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Characterization of Cellulolytic Activities of Environmental Bacterial Consortia from an Argentinian Native Forest

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Abstract Cellulolytic activities of three bacterial consortia derived from a forest soil sample from Chaco region, Argentina, were characterized. The phylogenetic analysis of consortia revealed two main highly supported groups including *Achromobacter* and *Pseudomonas* genera. All three consortia presented cellulolytic activity. The carboxymethylcellulase (CMCase) and total cellulase activities were studied both quantitatively and qualitatively and optimal enzymatic conditions were characterized and compared among the three consortia. Thermal and pH stability were analyzed. Based on its cellulolytic activity, one consortium was selected for further characterization by zymography. We detected a specific protein of 55 kDa with CMCase activity. In this study, we have shown that these consortia encode for cellulolytic enzymes. These enzymes could be useful for lignocellulosic biomass degradation into simple components and for different industrial applications.

Introduction

Cellulose is the most abundant form of photosynthetically fixed carbon in the biosphere. It is a fibrous polymer of glucose units that are linked by β-1,4-glycosidic bonds. The capacity to convert cellulose of plant cell wall in simple substrates is of crucial importance for the carbon cycle [11]. However, cellulose is notoriously difficult to hydrolyze enzymatically because it contains resilient glycosidic bonds [12]. It is crystalline and tightly associated with other polymers, including hemicelluloses, pectins, and lignin [25, 26]. The maximal rates of cellulose degradation come from a specific group of cellulolytic microorganisms that produce complex combinations of enzymes (cellulases, hemicellulases, and pectinases), which act synergistically to break down cellulose and its associated cell wall components [26, 35, 46]. In soil, it is likely that enzymes from many different organisms act cooperatively during cellulose hydrolysis [49]. A broad range of bacteria, fungi, and some invertebrates can produce cellulases [5, 25, 26, 41]. These enzymes belong to different sequence-based families of glycoside hydrolases (GHs) and can be classified into three classes: endoglucanases (endo 1,4-β-D-glucan-4-glucanohydrolase) that randomly attack cellulose chain and split β-1,4-glucosidic linkages, exoglucanases (exo 1,4-β-D-glucan-4-cellobiohydrolase) that release either cellobiose or glucose from the non-reducing end of cellulose, and β-glucosidase or cellobiase (β-D-glucoside glucohydrolase) that hydrolyze cellobiose and other soluble cellodextrins to glucose [38].

The imminent shortage of oil worldwide highlights the importance of developing renewable fuel alternatives [17]. The characterization of cellulolytic microbes for advanced ethanol production from lignocellulosic materials will provide a sustainable alternative to the global energy crisis. While the cost of cellulases remains a key barrier to production of biofuels, the availability of a wide range of naturally occurring lignocellulose-degrading enzymes increases the chances of successful enzyme optimization

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for industrial processes [12, 20, 36]. Microorganisms either isolated or associated in a consortium that ideally can produce biofuel should have specific features, such as the ability to degrade different lignocellulosic materials, resist different inhibitors of the saccharification process, and synthesize ethanol with high yield [1, 13]. Consequently, it is important to screen different ecosystems for highly efficient cellulolytic activities to obtain sugar from lignocelluloses. The aim of our study was to characterize the carboxymethylcellulase (CMCase) and total cellulase activities (FPase) of three bacterial consortia (heterogeneous colonies, more than one genus per colony), isolated from a pristine forest soil sample from Chaco, Argentina.

Materials and Methods

Soil Sample

A soil sample was collected from the surface layer (0–20 cm depth) of native forest soil from Chaco, Argentina (S 26° 52′ 0″ W 60° 43′ 48″) in the winter of 2010. The region is a plain of loess with forest patches, which has not been influenced by agronomic exploitation so far. The soil sample is classified as Udic Ustochreps fine loamy-silty, mixed, and hyperthermic. The physicochemical characteristics of the soil sample were analyzed at the Soil Analysis Laboratory (LabIS) from Soil Institute, CIRN, INTA. The organic matter content was analyzed with Walkley and Black semi micro method and pH was determined using a glass electrode in a 1:2.5 water slurry. The available phosphorous was analyzed with Bray and Kurtz method and total nitrogen was analyzed by total Kjeldahl nitrogen method.

Bacterial Samples

Three bacterial consortia (CH2, CH3, and CH4) were isolated and identified previously in our laboratory from a forest soil sample collected in the Chaco region, Argentina. These consortia were obtained by enrichment of culture medium with filter paper as the sole carbon source and successive passages of selection on solid medium containing CMC as previously described [41]. The resulting heterogeneous colonies (more than one genus per colony), known as consortia, were previously identified by the 16S rRNA sequence analysis as *Achromobacter* and *Pseudomonas* [41].

Phylogenetic Analysis

Almost complete 16S rRNA sequences were aligned using the MAFFT program version 6 [16] available online (http://mafft.cbrc.jp/alignment/server/). The sequences were compared using the specific ribosomal DNA Database Project (RDP; http://rdp.cme.msu.edu/) and confirmed by GeneBank using BLAST program (http://www.ncbi.nlm.nih.gov/). Reference sequences of *Achromobacter* spp. and *Pseudomonas* spp. available in GenBank were added. In addition, we estimated evolutionary distances between sequences by computing the proportion of nucleotide differences between each pair of sequences using the program MEGA5 [42].

Maximum likelihood (ML) trees were built assuming GTR + G model implemented in PhyML 3.0 [9], with 4 substitution rate categories. For this, we used a BIONJ starting tree and performed a search strategy combining two swapping operations: subtree pruning and regrafting (SPR) and the nearest neighbor interchange (NNIs). Branch support was calculated by Bootstrap, performing 1,000 resampling iterations. A sequence from a *Treponema* sp. (AB255897) was used to root the tree. The CH3 (CH2_3_10) sequence, similar to *Pseudomonas* sp. group, was deposited at GenBank (Acc. Number JX646698).

Culture Conditions

The bacterial consortia were grown in liquid medium I, supplemented with 1 % CMC or filter paper strip of 1 × 6 cm (50 mg) in order to determine the optimal period after growth conditions. Cultures were limited to one-fifth of the volume of culture flasks. Cultures were grown at 28 °C with vigorous aeration for a week. Growth was monitored by measuring the absorbance at OD_{600}.

Medium I, prepared as described by Hankin and Anagostakis [10] with some modifications, contained (grams per liter): K_2HPO_4, 1.67; KH_2PO_4, 0.87; NaCl, 0.05; $MgSO_4{\cdot}7H_2O$, 0.1; $CaCl_2$, 0.04; $FeCl_2$, 0.04; $Na_2MoO_4{\cdot}2H_2O$, 0.005; biotin, 0.01; nicotinic acid, 0.02; pantothenic acid, 0.01; and NH_4Cl, 1 supplemented with carboxymethylcellulose (CMC) low viscosity (M.W. 90.000) 1 % (w/v), as the sole carbon source.

Screening of Cellulolytic Activity

In order to assess the ability of the bacterial consortia to hydrolyze cellulose, we performed a qualitative assay of degradation on solid medium (medium I-agar 15 g/l), by flooding Petri dishes with 0.5 % CMC and 0.1 % Congo red for 15 min and then with 1 M NaCl for 15 min [14, 44]. Carboxymethylcellulose degradation was indicated by a clearing zone around the colonies and the enzymatic activity index (EAI) was estimated as the diameter (mm) of the zone of clearance plus the diameter of the colony divided by the diameter of the colony [4, 13]. A cut off value above 2.5 mm is generally recommended to consider a microorganism as a producer of cellulolytic enzymes [8, 24, 33].

Crude Enzyme Assays

Enzymatic activity (EA) and protein concentration were tested in culture supernatants and cell extract fractions. Ten milliliters of each culture were centrifuged at 12,000×*g* at 4 °C for 20 min. Culture supernatants were filtered through 0.2-µm filter (Ministart, Sartorius Stedim Biotech, USA).

Cell pellets were suspended in 500 µl of Tris buffer (pH 8.0), centrifuged at 12,000×*g* for 10 min, and the supernatant was discarded. The pellet was suspended in 600 µl of 0.1 M citrate buffer (pH 5.0) with 200 mg of zirconia–silica beads (100 µ) and lysis was performed in a homogenizer (FastPrep 24, MP Biomedicals, USA). Finally, the samples were centrifuged at 12,000×*g*, at 4 °C for 10 min and the supernatant was collected. Culture supernatants and extracts were used to assess CMCase and FPase activities. Protein concentration was measured by the bicinchoninic acid (BCA) kit following manufacturer's instructions (Micro BCA Protein Assay Kit, Thermo Scientific, USA), using bovine serum albumin (BSA) as a standard.

Enzymatic Assay

Cellulolytic activity on CMC and filter paper was tested by the dinitrosalicylic acid (DNS) reducing sugar assay according to the method described by King et al. [19]. Each test was performed by triplicate. The culture supernatant or cell extract without substrate and the substrate in buffer (without enzymes) were included as controls. The cellulase from *Aspergillus niger* was used (Sigma-Aldrich) as positive control. CMC (CMCase activity) and FP (FPase activity) assays were performed using 100 µl of sample (culture supernatant or cell pellets) and 100 µl of 2 % CMC or 1.60 mg of FP strip in 0.1 M phosphate citrate buffer pH 6.5, and incubated at 50 °C for 60 min. An additional assay was performed to measure FPase activity after 20 h under the same conditions described above.

Characterization of CMCase Activity

The effects of pH and temperature were analyzed. All assays were performed for 60 min. For pH analysis, assays were carried out at different pH values, ranging from 3.0 to 10.0 at 50 °C. Different buffers were employed to adjust conditions: 0.1 M citrate phosphate buffer (pH 3.0–6.0), 0.1 M phosphate (pH 7.0–8.0), and 0.2 M glycine–NaOH (pH 9.0–10.0). The EA was also tested at different temperatures ranging from 4 to 70 °C at pH 6.5.

Thermal and pH Stability of the Cellulolytic Enzymes

The cellulase heat stability in the crude supernatant was tested by preincubating enzyme samples at different temperatures ranging from 0 to 90 °C for 1 h. Then, the remaining activity was tested as described above. For pH stability, the crude supernatant was incubated at 4 °C for 16 h and pH ranging from 3.0 to 9.0. The citrate phosphate (pH 3.0–6.0) and phosphate (pH 7.0–9.0) buffers were used. Then, the remaining activity was tested at standard conditions at 50 °C for 1 h.

Zymogram

Bacterial consortia CH4 were grown in medium I supplemented with 0.2 % yeast extract and 0.5 % Avicel (Sigma-Aldrich) for 10 days at 28 °C. Cells and undegraded Avicel were harvested from cultures by centrifugation at 4 °C at 12,000×*g* for 20 min. The upper layer of Avicel was collected and dissolved in 50-mM citrate phosphate buffer. The sample was then sonicated by 3 pulses of 30 s each to release bound proteins in a Branson sonifier 250 (VWR Scientific, USA) and subsequently centrifuged. Then, 50 µl of the Avicel-binding protein fraction (10 µg of total protein) was resuspended in the same volume of 2× sample buffer (50 mM tris pH 6.0, 8.2 mM EDTA, 10 % glycerol, 0.025 % bromophenol blue, and 1 % SDS) and then heated at 100 °C for 2 min. The sample was centrifuged at 12,000×*g* for 2 min and 20 µl of the supernatant was loaded on 12 % SDS–polyacrylamide gel containing 0.2 % CMC. After electrophoresis, renaturation was carried out in gel by washes using 0.04 M Tris–HCl, pH 7.6. Then, the gel was incubated at 37 °C for 1.5 h and stained by Congo red as described above.

Statistical Calculations

A *t*-Student test for paired samples was performed with the software MedCalc version 9.0 (Mariakerke, Belgium). Every sample was analyzed in triplicate; mean values and standard deviations were calculated.

Results

Soil Sample

Three bacterial consortia were previously isolated from a forest soil sample from Chaco region, Argentina. The soil sample contained 5.69 % organic matter, available phosphorous 104.7 ppm, high capacity of cationic interchange (CEC) 23.4 mEq/100 g, and a fine loamy-silty texture. The pH of this sample was 5.8. Detailed information of physicochemical characteristics is shown in Supplementary Table 1.

In order to isolate novel cellulolytic bacteria, the soil sample was enriched with filter paper as the sole carbon source and successive passages of selection in solid

medium containing CMC. *Achromobacter* and *Pseudomonas* genera were both consistently identified in all colonies, despite the multiple passages of selection performed.

Phylogenetic Analysis

The phylogenetic analysis revealed two main highly supported groups including sequences of *Achromobacter* and *Pseudomonas* genera (Fig. 1). Within *Achromobacter*, two subgroups were recovered. A minor group gathered all sequences of CH2 consortium together with a reference sequence of *A. denitrificans*, with a bootstrap value (BS) of 96. Another group was composed of sequences from CH3 consortium and *A. xylosoxidans* / *A. marplatensis* reference sequences (BS = 84). Sequences from CH4 were included in a polytomy together with CH3 and CH2 groups (BS = 91). Regarding *Pseudomonas*, sequences from all three consortia were included in one group together with reference sequences of *Pseudomonas* spp. (BS = 84). Additionally, distances between sequences were estimated within each main group. In the *Achromobacter* spp. group, sequence distances ranged from 0 to 0.014. Within the *Achromobacter* CH3 group, values ranged from 0 to 0.002. In addition, distances between *Pseudomonas* spp. ranged from 0 to 0.007, with an overall mean distance of 0.002. Similarity values obtained from BLAST analysis ranged from 99 to 100 % for both *Achromobacter* spp. and *Pseudomonas* spp. sequences.

Qualitative Evaluation of Cellulolytic Activity

In order to assess the cellulolytic bacterial activity of CH2, CH3, and CH4 consortia, the colonies were grown on solid medium containing CMC. As expected, we observed CMC hydrolysis in all of them. However, the degradation zones differed among consortia. CH3 showed hydrolysis diameter (9.2 ± 0.7 mm) significantly greater than CH4 (7.3 ± 0.5 mm) and CH2 (3.4 ± 0.8 mm) ($P = 0.0187$ and $P = 0.0007$, respectively), whereas CH4 hydrolysis was significantly higher than CH2 ($P = 0.0020$) (Fig. 2).

Quantitative Evaluation of Cellulolytic Activity

Growth of all consortia was observed after 7 days of incubation in both broth selective media (medium I-CMC and medium I-Filter paper). FP total degradation was observed in CH4 culture, whereas partial degradation was observed in CH2 and CH3, which indicates that there is a higher cellulolytic activity in CH4 compared to CH2 and CH3. Moreover, total degradation of FP in CH3 culture was observed after 14 days (data not shown).

CMCase activity in crude enzyme solution was measured in the culture supernatant and cell extract from all bacterial consortia grown in CMC medium. EA was 0.044 ± 0.003 IU/ml and the specific enzymatic activity (sEA) was 0.140 ± 0.002 mg/ml in CH4 consortium. In CH2 and CH3 values of EA were 0.023 ± 0.001 and 0.026 ± 0.005 IU/ml, respectively, and the sEA was 0.058 ± 0.007 and 0.129 ± 0.004 IU/mg, respectively. CMCase activity was absent in the pellet extract in all samples tested.

Total cellulase activity was measured at two end-points, 1 and 20 h, at 7 days of culture. Release of reducing sugars was not observed at 1 h. Interestingly, at 20 h cellulolytic activity was observed in the culture supernatant of consortium CH4. The EA value was 0.0010 ± 0.0001 IU/ml, whereas the sEA was 0.0270 ± 0.0028 IU/mg. However, in CH2 and CH3 supernatants no EA was detected after 20 h of reaction.

Optimal Conditions and Stability of CMCase Activity

In CH2 and CH3 the CMCase activity was observed in a pH range of 4.0–8.0, although no significant differences among values in that range were observed ($P > 0.05$). This activity in CH4 consortium was observed in a pH range of 5.0–9.0, with two peaks of maximum activity at pH 6.0 and 8.0 (Fig. 3a–c). Maximum activity in CH2 and CH3 was observed at 50 °C, while the optimal temperature for CH4 was 37 °C (Fig. 4a–c). In addition, the pH stability in culture supernatant at various pH ranging from acidic to alkaline was analyzed. The CH2 consortium retained more than 70 % of EA at pH 4.0–9.0 after 1 h of incubation at 50 °C and showed more than 20 % of residual activity at pH 3.0 (Fig. 5a). In contrast, CH3 and CH4 were inactive at pH 3.0, but both consortia retained about 90 % of activity at pH 6.0 to 8.0 (Fig. 5b, c).

The thermotolerance of CMCase activity from the three bacterial consortia was analyzed. EA was completely lost above 30 °C (CH2) and 50 °C (CH3 and CH4). All consortia retained more than 90 % of remaining activity when the temperature of incubation was below these temperatures (Fig. 5d–f).

Zymogram

Based on the qualitative cellulolytic activity observed we selected the CH4 consortium for further characterization. Several protein bands with cellulolytic activity were identified using SDS-PAGE and Congo red staining. A major band with a molecular weight of 55 kDa was observed in the culture supernatant of this consortium (Fig. 6).

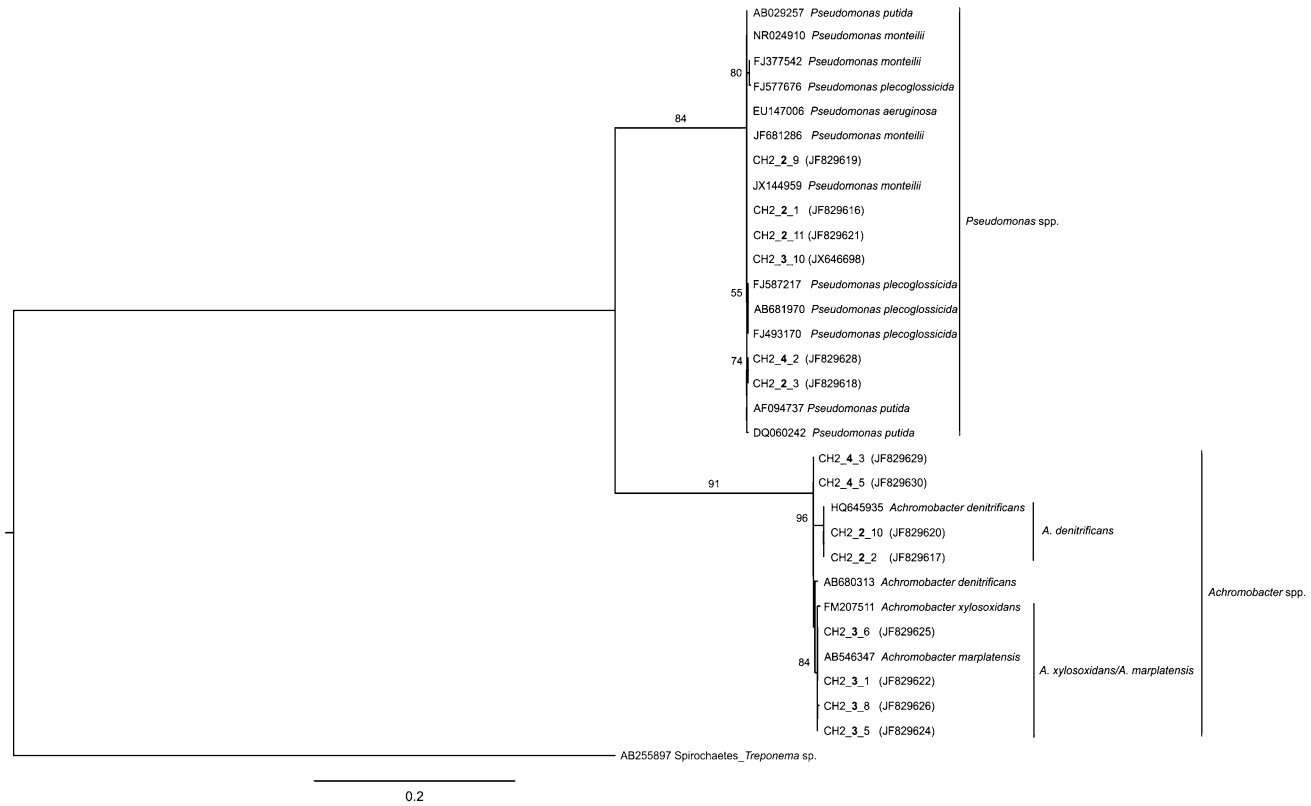

Fig. 1 Maximum likelihood tree obtained with PhyML using 16S rRNA sequences. *Numbers* above branches refer to *bootstrap* values. *Bars* represent principal groups as discussed in the text. The colony *numbers* of the consortia are showed in *bold*

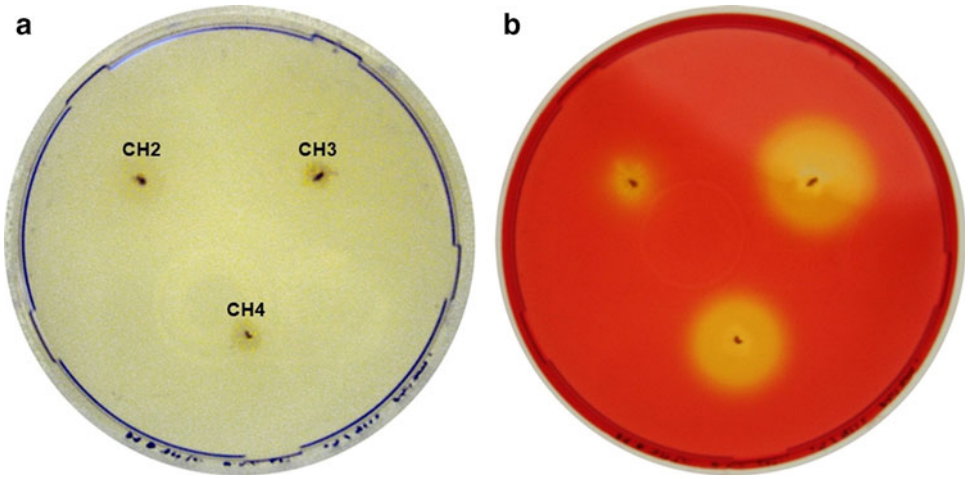

Fig. 2 *Congo red* staining of bacterial consortia showing CMCase activity on CMC plates. **a** Bacterial consortia grown on solid medium without staining. **b** Bacterial consortia produced clear hydrolytic zone after staining (Color figure online)

Discussion

Soil sample features described in this study are in accordance with the characteristics of the soils of the Chaco region, which shows high diverse ecosystem. This soil is a good source to isolate novel cellulolytic microorganisms as previously demonstrated [41].

We have assessed the cellulolytic activity of three bacterial consortia previously isolated in our laboratory from a forest soil sample from Chaco province, Argentina [41]. Heterogeneous colonies (more than one genus per colony), known as consortia, were obtained by enrichment of culture medium with filter paper as the sole carbon source and after successive passages of selection on solid medium containing CMC [41].

Two genera, *Achromobacter* and *Pseudomonas,* were identified in these consortia, both known as cellulolytic bacteria [23, 26, 30, 32, 48]. Our sequences, identified as *Achromobacter*, were clustered with *A. denitrificans*, *A. xylosoxidans*, and *A. marplatensis*. The cellulolytic activity

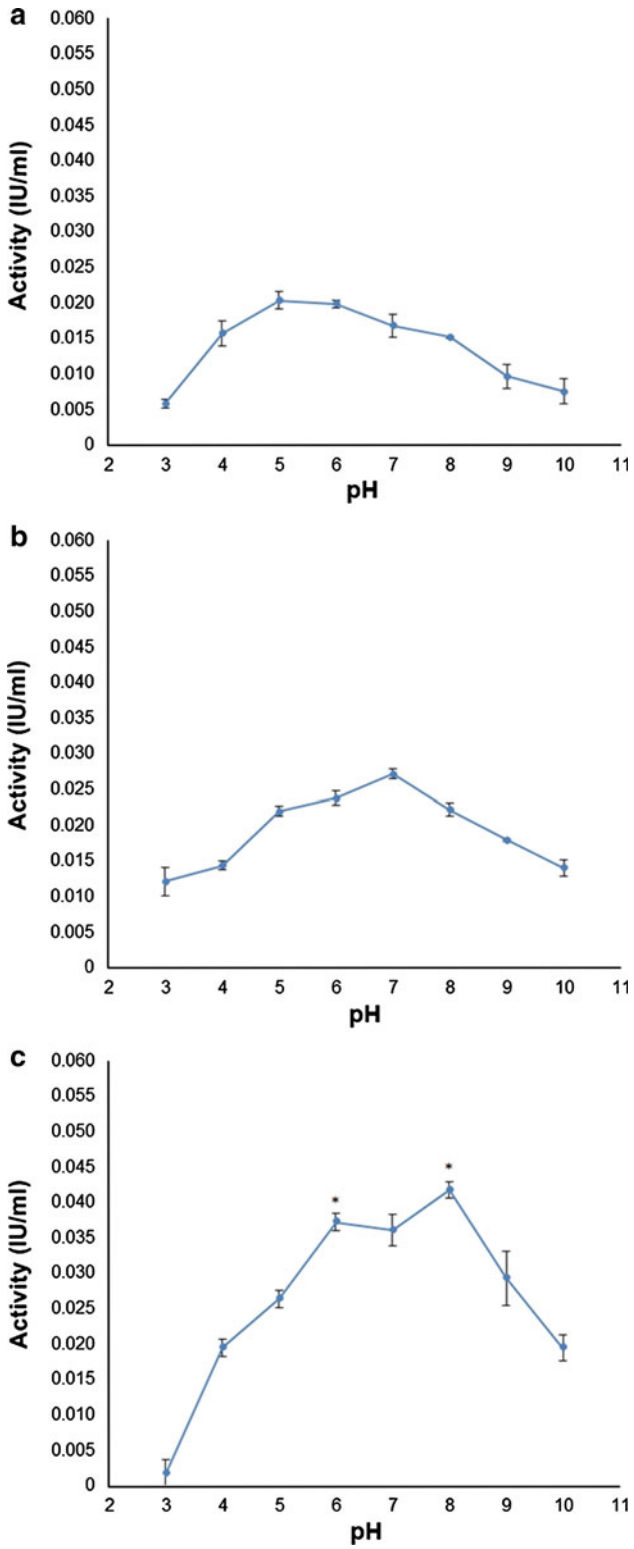

Fig. 3 CMCase activity in culture supernatants of bacterial consortia at different pH. **a** CH2, **b** CH3, and **c** CH4

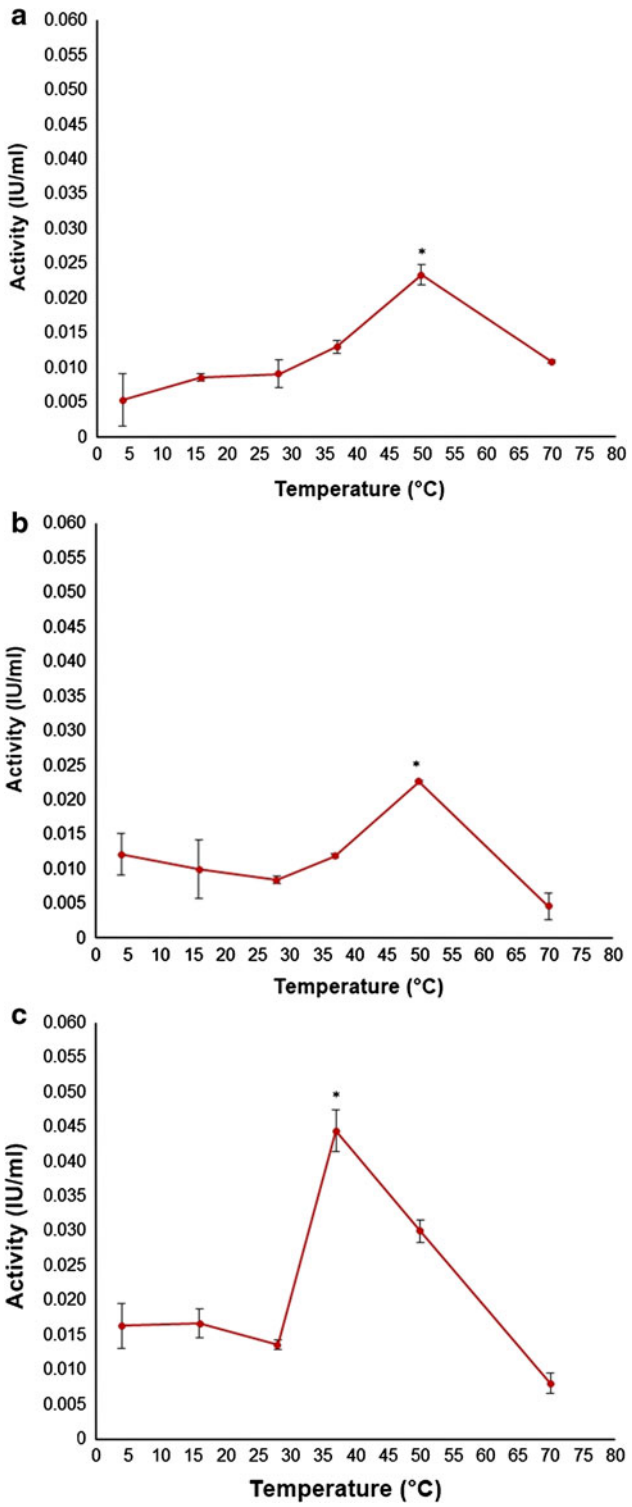

Fig. 4 CMCase activity in culture supernatant of bacterial consortia at different reaction temperatures. **a** CH2, **b** CH3, and **c** CH4

of these species has also been previously reported by Yang et al. [48] and Ghio et al. [6]. In their study they determined the cellulase and xylanase activity of a microbial community using switchgrass as carbon source. The *Pseudomonas* group included sequences from all three consortia, which belong to the *Pseudomonas* spp. group.

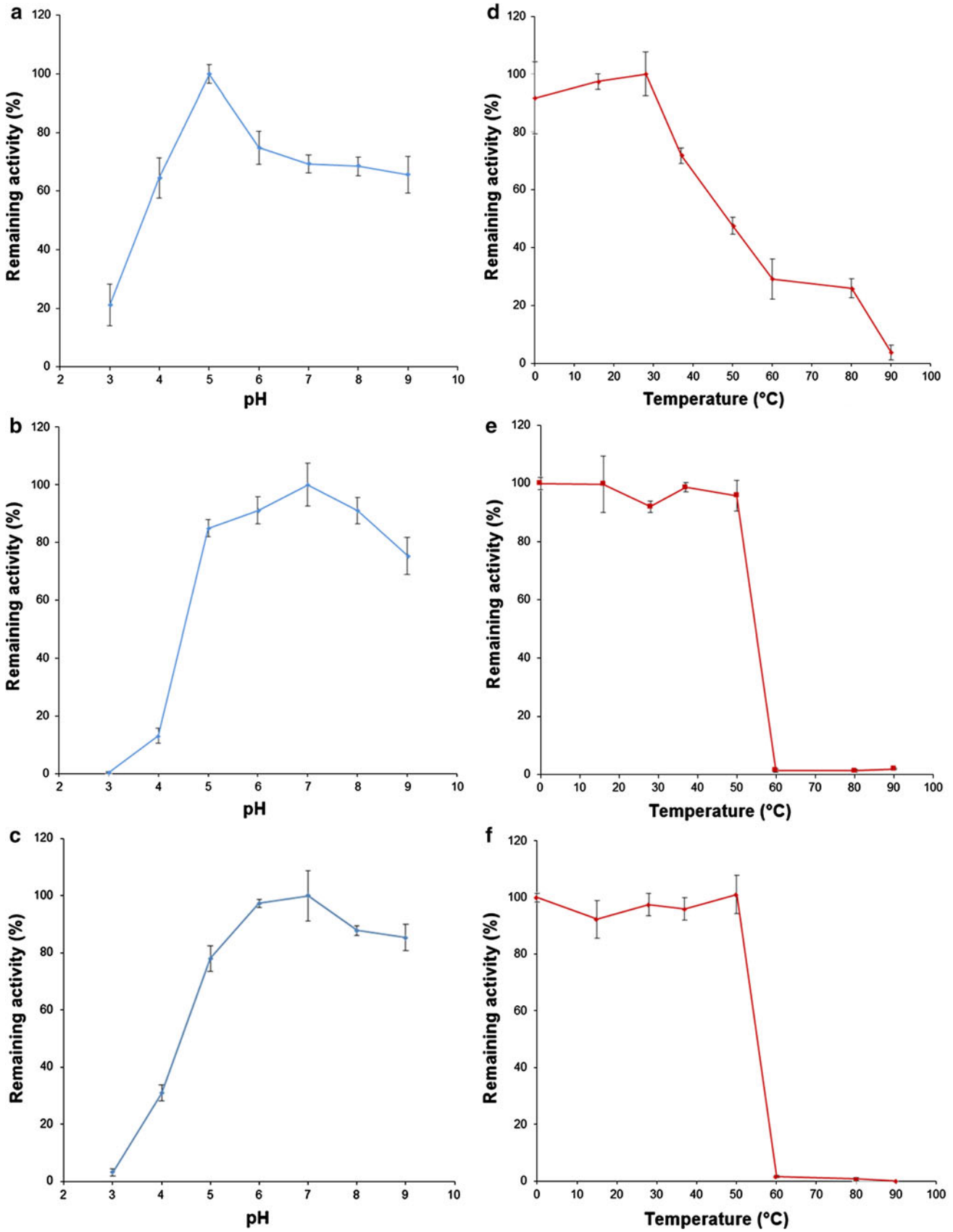

Fig. 5 CMCase activity stability. pH **a** CH2, **b** CH3, and **c** CH4. Thermal **d** CH2, **e** CH3, and **f** CH4

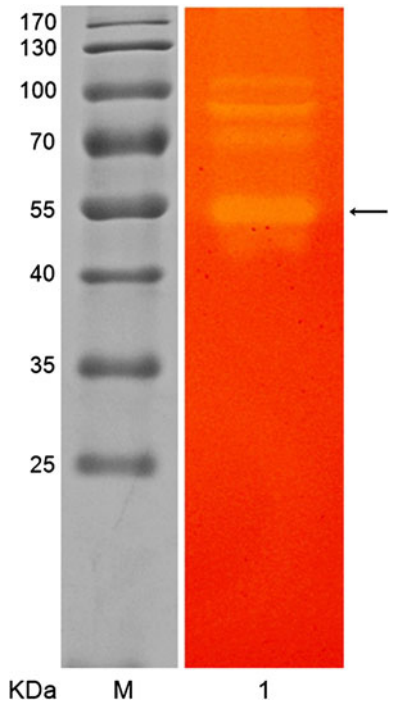

Fig. 6 Zymogram. *Lane 1* Avicel-adsorbed proteins; *Lane M* Standard molecular weight marker (PageRuler 26616, Thermo Scientific, Li). The *arrow* shows a protein of 55 kDa with CMCase activity

Many *Pseudomonas* species have been reported to degrade cellulose [13, 28, 29, 37], including *P. putida*. This species has cellulase, hemicellulase, and pectinase coding genes according to genomic analysis [7, 26, 45]. However, the cellulolytic activity of *P. putida* has not been biochemically characterized yet.

All three consortia were selected to assay cellulolytic activity in broth medium because they all showed EAI values higher than 2.5 mm on solid medium. In addition, a correlation between the degradation halo diameter and the degradative ability was observed. Despite differences in the ability to degrade CMC among consortia, specific enzyme activity values were similar between CH3 and CH4 bacterial consortia. Similar results have been reported in different organisms [3, 33]; however, it is difficult to compare the efficacy of the enzymes because of the variety of cellulosic media and organisms employed.

The ability of all consortia to grow both in CMC and in filter paper indicates that there are enzymes with CMCase and FPase (total cellulase) activities. In this sense, the inability to quantify FPase activity in CH2 and CH3 consortia was an unexpected result, since we found that these consortia showed an optimum growth in minimal media containing FP as the only carbon source (data not shown) and they were also able to degrade completely FP after 14 days. Several factors could explain this result. For instance, a rapid enzyme inactivation once the cell is disrupted or the absence of enzyme cofactors may cause the lack of this activity. Furthermore, there are other variables that need further optimization (and should be considered) such as time, buffer and pH. Consequently, further optimization under different conditions is needed.

The optimum pH of hydrolysis in CH2 and CH3 varied in a range while in CH4 consortium two peaks were observed. This observation could be explained by the presence of different enzymes in CH4. Furthermore, the zymogram assay showed several signals with different intensities and molecular weights, which would correspond to differences in enzyme concentration or affinity to Avicel substrate. Molecular weights are comparable to those reported for endoglucanases [18, 27, 34]. The 55-kDa band showed the main CMCase activity, which could be a useful characteristic for downstream processing. However, purification of these proteins should be performed to confirm their features and individual contribution to the total activity of the supernatants. In CH2 and CH3 consortia, one maximal temperature peak was observed at 50 °C. However, in the CH4 consortium the CMCase activity was optimal at 37 °C, while the value was abruptly reduced at 50 °C (Fig. 5). The enzymes that confer CMCase activity in CH3 and CH4 are sensitive to temperatures greater than 50 °C; however, this could not be considered as a disadvantage. Robustness of the cellulolytic enzymes is a key factor for industrial applications. The cost of cellulolytic enzymes is one of the factors determining the cost of a biocatalytic process and may be reduced if the optimal conditions for its production are reached [20, 25]. The utility cost of enzymatic hydrolysis is lower than that of acid or alkaline hydrolysis. As the enzymatic hydrolysis is usually conducted under mild conditions, pH 4.8 and temperature of 45–50 °C, the corrosion problem is avoided [39, 48].

Lignocellulose degradation in nature is a process carried out by a variety of microorganisms that are cooperatively associated to hydrolyze different fractions of this complex biopolymer. The process requires the application of various types of cellulolytic enzymes, which act synergistically [2, 15]. Bioconversion of lignocellulosic biomass to ethanol and other chemical products needs technologies to improve every step in the process [21, 22, 31, 40]. In order to produce renewable biofuel and other byproducts at competitive prices, some of the current technologies must be improved to compete with traditional production systems. Several factors that limit cellulase efficiency during the hydrolysis process are the recalcitrance of the substrate, end product inhibition, thermal inactivation of the native protein, nonspecific binding to lignin [47], and irreversible adsorption of the enzymes to the heterogeneous substrate [43]. Biotechnological innovation requires previous basic research to explore a variety of enzymes and microorganisms to improve the process.

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