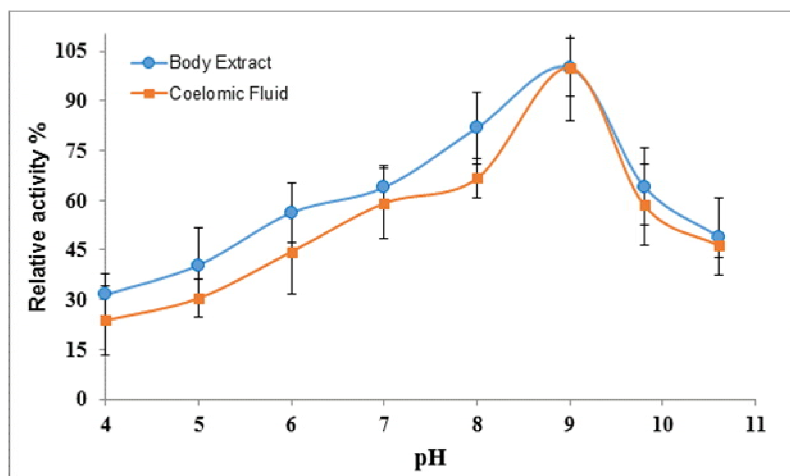

Fig. 1. The effects of different temperatures on cellulase activity from body extract and coelomic fluid.

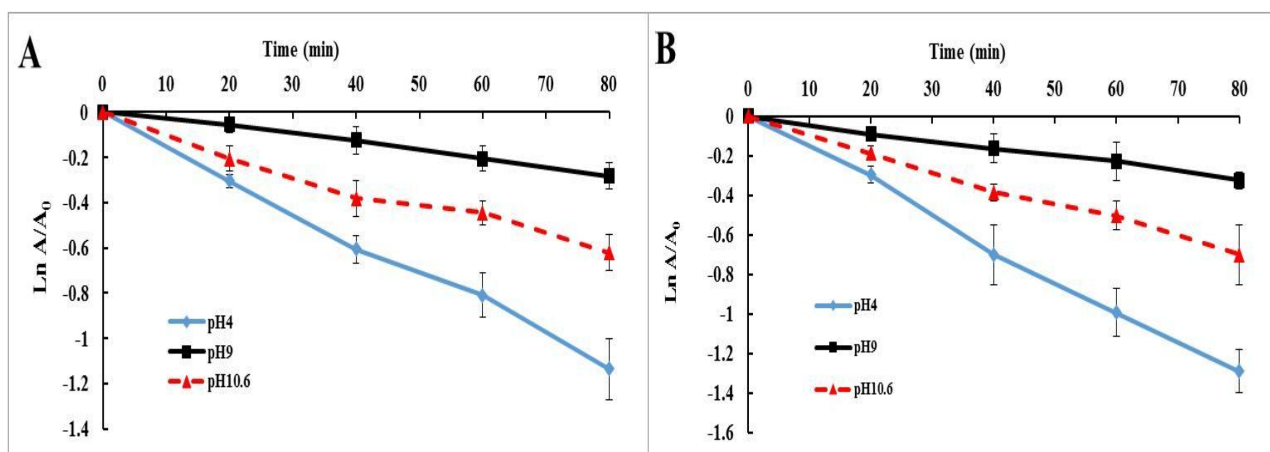

Fig. 2. The effects of various pH levels on the stability of cellulase from (A) body extract and (B) Coelomic fluid.

Allolobophora chlorotica equipped with an alkaliphilic cellulolytic enzyme.

The results of effects of various pH values (4, 9 and 10.6) on the stability of cellulase from coelomic body of the earthworm (Table 1) show that the enzyme is most stable at optimum pH (*i.e.* pH 9) and again its stability is more at alkaline than acidic pH.

Effect of Temperature on Cellulase Activity

The results of thermal effects on the cellulase activity from the body extract and coelomic body (Fig. 3) of the earthworm show that at lower temperatures the enzyme is less active while its activity increases as the temperature rises and reaches its maximum at 50 °C. This value (50 °C) was selected as the optimal temperature for the enzyme assay. However, when the temperature exceeds 50 °C, the enzyme activity abruptly falls and thus the lowest activity was recorded at 70 °C.

Thermal Deactivation and the Half-life of Cellulase

Figure 4 depicts the effects of thermal deactivation of cellulase from the body extract within the temperature ranges of 30, 40, 50, 60 and 70 °C. The slop of plot for each temperature (Fig. 4A and Table 2) indicates that cellulase

Table 1. The Slopes of pH Plot Obtained from pH Stability Plots

Source	pH	Slope (min^{-1})
Body extract	4	-0.0139
Body extract	9	-0.0036
Body extract	10.6	-0.0074
Coelomic fluid	4	- 0.0164
Coelomic fluid	9	-0.0036
Coelomic fluid	10.6	-0.0086

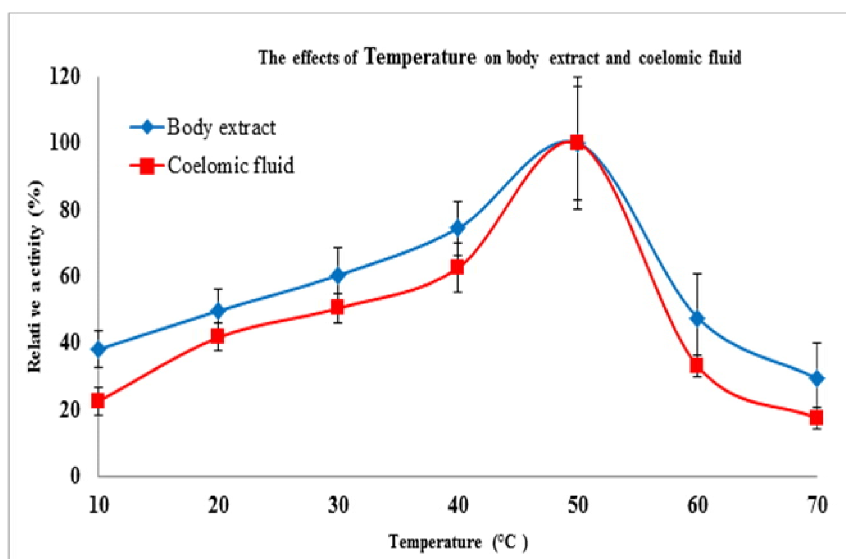

Fig. 3. The effects of different temperatures on cellulase activity from body extract and coelomic fluid.

activity of body extract is more stable at temperatures lower than the optimum temperature (*i.e.* 50 °C). The half-life of cellulase from body extract decrease with increase in temperature.

Regarding cellulase from coelomic fluid, results indicate that when the temperatures rise, the half-life of the enzyme decreases, and it becomes highly unstable. Results for cellulase from coelomic body is nearly similar to those of the body extract (Fig. 4B and Table 3).

Effects of Metal Salts and EDTA on Cellulase Activity

The effects of the metal salts KCl, $MgCl_2$, $HgCl_2$, NaCl, $FeCl_3$, $CaCl_2$ and EDTA on the cellulase activity (at three concentrations) is shown in Fig. 5A (for the body extract) and Fig. 5B (for the coelomic body). It was found that metal ions including KCl, $MgCl_2$, NaCl, $FeCl_3$ and $CaCl_2$ have stimulatory effects on the enzyme activity, whereas $HgCl_2$ and EDTA have inhibitory effects on it. The effect of an ion depends on its concentration. The activities in the presence of these compounds were assessed in comparison with a control sample lacking metal salts (100%).

DISCUSSIONS

Earthworms are the most important terrestrial

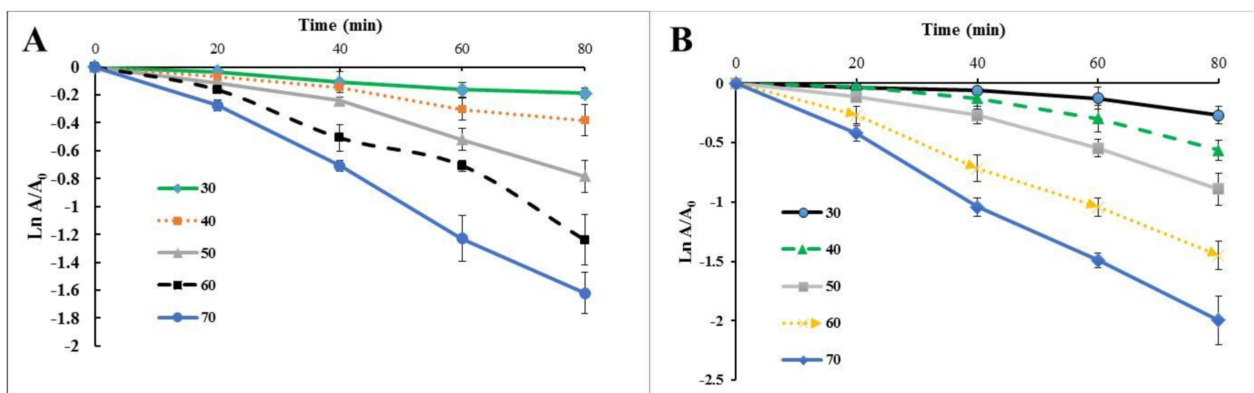

Fig. 4. The effects of various temperatures on the cellulase stability from (A) body extract and (B) Coelomic fluid.

Table 2. The Half-life Values of Cellualse from Body Extract at Different Temperatures Obtained from Thermal Deactivation Plots

Source	Temperature (°C)	k_d (min^{-1})	$T_{1/2}$ (min)
Body extract	30	-0.0025	277
Body extract	40	-0.0050	138
Body extract	50	-0.0099	70
Body extract	60	- 0.0151	45
Body extract	70	-0.0210	33

Table 3. The Half-life Values of Cellualse from Coelomic Fluid at Different Temperatures Obtained from Thermal Deactivation Plots

Source	Temperature (°C)	k_d (min^{-1})	$T_{1/2}$ (min)
Coelomic fluid	30	-0.0031	233
Coelomic fluid	40	-0.0070	99
Coelomic fluid	50	-0.0111	62
Coelomic fluid	60	- 0.0184	37
Coelomic fluid	70	-0.0253	27

invertebrates in terms of both biomass and activity and are ubiquitous in most soils worldwide. They have valued organisms with low-cost source of many bioactive molecules which can be applied to human and veterinary medicine. Earthworms are the sources of many enzymes such as cellulase. Currently, this enzyme is widely used

an increase in pH from acidic levels to neutral or alkaline ones will lead to a marked enzyme activity so that maximum activity can be observed at pH 9. Thus, pH 9 can be considered as the optimum pH of the cellulase activity, even though when the level of pH exceeds the optimum range, the activity abruptly falls. According to the results

obtained, cellulase is more active at alkaline than at acidic pH, however this effect is reversed- that is its activity falls when pH levels rise. In addition, based on the Figs. 1 , 2 and Table 1, the enzyme appeared to be more stable at the optimum pH value (pH 9) than at other pH levels, though the enzyme was stable at the three levels of pH tested, as time passed its stability reduced. This reduction in the enzyme stability occurred at faster rates at pH 4 than at alkaline pH. Thereby, it can be concluded that strongly acidic and/or strongly alkaline media while decreasing half-life, may cause the enzyme to be unstable.

The ability of enzymes to be active is associated with pH. In general, pH significantly affects the charges on the enzyme and its substrate so that pH changes may denature the protein structure of cellulase or inactivate it due to the appearance or removal of the on the substrate [14].

Here, the level of enzyme activity both in the body extract and coleomic fluid enhanced with increasing temperature and reached its maximum level at 50 °C (Fig. 3). Therefore, the 50 °C was considered as the optimum temperature for the enzyme activity. Therefore, it can be seen that the enzyme is moderately thermostable while show the highest activity in alkaline pH. At temperatures above the optimum level, the activity of the enzyme decreased so that at 70 °C the enzyme loss its activity. Moreover, the results obtained from the thermal deactivation of the enzyme (Figs. 3, 4 and Tables 2 and 3) showed that cellulase is most stable at the optimum temperature and at higher temperatures its stability drops, but at lower temperatures demonstrated a longer half-life. This is meant that at a temperature range of 30-70 °C, cellulase can withstand over a longer period of time, while when the temperature increases this time decreases and the enzyme goes through inactivation. In other words, at first the enzyme activity increases as a result of interactions between the enzyme and substrate however, as time passes the enzyme is denatured and loses its activity [15-17].

At the other extreme, the results of the effects of metal salts and EDTA on the enzyme activity (Figs. 5A and B) in the current study showed that $CaCl_2$, NaCl, $MgCl_2$, KCl have increasing effects on cellulase activity, but $HgCl_2$ and EDTA have inhibitory effects.The inhibitory effect of Hg^{2+} ions is possibly due to its binding to the thiol group, the interaction of carboxyl group and/or the imidazole group of amino acid [18]. This leads to irregular and unusual disulfide bonds in the protein and consequently reduced the activity of the enzyme. Furthermore, The inhibitory effect of EDTA can be possibly attributed to its chelator effects because EDTA can removes activator ions from the reaction assay volume which results in inhibition of cellulase activity [19].

Reviewing the literatures show that Xu and coworkers have purified a cellulase form blue mussel (*Mytilus edulis*) and they found an unusually broad optimum activity temperature range between 30 and 50 °C for the enzyme at pH 5.5 [20]. The mentioned study has reported that the enzyme activity dropped quite rapidly at temperatures above 50 °C. In another study conducted by Yan-Hong and colleagues, two novel endo-β-1,4-glucanases, EG45 and EG27, were isolated from the gastric juice of Mollusca (*Ampullaria crossean*) [21].

According to results, they have observed that optimum temperature ranges for the enzymes were between 50 and 60 °C and above 60 °C, the enzyme activity gradually reduced so that both enzymes were completely inactivated at 70 °C. In the previous study, using molecular cloning, they were able to characterize two endogenous cellulases, Ag-EGase I and Ag-EGase II from the mulberry longicorn beetle (*Apriona germari*). In another report, some researchers have purified a halostable cellulase from bacterium *Salinivibro* sp. NTU-O5. The optimum temperature of the later enzyme is 35 °C but as the temperature increases the enzyme activity decreases so that no activity can be detected at 70 °C. Based on the later report, the cellulase activity has significantly increased by adding Mg^{2+}, Na^{+}, K^{+} and Fe^{3+} but decreased by adding Hg^{2+} [22] .

In addition, by purification and characterization of a cellulalse from North Pacific krill *Euphausia pacifica* it was found that the optimum temperature of the enzyme was 40 °C at pH 6 [23]. The enzyme is stable in the pH range from 5-9, being most stable within the range of its pH optimum. The enzyme stability reduces at a highly acidic or alkaline pH, having the highest thermal stability within the 20-40 temperature range, and as the temperature is raised, the stability decreased [23]. The effects of various metal ions (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Al^{3+}, Co^{2+}) and anions (Cl^{-}, and CH_3COO^{-}) on two cellulases were conducted by. They

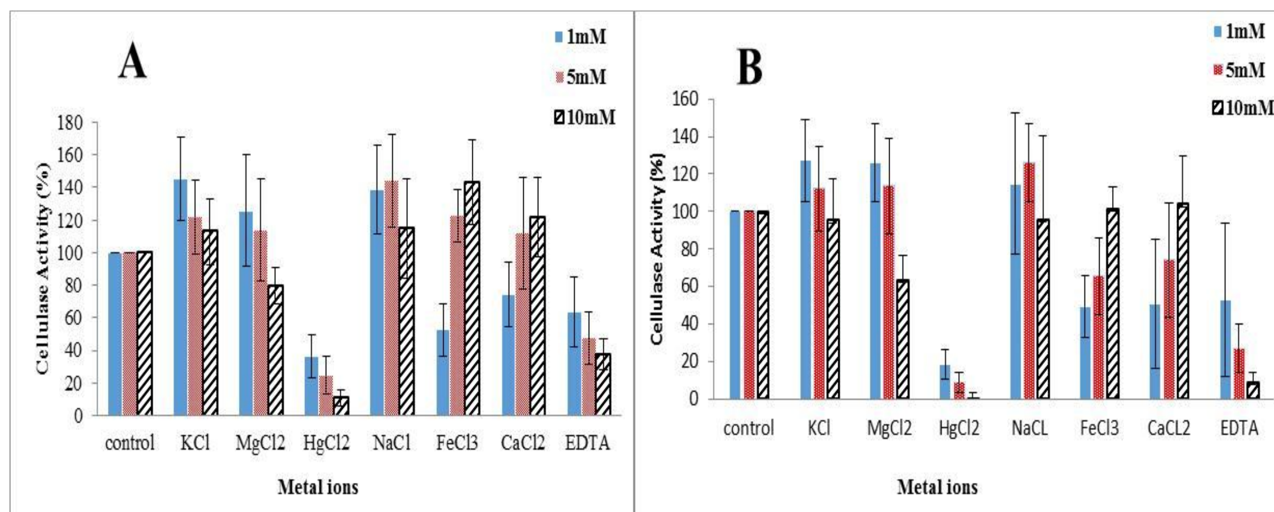

Fig. 5. The effects of metal salts and EDTA on cellulase activity (A) from the body extract and (B) from the coelomic body.

found that heavy metal ions such as Ag^+, Hg^{2+} and Mn^{2+}, showed a tendency to inhibit cellulase activity [24].

Similarly, the investigation of carboxymethyl cellulase from *Artemia salina* after the final purification, Showed that optimal temperature and pH values were found to be at 55 °C and 8.0, respectively. Additionally, previous study has reported that in the presence of $CaCl_2$ and $MgCl_2$, the enzyme has shown the highest activity and also inhibition of the enzyme activity occurred in the presence of EDTA and KCl. According to later study KCl inhibits the cellulase less than 20% [25].

According to a report, cellulase is composed of three main subgroups each with different molecular weights [25]. All three isoforms appear to be affected differently by temperature, pH and metal ions. Thus, different types of responses might be expected in different organisms. Moreover, a broad spectrum of cellulases under favored conditions has been recognized which have high activity at the optimum temperature and their activities decline with increasing temperature [25]. Different types of interactions take place within unpurified cellulases which may modify their activities. Nevertheless, our findings show the presence of a moderately thermostable cellulase with similar behaviors from coelomic fluid and body extracts of *Allolobophora chlorotica* for the first time which can be purified and characterized to be exploited in biotechnology.

CONCLUSIONS

According to our results, coelomic fluid and body extract contain a novel similar cellulase which can prove coelomic cellulase secrets from the earthworm intestine. The purification of this novel moderate-thermostable cellulase to exploit in biotechnology can be carried out from the low-cost source of the earthworm *Allolobophora chlorotica*.

Conflict of Interest Statement

The authors state that they have no conflict of interest.

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