Research Paper

Assessment of cellulolytic microorganisms in soils of Nevados Park, Colombia

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Abstract

A systematized survey was conducted to find soil-borne microbes that degrade cellulose in soils from unique ecosystems, such as the Superpáramo, Páramo, and the High Andean Forest in the Nevados National Natural Park (NNNP), Colombia. These high mountain ecosystems represent extreme environments, such as high levels of solar radiation, low atmospheric pressure, and extreme daily changes in temperature. Cellulolytic activity of the microorganisms was evaluated using qualitative tests, such as growth in selective media followed by staining with congo red and iodine, and quantitative tests to determine the activity of endoglucanase, β-glucosidase, exoglucanase, and total cellulase. Microorganisms were identified using molecular markers, such as the 16S rRNA gene for bacteria and the internal transcribed spacer region (ITS) of ribosomal DNA for fungi. Multivariate statistical analysis (MVA) was used to select microorganisms with high cellulolytic capacity. A total of 108 microorganisms were isolated from the soils and, in general, the enzymatic activities of fungi were higher than those of bacteria. Our results also found that none of the organisms studied were able to degrade all the components of the cellulose and it is therefore suggested that a combination of bacteria and/or fungi with various enzymatic activities be used to obtain high total cellulolytic activity. This study gives an overview of the potential microorganism that could be used for cellulose degradation in various biotechnological applications and for sustainable agricultural waste treatment.

Key words: cellulase, endoglucanase, β-glucosidase, exoglucanase, Páramo, High Andean Forest.

Introduction

The Nevados National Natural Park (NNNP) is one of the major natural regions in Colombia, covering 58,300 ha and bordered by the states of Quindío, Risaralda, Tolima, and Caldas. NNNP includes the ecosystems of perpetual snow (five volcanic craters, most of which are active), Superpáramo, Páramo, and the High Andean Forest (Van Wyngaarden and Fandiño, 2002). The páramos are the areas between the tree lines of the Andean Forest and the inferior limit of perpetual snow. These areas can be zoned by altitude as follows: Subpáramo (the transition zone between the montane forest and the open Páramo), Páramo (vegetation is dominated by pajonal-rosetal), and Superpáramo (the highest zone where vegetation is scarce) (Cuatrecasas, 1958). These high mountain ecosystems are considered extreme environments due to their high levels of solar radiation, low atmospheric pressure, and extreme daily changes in temperature, they are mainly found in Colombia, Ecuador, and Venezuela. The páramos are of great importance, they are unique ecosystems that contribute relevant functions in hydric regulation and maintenance of endemic biodiversity. Despite the importance of the NNNP's ecosystems, microbiological research on these biomes is limited and no studies on the cellulolytic activity of the microbial diversity associated with these ecosystems are available. These areas likely contain a community of extremophilic microorganisms that have evolved unique charac-

teristics to adapt to the uncommon conditions that can be found in these ecosystems. As cellulose is the most abundant renewable natural product in the biosphere (Bakare *et al.*, 2005; Feng *et al.*, 2007), cellulolytic microorganisms are fundamental for the transformation of cellulose into sugars that are essential nutrients for various organisms and for biofuels (Arifoglu and Ögel, 2000; Bhat and Bhat, 1997). Additionally, since the annual production of cellulose is estimated at 4.0 x 10^7 tons (Bakare *et al.*, 2005), large quantities of industrial and agricultural cellulosic waste have accumulated due to inefficient use (Kim *et al.*, 2003; Lee *et al.*, 2008). The NNNP's ecosystems could potentially support several microbes with novel cellulolytic enzyme activities and therefore the exploration of those communities could be useful for biotechnology as well as for ecological conservation.

The goal of this study was to conduct a survey for bacteria and fungi with cellulolytic potential, isolated from soils originating in such extreme environments as the Superpáramo, Páramo, and High Andean Forest of the NNNP in Colombia. To select for microorganisms with cellulolytic potential, qualitative cellulolytic activity was determined by culturing microorganisms in media containing cellulose as the only carbon source and using assays with congo red and iodine. Quantitative cellulolytic activity was also determined by measuring endoglucanase, exoglucanase, β-glucosidase, and total cellulase activities at different temperature conditions.

Materials and Methods

Isolation and culture of cellulolytic microorganisms

Soil samples were taken from NNNP-Colombia (Figure 1). Sampling was performed in the Superpáramo, Páramo, and High Andean Forest ecosystems. In total nine quadrants of soil were selected for sampling, based on the vegetation type. One quadrant in the Superpáramo (Valle de las Tumbas, N 4°54'10'' and W 75°21'35'', 4429 m.a.s.l), two in the Páramo (Alfombrales, N 4°51'51''and W 75°21'22'' 4300 m.a.s.l and six in the High Andean Forest (N 4°58'13'' and W 75°22'42'', 3464 m.a.s.l) For each quadrant four soil subsamples of 10 cm depth were randomly taken and pooled into a composite sample. Physicochemical parameters for each study soil sample were determined (data not shown).

Serial dilutions of soil samples were made and plated in the following culture media: 1. Bacterial Media: 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NH_4NO_3, 0.02 g $FeSO_4.7H_2O$, 0.05 g $Ca(NO_3)_2.4H_2O$, 15 g agar, and 10 g carboxymethylcellulose (CMC) per liter of distilled water with pH 7.0; 2. Fungal Media: as described above for bacterial media but with 50 mg Kanamycin, 50 mg Ampicillin, and pH 5.0. Bacterial cultures were incubated at 28 °C for 3 days, and fungal cultures were incubated at 20 °C for 7 days. Microorganisms were subsequently isolated, purified, and preserved.

Taxonomic characterization of isolated strains

For bacterial identification, the 16S rDNA sequence was amplified. Each colony was suspended in 200 µL of 2x
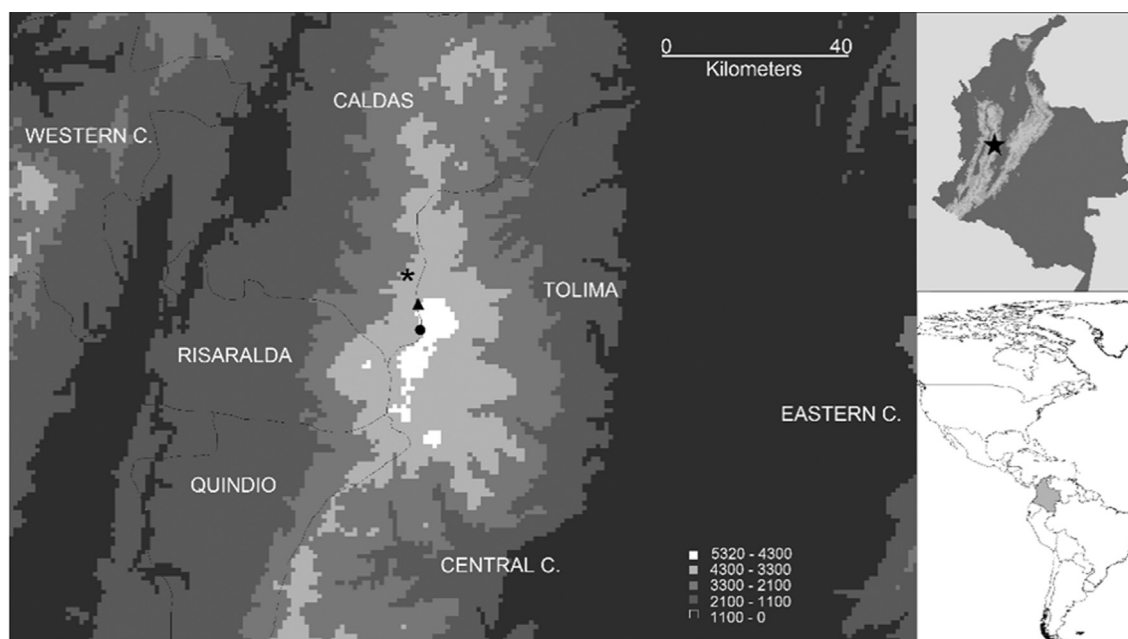

Figure 1 - The locations of the study sites used for sampling at NNNP. Symbols represent the sites in different ecosytems: Superpáramo (Valle de las Tumbas; triangle), Páramo (Alfombrales; circle) and High Andean Forest (asterisk). The different shades of gray represent the height in meters above sea level.

TE with 1% Tween 20, and boiled for 10 min and centrifuged at 14,000 rpm for 2 min and 5 μL of the supernatant was used for polymerase chain reaction (PCR). PCR was performed using primers 1492R and 27F following the condition described by Lane (1991). For fungi, DNA was extracted, and the internal transcribed spacer region (ITS) from rDNA was amplified using 5 μL of the sample in a final reaction volume of 50 μL. The reaction mixture contained 1x PCR buffer, 2.0 mM MgCl$_2$, 0.25 mM dNTP (all from Promega), 0.2 μM of primers ITS1 and ITS4, and 2.5 U/μL of highly efficient Taq DNA polymerase (Invitrogen) following the conditions described by Plaza *et al.* (2004). The obtained sequences were compared to sequences in NCBI GenBank and RDP databases using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990; Benson *et al.*, 2005).

Qualitative determination of cellulolytic activity

Qualitative determination of cellulolytic activity was achieved by growing the microorganisms in selective media and testing them with congo red and iodine. The first selective media was similar to the isolation media except that powdered cellulose was substituted for CMC. The second selection media contained 2 g NaNO$_3$, 1 g KH$_2$PO$_4$, 0.5 g MgSO$_4$.7H$_2$O, 0.5 g KCL, 0.1 g FeSO$_4$.7H$_2$O, and filter paper strips (Whatman N 1) per liter of distilled water.

The congo red test was performed by seeding the microorganisms by puncture of the 0.1% TSA base media containing 5 mL of 0.8% (w/v) agar and 0.1% (w/v) CMC. The bacteria were incubated at 28 °C for 24 h, and fungi were incubated at 20 °C for 7 days. Once the colonies had grown, 5 mL of 0.1% (w/v) congo red was added. After 15 min of incubation, excess congo red was removed, and 5 mL of 1 M NaCl was added. After another 15 min of incubation, the excess of NaCl was removed, and hydrolysis halos were measured (Guevara and Zambrano, 2006; Teather and Wood, 1982).

The iodine test was performed by seeding the microorganisms through puncture of the agar surface. The bacteria were incubated for at 28 °C for 24 h, and fungi were incubated at 20 °C for 4 days. Once the colonies had grown, iodine (Lugol's solution) was added to the Petri dish. After a 15 min incubation, the excess iodine was removed and hydrolysis halos were measured (Kasana *et al.*, 2008).

Quantitative determination of cellulolytic activity

The microorganisms that grew in selective media and tested positive for congo red and/or iodine were chosen for determination of endoglucanase, exoglucanase, β-glucosidase, and total cellulase activities. For endoglucanase activity, the bacteria and fungi were grown on solid media containing 1% CMC (reported in section 2.1) and incubated for 24 h and 7 days, respectively. The microorganisms were later inoculated in 5 mL of liquid media containing 1%

CMC. For exoglucanase and β-glucosidase activities, bacteria and fungi were replicated on solid media containing 1% powdered cellulose (reported in section 2.1) and incubated for 7 days and 15 days, respectively. The microorganisms were subsequently inoculated in 5 mL of liquid media containing 1% powdered cellulose. Total cellulase activity was determined by inoculating the microorganisms in 5 mL of minimal media containing 1 x 8 cm paper strips (Whatman N 1), equivalent to 1%. The cultures were incubated without shaking for 15 days in the case of bacteria and 30 days in the case of fungi. The cultures were subsequently centrifuged at 10,000 rpm for 10 min, and enzymatic activities were determined as described below.

β-glucosidase activity was evaluated by measuring released p-nitrophenol with several modifications. For this experiment, 180 μL of 1 mg/mL nitrophenyl-β-D-glucopyranoside (pNPG) in buffer was added to 20 μL of culture supernatant. After incubation, 80 μL of 2% sodium carbonate was added, and the absorbance was measured at 405 nm. The calibration curve was generated with 0-5 μmol of p-nitrophenol (Zhou *et al.*, 2008). The content of reducing sugars for endoglucanase, exoglucanase, and total cellulase was determined by the 3,5-dinitrosalicylic acid method (Miller *et al.*, 1960) with several modifications. Endoglucanase activity was determined by adding 50 μL of 1% CMC (w/v) to 50 μL of culture supernatant (Guevara and Zambrano, 2006). Exoglucanase activity was determined by incubating 15 μL of supernatant with 15 μL of 1% (w/v) p-nitrophenyl-β-D-cellobioside (pNPC) (Zhou *et al.*, 2008). Total cellulase activity was determined by adding 3 x 10 mm pieces of filter paper (equivalent to 1%) and 100 μL buffer to 100 μL of culture supernatant. Phosphate buffer (pH 7.0) was used for all determinations in bacteria, and acetate buffer (pH 5.0) was used for fungi. The respective mixtures were incubated at 40 °C and 28 °C for 1 h, and the reaction was stopped by refrigeration for 10 min. In all four cases, the protein content was determined by the Bradford method (Bradford, 1976). The enzymatic activity was expressed as the specific enzymatic activity, where 1 IU (international unit) of activity is defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent per minute under the assay conditions. Each IU was divided by the protein content to express the activity as the specific enzymatic activity (EEA) as measured in IU/mg protein.

Statistical analysis

The principal component analysis (PCA) and Ward's hierarchical cluster method were used as multivariate analysis tools that allowed analyzing the relationships between the different qualitative and quantitative cellulolytic enzymatic activities and the different microorganisms. This analysis allowed to select the strains that have the best enzymatic profiles, PCA and hierarchical clustering using Ward's method were implemented in R (version 2.10.0)

and Primer & Permanova (version 6). K-means was used to identify the number of groups and to perform cluster validation.

Results

Isolation and qualitative determination of cellulolytic activity

The soils from the three studied ecosystems had pH values in the range of slightly acidic to highly acidic: Superpáramo (pH: 5.9), Páramo (pH: 5.5) and High Andean Forest (pH: 4,2). The majority of samples had a notably high content of nitrogen and organic carbon, which is common in the Páramo ecosystems. However, the sample originating in the Superpáramo had the lowest content of nitrogen and organic carbon, falling in the category of "very low content". This result is in accordance with the scarce presence of vegetation in this ecosystem, where *Calamagrostis* sp. is predominant. All samples contained volcanic ash, which is expected given the presence of three active volcanoes in the study area. In total, 108 microorganisms (74 bacterial and 34 fungal isolates) with the capacity to degrade cellulose in the CMC form were isolated from the soils. Based on the cellulolytic activity of the isolates, 46 microorganisms (25 bacterial and 21 fungal isolates) were selected for further analysis. The identified bacterial isolates among the organisms with cellulolytic activity belonged to the genera *Pseudomonas*, *Streptomyces*, *Rhodococcus*, *Stentrophomonas*, *Variovorax*, *Serratia*, and *Janthinobacterium.* The identified fungal isolates belonged to the genera *Penicillum, Mortierella, Tolypocladium, Paecilomyces, Acremonium, Fusarium, Volutella, Hypocrea, Neonectria, Mucor, Aureobasidium,* and *Arthtinium* (Table S1 in the supplementary material).

Quantitative determination of cellulolytic activity

Microorganisms with positive results in the qualitative analyses were selected for determination of endoglucanase, exoglucanase, β-glucosidase, and total cellulase activity. The PCA results for all microorganisms showed that fungi have higher enzymatic activities than bacteria, all variables are positively correlated (Figure 2). The PCA and its correlation matrix (data not shown) show that the congo red and iodine tests have the highest correlation coefficients (0.8621) followed by the correlations between β-glucosidase and congo red (0.5730), endoglucanase and iodine (0.5395), and endoglucanase and total cellulase (0.5044). The PCA eigenvectors in the principal component (PC) 1 indicate that the variables with highest contribution to microorganism cellulolytic activity are iodine (0.50), congo red (0.48), and moderately, endoglucanase activity (0.40). Exoglucanase appears to be the variable with the lowest contribution to cellulolytic activity. Similarly, PCA PC 2 identified two groups of variables that are correlated. The first group includes endoglucanase (0.22), exoglucanase
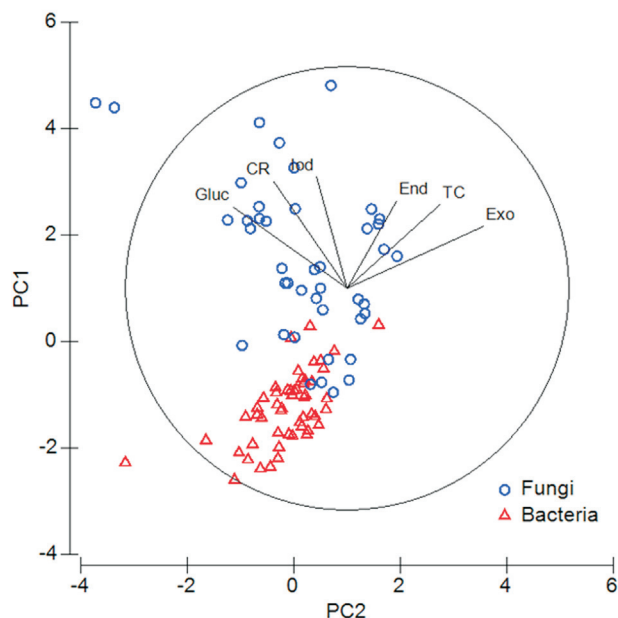

Figure 2 - Principal component analysis of bacteria and fungi. CR: congo red, Iod: Iodine, Gluc: β-glucosidase, End: endoglucanase, TC: total cellulase, Exo: exoglucanase.

(0.61), and total cellulase (0.42), and the second group includes β-glucosidase (-0.51), congo red (-0.13), and iodine (-0.33).

The hierarchical clustering analysis for all for all microorganisms shows the characterization of two groups indicating the respective intervals of enzymatic activity (Table 1a). The majority of fungi are in group 1, which presents higher enzymatic activities than group 2.

Bacteria originating from the Páramo samples had higher endoglucanase and total cellulase activities and larger congo red and iodine halos. The strains originating in the Páramo and High Andean Forest had high β-glucosidase activity. The bacterial PCA analysis shows lower correlations among the different variables (Figure 3). The only significant correlation was detected between congo red and iodine (0.8755). The eigenvectors of the PC 1 show that the variables contributing the most to the cellulolytic activity of the microorganisms are iodine (0.59) and congo red (0.59) and the endoglucanase activity contributes moderately (0.39) to cellulolytic activity. With respect to PC 2, two groups of variables are correlated. The first group includes β-glucosidase (0.48), endoglucanase (0.43), and total cellulase (0.36). The second group includes exoglucanase (-0.67), congo red (-0.11), and iodine (-0.04).

Table 1b shows the characterization of the different groups of bacteria. Group 1 comprises the strains with higher values of endoglucanase activity. Group 2 comprises the strains with higher exoglucanase and total cellulase activities. Group 4 comprises the strains with higher β-glucosidase activity.

Table 1 - Cluster characterization.

a. Bacterial and fungal clusters

Group	1	2
End	0.01-0.90	0.00-0.24
Exo	0.00-1.66	0.00-0.44
Glu	0.00-5.56	0.00-2.19
TC	0.03-0.25	0.00-0.11

b. Bacterial clusters

Group	1	2	3	4
End	0.00-0.24	0.00-0.09	0.00-0.13	0.00-0.05
Exo	0.00-0.05	0.00-0.38	0.00-0.24	0.00-0.00
Glu	0.00-0.16	0.00-0.13	0.00-0.13	0.01-0.53
TC	0.00-0.08	0.00-0.21	0.00-0.11	0.00-0.07

c. Fungal clusters

Group	1	2	3	4	5	6
End	0.08-0.17	0.05-0.17	0.33-0.51	0.09-0.89	0.30-0.44	0.11-0.51
Exo	0.00-1.66	0.00-0.44	0.15-0.49	0.14-0.59	0.07-0.41	0.00-0.33
Glu	3.11-5.56	0.00-2.19	0.03-1.30	0.15-1.53	1.00-1.91	0.00-1.29
TC	0.12-0.13	0.02-0.11	0.17-0.25	0.05-0.13	0.03-0.07	0.05-0.12

End: Endoglucanase, Exo: Exoglucanase, Glu: β-glucosidase, TC: Total cellulase. Enzymatic activities expressed in IU/mg of protein.
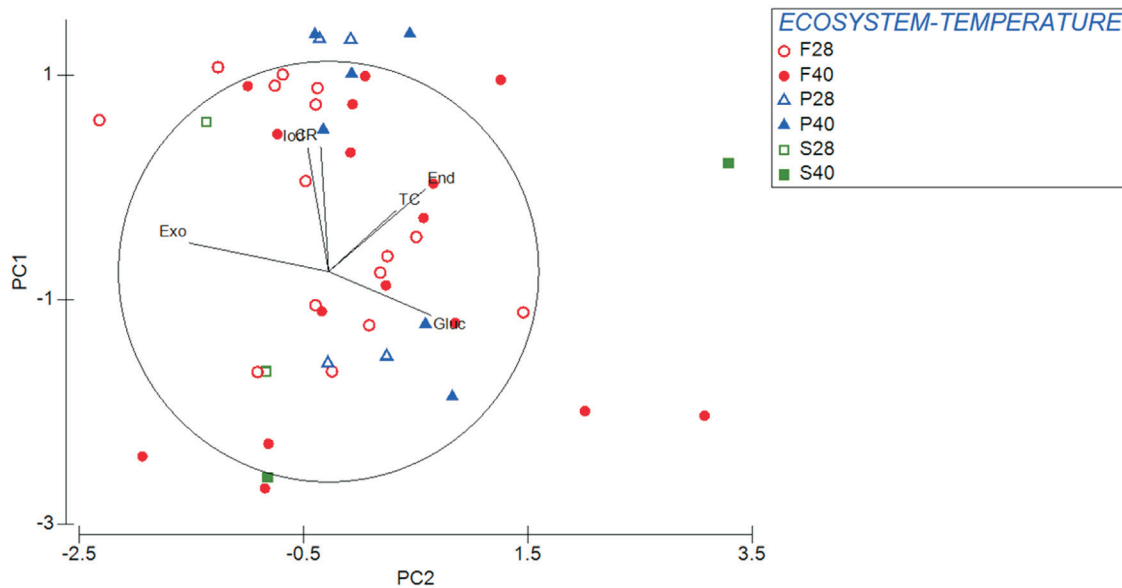

Figure 3 - Principal component analysis of isolated bacteria. CR: congo red, Iod: Iodine, Gluc: β-glucosidase, End: endoglucanase, TC: total cellulase, Exo: exoglucanase. F: High Andean Forest, P: Páramo, S: Superpáramo.

The fungi that exhibited the highest β-glucosidase, endoglucanase, congo red, and iodine activities originated in the ecosystems of the High Andean Forest (Figure 4). The PCA for fungi showed that congo red, iodine, β-glucosidase, and endoglucanase, as well as exoglucanase and total cellulose, are positively correlated in fungi (Figure 4).

Congo red and iodine showed the largest correlation coefficient (0.7650) followed by β-glucosidase and congo red (0.4721), and β-glucosidase and iodine (0.3564). The eigenvectors of PC 1 indicate that the variables contributing the most to cellulolytic activity in the isolated fungi are congo red (0.61) and iodine (0.60), and, to a moderate ex-
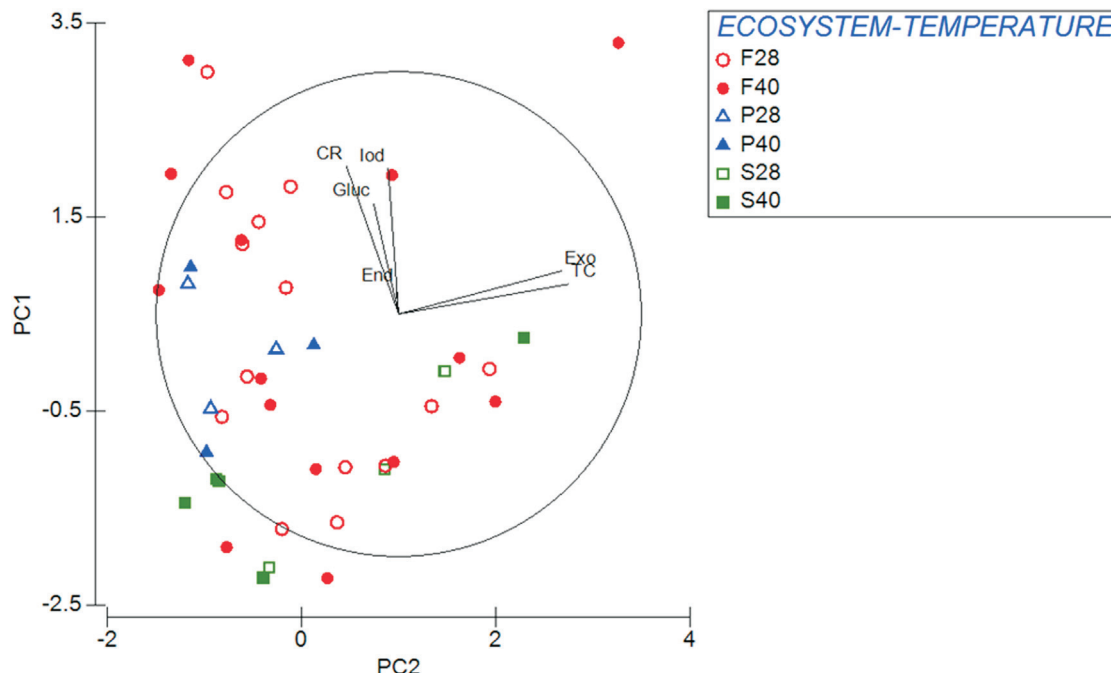
Figure 4 - Principal component analysis of isolated fungi. CR: congo red, Iod: Iodine, Gluc: β-glucosidase, End: endoglucanase, TC: total cellulase, Exo: exoglucanase. F: High Andean Forest, P: Páramo, S: Superpáramo

tent, β-glucosidase (0.46). The variable contributing the least to cellulolytic activity is exoglucanase. With respect to PC 2, two correlated groups could be observed. The first group includes exoglucanase (0.67) and total cellulase (0.70). The second group includes β-glucosidase (-0.10), endoglucanase (-0.01), congo red (-0.22), and iodine (-0.04).

The enzymatic characterization of the main groups from the cluster analysis is presented in Table 1c, where groups 3, 4, 5 and 6 show the highest endoglucanase activities. Group 1 comprises the highest exoglucanase and β-glucosidase activities. Group 3 comprises the highest total cellulase activity. Strains HpF (40 °C), HpF (28 °C), Ps3F (40 °C), Ps3F (28 °C), PtF-40, PtF (28 °C), and Pw3 (40 °C) showed high β-glucosidase, endoglucanase, congo red, and iodine activities. Strains PoS (40 °C), Ps2F (28 °C), and Pw2F (40 °C) showed the highest exoglucanase and total cellulase activities.

Table 2 shows fungi and bacteria that were selected for their high cellulolytic capacity. The generation of pools of these microorganisms is important for the optimization of cellulolytic activity.

Discussion

Isolation and qualitative evaluation of cellulolytic activity

The present study is the first report of cellulolytic microorganisms in the ecosystems of Superpáramo, Páramo,

and High Andean Forest in the NNNP. The genera isolated in this study have been reported by other authors for their cellulolytic activity, *e.g.*, *Pseudomonas* (Shastry and Prasad, 2002) *Streptomyces* (Gaitán and Perez, 2007; Grigorevski de Lima *et al.*, 2005; Gutiérrez *et al.*, 2008), *Rhodococcus* and *Penicillium* (Villalba *et al.*, 2004), and *Mortierella* (MartÃnez *et al.*, 2001; Valenzuela *et al.*, 2001).

The growth of these microorganisms on different culture media containing cellulose as a unique carbon source (CMC, powdered cellulose, or filter paper) varied among the various strains and species, depending on the media. This finding is supported by previous reports that showed that small variations in carbon sources, pH, macro- and micro-nutrients, temperature, and time of incubation induced differences in microorganism growth (Ahamed and Vermette, 2008; Arifoglu and Ögel, 2000; Grigorevski de Lima *et al.*, 2005; Hanif *et al.*, 2004; Ögel *et al.*, 2001).

Bacterial isolates were more abundant than fungi. However, fungi produced the larger congo red and iodine halos, which is indicative of a stronger cellulolytic potential compared to bacteria. A larger number of isolates associated with organic material was found in the samples originating in the High Andean Forest, which is consistent with the high content of decomposing wood and leaves that provides a more abundant cellulosic substrate in this area. Microorganisms with high reported cellulolytic activity like *Penicillium swiecickii*, *Penicillium glabrum*, *Penicillium* sp, and *Streptomyces* sp. were also found in the samples from the páramo and superpáramo. The presence of those

Table 2 - Microorganisms selected based on their higher cellulolytic potential.

a. Fungi

Enzyme	Fungi	SEA (IU/mg protein)	Temperature (°C)	Ecosystem
End	*Penicillium thomii*	0.72	28	F
		0.89	40	F
Exo	*Penicillium* sp	1.66	40	F
	3Penicillium swiecickii -3	0.59	40	F
Gluc	*Hypocrea pilulifera*	51.09	28	F
		55.58	40	F
TC	*Penicillium* sp -	0.25	28	F
	2Penicillium sp - 5	0.21	40	S

b. Bacteria

Enzyme	Bacteria	SEA (IU/mg protein)	Temperature (°C)	Ecosystem
End	*Pseudomona* sp -1	0.24	28	F
		0.16	40	F
Exo	*Streptomyces* sp -5	0.37	28	P
		0.38	40	P
Gluc	*Streptomyces* sp -13	1.27	28	F
		5.29	40	F
TC	*Streptomyces* sp - 4	0.21	28	P
		0.11	40	P

SEA: Specific Enzymatic Activity, End: Endoglucanase, Exo: Exoglucanase, Glu: β-glucosidase, TC: Total cellulase. Enzymatic activities expressed in IU/mg of protein. S: Superpáramo, P: Páramo, F: High Andean Forest.

microbes in those ecosystems with low plant diversity could be associated with the presence of *Calamagrostis* sp., which could provide input cellulose and therefore promote the existence and maintenance of these microorganisms.

Comparisons with previous reports

In this study, we report high activities, specifically, 0.72 and 0.89 IU/mg protein, for a strain of *Penicillium thomii* at 28 °C and 40 °C, respectively (Table 2). Studies from other authors also reported strains of *Acremonium cellulolyticus*, namely, C-1 and CF2612 (C-1 was subjected to mutagenesis), with β-glucosidase-specific activities at 30 °C of 1.7 and 2.2 IU/mg protein, respectively (Fang *et al.*, 2009). Furthermore, *Hypocrea pilulifera* has activities greater than 51.09 and 55.58 IU/mg protein at 28 °C and 40 °C, respectively (Table 2). Fang *et al.* (2009) also reported pFase (total cellulase) activities of 0.84 and 0.99 for the strains at 30 °C, which contrasts with our findings for the strains of *Penicillium* sp - 2 and *Penicillium* sp - 5 which had 0.25 and 0.21 IU/mg protein, respectively (Table 2). It is worth mentioning that these authors determined the protein concentration using the Lowry method and used a different methodology to assess β-glucosidase activity (Fang *et al.*, 2008). Conversely, a maximal specific endoglucanase activity of 0.5 IU/mg protein at 28 °C was reported for a strain of *Curvularia pallescens* (Freire *et al.*, 1999).

Specific endoglucanase activities of 0.45 and 0.7 IU/mg protein has also been reported in crude cellular extracts of *Tricoderma reseei* and in a mutant of the same strain at 50 °C, respectively (Gadgil *et al.*, 1995). Our report found higher activities (0.72 and 0.89 IU/mg protein at 28 °C and 40 °C respectively) for a strain of *Penicillium thomii* and (Table 2). This same strain showed higher total cellulase activities (0.25 and 0.21 IU/mg protein at 28 °C and 40 °C, respectively; Table 2) than the strain *Tricoderma reseei* and its mutant (0.04 and 0.07 IU/mg protein, respectively) at 50 °C (Ögel et al., 2001). Kaur *et al.*, 2007 reported values of up to 300 and 500 IU/mg protein of endoglucanase and β-glucosidase activities for strains of *Melanocarpus* sp. at 50 °C, respectively. These results are much higher than the results reported by most authors, including the present study.

The differences between the enzymatic activities reported by those previous studies, as well as our results, are likely caused by variations in experimental conditions. It has been observed that enzymatic activities are strongly influenced by different factors, such as the carbon source utilized (CMC, power cellulose, filter paper, or natural substrates), the brand or origin of the carbon source, pH variation, micronutrients in the culture media, the stage of the culture at the time of activity determination (stationary *vs.* exponential phase), and the temperature at which the enzymatic activity is assessed (Ahamed and Vermette, 2008; Arifoglu and Ögel, 2000; Freire *et al.*, 1999; Grigorevski de

Lima *et al.*, 2005; Hanif *et al.*, 2004; Karnchanatat *et al.*, 2008; Kaur *et al.*, 2007; Murashima *et al.*, 2002; Ng *et al.*, 2010). Additionally, different methodologies have been reported, for example, to determine the reducing sugars, which is typically performed by the dinitrosalicylic acid method (Ahamed and Vermette, 2008; Fang *et al.*, 2009; Freire *et al.*, 1999; Gadgil *et al.*, 1995; Grigorevski de Lima *et al.*, 2005; Hanif *et al.*, 2004; Karnchanatat *et al.*, 2008; Kaur *et al.*, 2007; Kaur *et al.*, 2006; Lo *et al.*, 2009; Murashima *et al.*, 2002; Ng *et al.*, 2010), although other reports have used the Nelson-Somogy method (Arifoglu and Ögel, 2000; Ghose, 1987; Ögel *et al.*, 2001). These differences are also found in determination of protein concentrations, with several authors using the Bradford method (Hanif *et al.*, 2004; Karnchanatat *et al.*, 2008; Kaur *et al.*, 2007; Kaur *et al.*, 2006; Lo *et al.*, 2009; Murashima *et al.*, 2002; Ng *et al.*, 2010), and others choosing the Lowry method (Arifoglu and Ögel, 2000; Fang *et al.*, 2009; Freire *et al.*, 1999; Lowry *et al.*, 1951; Ögel *et al.*, 2001).

Comparisons among different values of enzymatic activity are hampered because of the different methods of reporting such activity. Several studies determine the amount of protein and report the enzymatic activity in IU per mg of protein (specific enzymatic activity) (Arifoglu and Ögel, 2000; Fang *et al.*, 2009; Freire *et al.*, 1999; Hanif *et al.*, 2004; Karnchanatat *et al.*, 2008; Kaur *et al.*, 2007; Kaur *et al.*, 2006; Lo *et al.*, 2009; Murashima *et al.*, 2002; Ögel *et al.*, 2001; Pitson *et al.*, 1997; Souza *et al.*, 2010), while others report it as IU per mL (Ahamed and Vermette, 2008; Fang *et al.*, 2009; Kaur *et al.*, 2006; Ögel *et al.*, 2001). In other cases, the enzymatic activity has been reported in filter paper units (FPU) following the Ghose method (Ahamed and Vermette, 2008; Mohagheghi *et al.*, 1988). Other authors have reported relative activity in percentage units (Karnchanatat *et al.*, 2008; Kaur *et al.*, 2007; Murashima *et al.*, 2002; Ng *et al.*, 2010). To allow adequate comparisons, authors should report specific enzymatic activity, which is an indication of the efficiency of the enzymes and/or microorganisms. When the activity is reported in terms of IU/mL, it is not possible to conduct clear comparisons because a high enzymatic activity could be due to a large quantity of a cultured microorganism or a large amount of enzyme, rather than to high efficiency of the enzyme present in the extract. In addition, several reports do not distinguish between the endoglucanase activity and the total cellulolytic activity, and others do not determine the contribution of the three enzymes to the total cellulolytic activity.

Relationships among the assessed enzymatic activities

Researchers are questioning the optimal method to conduct functional screening for cellulolytic microorganisms, the best enzyme to indicate cellulolytic activity of a species, the relationships between the distinct enzymes that contribute to the total cellulolytic activity, and whether one enzyme competes with, generates synergies with, or actuates independently of the others.

Based on the PCA results presented in this study (Figure 2), we found that all variables were positively correlated. This finding indicates a coordinated action of the distinct enzymes involved in the degradation of cellulosic compounds. Therefore, the results obtained by congo red and iodine tests are in agreement with each other and with the action of β-glucosidase. This means that the rupture of glucose dimers is directly related to the hydrolysis produced by these two methods. However, several authors have suggested that endoglucanase activity, which randomly ruptures large cellulose chains, is the primary process for the establishment of total cellulolytic activity, which is consistent with the correlation between the two enzymes. It should be noted that exoglucanase activity does not significantly correlate with any other enzymatic activity, which might indicate that the production of glucose dimers due to the action of this enzyme is an independent or random process in relation to the other evaluated enzymatic activities.

However, the PCA in the bacterial group (Figure 3) shows that the endoglucanase and total cellulase activities in bacteria are positively correlated. This result is in agreement with the PCA for the composite of all microorganisms. Iodine and congo red for bacteria are also positively correlated, and their paired values are notably similar. The bacterial β-glucosidase and exoglucanase activity values show an inversely proportional trend, similar to the respective trend found in the PCA for the composite of all microorganisms.

There is a larger correlation among the different variables for fungi compared to bacteria (Figures 3 and 4). Multiple reports have suggested that correlations among the distinct enzymes that contribute to the total cellulolytic activity are not clear and vary depending on the context (Lo *et al.*, 2009; Ögel *et al.*, 2001). In this regard, it is worth mentioning that the results presented in this report do not indicate that the total cellulolytic activity is due to the combined action of the three distinct enzymes (endo, exo, and β-glucosidase), but is instead a more dynamic and complex process. Therefore, it is presumed that the existence or absence of correlations among the distinct enzymatic activities are mediated by conditions specific to the microorganisms, growth phase, pH, micronutrients, temperature, and carbon source among others factors. Therefore, it would not be appropriate to make an absolute generalization about direct or inverse correlations among the relevant enzymes, given that these relationships depend on the microorganism under evaluation and conditions that affect the enzymatic activity.

Conclusions

A systematized survey for microorganisms with cellulolytic potential was conducted using soils from the

NNNP. The majority of the isolated microorganisms originated from the High Andean Forest ecosystem, which is expected, given the associated vegetation and the different processes experienced by the organic material in this area. However, microorganisms with good cellulolytic capacities were found among the isolates originating in the Páramo and Superpáramo (25 bacterial and 21 fungal isolates; bacterial isolates belonged to the genera *Pseudomonas*, *Streptomyces*, *Rhodococcus*, *Stentrophomonas*, *Variovorax*, *Serratia*, and *Janthinobacterium*, and fungal isolates belonged to the genera *Penicillum*, *Mortierella*, *Tolypocladium*, *Paecilomyces*, *Acremonium*, *Fusarium*, *Volutella*, *Hypocrea*, *Neonectria*, *Mucor*, *Aureobasidium*, and *Arthtinium*). This makes these organisms interesting from an ecological and biotechnological viewpoint, given the extreme conditions in these ecosystems. Cellulolytic enzymes were characterized under pH 7 for bacteria and fungi, pH 5 and temperatures between 28 and 40 °C are considered optimum conditions for cellulolytic enzymes, however, at other temperatures and pH levels these microorganisms could also have good cellulolytic activity due to the extreme conditions in these ecosystems where microorganisms were isolated.

Fungi were confirmed to be better cellulolytic microorganisms than bacteria. None of the microorganisms simultaneously produced the highest cellulolytic activities for all relevant enzymes. Therefore, the use of pools of bacteria and/or fungi that include the best organisms for each specific enzymatic activity is suggested. This study also revealed that when all microorganisms are analyzed as a group, positive correlations are found among the distinct parameters evaluated (endoglucanase, exoglucanase, β-glucosidase, total cellulase, and hydrolysis halo detected by congo red and iodine). However, different correlations emerge when bacteria and fungi are analyzed as separate groups and here there is a general correlation among congo red, iodine, and β-glucosidase. Exoglucanase had a low correlation with any other parameter tested, which suggests that it would not be a good indicator of cellulolytic activity. The identification of hydrolysis halos using the congo red and iodine tests remain the methods of choice, due to these techniques' rapidity and validity for determining cellulolytic activity. Although, based on our experience, both methods seem adequate, we would suggest a more effective use of the iodine test because it is faster, less toxic, more economic, and easily accessible in multiple microbiology laboratories.

This work identified microorganisms with satisfactory cellulolytic potential based on assays that were developed. The assays used substrates of commercial or biotechnological interest and pools of select microorganisms or enzymatic extracts, focusing on those enzymes and parameters that were the best indicators of cellulolytic activity.

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