Arquivos de Ciências do Mar

PRELIMINARY STUDY ON CELLULOLYTIC ACTIVITY OF Neoteredo reynei (BARTSCH, 1920) (MOLLUSCA: BIVALVIA: TEREDINIDAE)

Estudo preliminar sobre a atividade celulolítica de *Neoteredo reynei* (Bartsch, 1920) (Mollusca: Bivalvia: Teredinidae)

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RESUMO

Este trabalho objetivou avaliar a presença de atividade celulolítica na microbiota isolada do trato digestório de Neoteredo reynei *e a presença de atividade celulolítica intrínseca do próprio animal. Para investigar atividade celulolítica na microbiota gastrintestinal de N. reynei, os moluscos foram dissecados assepticamente e amostras tanto do epitélio quanto do conteúdo gastrintestinal foram coletadas e, posteriormente, inoculadas em placas de ágar nutritivo para isolamento das colônias microbianas. Já para investigar a presença de atividade celulolítica intrínseca ao animal, alguns espécimes tiveram seu corpo dividido, arbitrariamente, em quatro regiões (região da concha, região anterior do ceco, região posterior do ceco e região das paletas) que se constituíram em quatro diferentes amostras. De outros espécimes foi coletado o conteúdo gastrintestinal para obtenção de uma quinta amostra (lúmen). Todas as amostras foram esterilizadas através de tratamento com os antimicrobianos tetraciclina e cetoconazol e, posteriormente, maceradas e submetidas à extração a frio com solução tampão fosfato de sódio 0,1 M, pH 6,0 (1:1; p/v) por 4 h. Em seguida, os extratos foram filtrados e centrifugados, sendo o sobrenadante utilizado nos ensaios. O ensaio de atividade celulolítica foi realizado em placas de ágar celulose pelo método de difusão radial. Quatro dos cinco isolados microbianos caracterizados mostraram atividade celulolítica. Da mesma forma, todas as amostras estéreis das secções do corpo do animal e do conteúdo gastrintestinal de N. reynei apresentaram atividade celulolítica. Assim, tanto a microbiota do hospedeiro quanto o próprio molusco parecem estar envolvidos na digestão da celulose.*

Palavras-chaves: Neoteredo reynei*, celulase, simbiontes, digestão, atividade celulolítica.*

ABSTRACT

This work aimed to assess the presence of cellulolytic activity in the microbiote isolated from the digestive tract of Neoteredo reynei *and also the presence of cellulolytic activity intrinsic to the animal. To investigate cellulolytic activity in the gastrointestinal microbiote of* N. reynei*, the mollusks were aseptically dissected and samples of both gastrointestinal epithelium and gastrointestinal content were collected and then inoculated in nutritive agar plates for isolation of bacterial colonies. In order to investigate the mollusk intrinsic cellulolytic activity, some specimens had their whole bodies arbitrarily cut into four regions (shell region, anterior cecum region, posterior cecum region and pallet region) which constituted four different samples. Other specimens had their gastrointestinal content collected to obtain the fifth sample (lumen). All samples were sterilized by treatment with the antimicrobials tetracycline and cetoconazol and then macerated and submitted to cold extraction with sodium phosphate buffer 0.1 M, pH 6.0 (1:1; w/v) for 4 h. Then, extracts were filtered and centrifuged. The supernatant was separated for the cellulolytic assays which were performed in agar cellulose plates by radial diffusion method. Four out of five microbial isolates showed cellulolytic activity. Likewise, all body sections and lumen sterile samples of N. reynei showed cellulolytic activity. Thus, both host microbiote and the mollusk itself seem to be involved in cellulose digestion.*

Key words: Neoteredo reynei*, cellulase, symbionts, digestion, cellulolytic activity.*

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1 INTRODUCTION

Cellulose is an insoluble polymer composed of glucose subunits linked together through β-1,4-glycosidic bonds. The degradation of this insoluble crystalline substance, in its native form, requires the action of different cellulases (Bergmeyer, 1974). The presence of true cellulases was verified in the digestive tract of many invertebrates that feed on wood and similar plant products. Nevertheless, in many cases, cellulose digestion is done by symbiotic microorganisms which inhabit host digestive tracts (Waterbury *et al*., 1983).

The Teredinidae is a family of highly specialized bivalves and constitutes the most representative group among wood-boring marine animals (Omena *et al*., 1990). Due to their particular lifestyle, body structure of teredinids presents morpho-physiological modifications such as an elongation of the posterior portion, alteration of its form and the development of new structures exclusive to this group (Lopes & Narchi, 1998). Anatomic specialization of ctenidia, labial palps and mantle proves that these animals permit that only small particles reach their mouths (Lopes *et al*., 2000). The occurrence of cellulase has been evidenced in few teredinids: *Bankia setacea* Tryon, 1865 (Boynton & Miller, 1927), *Teredo navalis* Linnaeus, 1758 (Dore & Miller, 1923) and *Teredo norvegica* Spengler, 1792 (Harington, 1921). This preliminary study aimed to verify the presence of cellulolytic activity in *Neoteredo reynei*, both of the microbiote isolated from the digestive tract and of the animal itself.

2 MATERIALS AND METHODS

2.1 Animal sampling

Neoteredo reynei was obtained from fallen stems of *Rizophora mangle* (red mangrove) at the Pacoti River estuarine area, Fortaleza (Ceará, Brazil). The animals were carefully taken out of their galleries, some were preserved in 70% ethanol for subsequent specific identification with the help of a stereomicroscope and specialized literature (Clench & Turner, 1946; Müller & Lana, 1986; Rios, 1994). Other animals were sorted into two groups where each of them was submitted to different treatments according to needs for the microbiote cellulolytic activity and animal intrinsic cellulolytic activity assays.

2.2. Microbiote isolated from *N. reynei* digestive tract

The specimens of *N. reynei* were aseptically dissected under laminar flux conditions with sterile surgical material. At dissection, components of the digestive tract were exposed, and portions of the gastrointestinal epithelium (GE) as well as aliquots of the gastrointestinal content (GC) were removed. GC aliquots were promptly diluted (1:10; 1:100 and 1:1000) in sterile 0.9% NaCl. These diluted samples and the GE samples collected with a sterile swab were plated in nutritive agar containing 4% NaCl (Soares *et al*., 1991). All the plates were then incubated at 37 ºC for 24 h. Analysis of morphological and cultural characteristics of colonies was conducted. Five isolates were selected and used for cellulolytic activity assay.

2.3 Body sections and luminal content of *N. reynei*

Five samples were obtained from *N. reynei* for cellulolytic activity assay. The whole body from some specimens was divided into four sections and arbitrarily named shell region, anterior cecum region, posterior cecum region and pallet region (Figure 1). Other specimens had their intestinal content taken out by washing it with 0.9% NaCl and this sample was named lumen. Both body sections and lumen were sterilized with tetracycline (EMS, Hortolândia, Brazil) and cetoconazol (EMS, Hortolândia, Brazil),

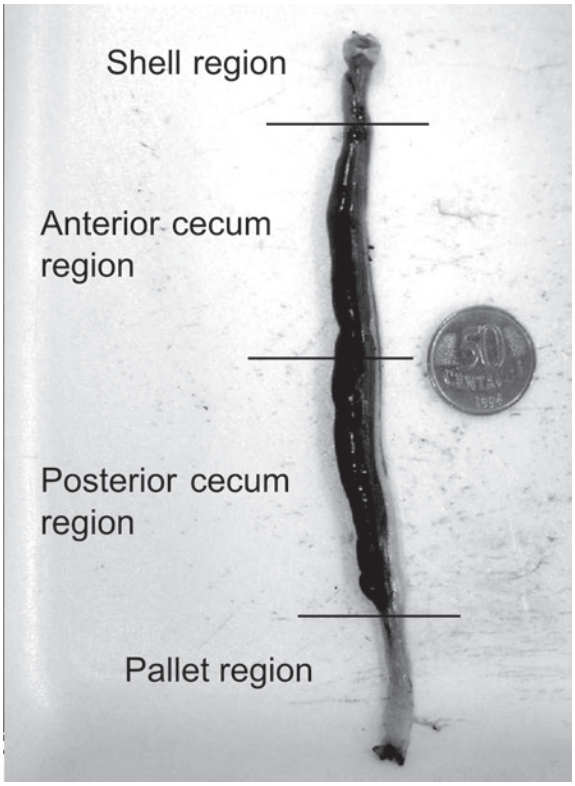

Figure 1 - Body sections (Shell region, Anterior cecum region, Posterior cecum region and Pallet region) of *Neoteredo reynei* utilized for cellulolytic activity assay.

for 12 h under refrigerated conditions. The body sections were macerated and homogeneized in 0.1 M sodium phosphate buffer, pH 6.0 (Greenfield & Lane, 1953) and submitted to agitation under refrigerated conditions for 4 h. The lumen and body section extracts were filtered and centrifuged at 15,000*g* for 15 min. The supernatant was separated and liophylized. Finally, the five samples were resuspended in minimal volumes of 0.1 M sodium phosphate buffer, pH 6.0.

2.3.1 Cellulolytic activity assay

Cellulolytic activity of microbiote isolated from GE and GC from *N. reynei* was verified through assays on plates with medium containing cellulose as the only carbon source. Inoculates used on the assay were standardized as 24-hour colony, grown in nutritive agar at 37 ºC. The bacteria were cultivated in cellulose agar and incubated for 24 h at 37ºC. Likewise, to evaluate the cellulolytic activity intrinsic to *N. reynei*, 30 µL of the liophylized extracts of both body sections and lumen were transferred to wells in cellulose agar plates, according to Ruegger & Tauk-Tornisielo (2004). The plates were submitted to thermal shock in oven (FANEM, São Paulo, Brazil) for 16 h at 50ºC. After this period, the plates were stained with 10 mL of Congo red staining solution for 30 min, following a wash with 5 mL of 0.5 M NaCl solution in 0.1 M Tris-HCl buffer, pH 8.0. Cellulolytic activity was shown by the formation of a clear halo surrounding bacterial growth area and around wells where body sections and lumen samples were inoculated (Nogueira & Cavalcanti, 1996). Cellulase from *Aspergillus sp.* (EC 3.2.1.4., Sigma-Aldrich Co. USA) was used as positive control and 0.1 M sodium phosphate buffer, pH 6.0 as negative control.

3 RESULTS

All collected animals were identified as members of the species *Neoteredo reynei* Bartsch, 1920 through observation of pallets (Figure 2-a_1,a_2,b), which are located in the posterior part of the animal, and by analysis of soft body parts morphology (Figure 2-c,d).

Twenty-eight isolates were obtained from the dissected animals, 15 from GE and 13 from GC. Five apparently different colonies were individually observed, two from the GE and three from GC. As to cultural characteristics, some differences were observed among the colonies. Table I summarizes the cultural and morphological profile of the studied isolates. Taking into consideration morphological characteristics, the microorganisms were identified as Gram-positive coccobacilli, Gram-positive bacilli, and Gram-positive coccus. Following the protocol adapted from Nogueira & Cavalcanti (1996), a positive result for cellulolytic activity was obtained in four out of the tested isolates (Figure 3). Among the positive isolates, two were obtained from GE and 2 from GC of

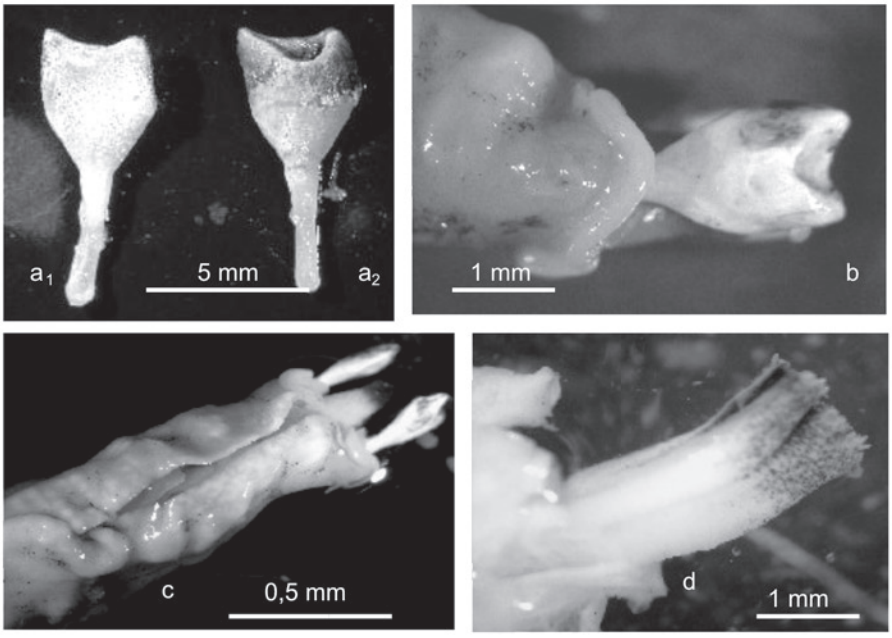

Figure 2- a1: Internal face of *Neoteredo reynei* pallet; a2: External face of *Neoteredo reynei* pallet; b: *Neoteredo reynei* pallet; c: *Neoteredo reynei* dorsal lappets; d: *Neoteredo reynei* siphon detail.

Table I -. Cultural and morphological profile of the isolates from *Neoteredo reynei* microbiote.

Isolates	Cultural characteristics	Morphological characteristics
GE_1	Fusiform, flat elevation, entire borders, no pigmentation and with smooth surface	Gram-positive coccus
GE_2	Rhizoid-shaped, elevated, filamented borders, beige pigmentation and dry surface	Gram-positive bacilli
GC_1	Circular, flat elevation, entire borders, beige pigmentation, shiny and smooth surface	Gram-positive coccobacilli
GC_2	Irregular form, convex elevation, wavy borders, no pigmentation and mucoid surface	Gram-positive coccobacilli
GC_3	Circular, flat elevation, wavy borders, white pigmentation, shiny and rough surface	Gram-positive bacilli

GE= Gastrointestinal epithelium isolates; GC= Gastrointestinal content isolates

the mollusk. Likewise, the inoculated plates showed degradation halos around the samples of all body sections and lumen from *N. reynei* (Figure 4).

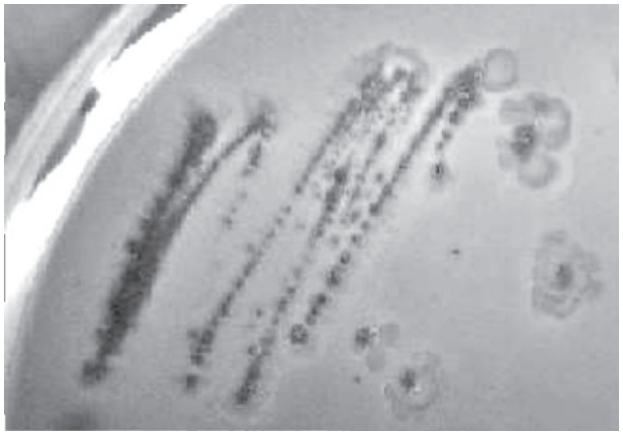

Figure 3 - Cellulolytic activity from one of the isolates from *Neoteredo reynei* digestive tract.

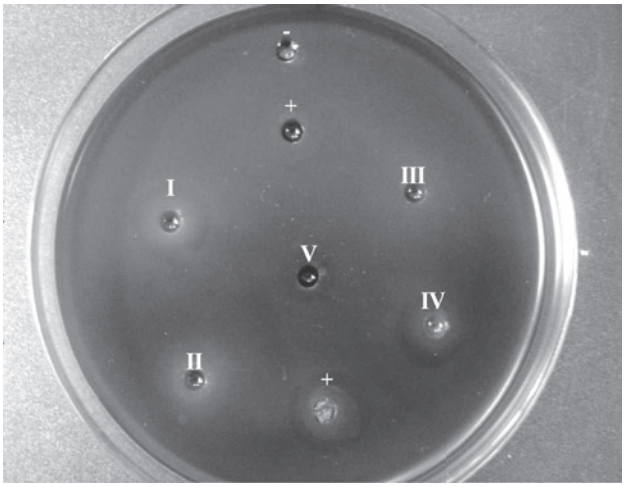

Figure 4 - Visualization of degradation halos showing cellulolytic activity. I - shell region, II - anterior cecum region, III - posterior cecum region, IV - pallet region, V - intestinal content; (+), positive control; (-), negative control.

4 DISCUSSION

The presence of cellulose-degrading symbiont bacteria has already been described for many invertebrates (Waterbury *et al*., 1983). Therefore, it is not surprising that microorganisms with this capacity are also found in the digestive tract of the wood-boring *Neoteredo reynei*. Besides, cellulolytic activity has also been reported for symbiont microorganisms in other teredinids (Sipe *et al*., 2000; Distel *et al*., 2002; Ahuja *et al*., 2004). It may be possible that even the microbial isolate from the gastrointestinal content which did not show cellulolytic activity might be involved in cellulose digestion since the assay was performed under laboratory conditions which do not simulate completely the natural conditions of the mollusk digestive tract. This bacterium, in its original environment, could make use of metabolites from other organisms and thereby become able of degrading such compound. However, this result does not allow us to establish that cellulose digestion in teredinids is exclusively carried out by symbiont microorganisms.

The degradation of cellulose in all samples of body sections and lumen from *N. reynei* was detected (Figure 4). The positive result in the shell region might be related to an abundant mucus production to help on the conduction of particles to the mouth and to give protection against friction (Beninger & St-Jean, 1997). Further investigation on this secretion is necessary to elucidate its involvement in cellulolytic activity. Likewise, other regions of body such as anterior and posterior cecum regions could also represent areas responsible for production of cellulase intrinsic to the animal itself. According to Greenfield & Lane (1953), sterile samples from the cecum epithelium of specimens of *Teredo* sp. have shown cellulolytic activity, being the prececal region more active than the postcecal.

A positive result in the pallets region may also be related to the production of cellulase by the animal itself and by endosymbionts present in the ctenidia. This association has been mistaken for a gland by Deshayes (1848), having been named "Deshayes gland". Distel *et al.* (2002) have shown the occurrence of endosymbiont bacteria in ctenidia cells of *Lyrodus pedicellatus*, in which *Teredinibacter turnirae* is predominant. This symbiosis could possibly be also responsible for an extracorporeal cellulolytic activity

that would facilitate ingestion of wood through the mouth in such mollusk. However, there is no description of this kind of digestion in teredinids. In addition, metabolites and animal feces eliminated from the body by the exhalant siphon, which is close to the pallets, may contribute to cellulose digestion as well. It is possible that remains of enzymatic material have been left inside the siphon, contributing to the positive result.

In the present research work, the lumen sample showed only a slight cellulolytic activity. This could be attributed to high dilutions of the lumen caused by extensive washing of the gastrointestinal tract. An intense cellulolytic activity was expected for this sample since all extracellular content should contain cellulose degrading enzymes.

5 CONCLUSIONS

These preliminary results demonstrated that the microbiote associated to *Neoteredo reynei* digestive tract show cellulolytic activity, therefore contributing to cellulose digestion of this mollusk. Cellulolytic activity was also shown for the teredinid itself, reinforcing the fact that there is an intrinsic production of cellulase by the mollusk. However, further studies must be performed in order to establish in which specific organs cellulase production occurs and to separate the animal from its endosymbiotic bacteria to guarantee the animal's contribution *per se* to the production of this enzyme.

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