# A Beta-glucosidase with Beta-xylosidase Activity from the Digestive Juice of the Land Crab *Cardisoma armatum*

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**ABSTRACT—** ***A bifunctional beta-glucosidase/beta-xylosidase enzyme from land crab Cardisoma armatum was purified to homogeneity by anion-exchange, size-exclusion, cation-exchange and hydrophobic interaction chromatographies and characterized to explore his suitability in biotechnology and to elucidate his role in biodegradation of plant material. The enzyme showed a single protein band and its relative molecular weight was estimated to be 69.81 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 115.24 kDa by gel filtration, indicating that the enzyme should be monomeric or homo-oligomeric enzyme. The specific activities towards para-nitrophenyl-beta-D-glucopyranoside and para-nitrophenyl-beta-D-xylopyranoside were 74.16 ± 2.10$^{-4}$ and 88.60 ± 2.10$^{-3}$ U/mg of protein, respectively. This enzyme was optimally active at pH 5.6 and at 60 °C, and was stable at pH range 4.6 to 6.6. The only substrates that were hydrolyzed by the purified enzyme were para-nitrophenyl-beta-D-glucopyranoside, para-nitrophenyl-beta-D-xylopyranoside, xylobiose, cellobiose and cellodextrins. The enzyme was specific for substrates with a beta-(1,4)-glucosidic linkages and was an exo-glycosidase. The catalytic efficiencies values for beta-D-1,4-glucoside and beta-D-1,4-xyloside were respectively 1666.67 and 132.89 mM$^{-1}$s$^{-1}$. The purified enzyme is a beta-glucosidase with beta-xylosidase activity, which takes effect after actions of endo-enzymes and exo-enzymes and it hydrolyzes cellobiose and xylobiose in glucose and xylose, respectively. However, the beta-glucosidase/beta-xylosidase catalyzed both hydrolysis and transglycosylation.***

**Keywords—** Bifunctional enzyme, beta-glucosidase, beta-xylosidase, land crab, *Cardisoma armatum*, transglycosylation

## 1. INTRODUCTION

*Cardisoma armatum* is a west africa land crab species which is living in a marshy and littoral middle [1]. Land crabs are defined as crabs that show significant behavioral, morphological, physiological, and/or biochemical adaptations permitting extended activities out of the water [2]. *Cardisoma armatum* just as a wide variety of crab are herbivorous. It means that these animals consume plant material [3] and are capable of assimilating significant cellulose and hemicellulose, which are there essential source of energy [3]. Land crabs such as *Gecarcoidae natalis* and *Discoplax hirtipes*, are able to assimilate 26-43 % of cellulose and 14-49 % of hemicellulose from a diet of brown leaf litter [4]. This assimilation can be attributed to the activities of cellulase and hemicellulase [5].

Generally cellulose enzymatic hydrolysis requires the synergistic action of endo-beta-1,4-glucanases (EC 3.2.1.4), exo-beta-1,4-glucanases or cellobiohydrolases (EC 3.2.1.91), and beta-1,4-glucosidases (EC 3.2.1.21). Endoglucanases randomly attacks internal beta-1,4-linkages in cellulosic chains, while exoglucanases liberate cellobiose from their edges. As the exoglucanase only attacks the ends of chains, its activity will be low unless endoglucanase is present to provide sufficient substrate. Beta-glucosidases then act on cello-oligosaccharides and cellobiose, liberating glucose [6, 7].

Cellobiohydrolases are also considered to be important because they are active towards crystalline cellulose and are able to displace individual chains from the surface of crystalline regions, making it accessible for enzymatic hydrolysis [6]. By contrast, endo-beta-1,4-glucanases primarily act on amorphous regions of native cellulose. Beta-Glucosidase is necessary not only because it liberates glucose but because it removes cellobiose, a known inhibitor of both endo-beta-1,4-glucanase and cellobiohydrolase activity [8, 9].

Next to cellulose, the total degradation of hemicellulose may necessitate the co-operation of two principal enzymes and several accessory ones. Endo-beta-1,4-xylanases (EC 3.2.1.8) randomly cleave beta-1,4-glycosidic linkages in the xylan main chain, releasing xylo-oligosaccharides, while beta-xylosidases (EC 3.2.1.37) release xylose from xylobiose and xylo-oligosaccharides. Accessory enzymes, including alpha-L-arabinofurosidase (EC 3.2.1.55), remove side groups from the xylan main chain [10, 11]. As beta-glucosidases cleave beta-1,4-linkages of cellobiose to generate glucose, beta-xylosidases hydrolyze beta-1,4-linkages of xylobiose to release xylose.

These enzymes are widely spread in nature, predominantly being produced by microorganisms such as fungi, bacteria and insects [12-14]. The existence of beta-glucosidases and beta-xylosidases in crab species has been proved [3, 5, 6]. However, no data have been published on the presence of bifunctional beta-glucosidase/beta-xylosidase enzymes from digestive juice of crab *C. armatum*. In this study, we investigated the purification and characterization of the beta-glucosidase with beta-xylosidase activity from land crab *C. armatum* in order to elucidate its role in the digestive tract and increase our knowledge on assimilation of cellulose and hemicellulose by this species.

## 2. MATERIALS AND METHODS

### *2.1 Chemicals*

Polysaccharides, oligosaccharides and *para*-nitrophenyl-glycopyranosides (*p*NP-glycopyranosides) were purchased from Sigma Aldrich. DEAP-Sepharose 4 Fast-flow, Sephacryl S-100 HR, CM-Sepharose CL-6B and Phenyl-Sepharose 6 Fast-flow gels were obtained from Pharmacia-LKB Biotech. The chemicals used for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. All other chemicals and reagents were of analytical grade.

### *2.2 Animal collection and enzyme extraction*

*Cardisoma armatum* were collected at the forest between the lagoon *Tchagba* and the great river Bandaman (Grand-Lahou, Côte d'Ivoire) and transported to the laboratory of University Nangui Abrogoua, Abidjan - Côte d'Ivoire. The crab samples were maintained for 72 hours without eating and were put on ice at last 30 min before dissection. Carapaces of crabs and all waste in the stomach were removed. The digestive juice was aspired with sterile syringe, pooled and centrifuged at 12,000 g for 30 min. The obtained supernatant constituted the crude extract, which was stored at - 20 °C.

### *2.3 Purification procedure*

The procedure of purification was performed at 18 °C. The crude extract (0.5 ml) was loaded onto a DEAP-Sepharose 4 Fast-flow column (2.5 × 5.6 cm) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.0). Unbound proteins were removed by washing the gel with the same buffer. Bound proteins were eluted with 40 ml of increasing discontinue gradient (0.5; 0.8 and 1 M) of NaCl dissolved in 20 mM sodium acetate buffer (pH 5.6). Fractions (2 ml) were collected at a flow rate of 141.18 ml/h. The active fractions were pooled and immediately concentrated by adding ammonium sulfate to 80 % final saturation at 4 °C for 16 h. After centrifugation at 10,000 g for 30 min, the precipitate was dissolved in 1 ml of 20 mM sodium acetate buffer (pH 5.6) and the resulting solution was loaded through a Sephacryl S-100 HR column (1.6 × 64 cm) that had been equilibrated with the same buffer. Proteins were eluted at a flow rate of 24 ml/h and fractions of 1 ml were collected. The pooled active fractions were extensively absorbed on a CM-Sepharose CL-6B column (2.5 × 5.3 cm) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.6). After washing the column with the same buffer, a 50 ml increasing discontinue gradient (0.3 and 1 M) of NaCl dissolved in 20 mM sodium acetate buffer (pH 5.6) was applied to the column. Fractions of 2 ml were collected at a flow rate of 315 ml/h and the active fractions were pooled. The pooled fractions from the previous step was saturated to a final concentration of 1.7 M ammonium sulfate and applied on a Phenyl-Sepharose 6 Fast-flow column (1.6 × 4.5 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 5.6) containing 1.7 M ammonium sulfate. After washing the column with two bed volumes of equilibration buffer, elution (flow rate, 170.52 ml/h; fractions, 1 ml) was carried out with a 15 ml decreasing discontinue gradient (1.7; 1; 0.5; 0.25; 0.1 and 0 M) of ammonium sulfate in 20 mM sodium acetate buffer (pH 5.6). The active fractions were finally dialyzed against 20 mM sodium acetate buffer (pH 5.6) and stored at 4 °C.

### *2.4 Protein essay*

Protein concentration was determined spectrophotometrically at 660 nm by the method of Lowry et al. [15] using bovine serum albumin as a standard.

### *2.5 Enzyme essay*

Under the standard test conditions, hydrolytic activity of beta-glucosidase or beta-xylosidase against *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside, respectively, were measured by the release of *para*-nitrophenol. An essay mixture (250 µl) containing 75 µl of *p*NP-glycopyranoside (5 mM) in 20 mM sodium acetate buffer (pH 5.6) with 50 µl enzyme solution, was incubated at 37 °C for 10 min. The reference cell contained all reactants except the enzyme. The reaction was stopped by adding 2 ml of sodium carbonate (2 %, w/v) and the absorbance of the essay solution was measured at 410 nm [16].

The oligo-saccharidase activity was carried out by measuring the amount of glucose or xylose liberated from 10 mM oligosaccharide (sucrose, gentiobiose, sophorose, xylobiose, cellobiose and cellodextrins) after incubation at 37 °C for 10 min in 20 mM sodium acetate buffer (pH 5.6). The amount of glucose was determined by the glucose oxidase-peroxidase method [17] after heating the reaction mixture at 100 °C for 5 min. The hydrolysis of xylobiose was assayed by HPLC after heating the reaction mixture at 100 °C for 5 min [16].

The polysaccharidase activity was assayed by the dinitrosalicylic acid (DNS) procedure [18], using 0.5 % (w/v) polysaccharide (beta-1,3-glucan, carboxymethylcellulose, xylan and starch) as substrate. The enzyme (50 µl) was incubated for 30 min at 37 °C with 170 µl sodium acetate buffer (20 mM, pH 5.6) and 80 µl polysaccharide. The reaction was stopped by adding 300 µl of dinitrosalicylic acid solution [18] and heating for 5 min in boiling water bath. The absorbance was read at 540 nm after cooling on ice for 5 min.

One unit (U) of enzyme activity was defined as the amount of enzyme, which released one µmol of *para*-nitrophenol or reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg protein (U/mg of protein).

### *2.6 Determination of molecular weights*

The apparent molecular weight (MW) of the native purified enzymes was determined by gel filtration on Sephacryl S-200 HR (0.6 × 32 cm). The standard proteins used for calibration were myosin (200 kDa), beta-galactosidase (116.25 kDa), phosphorylase B (97.40 kDa), bovine serum albumin (66.20 kDa) and amyloglucosidase (63 kDa).

The MW determination under denaturing conditions was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [19] using 10 % separating gel and 4 % stacking gel (Hoefer mini-gel system; Hoefer Pharmacia Biotech, San Francisco, USA). The enzyme sample was incubated for 5 min at 100 °C with SDS-PAGE buffers containing SDS and 2-mercaptoethanol. Proteins were stained with silver nitrate according to Blum et al. (1987) and the molecular weight (MW) of the purified enzyme was determined using the plot of *log* MW of standard protein markers versus their relative mobility.

### *2.7 pH and temperature optima*

The effect of pH on enzyme activity was determined by performing the hydrolysis of *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside (5 mM) in two buffers (20 mM) at various pH values (3.6-8.0). Buffers used were sodium acetate (pH 3.6-5.6) and sodium phosphate (pH 5.6-8.0). pH values of each buffer were determined at 37 °C.

The effect of temperature on purified enzyme activity was performed in 20 mM sodium acetate buffer (pH 5.6) over a temperature range of 30 to 80 °C by using *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside (5 mM) as substrate under the enzyme assay conditions.

### *2.8 pH and temperature stabilities*

The pH stability of the purified enzyme was studied in pH range of 3.6 to 8.0 in 20 mM buffers. Buffers used were the same as in pH and temperature optima study. After 1 h preincubation at 37 °C, aliquots were taken and immediately assayed for residual glucosidase or xylosidase activity. The thermal inactivation was determined at 37 and 60 °C in 20 mM sodium acetate buffer (pH 5.6). Then, aliquots were withdrawn at intervals and immediately cooled in ice-cold water. In the thermal denaturation tests, aliquots of enzyme solution were preincubated at different temperatures ranging from 30 to 80 °C for 15 min. Residual activities, determined in the three cases at 37 °C under the enzyme assay conditions, were expressed as percentage activity of zero-time control of untreated enzyme.

### *2.9 Substrate specificity and kinetic parameters*

Substrate specificity of the purified enzyme was determined by using several commercial grade substrates (*p*NP-glycopyranosides, sucrose, gentiobiose, sophorose, xylobiose, cellobiose, cellotriose, cellotetraose, beta-1,3-glucan, carboxymethylcellulose, xylan, starch).

Hydrolysis of *p*NP-glycopyranosides, sucrose, carboxymethylcellulose, xylan and starch was quantified on the basis of released reducing sugars similarly described in standard enzyme assay.

The eventual hydrolysis of gentiobiose, sophorose, xylobiose, cellodextrins and beta-1,3-glucan was analyzed onto a TLC silica gel plate 60 F254 (E. Merk AG, Darmstadt, Germany), using butanol/acetic acid/water (9:3.75:2.25, v/v/v) as the mobile phase system. Sugars were visualized with naphto-resorcinol staining as described by Brückner [20].

The kinetic parameters ($K_M$, $V_{max}$ and $V_{max}/K_M$) were determined in 20 mM sodium acetate buffer (pH 5.6) at 37 °C. $K_M$ and $V_{max}$ were determined from Lineweaver and Burk [21] plot using different concentration (0.5 to 5 mM) of *p*NP-beta-D-glucopyranoside and *p*NP-beta-D-xylopyranoside. Each experimental point was determined at least in triplicate and in all cases the initial rate was used for plotting.

### *2.10 Effect of chemical agents*

To determine the effect of various compounds (cations, detergents, sulphydryl specific and reducing agents) as possible activators or inhibitors of the purified enzyme, the enzymatic solution was preincubated at 37 °C for 30 min with 1 mM or 1 % (w/v) of the chemical agents and the activity was assayed under the enzyme assay conditions using *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside (5 mM) as substrate. Residual activities were expressed as percentage referred to control without chemical agents.

### *2.11 Transglycosylation reaction*

To test the capacity to catalyze transglycosylation reaction, the purified enzyme was incubated with 10 mM of glycosyl donor (*p*NP-beta-glucoside) and 50 mM of glycosyl acceptor (maltose or cellobiose) in 20 mM sodium acetate buffer (pH 4.6 or 5.6) at 37 °C for 16 h. The reaction was stopped by heating at 100 °C for 5 min. Aliquots (4 µl) were spotted onto a TLC silica gel plate 60 F254 as described previously and the transglycosylation products were visualized with naphto-resorcinol staining as described by Brückner [20].

## 3. RESULTS AND DISCUSSION

### *3.1 Purification of enzyme*

The purification procedure of enzyme from digestive juice of crab *C. armatum* involved four steps including anion-exchange, gel filtration, cation-exchange and hydrophobic interaction chromatographies as summarized in Table 1.

A single peak for beta-glucosidase activity and for beta-xylosidase activity was obtained on the anion-exchange chromatography (DEAP-Sepharose 4 Fast-Flow) used as the first step of purification (Fig. 1-A). Active proteins (pooled fractions 53-79) showing beta-glucosidase and beta-xylosidase activities were eluted at 0.8 M of NaCl and were subjected to a gel filtration chromatography using a Sephacryl-S-100 HR column. Purification on gel filtration showed a single peak of protein containing beta-glucosidase and beta-xylosidase activities (Fig. 1-B). Pooled fractions (118-131) after this step were subjected to a cation-exchange chromatography on CM-Sepharose CL-6B column. A single peak showing active proteins (fractions 1-17) of beta-glucosidase and beta-xylosidase were eluted with 0 M of NaCl (Fig. 1-C). The enzyme was subsequently purified by using an ultimate hydrophobic chromatography on a Phenyl-Sepharose 6 Fast-Flow column. The active proteins (pooled fractions 94-118) showing a single peak for beta-glucosidase and beta-xylosidase activities were eluted with 0.1 M ammonium sulfate (Fig. 1-D). After these steps, results showed that the purified enzyme from digestive juice of crab *C. armatum* should be a bifunctional enzyme with beta-glucosidasic/beta-xylosidasic activities.

Finally, the specific activities towards *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside were 74.17 and 88.60 U/mg of protein, respectively. These values are higher than those reported for other glucosidases [22, 23] and xylosidases [24, 25] already described.

Although DEAE-Sepharose chromatography led to a low purification factor, this step permitted the elimination of many pigments that are very abundant in the crude extract. The bifunctional enzyme was purified with overall yields of about 3.35%, enriched about 76.45-fold for beta-glucosidase activity and 91.34-fold for beta-xylosidase activity (Table 1). This low yield could be due to several fractionation steps used. The bifunctional enzyme showed a single protein band of silver staining on native polyacrylamide gel electrophoresis (Fig. 2-A).

**Table 1:** Purification procedure of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum.* Values given are the averages of at least of three experiments ± SE.

| Purification steps | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| ***Crude extract*** | | | | | |
| beta-glucosidase | $135.00\pm3.10^{-4}$ | $132.15\pm3.10^{-3}$ | $0.97\pm4.10^{-3}$ | 100 | $1\pm5.10^{-2}$ |
| beta-xylosidase | $135.00\pm3.10^{-4}$ | $112.23\pm3.10^{-3}$ | $0.83\pm5.10^{-4}$ | 100 | $1\pm5.10^{-2}$ |
| ***DEAP-Sepharose 4 Fast-flow*** | | | | | |
| beta-glucosidase | $31.54\pm1.10^{-5}$ | $37.50\pm2.10^{-3}$ | $1.18\pm2.10^{-3}$ | $28.37\pm1.10^{-3}$ | $1.22\pm2.10^{-4}$ |
| beta-xylosidase | $31.52\pm6.10^{-4}$ | $37.47\pm2.10^{-3}$ | $1.18\pm5.10^{-4}$ | $28.35\pm3.10^{-5}$ | $1.22\pm2.10^{-6}$ |
| ***Sephacryl S-100 HR*** | | | | | |
| beta-glucosidase | $0.28\pm2.10^{-6}$ | $5.53\pm1.10^{-3}$ | $19.75\pm3.10^{-6}$ | $4.18\pm2.10^{-5}$ | $20.36\pm3.10^{-4}$ |
| beta-xylosidase | $0.27\pm1.10^{-3}$ | $5.51\pm2.10^{-3}$ | $20.40\pm4.10^{-4}$ | $4.16\pm5.10^{-6}$ | $21.03\pm2.10^{-7}$ |
| ***CM-Sepharose CL-6B*** | | | | | |
| beta-glucosidase | $0.13\pm2.10^{-7}$ | $5.24\pm3.10^{-3}$ | $40.30\pm6.10^{-3}$ | $3.96\pm4.10^{-4}$ | $41.54\pm2.10^{-5}$ |
| beta-xylosidase | $0.11\pm5.10^{-4}$ | $5.21\pm4.10^{-3}$ | $47.36\pm3.10^{-5}$ | $4.94\pm2.10^{-6}$ | $48.82\pm1.10^{-4}$ |
| ***Phenyl-Sepharose 6 Fast-flow*** | | | | | |
| beta-glucosidase | $0.06\pm1.10^{-5}$ | $4.45\pm5.10^{-4}$ | $74.16\pm2.10^{-4}$ | $3.36\pm1.10^{-4}$ | $76.45\pm3.10^{-5}$ |
| beta-xylosidase | $0.05\pm4.10^{-7}$ | $4.43\pm6.10^{-3}$ | $88.60\pm2.10^{-3}$ | $3.35\pm3.10^{-5}$ | $91.34\pm1.10^{-6}$ |

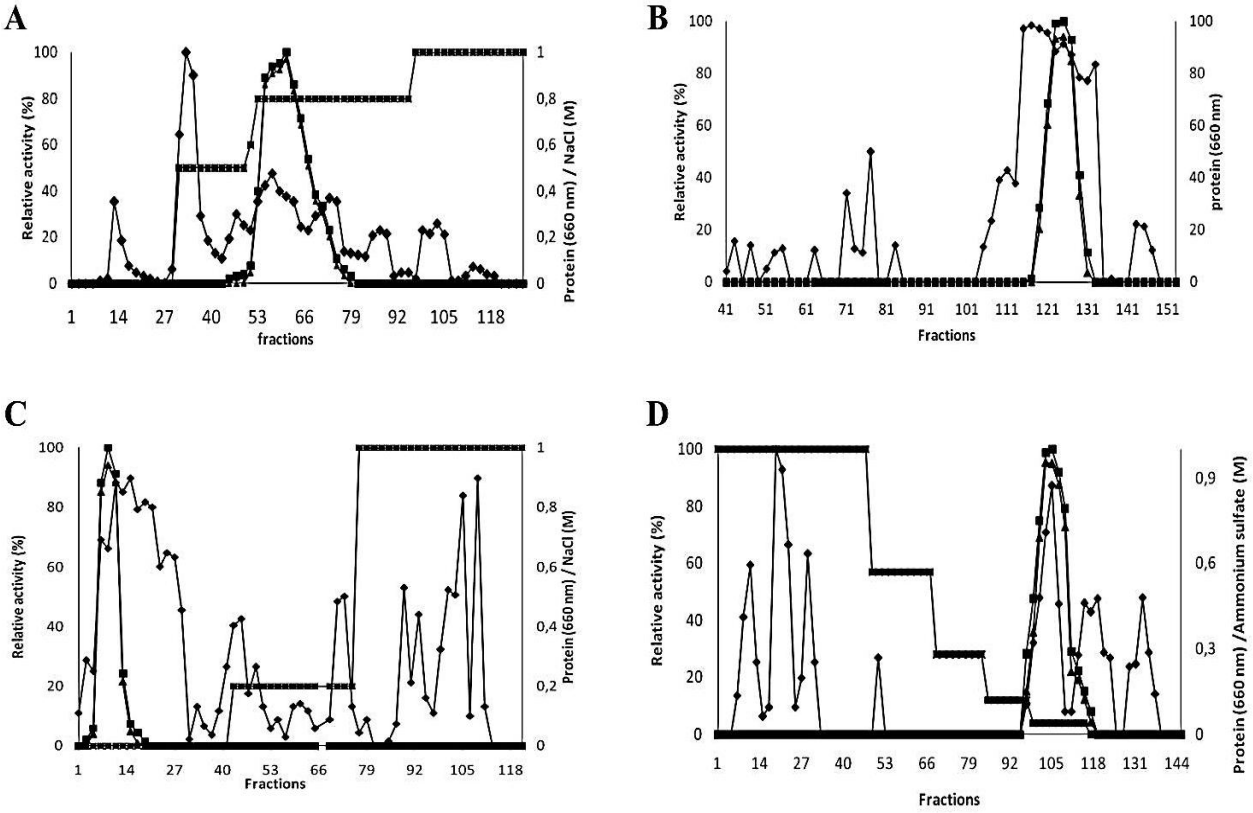


**Figure 1:** Chromatographic profiles of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum*. The enzyme activity was measured in 20 mM sodium acetate buffer pH 5.6 at 37 °C using *p*NP-beta-D-glucopyranoside or *p*NP-beta-D-xylopyranoside as substrate. (A) Anion-exchange chromatography on DEAP-Sepharose 4 Fast-Flow. (B) Size exclusion chromatography on Sephacryl S-100 HR. (C) Cation exchange chromatography on Carboxymethyl-Sepharose CL-6B. (D) Hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast-flow. Beta-glucosidase activity (■), beta-xylosidase activity (▲), chloride sodium or ammonium sulfate (▪) and protein contents (♦).

### *3.2 Molecular weight estimation*

SDS-PAGE profile of purified bifunctional enzyme beta-glucosidase/beta-xylosidase showed a single protein band and its relative molecular weight (MW) was estimated to be 69.81 kDa (Fig. 2-B; Table 2). On the other hand, beta-glucosidase/beta-xylosidase showed on gel filtration chromatography (Sephacryl S-200 HR) an approximately MW of 115.24 kDa (Table 2). A difference between these two MW suggests that beta-glucosidase/beta-xylosidase may exist as dimer, joined by a disulphide bond or Van der Waals forces. In comparison to other purified beta-glucosidases, the MW of beta-glucosidase with beta-xylosidase activity from crab *C. armatum* was lower than those from *Aspergillus oryzae* [26], *Gecarcoidae natalis* [6] and *Macrotermes bellicosus* [22], while this value was higher than those of *Achatina achatina* [27] and *Lodgepole Pine* [28].

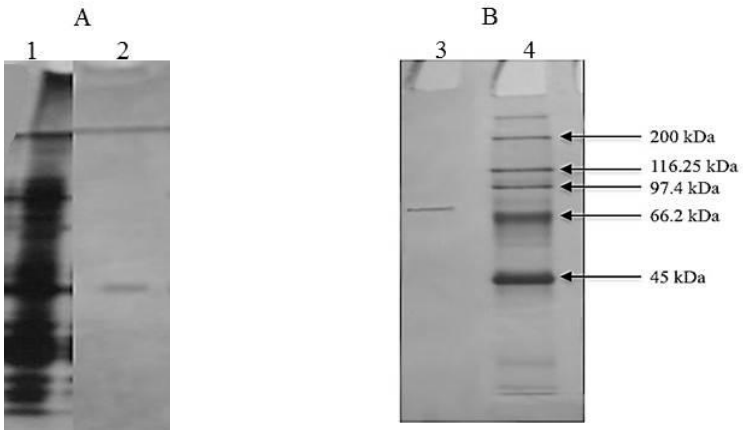


**Figure 2:** Polyacrylamide gel electrophoresis in native (A) and denaturing (B) conditions of the purified beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum*. Lane 1, crude extract; lanes 2 and 3, purified enzyme; lane 4, molecular weight markers.

**Table 2:** Some physicochemical characteristics of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum.*

| **Physicochemical properties** | **beta-glucosidase activity** | **beta-xylosidase activity** |
|---|---|---|
| Optimum temperature (°C) | $60 \pm 1.0.10^{-3}$ | $60 \pm 1.1.10^{-3}$ |
| Optimum pH | $5.6 \pm 2.0.10^{-2}$ | $5.6 \pm 2.2.10^{-2}$ |
| pH stability | $4.6 - 6.6 \pm 1.1.10^{-4}$ | $4.6 - 6.6 \pm 2.0.10^{-3}$ |
| Temperature coefficient ($Q_{10}$) | 1.68 | 1.27 |
| Activation energy ($kJ.mol^{-1}$) | 38.17 | 19.08 |
| Michaelis Menten equation | obeyed | obeyed |
| Molecular weight (kDa) | | |
| SDS-PAGE | 69.81 | 69.81 |
| Gel filtration | 115.24 | 115.24 |

### *3.3 pH and temperature dependences*

Activities of the bifunctional enzyme from crab *C. armatum* were analyzed for their hydrolytic pH and temperature dependences. Hydrolytic activities of the bifunctional beta-glucosidase/beta-xylosidase were maximal at 60 °C in sodium acetate buffer pH 5.6 (Table 2).

The pH optimum of 5.6 was comparable to values reported for beta-glucosidases from *Monascus purpureus* (pH 5.5) [29], *Abracris flavolineata* (pH 4.8-5.5) [30] and for beta-xylosidase from *Aspergillus ochraceus* (pH 3.0-5.5) [31]. This pH was lower than value (pH 6.0) obtained for beta-glucosidase/xylosidase from yak rumen metagenome [32] and for beta-xylosidase from *Thermotoga thermarum* [33]. At 25 °C, the purified bifunctional enzyme showed best stability over pH values ranging from 4.6 to 6.6 for 60 min at 37 °C (Fig. 3). Therefore, the optimum pH is a good compromise between activity and stability of the enzyme to perform hydrolysis of natural substrates and biosynthesis reaction over a long time.

The optimum temperature (60 °C) determined for the purified beta-glucosidase/beta-xylosidase was higher than those of beta-glucosidase/xylosidase from yak rumen metagenome (50 °C) [32] and beta-glucosidase from *P. carotovorum* (40 °C) [34]. However, thermophile beta-xylosidases were obtained by Michelin et al. [31] and Shi et al. [33] with optimal activity at 70 °C and 95 °C, respectively.

The thermal stability study has revealed that at 37 °C the beta-glucosidase/beta-xylosidase activities remained stable for 150 min (Fig. 4). At their optimum temperature (60 °C), these activities were less stable, showing half-life (50 % of activity) around 150 min and 130 min for beta-glucosidase and beta-xylosidase activities, respectively. However, they were lost about 30-40 % after 120 min of preincubation (Fig. 4).

The thermal denaturation shows that the bifunctional enzyme was fairly stable at temperatures up to 60 °C. At higher temperatures, their activity declined progressively as the temperature increased and the enzymes were completely inactivated at 80 °C (Fig. 5). In this context, running biotechnological processes at moderate temperatures would be advantageous for application of these enzymes.

Values of temperature coefficient ($Q_{10}$) calculated between 40 and 55 °C were 1.68 and 1.27 for beta-glucosidase and beta-xylosidase, respectively (Table 2). From Arrhenius plot (data not shown), the activation energy was found to be 38.17 $kJ.mol^{-1}$ for beta-glucosidase and 19.08 $kJ.mol^{-1}$ for beta-xylosidase (Table 2). These activation energies are comparable to that obtained (38.16-42.84 $kJ.mol^{-1}$) for beta-glucosidases from *M. bellicosus* [22]. Values of activation energy indicate the relative tendency of a failure mechanism to be accelerated by temperature. In this respect, the beta-glucosidase/beta-xylosidase from crab *C. armatum* should be top-grade tools for various catalyzing reactions since it is well known that enzymes are biocatalysts that speed up chemical reactions by lowering the required activation energy.

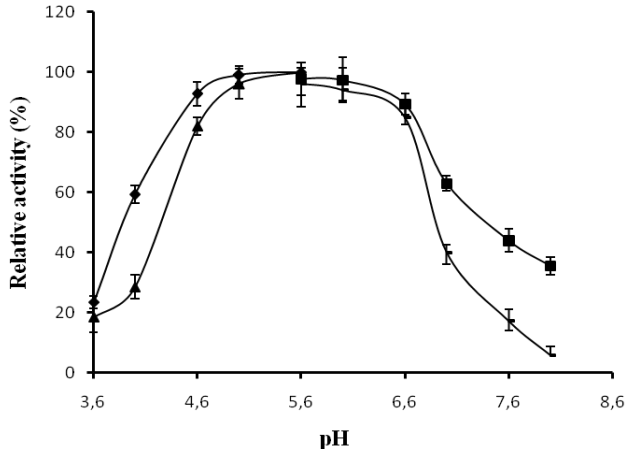


**Figure 3:** pH stability of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum*. Beta-glucosidase activity in acetate buffer (♦) and in phosphate buffer (■); beta-xylosidase activity in acetate buffer (▲) and in phosphate buffer (-).

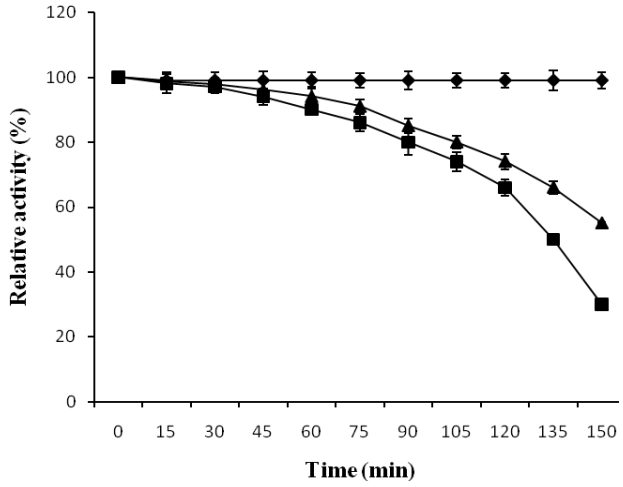


**Figure 4:** Thermal inactivation of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum*. Inactivation of the enzyme at 30 °C (♦); inactivation of beta-glucosidase activity at 60 °C (▲); inactivation of beta-xylosidase activity at 60 °C (■).

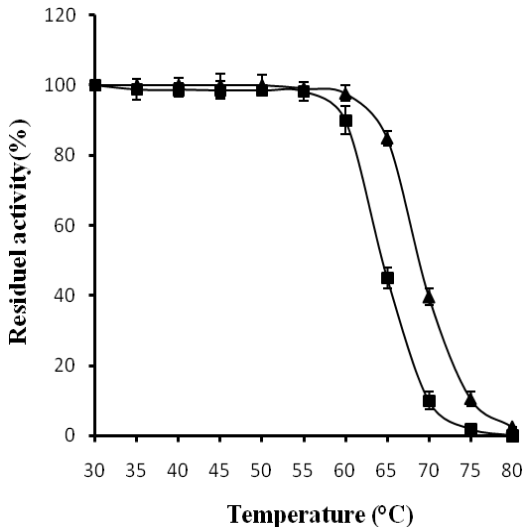


**Figure 5:** Thermal denaturation of beta-glucosidase (▲) / beta-xylosidase (■) from digestive juice of crab *Cardisoma armatum*.

### *3.4 Substrate specificity and kinetic parameters*

Enzyme from crab *C. armatum* was assayed for hydrolytic activity against a variety of natural and synthetic substrates to know if they were suitable to serve as substrates for purified enzyme (Tables 3 and 4). This enzyme was found to be inactive towards *p*NP-beta-D-galactopyranoside, *p*NP-alpha-D-galactopyranoside, *p*NP-alpha-D-glucopyranoside, sucrose, gentiobiose, sophorose, beta-1,3-glucan, carboxymethylcellulose, xylan and starch. Although, the enzyme was able to hydrolyze *p*NP-beta-D-glucopyranoside, *p*NP-beta-D-xylopyranoside, xylobiose and cellodextrins (cellobiose, cellotriose, cellotetraose) (Tables 3 and 4). However, high activity was obtained with *p*NP-beta-D-glucopyranoside. Similar observations were noted for beta-glucosidase from *Melanocarpus sp*. MTCC 3922 [35]. Purified enzyme seems to be a broad-specificity exo-glycosidase, although it could not hydrolyze sophorose, beta-1,3-glucan and gentiobiose, which contained beta-(1,2), beta-(1,3) and beta-(1,6)-glucosidic linkages, respectively. This was in disagreement with the work of Copa-Platino and Broda [36] in *Phanerochaete chrysosporium* and Riou et al. [26] in *Aspergillus oryzae*. From these results, it appears that the purified enzyme from crab *C. armatum* is a beta-glucosidase with dual activity against *p*NP-beta-D-xylopyranoside and with specificity from beta-(1,4)-glucosidic linkages.

The hydrolysis of cellodextrins by this enzyme could raise the problem of lack of cellobiohydrases in the cellulase system of some arthropods. Cellulase system of some arthropods combined endo-beta-1,4-glucanase and beta-glucosidase actions for complete hydrolysis of cellulose [37]. However, it remains to prove the lack of cellobiohydrolases in cellulase system of crab *C. armatum*.

The effect of substrate concentration on enzymatic activity was studied with *p*NP-beta-D-glucopyranoside and *p*NP-beta-D-xylopyranoside, which displayed the greatest activity. With these substrates, the enzyme obeyed the Michaelis-Menten equation (Table 2). The $K_M$, $V_{max}$ and $V_{max}/K_M$ values are reported in Table 5. The catalytic efficiency of bifunctional enzyme, given by the $V_{max}/K_M$ ratio is much higher for *p*NP-beta-D-glucopyranoside than *p*NP-beta-D-xylopyranoside (Table 5). These values were higher than those obtained for beta-glucosidases from *M. bellicosus* towards *p*NP-beta-D-glucopyranoside (66.66-560.64 U/mM) [22] and for beta-xylosidase from *A. ochraceus* towards *p*NP-beta-D-xylopyranoside (59 U/mM) [31]. Furthermore, beta-glucosidase/beta-xylosidase from *C. armatum* had a much more higher affinity for *p*NP-beta-D-glucopyranoside and *p*NP-beta-D-xylopyranoside (3 and 10.79 mM, respectively) compared to beta-glucosidase/xylosidase from yak rumen metagenome (0.164 and 0.03 mM, respectively) [32].

**Table 3:** Activities of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum* on synthetic chromogenic substrates. Values given are the averages of at least of three experiments ± SE.

| Synthetic chromogenic substrate | Relative rates of hydrolysis (%) |
|---|---|
| *p*NP-beta-D-glucopyranoside | 100 |
| *p*NP-beta-D-xylopyranoside | $98.5 \pm 2.5.10^{-5}$ |
| *p*NP-beta-D-galactopyranoside | 0 |
| *p*NP-alpha-D-glucopyranoside | 0 |
| *p*NP-alpha-D-galactopyranoside | 0 |

**Table 4:** Ability of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum* to hydrolyze (+) or not (-) natural and synthetic substrates.

| Substrate | Hydrolysis |
|---|---|
| Sucrose | - |
| Gentiobiose | - |
| Sophorose | - |
| Xylobiose | + |
| Cellobiose | + |
| Cellotriose | + |
| Cellotetraose | + |
| Beta-1,3-glucan | - |
| Carboxymethylcellulose | - |
| Xylan | - |
| Starch | - |

**Table 5:** Kinetic parameters of beta-glucosidase/beta-xylosidase from digestive juice of crab *Cardisoma armatum* towards *p*NP-beta-D-glucopyranoside and *p*NP-beta-D-xylopyranoside.

| Substrate | $K_M$ (mM) | $V_{max}$ (U) | $V_{max} / K_M$ (U/mM) |
|---|---|---|---|
| *p*NP-beta-D-glucopyranoside | 3 | 5000 | 1666.67 |
| *p*NP-beta-D-xylopyranoside | 10.79 | 1428.6 | 132.89 |

### *3.5 Effect of chemical agents on enzyme activity*

The effect of selective inhibitors or activators on beta-glucosidase with beta-xylosidase activity was examined. As regards the influence of cations, the results are presented in Table 6. Most of the cations tested had no significant or slightly inhibitory effect on the beta-glucosidase/beta-xylosidase activities, while $K^+$ and $Mg^{2+}$ slightly activated the beta-glucosidase activity by 25 % and 2 %, respectively. In addition, inhibition by EDTA (cations chelator) suggests that the bifunctional enzyme requires divalent metal cations to be fully active.

The effect of sulfhydryl-specific and reducing agents on the purified bifunctional enzyme showed that *p*CMB and DTNB displayed a strong inactivation on beta-glucosidase and beta-xylosidase activities. Some compounds tested, such as DL-dithiothreitol and urea were found to have no significant effect on the two enzymatic activities. However, L-cystein slightly activated beta-xylosidase activity whereas beta-glucosidase activity was slightly activated by beta-mercaptoethanol, suggesting the involvement of sulfhydryl groups in catalytic activity (Table 7). Beta-xylosidases from *Aspergillus phoenicis* [38] and *Streptomyces sp.* CH7 [24] have also been reported to have sulfhydryl groups involved in their activities. The sensitivity of these enzymes in the presence of sulfhydryl-specific agents led us to assume that –SH groups participate probably in catalysis activities of the enzymes.

Except cationic detergents (TTAB and HTAB) studied that displayed a strong inhibitory effect (up to 80 % inhibition) on beta-glucosidase and beta-xylosidase activities, most of detergents currently used for denaturing proteins showed by and large no significant effect on the two enzymatic activities (Table 8). These two enzymes had a common activator effect with P9-LE and P10-OE (anionic detergents), which showed the best stimulations. However, beta-glucosidase activity was also slightly stimulated by SDS, whereas beta-xylosidase activity was slightly enhanced by Lubrol Wx, Nonidet P-40 and ACS (Table 8). The stimulatory effects shown by these detergents make them useful when purifying beta-glucosidase/beta-xylosidase from digestive juice of crab *C. armatum*, since it is well known that detergents are used for protein solubilization before their purification.

**Table 6:** Effect of cations on beta-glucosidase/beta-xylosidase activities from digestive juice of crab *Cardisoma armatum*. Values given are the averages of at least of three experiments ± SE.

| Reagent | Concentration (mM) | Relative activity (%) | |
|---|---|---|---|
| | | beta-glucosidase | beta-xylosidase |
| None | 0 | 100 | 100 |
| $Na^+$ | 1 | $99.78 \pm 2.0.10^{-3}$ | $92.89 \pm 3.0.10^{-3}$ |
| $K^+$ | 1 | $125.03 \pm 2.1.10^{-3}$ | $97.81 \pm 4.0.10^{-3}$ |
| $Ba^{2+}$ | 1 | $89.25 \pm 2.3.10^{-3}$ | $87.97 \pm 3.5.10^{-3}$ |
| $Ca^{2+}$ | 1 | $92.05 \pm 2.7.10^{-3}$ | $84.14 \pm 2.0.10^{-3}$ |
| $Mg^{2+}$ | 1 | $102.10 \pm 3.0.10^{-3}$ | $98.88 \pm 4.1.10^{-3}$ |
| $Zn^{2+}$ | 1 | $71.78 \pm 3.7.10^{-3}$ | $74.31 \pm 4.5.10^{-3}$ |
| EDTA | 1 | $98.50 \pm 3.6.10^{-3}$ | $98.36 \pm 3.7.10^{-3}$ |

EDTA, ethylene diamine tetraacetic acid.

**Table 7:** Effect of sulphydryl specific and reducing agents on beta-glucosidase/beta-xylosidase activities from digestive juice of crab *Cardisoma armatum*. Values given are the averages of at least of three experiments ± SE.

| Reducing agents | Concentration (%) | Relative activity (%) | |
|---|---|---|---|
| | | beta-glucosidase | beta-xylosidase |
| None | 0 | 100 | 100 |
| L-cystein | 1 | 100 ± 00 | $103.45 \pm 3.2.10^{-3}$ |
| DL-dithiothreitol | 1 | $98.07 \pm 4.2.10^{-3}$ | $97.32 \pm 4.3.10^{-3}$ |
| *p*CMB | 1 | $7.30 \pm 4.1.10^{-3}$ | $2.54 \pm 4.7.10^{-3}$ |
| DTNB | 1 | $11.07 \pm 6.9.10^{-2}$ | $12.05 \pm 3.6.10^{-2}$ |
| Urea | 1 | $99.23 \pm 2.1.10^{-3}$ | $97.70 \pm 2.5.10^{-3}$ |
| Beta-mercaptoethanol | 1 | $108.07 \pm 3.4.10^{-2}$ | $98.04 \pm 4.6.10^{-3}$ |

*p*CMB, *para*-chloromercuribenzoate; DTNB, 5,5'-dithio-2,2' dinitro-dibenzoïc acid.

**Table 8:** Effect of detergents on beta-glucosidase/beta-xylosidase activities from digestive juice of crab *Cardisoma armatum*. Values given are the averages of at least of three experiments ± SE.

| | Detergents | Concentration (%) | Relative activity (%) | |
|---|---|---|---|---|
| | | | beta-glucosidase | beta-xylosidase |
| | None | 0 | 100 | 100 |
| Cationic | TTAB | 1 | $22.50 \pm 2.7.10^{-4}$ | $23.56 \pm 5.2.10^{-4}$ |
| | HTAB | 1 | $27.16 \pm 4.5.10^{-4}$ | $19.15 \pm 3.2.10^{-4}$ |
| Non ionic | Tween 80 | 1 | $98.46 \pm 2.5.10^{-2}$ | $97.32 \pm 2.3.10^{-2}$ |
| | Lubrol Wx | 1 | $99.23 \pm 3.3.10^{-2}$ | $105.36 \pm 3.4.10^{-2}$ |
| | Nonidet P-40 | 1 | $98.46 \pm 4.1.10^{-2}$ | $104.60 \pm 2.5.10^{-2}$ |
| Anionic | P9-LE | 1 | $108.85 \pm 3.6.10^{-2}$ | $104.21 \pm 3.3.10^{-3}$ |
| | P10-OE | 1 | $105.01 \pm 3.5.10^{-2}$ | $104.98 \pm 3.7.10^{-2}$ |
| | ACS | 1 | $99.23 \pm 3.0.10^{-3}$ | $101.91 \pm 4.3.10^{-3}$ |
| | SDS | 1 | $102.30 \pm 2.2.10^{-3}$ | $98.44 \pm 2.3.10^{-3}$ |
| | Sodium hexan sulfonate | 1 | $100.70 \pm 4.10^{-3}$ | $99.64 \pm 4.4.10^{-3}$ |

TTAB, Tetradecyl Trimethyl Ammonium Bromide; HTAB, Hexadecyl Trimethyl Ammonium Bromide; P9-LE, Polyoxyethylene 9-Lauryl Ether; P10-OE: Polyxyethylene 10-Oleyl Ether; ACS, Sodium cholate ; SDS: Sodium Dodecyl Sulfate.

### *3.6 Transglycosylation assay*

Transglycosylation is another interesting property of beta-glucosidase from *C. armatum*. The enzyme catalyzed this reaction in the presence of maltose as glycosyl acceptor (Fig. 6-A). In this case, the transglycosylation product was mainly a triose (Glucose-maltose). Contrariwise, no transglycosylation product was observed when cellobiose was used as acceptor (Fig. 6-B). This could be explained, on the one hand by the competitive action between the donor (*p*NP-beta-D-glucopyranoside) and the acceptor (cellobiose) which are both substrates for the enzyme, and on the other hand by the competition between hydrolysis and transglycosylation reactions. However, the ability to synthesize higher oligosaccharides of up to six glucose residues from disaccharides could greatly extend the biotechnological applications of this enzyme.

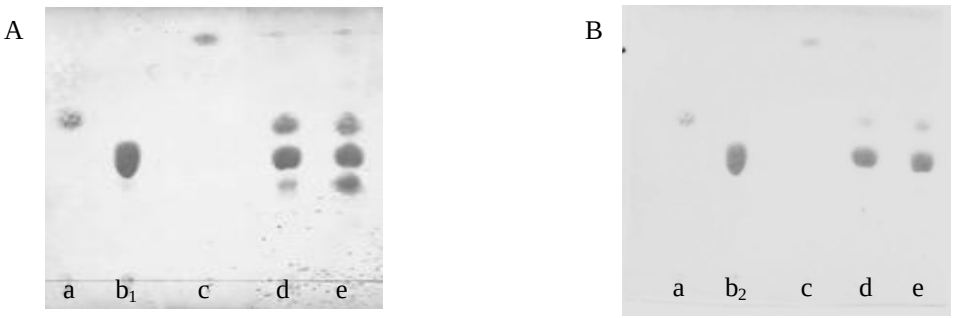


**Figure 6:** Thin layer chromatography plates showing separation of the oligosaccharides released when incubating beta-glucosidase/beta-xylosidase with *p*NP-beta-D-glucopyranoside 10 mM and (A) maltose 50 mM or (B) cellobiose 50 mM in 20 mM acetate buffer (pH 4.6 and 5.6). a: glucose; b1: maltose; b2: cellobiose; c: *p*NP-beta-D-glucopyranoside; d: reaction mixture at pH 4.6 ; e : reaction mixture at pH 5.6.

## 4. CONCLUSION

The present study finally showed that the digestive juice of land crab *Cardisoma armatum* produce a beta-glucosidase endowed beta-xylosidase activity, which was active in acid condition. This enzyme was a very stable enzyme mesophile in the plug acetate 20 mM with 37 °C, and possessed an exo-hydrolytic mode of action. The activity of this enzyme was found to be higher toward *p*NP-beta-D-glucopyranoside over *p*NP-beta-D-xylopyranoside and this enzyme catalyzed both hydrolysis and transglycosylation reaction. This study represents the first report describing beta-glucosidase with beta-xylosidase activity from crab *C. armatum.* The presence of this bifunctional enzyme (beta-glucosidase/beta-xylosidase) in the land crab *C. armatum* reveals its role in the hydrolysis of digestion products of plant material such as hemicellulose and potentially cellulose. All properties of beta-glucosidase/beta-xylosidase make it an advantageous biocatalyst for biotechnology and bio-industrial applications. Thus, this land crab species deserves further investigations in search of new enzymes.

## 5. ACKNOWLEDGEMENT

This work was supported by Ph.D. grant to the first author. The authors are grateful to Laboratory of Biocatalysis and Bioprocessing at the University Nangui Abrogoua (Abidjan, Côte d'Ivoire) for technical assistance.

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