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Cellulolytic activity of tropical marine rotifer (*Brachionus rotundiformis*)

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Abstract. Cellulose degrading enzymes are important in agriculture, aquaculture, feeds and food, drugs and biotechnology. Furthermore, the high cost of these enzymes and large market potential especially for bioethanol production provides a motivation to search for new enzyme source. This study is the first to characterize the cellulose degrading enzyme in the marine rotifer, *Brachionus rotundiformis*. The influence of pH, temperature and substrate concentration on cellulolytic activity was assessed using a water soluble substrate carboxymethyl cellulose (CMC), to determine the conditions for maximum enzyme activity. Maximum catalytic activity was at pH 5.0. The hydrolysis is effective in acid digestion and is in the range of intestinal pH of the rotifer. The observed optimum temperature for substrate hydrolysis (30.0°C) is in the temperature range (28.0-35.0 °C) of the rearing medium for the organism. Substrate concentration - activity plot revealed of K_m of 2.5 mg CMC/mL and a V_{max} of 178.6 µg glucose mg protein^{-1}hr^{-1}. *In vitro* hydrolysis of pure cellulose (filter paper) established that the crude enzyme has cellulolytic activity. It is highly recommended to pursue enzyme purification and further characterization using the protocol established in this study and be tested for the hydrolysis of lignocellulose plant material for fermentable sugar production.

Key Words: cellulase, *Brachionus rotundiformis*, carboxymethyl cellulose, K_m, V_{max} , filter paper.

Introduction. Cellulose is a polysaccharide synthesized by plants for food, structural organization and energy source. It is the most abundant biopolymer on earth and is made up of β 1-4 glycosidic linkages of glucose. The complete degradation of cellulose can be accomplished by the synergistic action of three major enzymes comprising the cellulase complex that includes endo-β-1, 4 glucanase (EG), the exo- β-1,4 cellobiohydrolase (CBH) and the β-glucosidase (EC). Specifically, EG hydrolyses the inner β-1, 4 bonds in cellulose chains preferentially in soluble derivatives like carboxymethylcellulose (CMC) hence its activity is frequently referred as CMCase. CBH release cellobiose from non-reducing ends of cellulose while β-glucosidase which hydrolyses cellobiose and longer cellulose chain to glucose (Percival-Zhang et al 2006; Palais et al 2010).

Cellulase and amylase which both belong to the carbohydrase family, is the second most extensively used enzyme in starch, textile, detergent and baking industry. In textile processing, cellulase is used for cotton softening and denim finishing to achieve the stone washed look; in detergent business, for cleaning, color care and anti-deposition in cotton; and for the pulp and paper trade, it is used for de-inking, drainage improvement and fiber modification; in drugs it is used to separate enantiomers from racemic mixtures (Kirk et al 2002; Percival-Zhang et al 2006). The demand for cellulase is also expected to increase dramatically as the use of a combined cellulolytic enzyme (enzymatic hydrolysis) has been identified as the method with greatest potential for cost reduction for bioethanol production (Oppert et al 2010). The high cost of cellulases and

large market potential especially for bioethanol production provides a motivation to search for new enzyme source (Sun & Cheng 2002; Percival-Zhang et al 2006).

A variety of organisms are capable of producing cellulose degrading enzymes (Watanabe & Tokuda 2001; Oppert et al 2010), and a growing number of researches have focused on their presence in tissues of aquatic invertebrates (Kühle & Kleinow 1985; Chang et al 1997; Rehman et al 2009; Oppert et al 2010; Palais et al 2010). The current study focused on the marine rotifer *B. rotundiformis* as a cellulase source. The organism is well adapted in tropical conditions as it thrives in warm waters (28 – 35°C). It feeds on foods with high cellulose content like microalgae. It has a high reproduction rate, high rearing density and renewable for it is harvested in just four days (Chew & Lim 2006).

Characterization of enzymatic activity usually involves assays relating to its basic properties such as effect of pH, temperature and enzyme substrate affinity that promotes optimum its activity. Knowing these conditions is essential to develop optimized assay protocols intended for measuring the reaction rates. Characterization is also important if the enzyme is to be purified or used in industrial application such as the production of fermentable sugars from cellulosic material for bioethanol production, for animal feed ingredient and food. The present study was undertaken to provide information on the basic properties (effect of pH, temperature, substrate concentration, K_m and V_{max} elucidation) of cellulose degrading enzymes in present *B. rotundiformis*, as well as its hydrolytic activity to a pure cellulose material (Whatmann No 1 filter paper).

Material and Method

Rotifer collection and extract preparation. The S (small) type marine rotifer *B. rotundiformis* was cultured with *Tetraselmis sp* at Institute of Aquaculture CFOS Hatchery. It was collected and concentrated by passing through a 60 µm mesh plankton nets, rinsed with distilled water and stored at -80°C until use. The extraction process was performed at 4.0°C. The collected rotifer was washed and added with 0.05 M phosphate buffer pH 7.0 in 1:15 ratio (weight to buffer volume). The level of dilutions was chosen to ensure that the amount of product formed is i) in the linear range of the reaction (initial velocity) and ii) only the amount of enzyme will be limiting. The mixture was homogenized in Glascol tissue homogenizer for 10 minutes. The resulting crude homogenate was centrifuged at 10000 rpm for 10 minutes and the supernatant filtered. The collected extract was used in subsequent enzyme assays.

CMCase Assay. The effect of pH, temperature and substrate concentration was conducted by modifying the methods described by Traifalgar et al (1999) and Palais et al (2010). The reaction cocktail mixture consists of the 0.2% substrate CMC, working buffer and diluted enzyme to a total volume of 3.0 mL. Enzyme reaction was allowed to proceed for 3 hours at a controlled temperature in a waterbath. The reaction was initiated with the addition of the enzyme and stopped with 3.0mL of 3-5 Dinitrosalycylic acid (DNS reagent) (Miller 1959). A blank mixture was prepared in a similar manner but the enzyme was added after the reaction was halted (upon addition of DNS). The formation of reduced DNS was determined spectrophotometrically at 540 nm. The amount of reducing sugar formed was determined using glucose solution standard curve.

Effect of pH. The optimum pH for CMCase activity was determined using different buffer systems for the reaction mixture: 0.2 M of CH_3COOH / $NaCH_3COO$ - buffer (pH 3.0-5.5), 0.2 M $NaHPO_4$ / Na_2PO_4 (pH 6.0 – 8.0) and 0.2M $NaHCO_3$ – Na_2CO_3 buffer (pH 8.5-11).

Effect of temperature. The influence of temperature on the hydrolytic rate was determined by conducting the reaction at different incubation temperatures ranging from 30°C - 60°C.

Effect of substrate concentration. The substrate concentration and activity relationship was investigated at five levels of substrate concentrations ranging from 0.1 –

1.0 g CMC/100mL. The resulting enzyme activity was plotted against the increasing levels of substrate concentration, linearized by Hanes equation and the K_m and maximum velocity (V_{max}) were determined.

Hydrolysis of filter paper. The hydrolysing ability of the cellulase obtained from *B. rotundiformis* was tested using pure cellulose (filter paper, Whatmann No 1). Two milligrams of the sample and 700µL of enzyme extract was added with sufficient buffer (pH 5.0) to 3.0 mL final volume. Three hours of hydrolysis was conducted in a thermo-controlled water bath at 30.0°C.

Total protein content was measured following the method described by Bradford (1976). Specific activity of the crude enzyme extract was expressed as µg reducing sugar formed per hour per milligram protein (µg glucose mg^{-1} hr^{-1}).

Results and Discussion

Effect of pH. CMCase hydrolytic activity was observed to increases linearly with pH (Figure 1) until a maximum activity peaked at pH 5.0. Further increase in pH caused a sharp decline in hydrolytic activity up to the basic pH range. For a fixed temperature and substrate concentration enzymes have an optimum pH or pH range where activity is at maximum. It is a consequence of the amino acid side chains which behaves as a weak acid or base. The observed maximum activity at pH 5.0 may reflect the area where cellulose degradation takes place in the organism. Hence it is necessary to compare the experimental enzyme – pH activity with the actual pH value of the digestive tract of *Brachionus*. Kuhle & Kleinow (1985) estimated the pH value of *Brachionus* using yeast cell stained with indicator dye. The pH value of the "stomach" was found to be at pH 7.0 – 8.0 while in the "intestine" is between pH 5.0-6.0. The optimum pH obtained from the experiment is 5.0, though 92.5% of the activity is retained at pH 4.6 and reduced to almost half (55.7%) at pH 5.8. The result of this study is indicative that crude enzyme appears to be effective in acid digestion and is in the range of intestinal pH of the rotifer.

In contrast, Chang et al (1997) using a partially purified enzyme sample from *B. plicatilis* reported a CMCase enzyme activity with optimum activity at a pH range of 6.0 to 8.0 while Kuhle & Kleinow (1985) using similar species reported that the optimum CMCase activity is at pH 6.3. It is interesting to note that while belonging to the same genus, *B. rotundiformis* has a an acidic optimum pH and outside the range reported for *B. plicatilis*. In the present study, the result in pH optimum of CMCase in rotifer is comparable to those found in other terrestrial invertebrates including red flour beetle with an activity optimum at pH 4.8 (Rehman et al 2009), the land crab *Gecarcoidea* natalis at pH 5.5 and *Discoplax hirtipes* with pH optimum at 5.5-7.0 (Linton & Greenway 2004). Also in freshwater bivalve *Dreisenna polymorpha* a CMCase operating at maximum in the acidic range (pH 5.0 – 6.0) (Palais et al 2010) was reported and is in agreement with the results of the present findings.

Effect of temperature. The temperature dependence of CMCase activity is illustrated in a bell shaped profile in Figure 2. Highest CMCase activity was at 30.0 °C. Activity decreased markedly beyond this temperature.

Under the present experimental condition the observed optimum temperature (30.0°C) is in the temperature range where *B. rotundiformis* is commonly reared, that is from 28.0-35.0 °C (Chew & Lim 2006). The optimum temperature observed in the present study is in good agreement to that reported by Chang et al (1997) for *B. plicatilis* indicating optimum enzymatic activity at 37.0 °C. Other organisms display an optimum temperature above the normal physiological range of operation for the organism*: Dreisenna polymorpha* diverticulate at 50.0°C (Palais et al 2010), *Acremonium cellulolyticus* (Kansarn et al 2000) at 60.0°C, *Chaetomium thermophile* var *dissitum* at 45.0-50.0°C (Eriksen & Goksöyr 1976), *Cellulomonas sp* (Emtiazi & Nativi 2004) at 50.0°C, red flour beetle at 40.0°C (Rehman et al 2009).

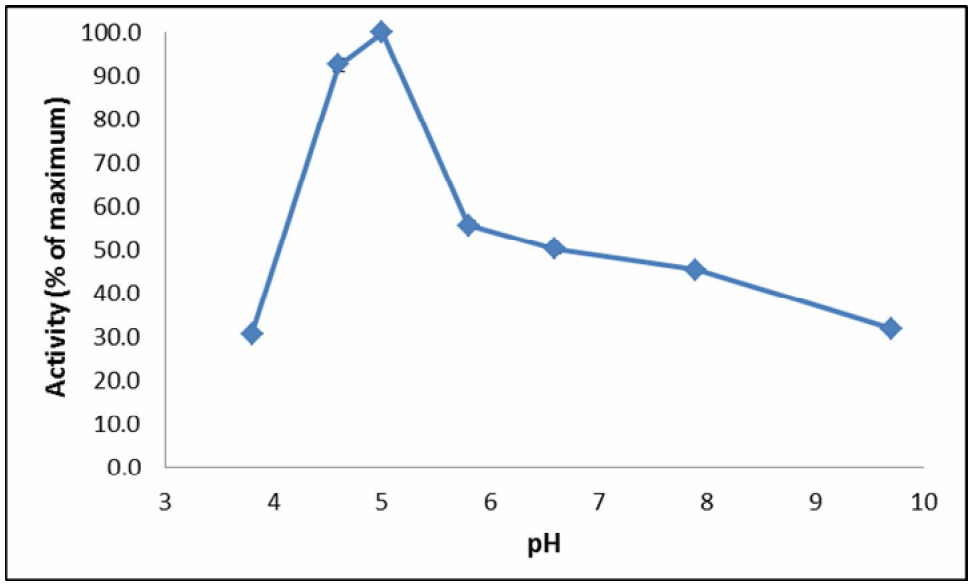

Figure 1. Effect of pH on CMCase activity of *Brachionus rotundiformis* extracts with maximum activity at pH 5.0. Each point represents the mean of three replicates ±SD.

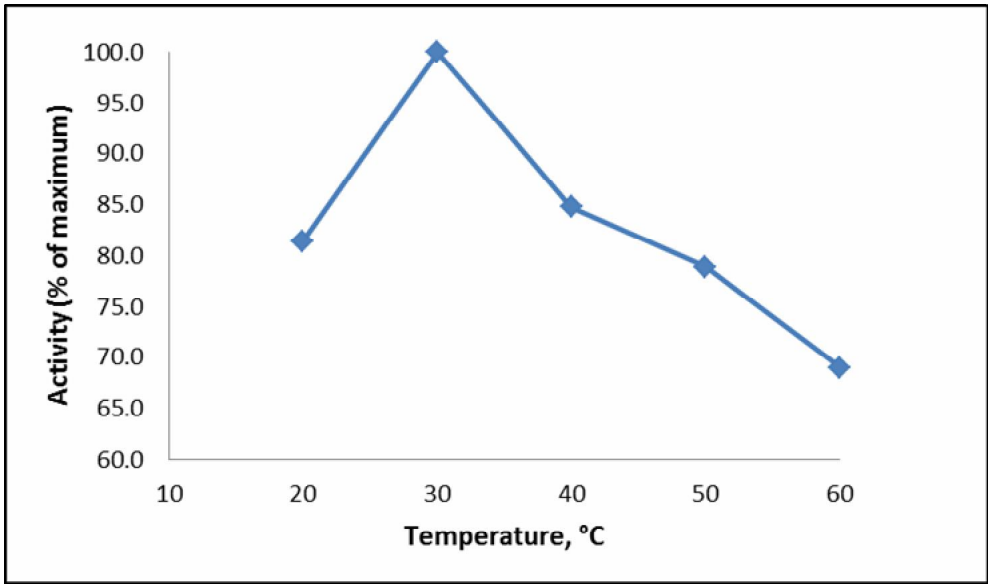

Figure 2. Effect of temperature on CMCase activity of *B. rotundiformis* extracts. Maximum activity was observed at 30.0°C. Each point represents the mean of three replicates ±SD.

It is commonly observed in most studies that the optimum temperature for *in vitro* experiment does not necessarily reflect the physiological range of operation of most enzymes (Clark et al 1987; Sabapathy & Teo 1994; Traifalgar et al 1999). Prolonged exposure to temperature greater than the ambient of the natural habitat could result in thermal denaturation of the enzyme. Too much heat can disrupt the noncovalent interactions that maintain the enzyme's three-dimensional structure. The consequent unfolding, or denaturation, of the polypeptide chains is accompanied by the rapid loss of catalytic activity.

Effect of substrate concentration. The activity of CMCase is influenced by the substrate concentration. For a fixed enzyme concentration, a linear increase of product formation with increasing substrate concentration was generally observed. However at a certain concentration of substrate, a plateau of the activity-substrate concentration curve could be observed, indicating that all of the binding sites of the enzymes are saturated with the substrate and no further increase of the hydrolytic activity. Linear

transformation using Hanes plot (Figure 3) estimates the K_m and V_{max} for the enzyme. The K_m estimates the amount of substrate needed to reach half the maximal velocity. It is a measure of affinity of the enzyme to its substrate and the concentration of the substrate in the environment. If the K_m is low, the enzyme is able to quickly adapt to the influx of substrate by processing it. For a fixed enzyme concentration, there is an increase in product formation with an increase in substrate concentration. A saturation point is reached if there is no product formation. At this point, it can be inferred that the active sites are filled with substrate molecules or it crowds or binds to other parts of enzymes. There is little information for the K_m and V_{max} values of CMCase for *Brachionus* genus. In the present study, the Hanes plot for the linear transformation of the Michaelis-Menten equation (r^2=0.96), estimates the K_m at 2.5 mg/mL and V_{max} of 178.6 µg glucose mg protein^{-1}hr^{-1}. The advantage of the cellulase from *B. rotundiformis* with a low Km value is that it can process the influx of substrate to form the enzyme substrate complex then the products, but it will also reach saturation point quickly.

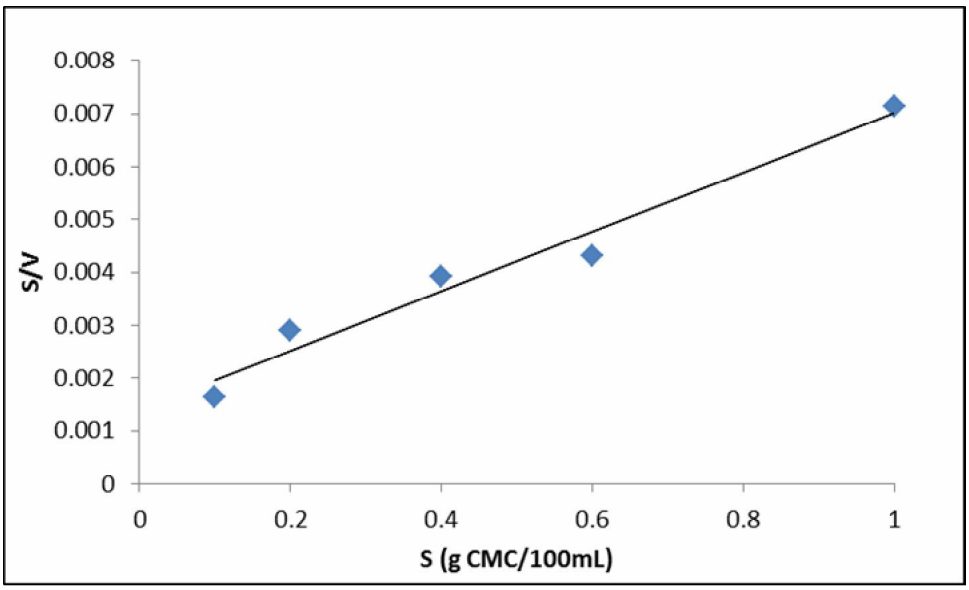

Figure 3. Hanes plot (substrate concentration vs substrate concentration/activity for CMCase r^2=0.96; Km (2.5 mg CMC /mL) and Vmax (178.6 µg glucose mg^{-1}hr^{-1}).

The crude enzyme was able to hydrolyze a pure cellulose filter paper (Whatmann No 1) with a specific activity of (57.0 µg glucose mg^{-1}hr^{-1}). This result established that a crude digestive homogenate has cellulolytic activity. Filter paper is hydrolysed with ease as it is 99.0% pure cellulose and is amorphous in origin, thus very susceptible to enzymatic attack. In contrast to a real plant material where cellulose is highly protected by lignin, hydrolysis for bioethanol production is difficult unless a pre-treatment step is employed. A pre-treatment step removes lignin, disrupts the crystalline structure of cellulose making it more susceptible for enzymatic attack. Pre-treatment are either chemical or physical and should be tailored for a specific biomass.

Conclusions. The following properties of *B. rotundiformis* cellulase was elucidated in the study: maximum catalytic activity at pH 5.0 and 30.0°C was found to be the optimum temperature for this reaction. Substrate concentration - activity plot revealed of K_m of 2.5 mg CMC/mL and a V_{max} of 178.6 µg glucose mg protein^{-1}hr^{-1}. The crude enzyme has a positive activity (57.0 µg glucose mg protein^{-1}hr^{-1}) towards pure cellulose, proving that the crude digestive homogenate has cellulolytic activity. It is highly recommended that: (1) the crude enzyme be tested for a specific application like hydrolysis of lignocellulosic material for fermentable sugar production and compare its performance with commercial cellulase;

(2) purify the crude enzyme using the protocols established in the experiment to determine other properties like molecular weight;
(3) assay the other two enzymes that comprises the cellulase complex, the exo- β-1,4 cellobiohydrolase (CBH) and the β-glucosidase (EC).

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