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Biodegradation of cellulose and xylan by a paddy pest, *Oxya chinensis*

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ABSTRACT

Oxya chinensis Thunberg is a very serious paddy pest in Pakistan and is a major cause of rice crop deterioration. Total cellular protein extract was able to degrade plant biomass from sugar cane bagasse, as observed under microscope. Its endoglucanase and xylanase activities were characterized. The endoglucanase activity showed two pH optima 2.0 and 7.0 at 50-60°C. Endo-beta-1,4-glucanase activity enhanced by calcium ions. A reducing agent beta-mercaptoethanol inhibited the enzyme activity, showing the involvement in stabilizing the three-dimensional structure of the molecule. The enzyme product was identified as glucose by thin layer chromatography. For xylanase the pH optimum was 4.2 and 7.0-8.0. $CaCl_2$ and 2-mercaptoethanol are proven to be activator in lower concentration for xylanase activity.

Key words: Oxya chinensis, cellulose degradation, cellulase, xylanase.

INTRODUCTION

Oxya chinensis is one of the most common and widespread insects in Asia and is abundantly found in the rice paddies, in sugar cane and other graminaceous plants. Because this organism causes extensive damage in an agriculturally important sector, this species has received much attention at different levels studies throughout the world [1-5]. Another grasshopper, *Zonocerus variegatus*, has been studied for morphometric data and enzyme activities in the femoral muscles[2,5]. The insect is totally dependent on crops for its nutritional requirements. Plant biomass is mainly composed of cellulose and hemicelluloses. These polysaccharides are resistant to degradation, and the insects themselves do not secrete all of the digestive enzymes to hydrolyze β-linkages in the polymer. Rather, much of the hydrolysis of these polysaccharides is carried out by enzymes produced by the microbial symbionts, or associated microbes [6-8].

Recently, it has been observed that insects produce their own cellulases by expressing indigenous cellulase gene [9-12]. Cellulose hydrolysis is of two types: acidic hydrolysis and enzymatic hydrolysis.

During acidic hydrolysis in industries, cellulose is treated with dilute acid. Acid treatment softens the cellulose and results in the complete hydrolysis of cellulose to D-glucose units.

Enzymatic hydrolysis involves a series of enzymes, cellulases, which act in a synergistic way to completely hydrolyze cellulose to simple sugars. These enzymes are highly specific for their substrates. The product of one enzyme acts as a substrate for the other enzyme and in this way cellulase system completely degrades cellulose to simple sugars useable by animals. Cellulases are O-glycosyl-hydrolases (GHs) that hydrolyse β-1,4-glucosidic bonds in cellulose. They are found in all kingdoms, predominantly in prokaryotes and fungi [13]. Interest in these enzymes has increased in recent decades due to their important role in the global carbon cycle, as well as their use in alternative fuel production and other industries [14]. The cellulolytic enzymes are members of a superfamily of GHs (glycoside hydrolases), with more than 2200 known protein sequences. To date, the GHs have been classified into more than 80 different GH families based on their amino acid sequence similarities [15-17]. Cellulases can be classified into three types: endoglucanases (1,4-β-D-glucan 4-glucohydrolase, EC 3.2.1.4), exoglucanases (β-1,4-glucan cellobiohydrolase), and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21). Endoglucanases cut cellulose chains at random positions in less crystalline regions, creating new chain ends. Extreme endoglucanases, often called CM-cellulases (carboxymethyl-cellulases) have little activity towards crystalline cellulose, but hydrolyse readily CMC, acid-swollen cellulose and even barley β-glucan in a random fashion, resulting in a rapid fall in the degree of polymerisation [18]. Exoglucanases release cellobiose units mainly from the chain ends (either from reducing end or from non-reducing end). Their action results in the rapid release of reducing sugars but little change in polymer length. Exoglucanases degrade preferentially crystalline cellulose in a progressive manner. Cellobiose is subsequently hydrolysed by *β*-glucosidases to glucose [19]. A complete understanding of the enzyme system involved in the cellulose and hemicellulose degradation by the insect will help to protect fields from pest attack by masking the enzyme active sites or in turn the enzymes isolated from insect may find a biotechnological importance, as not much attention has been paid in this regard. It is pertinent to mention that till now the work was done on beetle or sea animal cellulases, but there is no attempt to study the biodegradation of plant biomass by *O.chinensis*.

MATERIALS AND METHODS

The rice grasshoppers, *Oxya chinensis*, were collected from the local fields of Punjab University, New Campus, Lahore during the month of April in the morning, washed, dried and then preserved in freezer for future use.

Freshly collected insects (5.0 g) were ground in 150 ml of Tris-HCl buffer (pH 8.5, 0.05 M), filtered through a thick sieve and then centrifuged. The supernatant was used as a source of enzymes as previously described [12, 20]. Protein concentration was estimated by Bradford method [21]. To check the hydrolysis of plant biomass by the protein extract of *O.chinensis*, 5ml

of the enzyme source containing 1 mg of protein was mixed with 5 ml of buffer pH 7.5, 0.1M (Tris-HCl) containing 0.1 g powdered sugar cane bagasse, the reaction mixture was kept in a shaker at 50 °C overnight at 250 RPM. Next morning the residual substrate was viewed under the microscope and was photographed. (The sugar cane bagasse substrate was prepared as follows. 100 g of sugar cane bagasse was obtained from a local fruit shop of sugar cane juice, washed with distilled water and dried. After drying, bagasse was ground in a grinder and a powder was obtained. The powdered sugar cane bagasse was passed through a sieve and was stored in a dried bottle till further use).

CMCase assay was performed as described by Sami et al. [22].

For xylanase assay, the reaction mixtures, comprising of 2ml of 2% xylan from birch wood, 1ml of buffer of either pH 4.2 or pH 7.0, 1ml of distilled water and 100µl of enzyme, were incubated at different temperatures for 90 minutes. DNS (3-5 dinitrosalicylic acid reagent), 3 ml, was added to each reaction mixture. The mixtures were filtered to remove any insoluble particle and heated in boiling water bath for 15 minutes and absorbance was taken at 546 nm against blank to estimate the reducing sugars. Optimum assay pH was determined for both endoglucanase and xylanase activities. pH range was from pH 1.0 to pH 10.2. HCl-KCl buffers in the pH range 1.0 to 2.2; Citric acid- Na_2HPO_4 buffers in the pH range 2.6 to 7.6; KCl-H_3BO_3- NaOH buffers in the pH range 8.2 to 10.2 were prepared.

Paper chromatography was performed using Whatman filter paper No.1 . Sample hydrolysates were prepared and spotted on the filter paper. The reference sugars as glucose and cellobiose (1%) were also applied on the filter paper. The chromatograms were run at room temperature for 4 hours.

Products were identified as described previously [22] by dipping the chromatogram in $AgNO_3$ solution. The chromatograms were then air dried and sprayed with NaOH solution. Black spots were appeared against the light greenish brown background.

RESULTS

Hydrolysis of plant tissues (sugar cane bagasse) by protein extract of *O.chinensis*

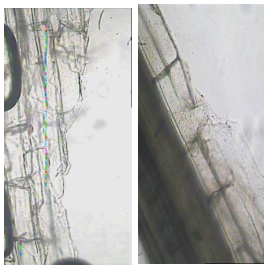
To check the effect of total protein extract on the plant tissue, sugar cane baggasse was incubated with the total protein extract for 24 h at 50 °C with constant shaking to facilitate the reaction. A blank was similarly prepared and buffer pH 8.5 0.1M was used instead of protein extract. Micrographs of 1%bagasse before and after treatment with *O.chinensis* total protein are shown in Fig. 1 (a,b). It was clearly visible that the plant tissues composed of cellulose and xylan were degraded as compared to the untreated tissue. This was an indication of the presence of cellulose and xylan hydrolyzing enzymes in the total protein extract of the insect. Carboxymethylcellulose hydrolysis by *Oxya chinensis* was measured at different pH values ranging from 1 to 10.2 at 50°C. It was noticed that the enzyme showed two pH optima: a sharp pH optimum was obtained at pH 2, while a broad range pH optimum was observed between 6-8 with maximum activity at pH 7.0, as shown in Fig.2. The activity of endo-beta-1,4-glucanase at

different temperatures was determined (4ºC to 80ºC). 3% CMC was used as substrate. For CMCase, maximum activity was obtained at 60°C for pH 2.0 and 50ºC at pH 7.0 (Fig.3).

Effect of substrate concentration on endoglucanase activity was determined. For this assay enzyme concentration was kept constant and amount of substrate was changed from 100 µl to 3.0 ml (3%) at pH 2.0, 2.2 and 7.0 at 50ºC. The effect of substrate concentration is shown in Fig.4.

The effect of 2-mercaptoethanol on the activity of endoglucanase was determined at different concentrations of 2-mercaptoethanol (0.1% to 0.5%). This test was also performed at two temperatures i.e., 50°C with pH 2.0 and pH 7.0 and 60°C with pH 2.0 and 7.0. There was a constant decrease in endoglucanase activity with increase in 2-mercaptoethanol concentrations (Fig.5).

Effect of $CaCl_2$ on the activity of endoglucanase was studied. For this test 0.1 M $CaCl_2$ was used. Different concentrations of $CaCl_2$ were used in reaction mixture. It was observed that Ca ions served as an activator of the enzyme activity (Fig 6). End product of the enzyme action was identified as glucose, when hydrolysate after 24 h of reaction was analyzed by thin layer chromatography (Fig.7). Xylanase assay was performed at different pHs ranging from 1.0 to 10.2. Reaction mixture consisted of 2 ml 2% xylan, 1 ml distilled water, 1 ml buffer and 50 µl of crude enzyme. Incubation period was 90 minutes and temperature was 50°C. Xylanase activity showed a broad pH range for its activity e.g 4.2 and 6-8 with an optima of 7.0. (Fig.8). Xylanase activity was checked in the temperature range from 4°C to 80°C at pH 7.0 & pH 4.2. Maximum xylanase activity was at temperature 50°C for pH 7.0 (optimum pH), where as for pH 4.2 two peaks were obtained, one at 50°C and other at 70°C with comparatively higher activity at 70°C (Fig.9). In order to determine the effect of 2-mercaptoethanol on xylanase activity, 0.1% to 0.5% solutions of 2-mercaptoethanol were used under the optimum conditions of temperature and pH. A concentration of 2% xylan was used, results showed that there was an increase at lower concentrations(0.1%) but at higher concentrations of reducing agent, a decrease in activity was observed (Fig 10). For the effect of Calcium chloride for the effect on xylanase activity, there was an increas till concentration of 6.25µM $CaCl_2$ and inactivation was observed at higher concentration (Fig.11).



a **b**

Fig: 1 Hydrolysis of cellulose and hemicelluloses by cellulases and xylanases of *O.chinensis.* Sugar cane baggasse was used as a substrate. a.treated. b.untreated

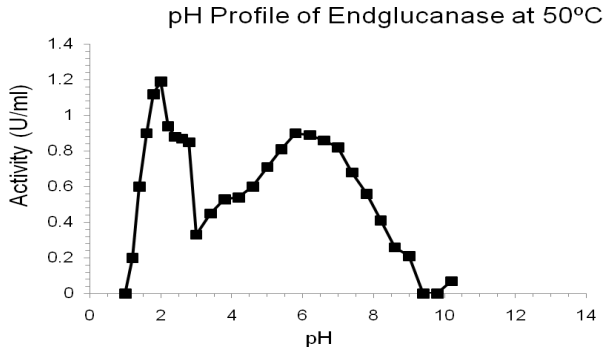


Fig.2 pH Profile of CMCase at 50ºC

pH range was 1.0 to 10.2. Reaction mixture consisted of 2 ml 3% CMC, 1 ml water, 1 ml buffer of respective pH and 50 µl of enzyme, Incubated at 50ºC for 90 minutes. Maximum activity was obtained at pH 2.0 and a broad peak was in the pH range 4.0-7.0

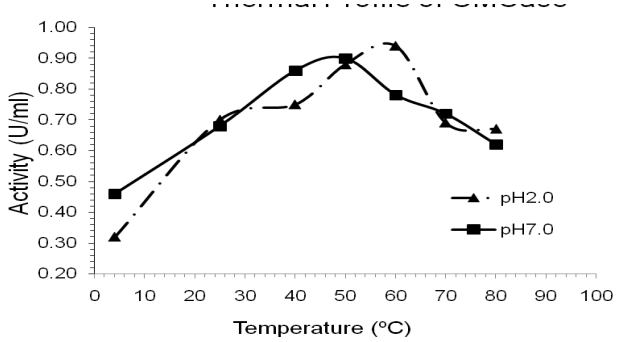


Fig.3: Thermal profile of CMCase

The reaction mixture consisted of 2 ml 3% CMC,1 ml buffer of either pH 7.0 (■) or pH 2.0 (▲), 1 ml water and 50 µl enzyme. Incubation period was 90 min. and temperature range was 4ºC to 80ºC. For pH 7.0 maximum activity was given at 50ºC and for pH2.0, at 60ºC.

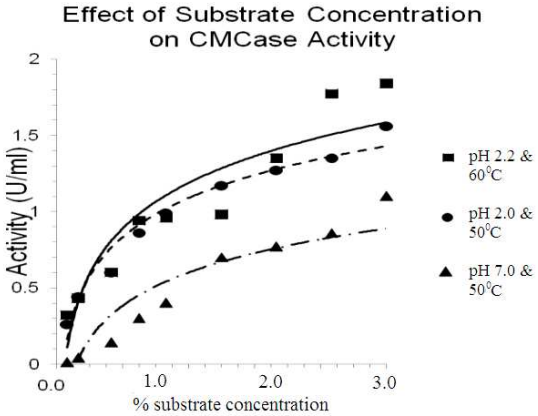


Fig.4: Effect of substrate concentration on CMCase activity

The reaction mixture consisted of 3 ml of CMC in the conc. range 3%-, 1 ml buffer, 1 ml water and 50 µl enzyme. Incubation time was 90 minutes. In each of the three sets of data, the activity increased logarithmically

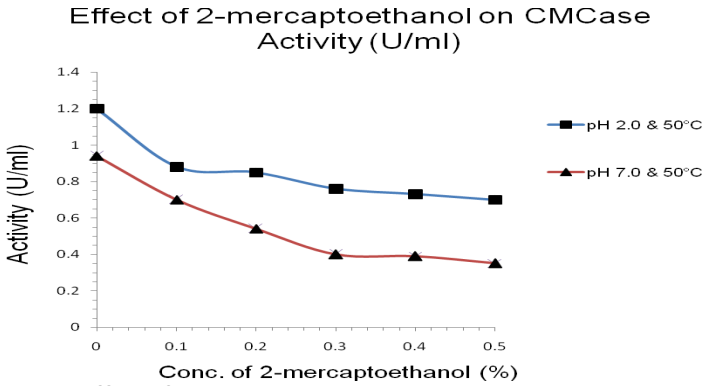


Fig.5: Effect of 2-mercaptoethanol on CMCase activity at 50°C

Conc. of 2-mercaptoethanol was in the range 0.1%-0.5%. The reaction mixture consisted of 1 ml of buffer, 1 ml of water, 50 µl of enzyme and 2 ml of 3% CMC. Temperature was 50ºC & time period was 90 min. at pH 2.0 (■) & pH 7.0 (▲)

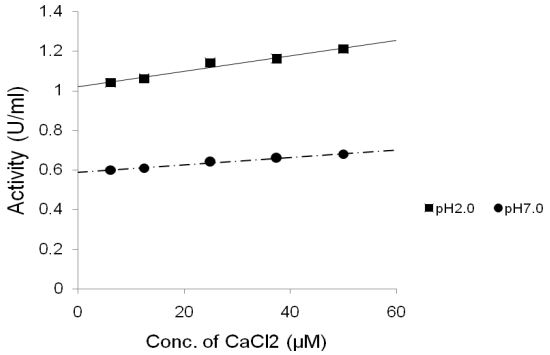


Fig.6: Effect of $CaCl_2$ on CMCase activity at 50°C for pH 2.0 & pH 7.0

The reaction mixture consisted of 1 ml of buffer, 1 ml of water, 2 ml of 3% CMC and 50 µl of enzyme at different conc. of $CaCl_2$ solution

A b c

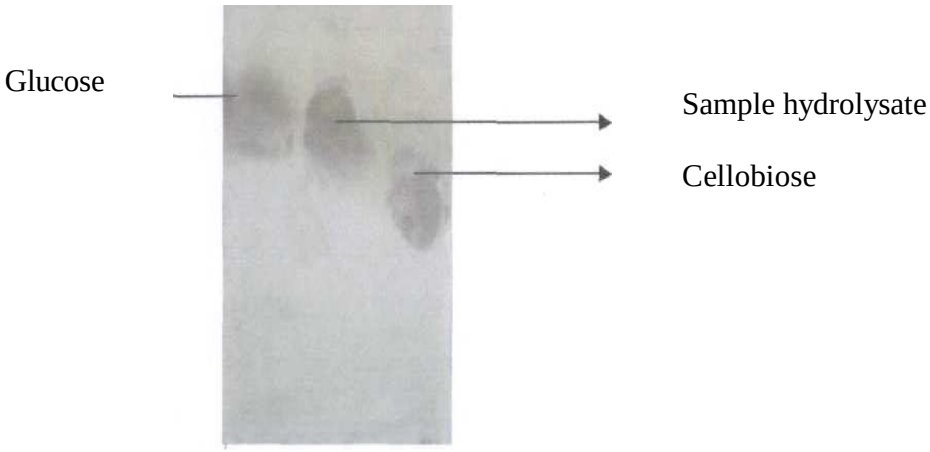


Fig.7 Identification of endo-beta-1,4-glucanase activity on paper chromatogram

Hydrolysate after 20 h of incubation was loaded onto the chromatogram and was stained with silver nitrate. End product was appeared to be glucose

A: cellobiose b: glucose c; enzyme hydrolysate. The end product of endo1,4-beta glucanase was identified as glucose

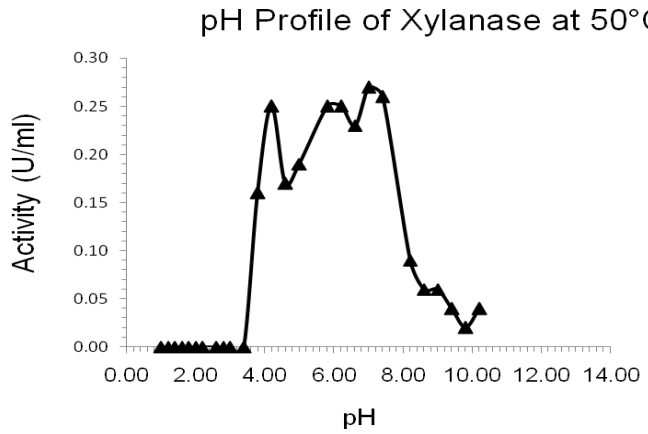


Fig.8: pH Profile of xylanase at 50°

pH range was 1.0 to 10.2. Reaction mixture consisted of 2 ml 2% xylan, 1 ml water, 1 ml buffer of respective pH and 50 µl of enzyme. Incubated at 50ºC for 90 minutes. Absorbance was taken at 546 nm to measure the conc. of reducing sugars by DNS method

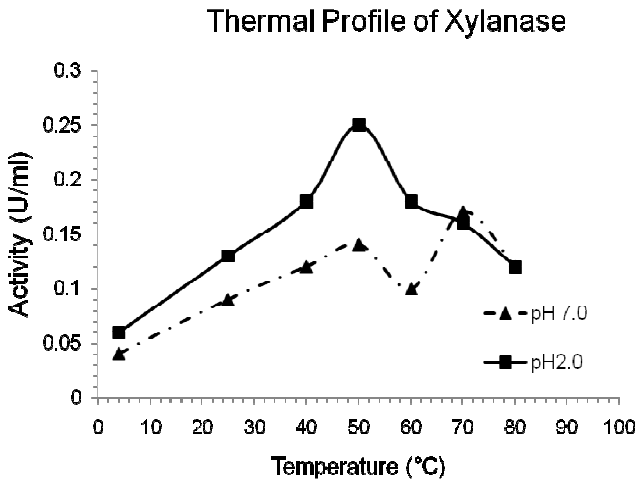


Fig.9 : Thermal profile of xylanase

The reaction mixture consisted of 2 ml 2% xylan, 1 ml buffer of pH 7.0 (■) or pH 4.2(▲), 1 ml water and 50 µl enzyme. Incubation period was 90 minutes and temperature range was 4ºC-80ºC. At pH 7.0 optimum temperature was 50ºC and for pH 4.2 it was 70ºC

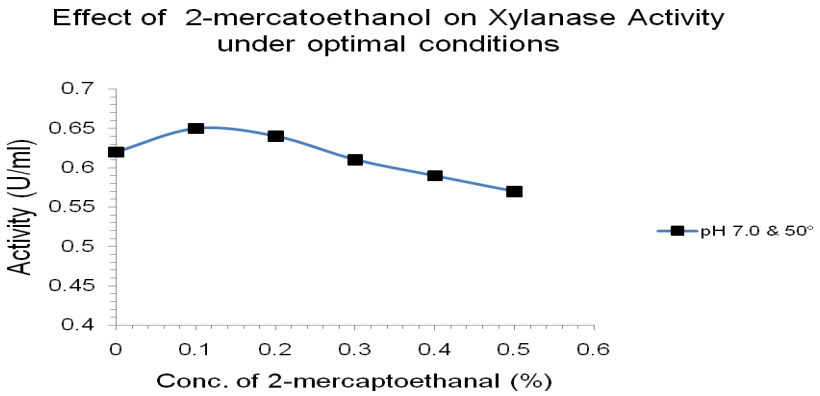


Fig.10: Effect of 2-mercaptoethanol on xylanase activity
Reaction was carried out at its optimal pH and temperature i.e., 7.0 and 50ºC respectively. The activity first increases, reaches its max. value and then decreases with an increase of conc. of 2-mercaptoethanol

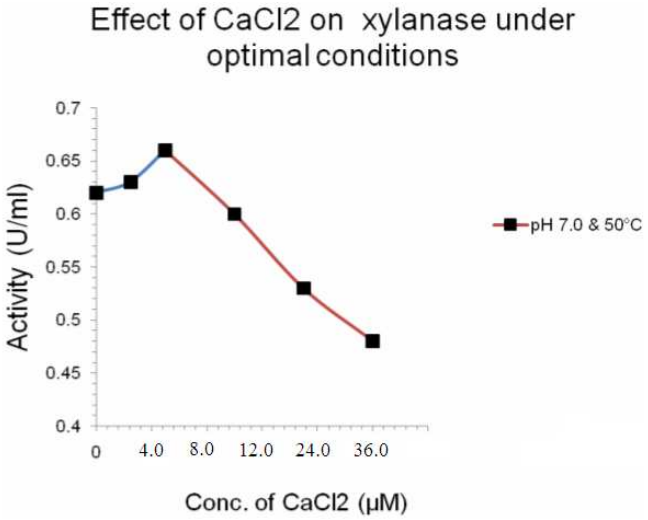


Fig. 11: Effect of $CaCl_2$ on xylanase activity under optimal conditions of pH (7.0) and temperature (50 ºC)
The reaction mixture consisted of 1 ml of buffer, 1 ml of water, 2 ml of 2% xylan, 50 µl of enzyme and different conc. of $CaCl_2$. Incubated at 50°C for 90 minutes

Neem as Natural Inhibitor of Cellulase

In order to check the inhibitory effect of Neem on CMCase activity of different types of insects; crude extract of its leaves was used. Extract of Neem was prepared in distilled water as well as in methanol. CMC agar plates were used in order to check the activity of CMCase of *O.chinensis*. 34% of CMCase activity was inhibited by *Azadirachta indica* (Neem) extracts.

DISCUSSION

Insects are one of the major population of the organisms on the earth and play a pivotal role in the maintaining the environmental conditions by recycling most of the important elements. Carbon, hydrogen and oxygen, as they are the major components of the plant biomass, utilized as feed by number of the insects. Plant biomass is mainly composed of cellulose, hemicelluloses

and pectin. Insects used complete enzyme systems for the hydrolysis of the bioorganic polymers, they could be exogenous or endogenous. Exogenous enzyme system could be provided by the microbes (pathogenic and non-pathogenic) present in the surrounding which generate a consortium of enzymes required for the hydrolysis of plant biomass. Endogenous sources could be in the salivary glands, gut of the insect either expressed by the insect [11]. or by symbiotic microbes [11,23]. We have previously shown that *Oxya chinensis* produce endo-beta-1,4-glucanase activity [23]. Grasshoppers mainly feed on leaves so they have mouth parts that slice through leaves. Cellulose and hemicelluloses constitute the plant biomass as main plant cell wall components. To note the effect of total protein extract on the plant biomass, the protein extract was incubated with the enzyme source and it was clearly visible that the proteins were able to damage the plant tissue as shown in Fig.1, indicating the presence of a complete system of enzymes for the destruction of plant biomass. As cellulose fibers are packed in the xylan fibres, thus their hydrolysis (as shown in Fig.1 under the microscope) was a clear indication that both cellulase and xylanase are present in the enzyme system of the insect. Glycohydrolase family 9 of carbohydrases has been reported to have cellulases able to hydrolyse cellulose and Xylose as well. Grasshoppers, are completely dependent upon plant biomass for their nutritional requirements like other herbivorous insects. They use graminacious plants as their food e.g., rice, corn etc. The endoglucanase activity was characterized for optimum pH, temperature, effect of substrate concentration, effect of 2-mercaptoethanol and calcium chloride. There were two pH optima noted for the enzyme 2.0-and 7.0 (Fig. 2). All animal cellulases reported until now have optimal activity under the weak acidic conditions. It has been reported that the gut of insects has acidic pH, the acidic pH opti-ma could be related to the acidic environment of the gut. Cellulose could be hydrolyzed to its components with the acids and the treatment with acids lead to generate ends for enzymatic hydrolysis [24]. Previously, Sugimura et al., [25] in the studies on the isolation of cellulase from beetles showed that optimum pH for the highest activity of cellulase from the larval gut of *P. hilaris* against CMC was 5.5. *Apriona germari* showed maximum activity at pH 6.0 against CMC [26]. The recombinant Ag-EGase III, isolated from mulberry longicorn beetle showed highest activity at pH 6.0 [27]. We have reported the pH optima 7.8 of red pumpkin beetle *Aulacophora foveicollis*. Due to these highly acidic pH optima, endoglucanase is able to hydrolyze cellulose into simple sugars completely by itself. Rice hulls are considered waste materials because of their low value as animal feed due to poor digestibility, peculiar size, little bulk density, high ash/silica contents, and abrasive characteristics. They contain about 36% cellulose and 12% hemicelluloses. The acidic pH optima of the enzyme could be reason for the hydrolysis of cellulose by the insect. This showed that highly acidic endoglucanase does not require exoglucanase activity which is required for generating the ends of the polymer. So far there is no report on the acidic hydrolysis by an endoglucanase present in the total cellular protein extract of the insect. Acidic hydrolysis is equivalent to exoglucanase activity as it softens the cellulose producing free ends. Rice husk has hard crystalline structure and it requires strong acidic conditions for softening and degradation. *Oxya chinensis* has highly acidic pH optima of endoglucanase so it is able to hydrolyze rice husk very efficiently. It is the first endoglucanase showing highly acidic pH optima i.e., 2.0. with optimum temperature at 60°C (Fig.3). Two different pH optima could be a result of expression of more than one genes or presence of another catalytic site in the enzyme primary structure, high acidic pH may not be required at further stages of cellulose and hemicelluloses hydrolysis. Multiple forms of endoglucanases have earlier been reported by us, comparison to microbial cellulases [28]. The effect of 2-mercaptoethanol on endoglucanase was also studied. 2-

mercaptoethanol caused a sudden decrease in endoglucanase activity initially which was followed by a slight decrease in activity (Fig.4). This indicates the involvements of sulfur containing amino acid residues. 1µM sol. of $CaCl_2$ caused a rise in endoglucanase activity with increase in its conc. (Fig.5), perhaps due to the calcium binding cleft near the active site of the enzyme. Effect of substrate conc. on endoglucanase activity was also studied. The rate of reaction rise linearly as substrate concentration increased and then began to level off and approached a maximum at higher substrate concentrations. This was the enzyme's equilibrium stage at which the rate of forward reaction was equal to the rate of backward reaction. Thus endoglucanase obeys Michaelis-Menten kinetics (Fig.4). To determine the kinetic parameter of the enzyme Km and Vmax purification of the enzyme is required, as at this stage a number of contaminants could affect the two parameters. Hydrolytic product of the enzyme was identified as reducing sugar, mainly glucose, as shown in Fig.7. Xylan is an important constituent of hemicelluloses. Xylanase makes cellulose accessible for hydrolysis by cellulase by degrading xylan. Xylanase of *Oxya chinensis* was also characterized. The hemicelluloses in rice hulls contain both arabinoxylan and xyloglucan. However, rice hulls also contain high quantities of lignin (16%) and ash (20%) which complicates its use as a lignocelluloses feedstock for conversion to ethanol [29]. Optimum pH of xylanase were 4.2 and 7.0 although a broad peak was obtained in the pH range acidic to alkaline pH range. Optimal temperature was 50°C (Figs 8, 9). Effects of 2-mercaptoethanol and $CaCl_2$ on xylanase activity were studied (Figs 10, 11). In both cases xylanase activity initially increased, then a decrease was observed. Involvement of cysteine has been demonstrated for *Apriona germari* cellulase [30]. It was suggested that the active site is clipped by the presence of disulfide bridges and could be relaxed by the addition of reducing agent. The higher concentrations of 2-mercaptoethanol destabilized the enzyme three-dimensional structures and led to a decrease in activity. We have previously reported the associated microbes present in the total cellular extract of the insect [20]. Microbial flora of *Oxya chinensis* was identified and included the following bacterial species: *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus spp.* All these bacterial species are plant pathogenic and nothing of them is a potent cellulase producer. It is not confirmed whether they are inhabitants of *Oxya*'s gut or just present on its body surface. At present GH family 9 has been studied in detail with reference to plants, including tomato *(Solanum lycopersium)* from which greatest number of endoglucanases has been studied [31]. Apart from this Arabidopsis and Oryaza sativa have 22 and 23 genes identified from the genome respectively, for the expression of cellulases protein [32, 33].

Conclusively, *Oxya chinensis* produced an efficient and complete enzyme system able to hydrolyze rice leaves as well as rice husk and so able to feed on this economically important crop. Multiple forms of enzymes produced by this insect lead to total destruction of plant biomass. It was intresting to observe that extract of Neem *Azadirachta indica* (Neem) leaves was able to inhibit cellulose hydrolyzing activity to some extent [20]. Thus, neem extract could be used as a soft insecticide for protecting the crops. On other hand, plant biomass hydrolyzing enzyme activities of *Oxya chinensis* could be used for the bioconversion of cellulose into its sub units, glucose. It is required to completely study the characteristics of the enzyme system designed for the biodegradation of plant biomass. Amino acid sequence of the protein and its catalytic features will be of immense importance for biotechnological application of the enzyme system produced by *Oxya chinensis.*

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