# Distribution of Cellulase Activities in *Acetes* Shrimps Living in the Matang Mangrove Forest Reserve, Malaysia

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**ABSTRACT** For the purpose of investigating the cellulose degradation mechanism in mangrove estuaries, cellulase activities of three *Acetes* shrimps (*Acetes sibogae*, *A. japonicus* and *A. indicus*) were examined in the Matang Mangrove Forest Reserve (MMFR). As a result of cellulose plate assay measurement, we detected the activities from all three species. SDS-PAGE zymographic analysis revealed that all *Acetes* shrimps demonstrated active bands at approximately 25 kDa and higher than 100 kDa. In addition, by quantification of reducing sugar production, hepatopancreas samples of *Acetes* showed significantly higher cellulase activity compared to whole body samples suggesting their endogenous origin. Our findings that cellulase activities are widely distributed among the *Acetes* shrimps living in the MMFR, suggests that mangroves are indirectly utilized by higher trophic consumers.

(**Keywords:** cellulase, mangrove, *Acetes*, hepatopancreas)

## INTRODUCTION

There is a unique ecological system in mangrove areas which develops extraordinary high biological productivity [1]. Roots of mangrove trees function as refuges for larval fish and mangrove litter is well utilized as carbon source for some organisms living in the mangrove estuary [1]. The Matang Mangrove Forest Reserve (MMFR), situated on the northwestern coast of Peninsular Malaysia, is reputed to be the world's best managed mangrove forest. The reserve is the largest single tract of mangrove forest in Peninsular Malaysia (40,151 ha) where abundant *Acetes* shrimps are well fed by the larger predators [1]. Thus, it is important to validate the food chain derived from mangrove in terms of nutritional aspect to maintain sustainable utilization of fishery resources. Despite the elaborate efforts of fatty acid analysis and stable isotopic analysis to elucidate the food chain in the mangrove ecosystem, biochemical degradation process of mangrove litter still remains unclear [1-7].

Cellulose, most abundant organic substance on the earth, is a high molecular weight compounds composing of glucose bound by β-1,4 linkages [8, 9]. Cellulose is a main component of plant cell wall and makes rigid superstructure known as cellulosemicrofibril. Thus, cellulose is resistant to degradation. Cellulase, which is a collective name of enzymes degrading cellulose, is classified into two subtypes such as an endo-β-1,4-glucanase (EC 3.2.1.4.) and a cellobiohydrolase (EC3.2.1.91.) [9, 10]. Glucose dimmers or oligosaccharides generated by these enzymes are further degraded by β-glycosidase (EC 3.2.1.21.) into glucose which is well utilized by various organisms [9]. Most studies on cellulase are focused on endo-β-1,4-glucanase, thus we used "cellulase" to indicate endo-β-1,4-glucanase in the present study. Recently, cellulase genes located on the chromosomes of some crustaceans, abalone and bivalves have been reported [11-15]. Also, Niiyama and Toyohara [16] detected the widespread presence of cellulase and hemicellulase activity among aquatic benthos, whereas King et al. [17] proposed self wood-digesting system of an aquatic isopod *Limnoriidae*. These findings suggest that aquatic invertebrates are possibly involved in cellulase degradation in the environment and subsequently play ecologically important roles in the aquatic food chain.

In the present study, in order to validate the implication of these animals in cellulose degradation, we made an

attempt to detect cellulase activities from *Acetes* shrimps in the MMFR.

## MATERIALS AND METHODS

### Animals

Sampling locations in the Matang Mangrove Forest Reserve are shown in Fig. 1. Sampling was carried out at two stations named R2 and C3 according to the locations of Tanaka et al. [18]. Station R2 is located in a place near the mudflat, where is approximately 2 km off from the shore. Station C3 is located in the creek. We collected three dominant species of *Acetes* shrimps (*Acetes japonicus* at Station R2, *A. sibogae* at Station C3 and *A. indicus* at Station R2) in July 29, 2010 and September 28, 2012. A small hand dip net and a sledge net were used to collect the samples and the shrimps were pick up artificially from the net. *A. indicus* and *A. japonicus* were known to be abundant around offshore, whereas *A. sibogae* has a tendency to be distributed in the middle to upper reaches [19]. Samples were kept on ice in the field and frozen (-32°C) in the laboratory until analysis. In the laboratory, the carapace length was measured as the standard length (Table 1). All procedure was based on Tanaka et al. [18].

### Measurement of cellulase activities by agar plate assay

According to the method of Sakamoto and Toyohara [14], 1.5 % agar plate containing 0.5 % carboxymethylcellulose (CMC, Sigma) was prepared. For measurement of enzymatic activity, intestine (approximately 3 cm in length) was picked out from a few individuals of each *Acetes* shrimps and homogenized (2 µl). The homogenate was prepared by grinding samples using a plastic pestle in a plastic tube. The prepared samples were placed on the agar plate, and the plates were incubated at 37°C for overnight. Then, these plates were stained by 0.1 % Congo Red for 3 h followed by destaining with 1 M NaCl. Cellulase activity was detected as an unstained circle.

### Analysis of molecular elements of cellulases by SDS-PAGE zymography

According to the method of Beguin [20], 7.5 % polyacrylamide gel containing CMC was used for the detection of molecular species of cellulases. In order to detect clear band, concentration of CMC is optimized to 0.5 %. Intestines of each species of *Acetes* dissected from two or three individuals were mixed and used for analysis. For electrophoresis, 10 µl of phosphate buffered saline (PBS) containing 140 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$ (pH 7.4) and 2 µl of 6×SDS sample buffer containing 0.6 M Tris-HCl (pH 6.8), 60% glycerol, 6% SDS and 0.06% bromophenol blue were added to the homogenate (3 µl) of *Acetes* shrimps. After electrophoresis, the gel was washed by 0.1 M acetate buffer (pH 5.5) containing 0.1% TritonX-100 for 30 min and soaked into 0.1 M acetate buffer (pH 5.5). After incubation at 37°C for overnight, the gel was stained and destained as described above. Destained gel was soaked into 1 M acetate to detect active bands clearly. Active bands were detected as unstained transparent bands.

### Colorimetric measurement of cellulase activity

Hepatopancreas was dissected out from 50 specimens for two *Acetes* species (*A. sibogae* and *A. indicus*) which were collected in September 28, 2012, and homogenized in 40 µl PBS buffer. Also, whole body of three individuals for each species was homogenized in 1 ml PBS buffer. The supernatants of the homogenate samples, namely enzyme solution, were used for further analysis. Protein concentration of the enzyme solution was measured by the method of Bradford [21], and adjusted at 1 mg $ml^{-1}$ with PBS buffer and stored at -32°C until the measurement. Bovine serum albumin was used as the standard for the determination of the protein concentration.

According to the method of Niiyama and Toyohara [16], 5 µl enzyme solution, whose protein concentration was 1 mg/ml, was mixed with 5 µl of 1 M sodium acetate buffer (pH 5.5), and 40 µl of the substrate solution (CMC). After the incubation at 37°C for 10 min for the enzymatic reaction, mixtures were heated at 100°C to terminate the reaction. The amount of reducing sugar produced was measured by the tetrazolium blue method by the absorbance at 660 nm according to Jue and Lipke [22]. D-Glucose was used as the standard for the determination of the amount of reducing sugar.

## RESULTS

### Occurrence of cellulase in *Acetes* shrimps

As shown in Fig. 2, cellulase activities were detected by cellulose plate assay as unstained circles from all the

three species of *Acetes*. Paticulary, *A. sibogae* showed clear unstained circle whereas *A. japonicas* and *A. indicus* showed faint and small circles, suggesting that *A. sibogae* has significant higher cellulase activity than the others.

By SDS-PAGE zymography, three species of *Acetes* commonly exhibited two clear active bands (Fig. 3). *A. sibogae* exhibited 200 kDa and 26 kDa bands, 150 kDa and 24 kDa bands in *A. japonicus*, and exhibited 120 kDa and 24 kDa bands in *A. indicus*.

### Differentiation of cellulase activity among *Acetes* shrimps

As shown in Fig. 4, reducing sugar produced by cellulase was quantified in the two species of *Acetes* (*A. sibogae* and *A. indicus*). Hepatopancreas samples represented significantly higher enzymatic activity compared to the whole body samples. (Whole body of *A. indicus*: 0.02985 μmol $min^{-1}$ mg $protein^{-1}$, hepatopancreas of *A. indicus*: 0.927 μmol $min^{-1}$ mg $protein^{-1}$, whole body of *A. sibogae*: 0.0154 μmol $min^{-1}$ mg $protein^{-1}$ and hepatopancreas of *A. sibogae*: 0.531 μmol $min^{-1}$ mg $protein^{-1}$).

## DISCUSSION

We have successfully detected cellulase activities from all the three species of *Acetes* examined, suggesting that the *Acetes* shrimps living in the mangrove estuary have cellulase as digestive enzyme (Figs. 2, 3 and 4).

This is the first report to prove on the occurrence of cellulase in *Acetes* shrimps, although cellulase activities including the reports of endogenous genes encoding cellulase have been already reported from several crustaceans such as crayfishes and crabs [11, 23-26]. In addition, the occurrence of multiple forms of cellulases is also found from all species of *Acetes*, although assimilation and digestion of cellulose in mysids have been studied and their endogenous occurrence of cellulase has been previously speculated [27-29]. The hepatopancreas samples demonstrated significantly higher enzymatic activity compared to the whole body samples in the two species tested, supporting the speculation of endogenous occurrence.

By means of SDS-PAGE zymographic analysis, all three species of *Acetes* demonstrated two active bands; one is approximately 25 kDa and the other is higher than 100 kDa as shown in Fig. 3. In crustaceans, the molecular weight of cellulases, 40 kDa (*Cherax quadricarinatus*), 53±3 kDa and 52 kDa (*C. destructor*) and 52 kDa (*Gecarvoidea natalis*) have been previously reported [23, 26], and these values differed from those found in the present study (Fig. 3). This result suggests that cellulases of *Acetes* shrimps are possibly novel and specific. It requires further study to determine whether these active bands of the *Acetes* shrimps are derived from enzymes encoded on the chromosomal genes as in the case of *Cherax quadricarinatus* [11] or from enzymes of parasitic microorganisms. We are now trying to isolate genes encoding these cellulases to determine their origin.

Our study has suggested that the dominant *Acetes* shrimps in the MMFR have endogenous cellulase because hepatopancreas samples represented significantly higher values compared to the whole body samples (Fig. 4). Hepatopancreas is known as the secretary organ in arthropods, mollusks and fish. In crustaceans, it provides the functions of both liver and pancreas in mammals; production and secretion of several digestive enzymes, uptake of nutrition, and storage of carbon source [30]. Thus, the presence of symbiotic microorganism is unlikely to produce cellulase in hepatopancreas, as previously reported by King et al. [17].

Among the *Acetes* shrimps tested in the present study, *A. sibogae* is a dominant species in the inner mangrove estuary, while *A. japonicas* and *A. indicus* are mainly distributed from the river mouth to the shallow coastal area [18]. In the previous studies, fresh water shrimps exhibited higher cellulase activities than seawater shrimp species [31]. Interestingly, a similar result was found in the present study on the result of plate assay, in which cellulase activity level is higher in *A. sibogae* than the other species (Fig. 2).

The three species of *Acetes* studied in the present study are assumed to play ecologically important roles in the estuaries of the MMFR as foods for other higher consumers in the food chain since the main prey items of John's snapper (*Lutjanus johnii*) and banana prawn (*Fenneropenaeus merguiensis*) are *Acetes* shrimps (the volumetric composition of *Acetes* shrimps constituted 37–43% of total stomach contents of John's snapper, and 40–50% of stomach contents of banana prawn captured in shallow coasts were mysids and *Acetes* shrimps) [32-34]. Moreover, juvenile John's snapper migrate into the upstream mangrove area where *Acetes* shrimps were more abundant [18]. Detection of enzymatic activity could confirm the food source speculation by gut content

investigation or stable isotope analysis, and contribute significantly to the determination of food web in the mangrove estuary.

This is the first report showing that *Acetes* shrimps species in the MMFR have significant cellulase activity. Also, these species were speculated to have endogenous cellulase in their hepatopancreas, which has the common character in molecular size. These findings strongly suggest that *Acetes* shrimps can digest cellulose as their food source, and subsequently, mangroves are indirectly utilized by higher trophic consumers. To validate the assimilation of cellulose derived from mangroves, further studies are required coupled with examination of stomach contents of *Acetes* shrimps and stable isotopic analysis.

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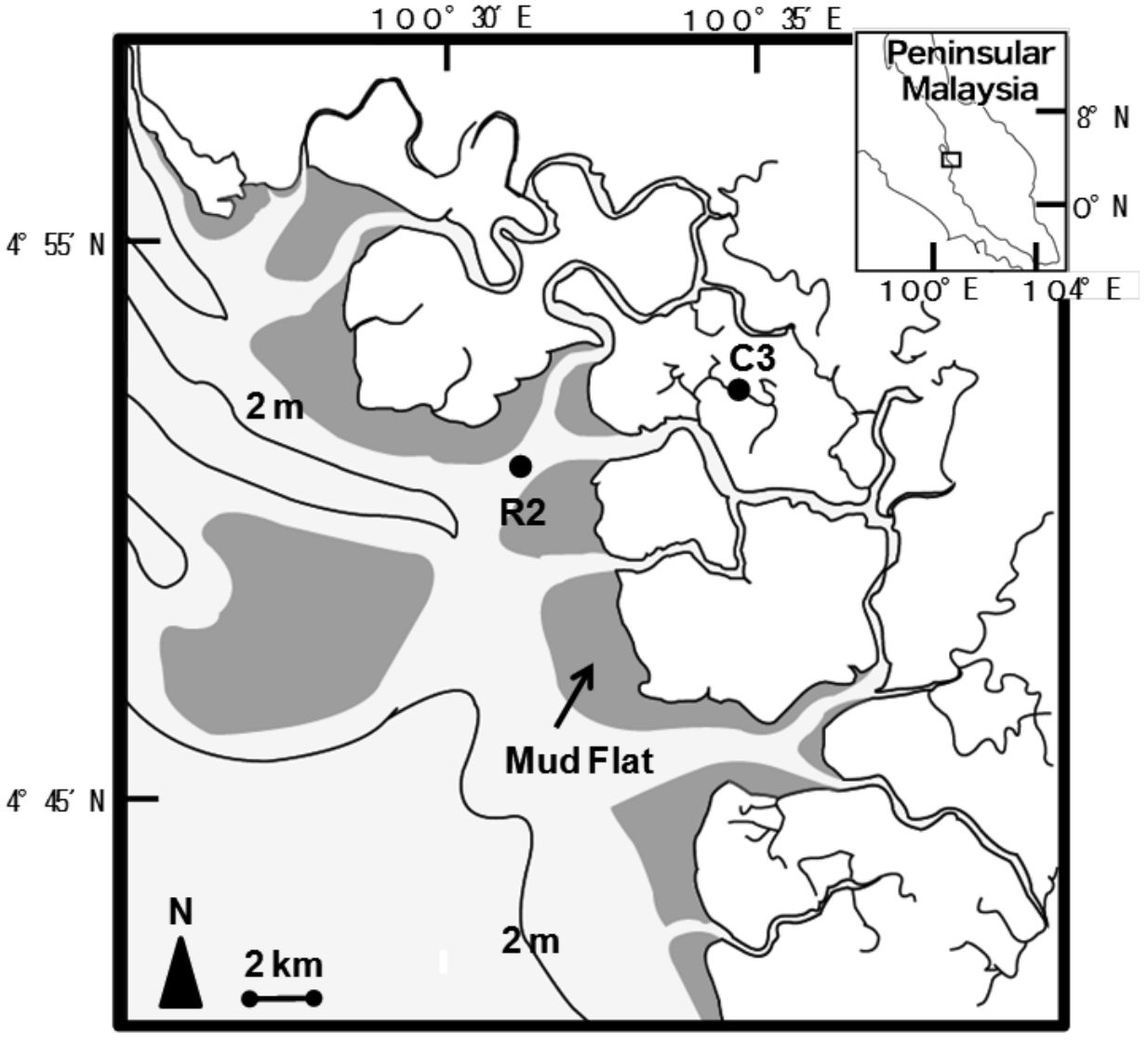


**Figure 1.** Sampling sites (filled circle) of *Acetes* shrimps in the Matang Mangrove Forest Reserve in the west of Peninsular Malaysia.

**Table 1.** *Acetes* shrimps used in the present study. Three species of *Acetes* were collected on the below date in the MMFR.

| Order | Species | Site | Date | Carapace Length (mm) |
|---|---|---|---|---|
| Decapoda, Sergestidae | *Acetes indicus* | R2 | July 29, 2010 | 2.5-4.5 |
| | | R2 | Sep. 28, 2012 | 5.0-6.5 |
| | *Acetes japonicus* | R2 | July 29, 2010 | 3.0-4.5 |
| | *Acetes sibogae* | C3 | July 28, 2010 | 2.5-4.5 |
| | | C3 | Sep.28, 2012 | 2.5-4.5 |

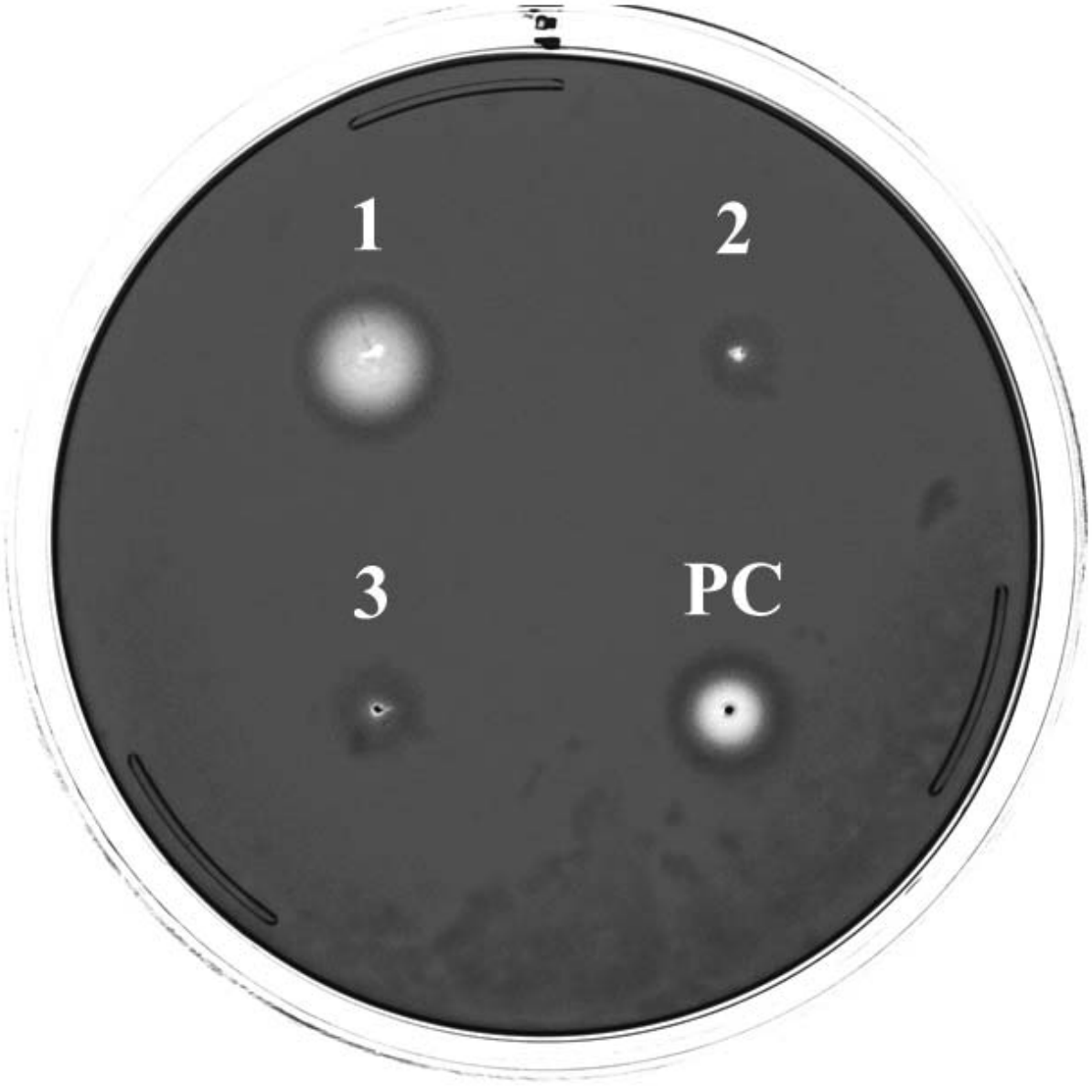


**Figure 2.** Detection of cellulase enzymatic activity by Plate Assay Analysis of *Acetes* shrimps collected in July 2010. Cellulase activity was detected as unstained circle by Congo Red. 1: *Acetes sibogae* 2: *Acetes japonicus* 3: *Acetes indicus.* Commercially obtained cellulase (MP Biomedicals) was used in PC (Positive control).

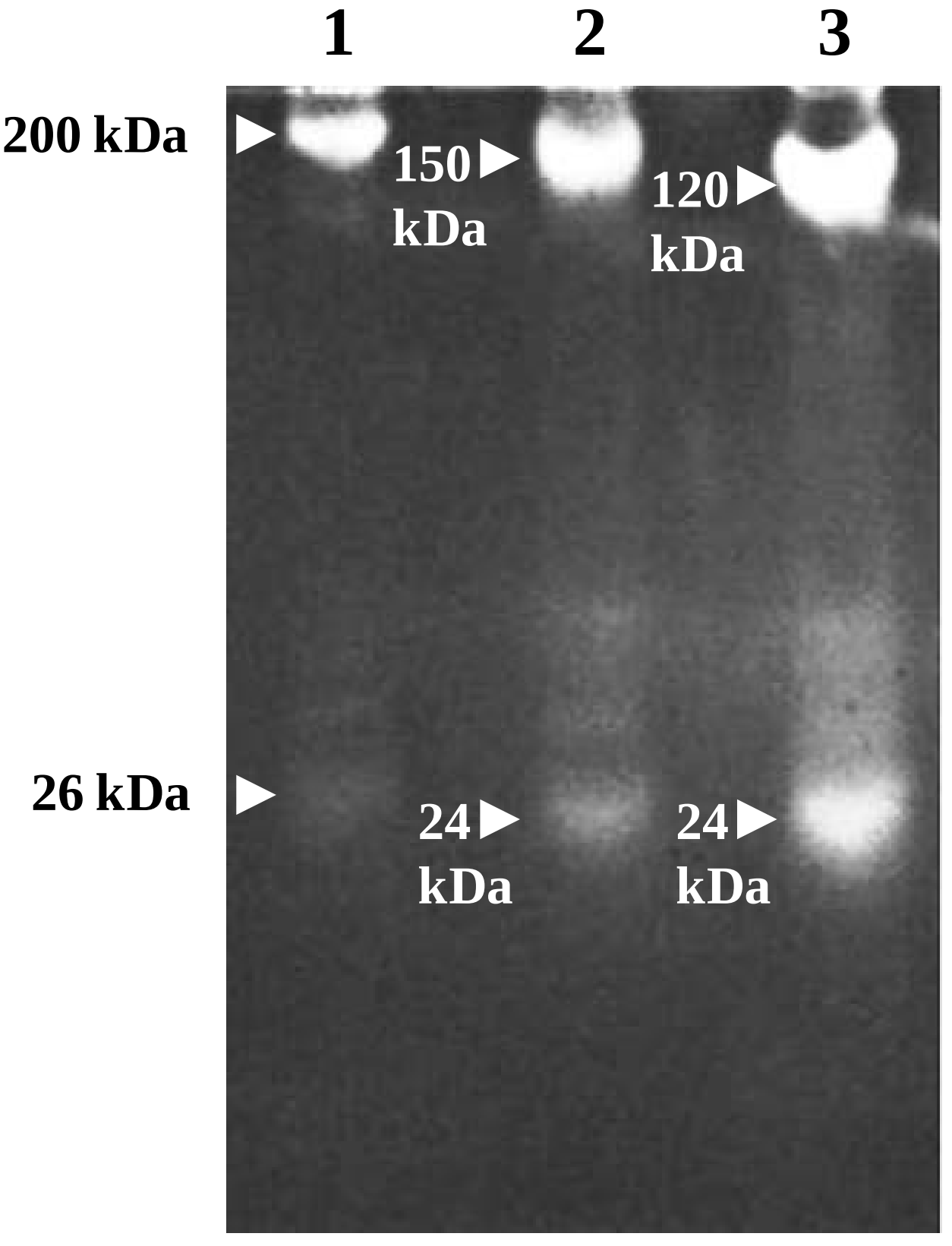


**Figure 3.** Zymographic analysis of *Acetes* shrimps collected in July 2010. Cellulase enzymatic activity was detected as unstained band by Congo Red. 1: *Acetes sibogae* 2: *Acetes japonicus* 3: *Acetes indicus*

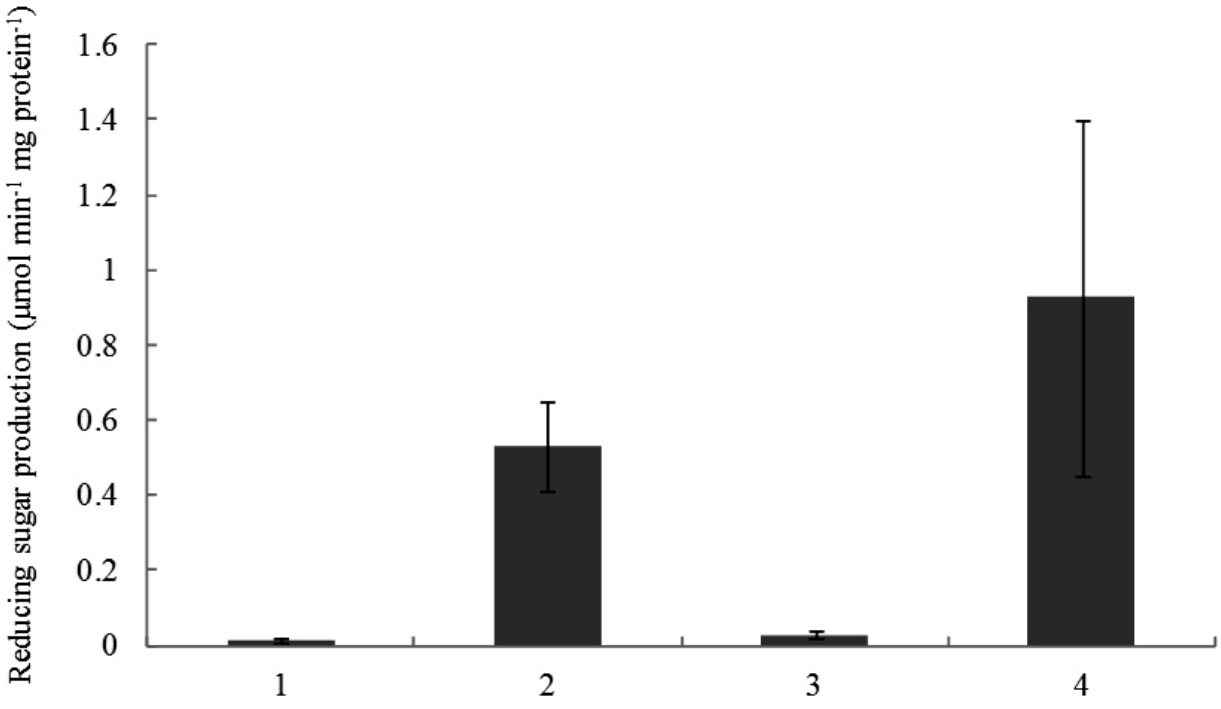


**Figure 4.** Quantification of reducing sugar production by cellulase of *Acetes* shrimps collected in Sept. 2012. Reducing sugar production was measured after incubation of the tissue extract with CMC (Carboxyl methyl cellulose) for ten minutes. 1: *Acetes sibogae* whole body 2: *Acetes sibogae* hepatopancreas 3: *Acetes indicus* whole body 4: *Acetes indicus* hepatopancreas. Bar represents mean ±SD.