Functional screening for cellulolytic activity in a metagenomic fosmid library of microorganisms associated with coral

F.M.O. Sousa, S.R. Moura, C.A. Quinto, J.C.T. Dias, C.P. Pirovani and R.P. Rezende

Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil

Corresponding author: R.P. Rezende
E-mail: rezende.rachel@gmail.com

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ABSTRACT. Cellulases are enzymes that degrade cellulosic materials. Cellulose is the most abundant renewable carbon resource on Earth, and cellulases are used in various industrial sectors. Although cellulases are obtained from a variety of sources, this is the first description of cellulolytic activity isolated from a coral metagenomic library. A metagenomic fosmid library of microorganisms associated with the coral *Siderastrea stellata,* comprising 3552 clones, was screened for cellulolytic activity; this allows access to non-cultivable microorganisms by exploiting the full biotechnological potential. Clones were grown on LB agar plates supplemented with 0.5% carboxymethylcellulose and cellulase positive clones revealed by staining with Congo red. Using this approach, six positive clones with cellulolytic activity were identified. The enzymatic index (EI) of the positive clones was calculated by the ratio between the hydrolysis zone diameter and colony diameter. All

positive clones had an EI greater than 1.5. Digestion of the DNA isolated from the six positive clones, using the *Hin*dIII restriction endonuclease, revealed different restriction patterns in each clone, indicating that the DNA of each clone is different. There is a growing interest for new cellulolytic enzymes in various industry sectors. Here, we present the initial selection of potential clones for cellulose degradation that could be targets for future studies of enzymatic characterization.

Key words: Coral; Metagenomic; Cellulase; Carboxymethylcellulose; Activity Gel

INTRODUCTION

The marine environment hosts a great biodiversity. The corals are an example of a system with high diversity. They are filter holobiont organisms and live with a wide variety of associated microorganisms (Lins-de-Barros et al., 2010). Of the different species of coral along the Brazilian coast, *Siderastrea stellata* is an endemic species, abundant on the southern coast of Bahia (Castro and Pires, 2001). Microorganisms are natural producers of enzymes with the ability to adapt different environmental conditions. The capacity of microorganisms to adapt comes from the ability of their enzymes to tolerate different environmental conditions (Suenaga, 2015).

Data indicate that more than 99% of microorganisms cannot be cultured in laboratory (Sharma et al., 2005). Metagenomic analysis is emerging as an effective alternative allowing direct access to the DNA of bacterial communities present in a given environment independent of cultivation. Metagenomics makes it possible to more widely exploit the biotechnological potential of microorganisms in the environment (Handelsman et al., 1998).

Cellulases are capable of hydrolyzing cellulose, the most abundant renewable biomass on the planet (Sandgren et al., 2005). Cellulases have applications in various industrial sectors including biofuels, feed, agriculture, textiles, and pulp and paper (Sharada et al., 2014).

Coral inhabits a saline and alkaline environment. It is possible that the enzymes of the microorganisms present in the coral microenvironment tolerate these characteristics. Therefore, the cellulases obtained from a saline and alkaline environment should be adapted to these conditions. There are no reports in the literature of studies involving cellulase research in metagenomic libraries of microorganisms associated with coral. Therefore, we screened the *S. stellata* metagenomic library for new cellulolytic enzymes.

MATERIAL AND METHODS

Metagenomic library

The metagenomic library was constructed using *Siderastrea stellata* coral samples collected in Camamu Bay (-13°57'49.248", -38°56'0.132") and send to the laboratory under refrigeration (Quito CA, unpublished results). The specimens were macerated, and then agitated for 24 h in sodium phosphate buffer, pH 7.5 containing 1% sodium pyrophosphate. The sample was centrifuged at 10, 000 *g* for 15 min. DNA was extracted from the supernatant using the E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA). The

metagenomics library was constructed using the CopyControl™ Fosmid Library Production Kit with the pCC1FOS Vector™ (Epicentre Biotechnologies, USA) following the manufacturer instructions. The metagenomic DNA of microorganisms associated with *S. stellata* was cleaved into fragments approximately 40 kb in length. DNA ends were repaired and the DNA cloned into the pCC1FOS vector. Later, transformants were made by introducing the DNA into host cells of *Escherichia coli* EPI300™ -T1R TransforMax™ (Epicentre Biotechnologies). Clones were selected on solid Luria-Bertani (LB) supplemented with 12.5 mg/mL of chloramphenicol. Clones were stored on 96 well plates containing LB broth with chloramphenicol 12.5 mg/mL and 20% glycerol at -80°C.

Screening for cellulolytic activity

Cellulolytic activity was evaluated by testing the hydrolysis of carboxymethylcellulose (CMC, Sigma) as revealed with Congo red (Teather and Wood, 1982). Initial tests were conducted on pools of clones. Pools with positive results were selected for individual screening. Clones for individual screening were cultivated on LB agar plates supplemented with 0.5% CMC, 0.01% L-arabinose, and chloramphenicol (12.5 mg/mL). Plates were incubated at 37°C for 5 days. To assess CMC degradation, plates with grown clones were stained with an aqueous solution of 0.1% Congo red for 10 min and washed with a 1 M NaCl solution. Positive results were identified by the presence of orange halos around the colonies.

Digestion of fosmidial DNA

Fosmidial DNA was extracted from six positive clones as previously described (Sambrook et al., 1989). The DNA was evaluated for quality and quantity using a NanoDrop® 2000 spectrophotometer. Isolated fosmid DNA was digested overnight at 37°C with *Hin*dIII restriction endonuclease (Life Technologies), following the manufacturer's instructions. Enzymatic digestion was verified by agarose gel electrophoresis (1% w/v). Electrophoresis was performed in TAE 1X buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) at 80 V for 1 h. GeneRuler 1-kb DNA ladder (Fermentas) was used as standard molecular weight marker. Gel was visualized under ultraviolet light in Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Eastman Kodak, USA).

Enzymatic index

The determination of enzymatic activity was represented by enzymatic index (EI). The EI was calculated by the ratio between the hydrolysis zone diameter and colony diameter. Measurements were made with a caliper. All measurements were made in triplicate.

Crude extract production

Crude extract was obtained from cultivation of the clones in LB medium supplemented with 0.5% CMC, 0.01% L-arabinose, and chloramphenicol (12.5 mg/mL), grown for 5 days at 37°C and 180 rpm. Samples were centrifuged at 10,000 *g* for 10 min under cooling. Cells were discarded and the supernatant used as crude extract to evaluate the cellulolytic activity.

Activity gel

Presence of cellulolytic enzymes in crude extract of positive clones was confirmed by activity gel. The gel was done according to Alfenas (1998) with some modifications. Proteins were separated by polyacrylamide gel electrophoresis 12% (w/v) in semi-denaturing conditions (Laemmli, 1970). After 2 h of running at 150V, the gel was immersed in an aqueous solution of 1% agarose containing 1% CMC and incubated at 45°C for 1 h. The gel was stained with 0.1% Congo red solution and washed with 1 M NaCl.

RESULTS AND DISCUSSION

We screened 3552 coral metagenomic library clones for cellulolytic activity. We identified six clones with cellulolytic activity, accounting for 0.164% of the metagenomic library (Figure 1). Several studies have used CMC as a substrate for the screening of cellulolytic enzymes, in the absence of cellulose enrichment from libraries obtained from different substrates (Table 1). Compared to similar studies (Table 1), it can be seen that our metagenetic library produced a relatively high yield of positive clones.

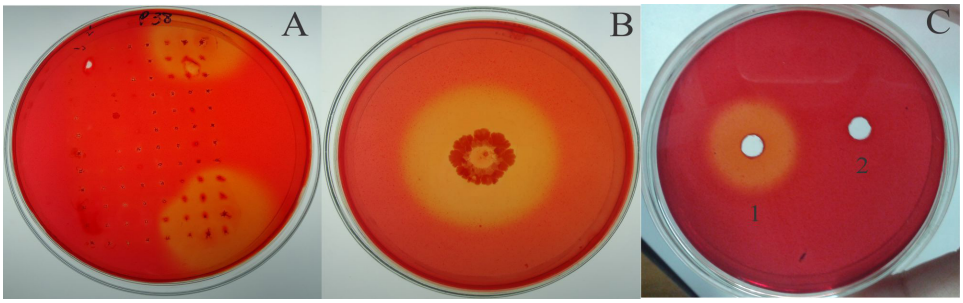

Figure 1. Activity of coral metagenomic library using carboxymethylcellulose as substrate. **A.** Pool coral library with several clones expressing cellulolytic activity; **B.** clone 19A1; **C.** crude extract of the clone 19A1 (1) and crude extract of *Escherichia coli* TransforMax™ EPI300™ -T1R (2).

Table 1. Comparison of different screening of metagenomic libraries without enrichment that use as carboxymethylcellulose substrate.

Reference	Sample	Number of clones	Size of the insert (kb)	Vector	Positive clones	%
Rees et al. (2003)	Crater Lake	36,000	3.4	phage λ	1	0.003
Feng et al. (2007)	Rabbit Cecum	32,500	31.5	Cosmid	4	0.012
Pang et al. (2009)	Compost Soil	100,000	33	Cosmid	4	0.004
Liu et al. (2011)	Soil	3,024	75	BAC	1	0.033
Zhang et al. (2013)	*Bursaphelenchus xylophilus*	5,000	3	Puc118	6	0.12
Gonçalves et al. (2015)	Mangrove	1,824	40	Fosmid	1	0.055
This study	Coral	3,552	40	Fosmid	6	0.169

Metagenomic analyses involving different genetic sources and vectors have been performed (Rees et al., 2003; Feng et al., 2007; Pang et al., 2009; Liu et al., 2011; Zhang et al., 2013; Gonçalves et al., 2015). Several of these studies have involved libraries with a large number of clones. Pang et al. (2009) analyzed a cosmid library of 100,000 clones derived from compost soil, and found that 0.004% of the clones showed cellulolytic activity. In contrast,

Zhang et al. (2013) screened 5000 clones from a *Bursaphelenchus xylophilus* library and found 0.12% were positive for cellulolytic activity. Taken together these results suggest that a large number of clones are not an absolute requirement for obtaining good results from functional screening.

Digestion of DNA from positive clones revealed different restriction fragment patterns (Figure 2). This indicates that our library generation consists of a randomly cloned DNA, and that the clones able to hydrolyze CMC are distinct.

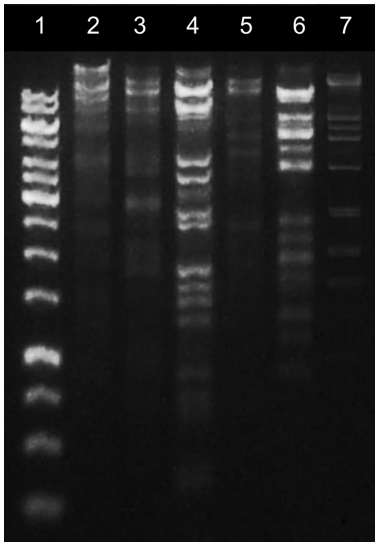

Figure 2. Profile digestion with *Hin*dIII restriction endonuclease of six positive clones for cellulolytic activity. The samples are distributed in the following order: *lane 1*, GeneRuler 1-kb Leader (Fermentas); *lane 2*, 19A1; *lane 3*, 19H3; *lane 4*, 19H12; *lane 5*, 38G12; *lane 6*, 38H10; *lane 7*, 18H9.

Measurement of the EI is a common practice and considered a simple, fast, and effective measure for enzymatic activity screening. The EI of positive clones was measured and all clones showed an EI greater than 1.5 (Table 2). EI can also be used as an initial screening tool for the selection of enzymes with higher potential activity (Florencio et al., 2012).

Table 2. Enzymatic index of the positive clones of coral metagenomic library.

Clones	Enzimatic index*
18H9	1.5
19A1	2.2
19H3	2.68
19H12	2.3
38G12	2.0
38H10	2.0

*Average of the index obtained in triplicate.

To confirm the presence of cellulolytic enzymes, we performed a polyacrylamide gel activity assay in semi-denaturing conditions incubated with CMC (Figure 3). This analysis revealed that the crude extract from the six positive clones did possess cellulase activity. Consistent with the EI results, clone 19H3 showed the largest hydrolysis zone.

Cellulases are versatile enzymes that are used in a range of industrial processes. Studies involving the search for enzymes from extreme environments, including the marine environment, have intensified. Kimes et al. (2010) analyzed microorganisms associated with coral using microarrays and demonstrated the presence of genes related to cellulose degradation. Martin et al. (2014) provided the first description of finding cellulose in a marine metagenome when they used samples of *Ascophyllum nodosum* algae to build a metagenomic library. However, to our knowledge, there are no reports of cellulolytic enzymes from metagenomic libraries of microorganism associated with coral.

Figure 3. CMCases activity gel. The proteic extract of clones was distributed in the following order: *lane 1*, 18H9; *lane 2*, 19a1; *lane 3*, 19H3; *lane 4*, 19H12; *lane 5*, 38G12; *lane 6*, 38H10.

Studying enzymes from different sources allows us to identify and isolate enzymes adapted to the specific, and sometimes extreme, environments from which they were obtained (Suenaga, 2015). Corals live in environments with high salinity and an alkaline pH. Therefore, enzymes obtained from this environment are likely to be able to withstand conditions of high salinity and pH. This is important when considering industrial applications, as this sector often requires enzymes that can operate well under extreme conditions.

CONCLUSION

Here, we identified that microorganisms associated with corals are a good source of cellulolytic enzymes. In screening a relatively small library of 3552 clones, we were able to identify a large number of putative cellulases. Given the growing interest for new cellulolytic enzymes for industrial applications, these clones may be useful in future enzymatic characterization studies.

Conflicts of interest

The authors declare no conflict of interest.

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