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CELLULASE ACTIVITIES DURING DECOMPOSITION OF A SUBMERGED AQUATIC MACROPHYTE (*UTRICULARIA BREVISCAPA*): A MICROCOSM ASSAY

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ABSTRACT

Decomposition of lignocellulosic detritus at enzymatic level is the rate-limiting step during aquatic macrophyte decomposition. Assays were carried out to evaluate cellulase activity during decomposition of *Utricularia breviscapa*; the assays included the following C-sources: leachate, lignocellulosic matrix and integral detritus. The incubations comprised *U. breviscapa* C-sources added to Óleo lagoon (21º 36'S and 47º 49'W) water maintained in the dark under anaerobic condition at 15ºC, 20ºC, 25ºC and 30ºC. Cellulase activity were monitored during 180 days, with the activity showing a time evolution that depended on the temperature and type of detritus: (i) cellulase activity tended to occur earlier with increasing temperatures, but it was less intense than at lower temperatures, (ii) no activity was observed in incubations with leachate, (iii) cellulase activity was observed in the incubation with integral detritus and lignocellulosic matrix and (iv) higher cellulase activity was observed in the incubations with integral detritus at 15ºC ($P < 0.05$).

Key words: cellulase, *Utricularia breviscapa*, cellulose

INTRODUCTION

Most of the biomass from aquatic macrophytes in the littoral zones of shallow aquatic systems enters the pool of particulate and dissolved organic matter after senescence and death. Metabolism associated with particulate and dissolved organic matter provides essential energy for the operation and metabolic stability of the entire ecosystem (39). Decomposition of aquatic plants is an important process for the input of carbon and energy and therefore the microbial metabolism associated with dead plant biomass could represent a major route for ecological recovery of organic matter in these environments. The decomposition rates in the aquatic environment depend on abiotic factors including nutrients (19), supply of electron acceptors (9), hydroperiod (38), temperature (23) and pH (20). Other factors such as plant detritus availability, chemical composition and morphological structure (14), nutritional C:N:P ratio of plant (12), molecular mass (4), origin (16), as well microbiota metabolic activity, biomass and diversity (31) also affect the decomposition rates. Hence, decomposition of organic matter occurs via multiple enzymatic reactions involving a variety of organisms and oxidants, in addition to a number of intermediate compounds (37).

Microorganisms mediate decomposition by utilizing a wide variety of organic compounds under diverse environmental conditions, extracting energy from organic compounds by fermentation, anaerobic and aerobic respiration. Microbial microorganisms release enzymes into the surrounding environment in order to degrade macromolecular and insoluble organic matter prior to cell uptake and metabolism (5). The biological hydrolysis of cellulose depends upon endoglucanase, exoglucanase and β-glucosidase, acting synergistically on structural polysaccharides (2), generating low-molecular weight reducing sugars (30). Interest in cellulase arises because it is an important enzyme related to the decomposition of lignocellulosic material. We hypothesized that abiotic (*i.e.* temperature) and biotic factors (type of C- source of detritus) also governed cellulase activity by microheterotrophs organisms once the

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decay rates of lignocellulosic material are mainly affected by these factors. In order to distinguish these factors, the present study was conduct to evaluate cellulase activity, under different temperatures, resulting from mineralization of organic C-sources from *Utricularia breviscapa*: leachate, lignocellulosic matrix and integral detritus. This study bridges gap at molecular level study of enzyme activity and environmental factors, providing baseline information on the performance of microbial assemblages associated with detritus from *U. breviscapa*.

MATERIALS AND METHODS

Sampling Site

Óleo lagoon (21º 36'S and 47º 49'W) is one of the many oxbow lagoons in the Mogi-Guaçu river floodplain situated within the Natural Reserve of Jataí (21º 33' to 21º 37'S and 47º 45'to 47º 51'W; Luiz Antonio, São Paulo, Brazil). It is a shallow (Z_{max} *ca.* 5.5 m), small (17.800 m^2), acidic (pH: 5.49 ± 0.65) lagoon with relatively low concentrations of dissolved oxygen (3.57 ± 2.18 mg L^{-1}) and dissolved organic carbon (3.05 ± 0.98 mg L^{-1}). The annual water temperature usually varies from 18ºC ± 2 (July) to 30ºC ± 1 (January). It has been classified as a seepage lagoon. The littoral zone of this environment is intensively colonized by aquatic macrophyte (27).

Water sampling and plant material sampling

Water samples (20 L), taken at 1 m depth intervals, were collected on June, 2002 from littoral and pelagic zone with 5 L Van Dorn underwater samplers at three distinct depths (surface: 0.5 m; middle: 2.0 m and bottom: 4.0 m). Water samples were mixed in a polyethylene container in order to get vertically integrated indigenous microorganisms samples. Immediately after collection, the samples were pre-filtered through a cellulose ester membrane (0.45 mm pore size, Millipore). Entire fresh living mature samples were manually collected on January, 2001 (summer); May, 2001 (autumn); June, 2001 (winter) and November, 2001 (spring) from the littoral lagoon zone; this procedure was adopted in order to avoid differences in the chemical composition of *U. breviscapa* structures caused by the phenological status. The plants were washed within the lagoon. In the laboratory, the plants were washed with tap water to remove periphyton, sediment particles and coarse material (26). After washing, the plant material was oven-dried (50ºC), grounded (0.2 cm > diameter > 1.32 cm) and homogenized. Prior to the assays, aqueous extractions were performed in the integral plant fragment, to obtain the lignocellulosic (LC) matrix and leachate. The extraction comprised addition of 100 g dry weight of grounded plant fragments in a flask containing 1 L of distilled and sterilized water (25). After 24 h of cold aqueous extraction (4ºC), LC matrix was fractionated from leachate by filtration through cellulose ester membrane (0.45 mm pore size, Millipore). Prior and after the aqueous extractions, the carbon contents of integral detritus and LC matrix were quantified using a Carlo Erba CHN elemental analyzer (model EA1110, Milan, Italy). The initial concentrations of carbon from leachate incubations were measured by Pt-catalyzed non-dispersive combustion and detection in infrared gas analysis (Shimadzu, TOC-5000A analyzer, Tokyo, Japan).

Incubations

Incubations (400 ml) were prepared adding integral detritus, LC matrix or leachate and lagoon water in the proportion of 3336.3 mg C L^{-1}, 3648.3 mg C L^{-1}, 122.2 mg C L^{-1}, respectively. The incubations (n = 2) were maintained under anaerobic conditions in the dark at 15.3 ± 1.2ºC, 20.8ºC ± 1.2ºC, 25.7ºC ± 1.7ºC and 30.3ºC ± 1.3ºC. The anaerobic conditions were obtained by sparging with N_2 during 30 min.

Crude extract and enzyme assay

On sampling days (1, 3, 6, 9, 12, 18, 24, 30, 45, 65, 85, 125, 155 and 180), the incubations maintained at controlled temperature and anaerobic conditions were homogenized and subsamples (10 ml) were centrifuged (3.000 × g, 45 min, 4ºC) for measuring cellulase activity in the supernatants. Cellulase assays were performed in duplicate for each treatment. Cellulase (C1 = endoglucanase - EC: 3.2.1.4 and exoglucanase - EC: 3.2.1.91) activity was determined by measuring the release reducing sugar acting on crystalline cellulose (22) and the concentrations of reducing sugars were quantified (35). One unit of cellulase activity was referred as the amount of enzyme that liberates 1 µmol of reducing sugar under the assay condition. Cellulase activities were expressed as mol per remaining carbon (g detritus) resulting from *U. breviscapa* decomposition and calculated as activity-day (15). Cumulative cellulase activities were calculated by integrating the enzyme activities over time. The temporal variations of cumulative cellulase activities were fitted to a sigmoidal curve using non-linear regression (iterative algorithm of Levenberg-Marquardt) following Press *et al.* (28). The coefficient of enzymatic activity (k_p) values were derived from the sigmoidal fittings.

Data corresponding to accumulated cellulase activity were submitted to non-parametric analysis (Kruskal-Wallis test). The level of statistical significance was 0.05, unless otherwise specified in the test.

RESULTS

Cellulase activity was observed in the incubations with integral detritus and LC matrix (Fig. 1). No cellulase activity was detected in the incubations with the leachate. For higher temperatures cellulase activity occurs earlier. For instance, at 30.3ºC cellulase activity was observed on 12th and 9th day in the incubation with integral detritus and in the LC matrix, respectively. At 15.3ºC, both integral detritus and LC matrix displayed cellulase activity on the 45th day of incubation. However, at higher temperatures cellulase activity was not enhanced, being

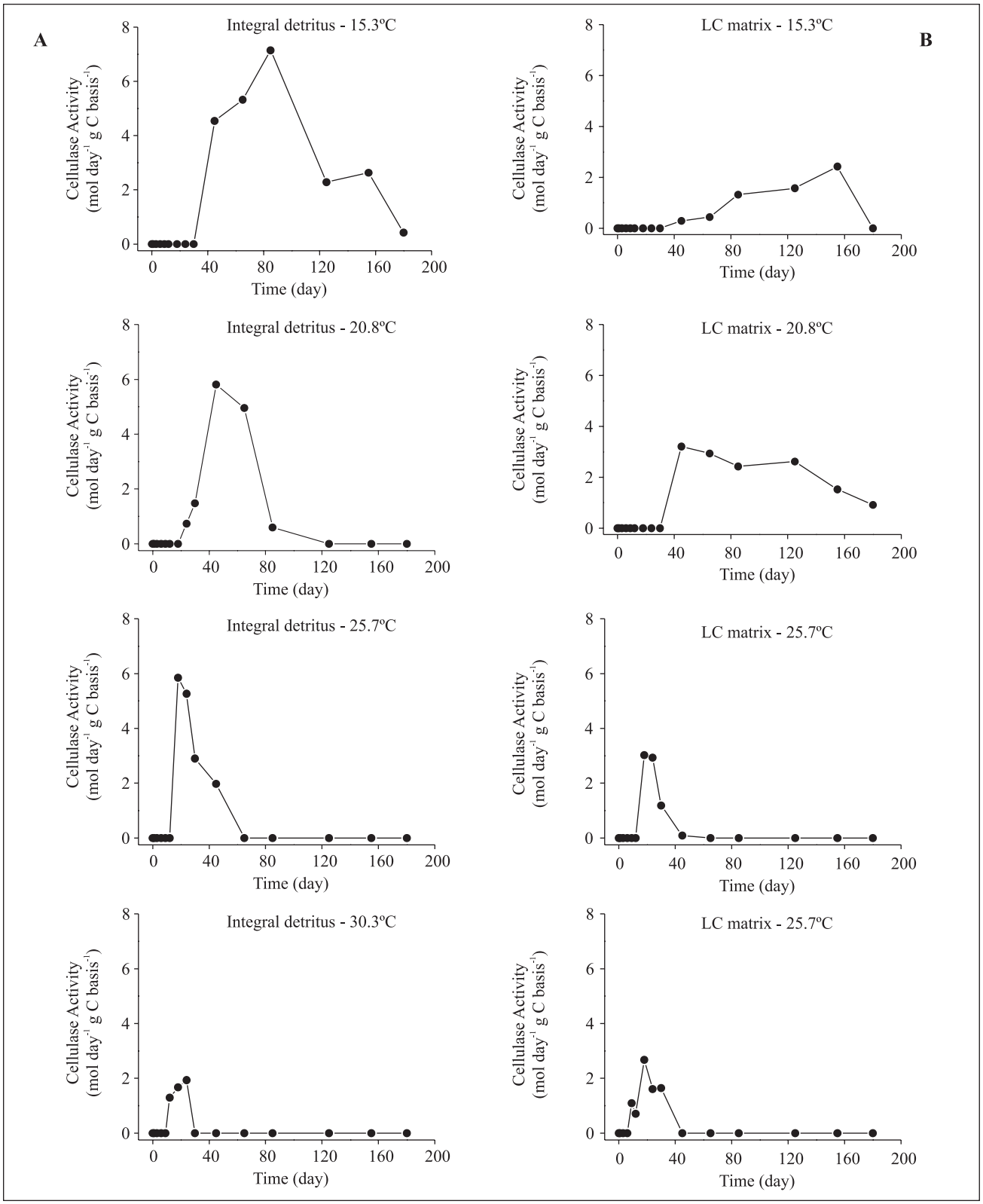

Figure 1. Cellulase activities from incubations with integral detritus (A) and LC matrix (B) decomposition under anaerobic condition and different temperatures.

markedly reduced at 30.3ºC as shown in Fig. 1. Cellulase activity increased in the early stage of incubation, reached a maximum and then decreased gradually. Maximum cellulase activity in the integral detritus incubations were (C-basis): 7.2 (15.3ºC), 5.8 (20.8ºC), 5.3 (25.7ºC) and 1.9 mol day^{-1} (30.3ºC). For the LC matrix, these values at the same temperature were 2.4, 3.2, 3.0 and 2.6 mol day^{-1} C basis^{-1}, respectively. On average, cellulase production was 1.83 higher for the integral detritus, except for 30.3ºC, where similar cellulase activities were observed for LC matrix and integral detritus.

During decomposition of integral detritus (Fig. 2A), the increment of incubation temperature decreased the maximum cumulative cellulase activity and increased k_p (i.e. coefficient of enzymatic activity increment). The maximum cumulative cellulase activities were 538.5 (15.3ºC), 237.5 (20.8ºC), 129.8 (25.7ºC) and 27.5 mol C basis^{-1} (30.3ºC), with corresponding k_p of 0.005, 0.107, 0.171 and 0.329 day^{-1}. For all incubation the determination coefficients (r^2) of these fittings were 0.99. The behavior described for the integral detritus was different for the LC matrix, which presented an increasing cumulative cellulase activity from 15.3ºC (195.4 5 mol C basis^{-1}) and reached the maximum at 20.8ºC (326.5 mol C basis^{-1}). In the range between 25.7 and 30.3ºC, these values were practically the same (49.4 mol C basis^{-1}). k_p was 0.044 day^{-1} at 15.3 and 20.8ºC, with a maximum at 25.7ºC (0.259 day^{-1}), then decreasing to 0.196 day^{-1} at 30.3ºC.

Using the accumulated values of cellulase activities, the one-way ANOVA analysis showed significant differences between activities in integral detritus at 15ºC ($P < 0.05$) and 30ºC. This statistical analysis did not indicate significant differences between the cellulase measured from LC matrix independently of temperature incubation ($P > 0.05$).

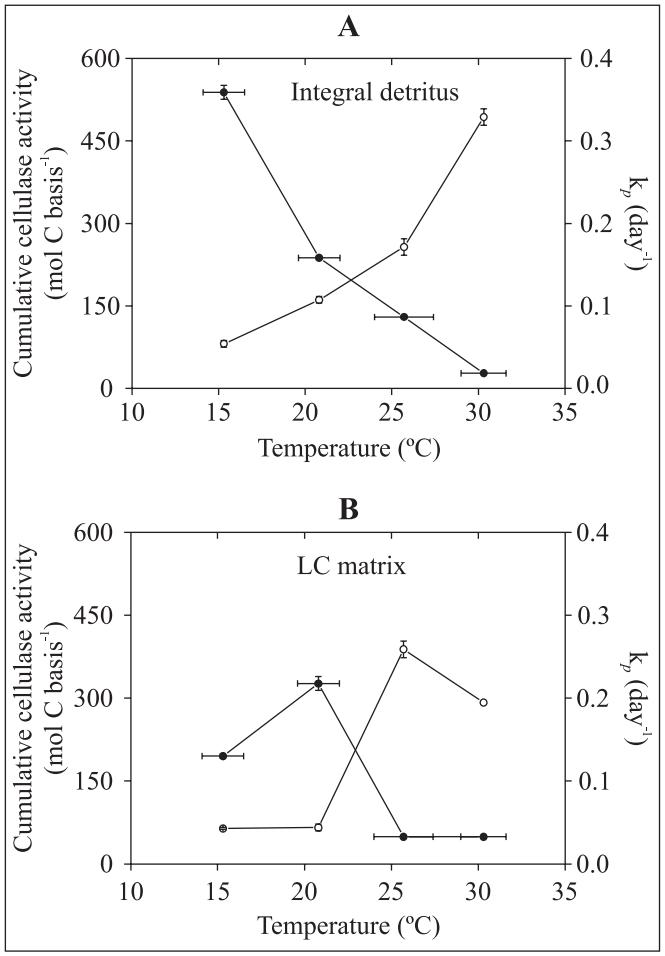

Figure 2. (●) Cumulative cellulase activity (mol C basis^{-1}) and (○) k_p (day^{-1}) of (A) integral detritus and (B) LC matrix in function of incubation temperature.

DISCUSSION

The present study allows comparison of cellulase activities under different temperature, for different C-sources detritus. These results can give insights into the relative importance of different thermal regimes in lignocellulosic material processing in aquatic environments. In this context, during *U. breviscapa* decomposition, lignocellolosic residues comprised the major remaining portion of the integral detritus and LC matrix incubations (10). As microorganisms depolymerize structural materials, a multi-enzymatic process that operates outside the cell mediates detritus microbial degradation (5) and the most important enzymes related to particulate detritus decomposition are those involved in the degradation of lignocellulosic materials (34).

In relation to leachate experiments, the lack of cellulase activity could be attributed to the absence of a cellulose source, owing to a potential inducer of cellulase production. Thus, the cellulase systems of the cellulose-decomposing microorganisms could be an adaptive or inductive system (1). Cellulase activities were only observed in incubations with integral detritus and LC matrix. The anaerobic bacteria can utilize a large multi-enzyme complex (cellulosomes), which operates at the cell-surface interface (18), while anaerobic fungi seem to have large cellulosome-like enzyme complex (11). Within each system an efficient complex of endocellulases and cellulases can be found. In the cellulose degradation by fungi exo and endocellulase cooperate synergistically for an effective degradation (7). The efficiency of cellulosomas in anaerobic microorganisms with regard degradation of crystalline cellulose is dependent on both endo/exocellulase synergism and clustering of the complex to the substrate surface (3). Symbiotic associations or consortia also raise the efficiency on cellulose degradation in anaerobic environments (*e.g.* sediment and rumen), that comprises primary cellulolytic species with secondary and ancillary microorganisms associated with the cellulose-decomposing microbiota (21).

The time requirement for the beginning of enzymatic activities was probably related to the first utilization of non-structural compounds released during physical leaching. According to Sala and Güde (31), during decomposition of the aquatic macrophyte *Potamogeton pectinatus* a succession of exoenzymatic activities involved in the hydrolysis of carbohydrates started with the hydrolysis of small and non-structural carbohydrates (*e.g.* starch), which derived from physical leaching. After the disappearance of dissolved carbohydrates, the hydrolysis of larger structural polysaccharides such as cellulose and hemicellulose took place. The utilization of simple compounds by decomposers is more energetically efficient than the uptake of complex polymers, so the degradation of simple carbohydrates might precede that of large polymers (24).

Sala and Güde (31) reported that the initial stage of degradation of the aquatic macrophyte *P. pectinatus* was characterized by a higher growth of free bacteria, probably due to the utilization of non-structural carbohydrates. A second stage was characterized by the increase of attached bacteria. However some studies have shown that physical contact of microorganism cells with cellulose substrate was not essential for cellulose degradation, since the products of cellulose degradation must be present in the medium (17). Herein, these observations are probably related to the necessary time to the predominance of attached microorganisms in the integral detritus and LC matrix incubations to initiate the effective cellulose degradation. With regard to the attachment of decomposing microbiota on the substrate, physical contact (*e.g.* bacterial adherence mechanisms: capsular material, fimbriae or cell surface attached enzymatic components; fungal adherence mechanisms: penetrating hyphae) between cells and cellulose fibers might be necessary for induction of cellulolytic enzymes (7,8).

The delay in appearance of cellulase activity for temperatures between 15.3 and 20.8ºC, in comparison to the range from 25.7 to 30.3ºC, was probably related to the decrease in metabolic activity of decomposing microorganisms acting on non-structural polysaccharides. Probably the slower metabolic activities increased the initial time of microbial attack on lignocellulosic compounds.

The higher production of cellulase activity from decomposition of the integral detritus was probably related to the presence of leachate that is rich in nutrients (P and N) and vitamins. The presence of these substances did not reduce the potential of limiting factors related to the growth of microorganisms. In this context, according to Sala *et al.* (32), bacterial response was reflected in 10-fold increase in bacterial production after nutrient (P) addition in a long-term microcosm experiment.

The intensity of cumulative cellulase activities for integral detritus and LC matrix decreased at higher temperatures. A possible explanation is that the increase of metabolic rates by these microorganisms due to increasing temperatures favored concurrent processes (*e.g.* xylanase production) related to degradation of structural polysaccharides. Cellulase systems are always associated with related polysaccharide hydrolyses, particularly xylanases, due to its close associations with hemicellulose (36). Xylose, the end-product of hemicellulose degradation, hampered cellulolytic activity in *Cellulomonas* sp., indicating inter-relationship between the systems regulating cellulase and xylanases (29). Other possibilities are: (i) the indigenous inoculum was composed by a population well adapted to the lower range of temperatures. In this context, it is assumed that the enzymatic activity was proportional to the microorganisms population (6), (ii) the rise in temperature increased the consumption of co-factors related to cellulase production (*e.g.* thiol-binding agents and ions calcium), (iii) the range of pH values was not adequate for an efficient development of cellulase enzymes and (iv) the temperature increased the rates of biochemical (enzyme-substrate) reactions.

Upon increasing the temperature, k_p associated with cellulase formation for integral detritus and LC matrix, tended to increase. According to Shiah and Ducklow (33) the elevation of the temperature accelerates most physiological processes, *e.g.* the microbial metabolism and consequently cellulase production. Even though k_p was higher at 20.8ºC than at 30.3ºC in the LC matrix, the values had the same order of magnitude.

Cellulase activities were usually assayed *in situ* experiments, where litter bags techniques were employed (15, 13). The lack of data under controlled conditions assays makes it difficult to compare studies on cellulase activity. The obtained measurements of cellulase activity could be used to provide useful information in nature and to assess the potential for cellulose biochemistry transformations. Also this assessment can be used to define the nutritional status or metabolic/physiologic diversity of populations under certain environmental conditions. In summary, we may conclude that the cellulase activities showed a temporal pattern governed by abiotic (temperature) and biotic (type of C-source): (i) cellulase activity tended to occur earlier with increasing temperatures, but it was less intense than at lower temperatures, (ii) no activity was observed in incubations with leachate, (iii) cellulase activity was observed in the incubation with integral detritus and LC matrix and (iv) a higher production was observed in the cellulase activity in the incubations with integral detritus at 15ºC. We suggest that investigation of cellulase activity and the effects of temperature on extracellular hydrolytic enzymes should be useful in understanding mechanisms that control lignocellulosic detritus processing.

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RESUMO

Atividades da celulase durante a decomposição de uma macrófita submersa (*Utricularia brevicapa*): um ensaio em microcosmo

A decomposição dos detritos lignocelulósicos em nível enzimático é uma etapa limitante da decomposição de macrófitas aquáticas. Experimentos foram realizados para avaliar a atividade da celulase durante a decomposição de diferentes fontes de carbono provenientes de *Utricularia breviscapa*: lixiviado, matriz lignocelulósica e detritos íntegros. As incubações compreenderam as fontes de carbono de *U. breviscapa* adicionadas à água da lagoa do Óleo (21º 36'S e 47º 49'W) mantida no escuro à 15ºC, 20ºC, 25ºC e 30ºC. As atividades da celulase foram monitoradas durante 180 dias, apresentando uma evolução temporal dependente da temperatura e do tipo de detrito: (i) a atividade da celulase tendeu a ocorrer mais cedo nas temperaturas mais elevadas, porém foram menos intensas, que nas temperaturas mais baixas, (ii) nenhuma atividade foi observada nas incubações com lixiviado, (iii) as atividades da celulase foram observadas nas incubações com detritos integrais e com matriz lignocelulósica e (iv) as atividades da celulase foram mais elevadas nas incubações com detritos integrais à 15ºC ($P < 0,05$).

Palavras-chave: Celulase, *Utricularia breviscapa*, celulose

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