Research article

Hydrolytic enzyme activity enhanced by Barium supplementation

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Abstract: Hydrolysis of polymers is a first and often limiting step during the degradation of plant residues. Plant biomass is generally a major component of waste residues and a major renewable resource to obtain a variety of secondary products including biofuels. Improving the performance of enzymatic hydrolysis of plant material with minimum costs and limiting the use of additional microbial biomass or hydrolytic enzymes directly influences competitiveness of these green biotechnological processes. In this study, we cloned and expressed a cellulase and two esterases recovered from environmental thermophilic soil bacterial communities and characterize their optimum activity conditions including the effect of several metal ions. Results showed that supplementing these hydrolytic reactions with Barium increases the activity of these extracellular hydrolytic enzymes. This observation represents a simple but major improvement to enhance the efficiency and competitiveness of this process within an increasingly important biotechnological sector.

Keywords: barium; extracellular enzyme activity; hydrolytic activity; cellulose; esterase

1. Introduction

Currently, there is an increasing interest [1] in the development of sustainable biotechnological processes which use renewable resources [2,3]. An example is the possibility to produce biofuels (i.e., biogas and biodiesel) from plant biomass [4]. Plant biomass is constituted largely by polymers

such as cellulose and lignin. Complete degradation of these polymers is generally a limiting step to achieve high efficiency and maximize productivity [4,5].

The enzymatic hydrolysis of cellulose can be processed by cellulose-degrading microorganisms or the enzymes they produce, e.g, cellulases. Esterases, in combination with cellulases, have been proposed to complement lignin digestion due to ester linkages present within this complex molecule [6]. Increasing temperature has been reported to contribute to lignin and cellulose degradation by thermophilic enzymes [2,7] which suggests that searching for thermophilic enzymes with novel or enhanced functionality can conduce to better overcome some of these limitations [2]. Maximizing the activity of these enzymes is critical to achieve a reduction of costs by decreasing the consumption of enzymes in the process and improving the efficiency of the treatment.

Previous studies, have shown that a scarce presence of trace metals can be limiting the growth of microorganisms during the treatment of plant biomass and waste residues for the production of biogas and other biofuels [8,9]. In this study, we identify and characterize three extracellular enzymes and focus on evaluating the potential of several metal ions to inhibit or increase hydrolytic enzyme activities as a proxy to enhance the efficiency of these enzymes and better understand the role of microorganisms during large scale processes of biofuel production or other biotransformations based on the use of sustainable resources from plant materials.

2. Materials and Methods

2.1. Samples

Soil samples were collected in sterile containers around "La Dulce" freshwater pond (Doñana National Park, Spain; location 36º 58.84'N 6º 29.23'W) [10] in September 2011 and May 2012. The samples were collected from the top 5 cm of soil. They were sandy soils with near neutral pH and an organic content of around 20% by weight. The samples were enriched at 60 °C for 24 h in nutrient broth (Difco, USA) to select the thermophilic bacterial community. Concentration of metal ions in soil samples were determined by an inductively coupled plasma-optical emission spectrometer (ICP-OES) at the analytical service of IRNAS-CSIC.

2.2. DNA extraction, sequencing and sequence analysis

Genomic DNA was extracted using the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) and sequenced by shotgun pyrosequencing in a 454 GS FLX+ machine with titanium chemistry (Roche, Basel, Switzerland) according to the manufacturer's instructions. The obtained reads constituted metagenomes from the selected thermophilic communities at the studied samples. Read assemblage was carried out using the Newbler assembler (Roche). Coding regions (CDS) were detected with the software FragGeneScan [11] and these CDSs were putatively annotated using Blastp and the nr database from GenBank (National Center for Biotechnology Information, NCBI; http://www.ncbi.nlm.nih.gov). We identified and cloned genes encoding enzymes of potential interest for the processing of biomass and plant residues (i.e., cellulase and esterase). Specific primers were designed for cellulase and esterases genes based on the obtained sequences and related sequences from GenBank database around the putative start and stop codons. Alignments between the selected sequences from the metagenomes and related database

sequences was carried out using ClustalW [12] under the suite BioEdit Sequence Alignment Editor 7.1.9 (www.bioedit.co.uk).

2.3. Cloning

A cellulase gene was amplified using the pair of primers: 5'-**ATG**GCGAAGTTG GATGCAA-3' and 5'-AT<u>CCTGCAGG</u>**TTA**GTCAAATGTAATTTCTTTCACTT-3'. An esterase gene denominated Est1 was amplified using the primers: 5'-**ATG**AAATTAGTTTCACCAAAACCA-3' and 5'-AT<u>CCTGCAGG</u>**TTA**CCAATCTAATGATTCTAAAAACTC-3'. A second esterase gene denominated Est2 was amplified with primers: 5'-**ATG**GTTCCATTATGGGAG TCAC-3' and 5'-AT<u>CCTGCAGG</u>**TTA**TTTTCTTAACCATGTCTCGC-3'. The restriction site *Sbf*I was incorporated into reverse primer sequences (underlined nucleotides). Start and stop codons are indicated in bold. The PCR amplification was carried out with the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) for 35 cycles (98 °C for 10 s; 55 °C or 60 °C for 15 s for Est2 or cellulase and Est1 sequences, respectively; and 72 °C for 1 min). Amplified PCR fragments were cleaned using ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA, USA), digested with *Sbf*I and ligated into pMAL-p5x vector (New England BioLabs) using T4 DNA Ligase (3 h at 20 °C). Previous to the ligation, the vector was digested with *Xmn*I and *Sbf*I restriction enzymes and purified with JetQuick DNA Purification Kit (Genomed, Löhne, Germany). DNA concentrations were estimated using a NanoDrop 2000c Spectrophotometer. Ligation products were transformed into *E.coli* ER2523 cells and the transformants were selected on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin. Clones were screened by colony-PCR using the pairs of primers described above and subjected to the following thermal cycles: 1 initial denaturation step at 95 °C for 3 min, 35 cycles at 95 °C for 15 s, annealing at 55 °C or 60 °C and extension at 72 °C for 1 min. The last cycle was followed by 2 min extension at 72 °C. The accession number for the DNA sequences of the studied genes are KP823432, KP823433 and KP823434.

2.4. Overexpression and purification of the recombinant enzymes

Transformants were grown in LB broth containing 2% glucose and 100 μg/mL ampicillin to an optical density of about 0.5 at 600 nm. The expression of the recombinant proteins was induced by adding IPTG to a 1 mM final concentration and cultures were incubated with shaking (150 rpm) at 37 °C for 4 h. The recombinant proteins were purified using affinity chromatography using an amylose resin according to pMAL-p5x usage manual (New England BioLabs).

2.5. Enzyme activity assays

Cellulase activity was determined spectrophotometrically by mixing 0.5 mL of enzyme solution with 0.5 mL of 0.5% w/v carboxymethyl cellulose in 50 mM phosphate-citrate buffer (pH 5) for 60 min at the temperature to be assayed (in the range 30 °C–80 °C). The reaction was stopped by adding 3 mL 3,5-dinitro salicylic acid (DNS) reagent. The mixture was heated at 100 °C for 10 min and cooled on ice. Optical density at 540 nm was determined using glucose as a standard. Samples and treatments were carried out in triplicate. Controls lacking enzyme were performed to evaluate

potential non-enzymatic hydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars per min.

Esterase activity was determined spectrophotometrically by measuring the amount of *p*-nitrophenol released during the esterase-catalyzed hydrolysis of *p*-nitrophenyl butyrate. A stock substrate solution containing 10 mM *p*-nitrophenyl butyrate was prepared in acetonitrile, ethanol and 50 mM of Tris-HCl (pH 8.0) at a ratio of 1:4:95 (in volume), respectively. The reaction was initiated by the addition of purified enzyme to a diluted substrate solution at a final concentration of 0.5 nM. The mixture was incubated at the temperature to be assayed (in the range 30 °C–80 °C) for 30 min and stopped by cooling on ice for 10 min. The enzyme activity was immediately measured at 405 nm using a NanoDrop 2000c Spectrophotometer. Samples and treatments were carried out in triplicate. Controls lacking enzyme were performed to evaluate potential non-enzymatic hydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min.

2.6. *Effect of temperature and pH on enzyme activity*

The effects of temperature and pH on cellulase and esterase activities were determined using the standard enzyme assays described above. Assays were carried out in triplicate. Assayed temperatures were 30 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C. Assayed pHs for cellulase activity were from pH 4 to 8 using 50 mM phosphate-citrate buffer. Assayed pHs for esterase activity were from pH 5 to 11 using the following buffers: 50 mM acetate buffer (pH 5), 50 mM Tris-HCl buffer (pH 7–9) and 50 mM Na_2HPO_4-NaOH buffer (pH 11). The pH was adjusted for each assay at the corresponding temperature.

2.7. *Effect of metal ions on enzyme activity*

The effect of metal ions on cellulase and esterase activities was determined by adding chloride salts of Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Ba^{2+} metal ions to the substrate solutions at final concentrations of 1 and 10 mM. Enzyme activity in control solutions lacking supplementing metal ions was defined as the 100% level. Enzymatic activity assays were performed under the established conditions described above at the optimum temperature and pH conditions for each enzyme.

3. **Results and Discussion**

3.1. *Effect of temperature and pH on enzyme activity*

Enzymatic activity of the environmental hydrolytic enzymes retrieved from natural thermophilic bacterial communities existing in soils was analyzed in a broad temperature range, 30 °C–80 °C (Figure 1). The optimum Est1 and Est2 activities were observed between 60 °C and 70 °C, and decreased significantly at temperatures below 40 °C and above 80 °C (Figure 1A). Moreover, the analyzed cellulase showed maximal activity in the temperature range of 40–50 °C and decreased sharply above 80 °C (Figure 1B).

These enzymes, esterases and cellulase, were active in a broad pH range (pH 5 to 11 for the esterases and pH 4 to 8 for the cellulase) (Figure 2). Est1 and Est2 exhibited optimum enzymatic

activities under alkaline conditions at pH 8–9 (Figure 2A). Esterase activities were drastically reduced below pH 5 and above pH 9. Highest cellulase activity was observed at pH 5 (Figure 2B). These enzymes prove to be valuable for their use in processes at high temperature and different pH values.

Optimum temperature for the studied esterases presented similar optima of temperature and pH than, for instance, a carboxylesterase from *Geobacillus* sp. ZH1 [13] among other thermophilic esterases [14,15,16]. A cellulase from *Trichoderma* sp. IS-05 [17] showed optimum temperature slightly higher (60 °C) than the one studied in this study (40 °C–50 °C); the optimum pH for this cellulase was more acidic (pH 3) than the cellulase retrieved from the environment during this work (pH 5). Similar behavior as a function of temperature and pH was observed for a cellulase obtained from *Bacillus thuringiensis* [18]. The enzymes obtained and analyzed during this study show typical patterns of activity over a broad range of temperature and pH for thermophilic cellulases and esterases [2,13–16] previously reported. Consequently, they represent potential candidates to be tested for biotechnological applications [3,7], such as during enzymatic treatments of plant materials (i.e., cellulose and lignin), and as possible complement to the activity of previously described enzymes under a range of temperature and pH conditions or the evaluation of their capability to degrade different types of plant materials.

3.2.   *Effect of metal ions on enzyme activity*

The effects of metal ions on cellulase and esterase activities are shown in Table 1. Interestingly, the addition of Ba^{2+} to the reactions of hydrolysis catalyzed by these enzymes showed a significant increase of the enzymatic activity at 10 mM Ba^{2+}. At this concentration, the enhancing effect of Barium on Est1 and Est2 activities were 79 and 28 percentage points, respectively, higher than in the unsupplemented control. Cellulase activity in the presence of 10 mM Barium showed an enhancement of 54 porcentual points of the activity observed under unsupplemented conditions. A previous study on bacterial cellobiohydrolases has reported a slight activity enhancement by Ba^{2+} [24], and a fungal cellulase was shown to be inhibited by Ba^{2+} and other metal ions [17]. Zhu et al. [13] has shown that Ba^{2+} could lead to moderate enhancement of the activity of an esterase from a deep-sea *Geobacillus* species at 10 mM concentration. In spite of these results, in these studies the effect of Barium has been left out and barely discussed. There are multiple studies on the effect of metal ions on proteases because the activity of a large number of proteases has been reported to be metal dependent [19,20]. The effect of Ba^{2+} has been reported to induce increased activity by several bacterial proteases [21,22]. To our knowledge, no previous reports have highlighted the potential of the enhancing effect of Ba^{2+} on various extracellular hydrolytic enzymes (other than proteases) from soil microbial bacterial communities.

In order to compare the activity of these enzymes when supplemented with several metals, other metal ions were tested. Significant inhibition on cellulase and esterase activities at 1 mM and 10 mM concentrations were observed with most metal ions (Table 1). Cu^{2+} at a concentration of 10 mM was the most inhibitory metal ion in every assay reducing cellulase activity down to 6.9% and the activities of the esterases down to 26% and 17.5%. Previous studies [13,18,23,24] have shown an inhibition of hydrolytic enzyme activities by a number of metals ions including Cu^{2+} and Zn^{2+}. A highly metal-resistant thermophilic esterase from the Red Sea showed resistance to various metal
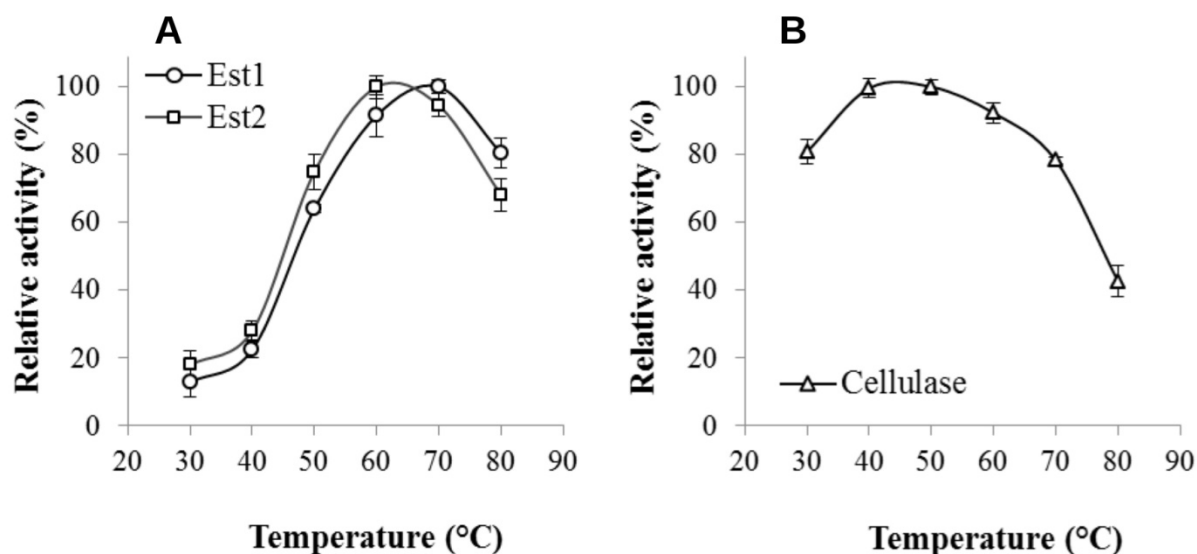
Figure 1. Effect of temperature on esterase and cellulase activities. Esterase (A) Est1 (○) and Est2 (□) activities, (B) cellulase (Δ) activity. Data points represent the mean of three replicates and their bars a standard deviation.
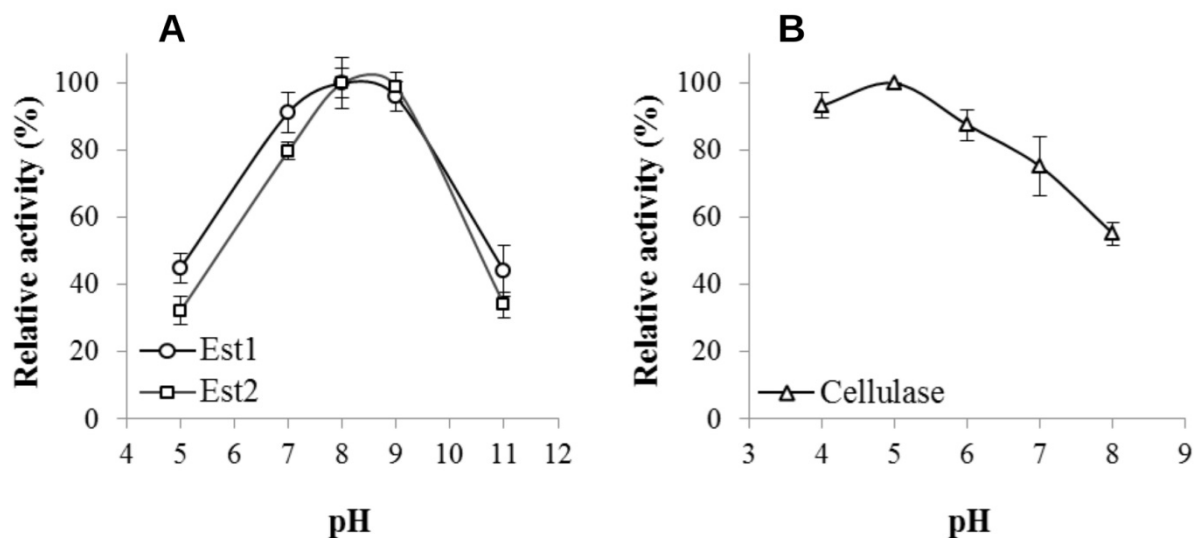

Figure 2. Effect of pH on esterase and cellulase activities. Esterase (A) Est1 (○) and Est2 (□) activities, (B) cellulase (Δ) activity. Data points represent the mean of three replicates and their bars a standard deviation.

inhibition by Cu^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} at 1 mM and 10 mM concentrations. As a result of soil content of these metals (Table 1), our results suggest that Iron could be inhibiting thermophilic extracellular enzyme activity at the sampled environment due to the high content of this ion at this location [10] although addition of Barium would result in higher extracellular enzyme activities.

Table 1. Effect of metal ions on recombinant cellulase and esterase (Est1 and Est2) activities obtained from soil thermophilic bacterial communities. Data are expressed as percentages of the activity in the control (lacking supplemented metals) that considered 100%. The environmental concentrations of those metals are also presented*.

Metal ions	Concentration (mM)	Relative activity (%)			Environmental concentrations (mM)*
		Est1	Est2	Cellulase	
Control		100.0 ± 4.1	100.0 ± 6.0	100.0 ± 5.8	
Zn^{2+}	1	69.9 ± 7.4	76.1 ± 6.3	73.5 ± 7.9	0.54/0.11
	10	24.7 ± 8.3	50.2 ± 7.1	29.0 ± 6.0	
Cu^{2+}	1	65.8 ± 6.2	59.0 ± 8.1	41.6 ± 4.6	0.21/0.05
	10	26.0 ± 7.9	17.5 ± 6.8	6.9 ± 5.6	
Fe^{2+}	1	67.8 ± 4.5	72.9 ± 6.6	70.2 ± 8.2	392.18/152.15
	10	40.4 ± 7.6	59.0 ± 8.1	30.8 ± 5.6	
Co^{2+}	1	94.9 ± 8.7	91.6 ± 5.2	55.3 ± 4.2	0.08/0.05
	10	57.5 ± 5.4	46.6 ± 15.4	40.1 ± 5.8	
Ba^{2+}	1	111.3 ± 6.5	106.8 ± 5.6	96.9 ± 4.4	0.58/0.42
	10	179.1 ± 5.7	128.3 ± 7.5	153.7 ± 2.8	
Ni^{2+}	1	80.8 ± 6.4	102.0 ± 11.7	68.9 ± 5.6	0.11/0.06
	10	69.2 ± 8.9	59.8 ± 8.0	50.4 ± 7.7	

*: Concentration (mM) of ions in the environmental samples where the enzymes were retrieved. Values on the left are for the sample collected on September 2011 and values on the right of the dash was for a sample collected on May 2012.

3.3. Relevance and enhancement of extracellular hydrolytic activity

The availability of trace metals is a requirement for the growth of microorganisms because some of them are required as cofactors for the activation of a number of intracellular enzymes [19,20] which are central to their metabolism. Around 30% of enzymes might require the tight binding of metal ions [19]. However, in this study, we analyze the effect of trace metals on the first and limiting step in the process of microbial degradation of plant polymers. This step corresponds to the action of extracellular hydrolytic enzymes in charge of decomposing large polymers into smaller molecules and monomers which then can be taken up by the microorganisms and so they can proceed further into microbial metabolism. Most extracellular hydrolytic enzymes (i.e., cellulase and esterases) do not require specific trace metals to show catalytic activity and generally after they have been synthesized they carry out spontaneous folding once outside the cell [19,20].

Although the effect of trace metals on microbial metabolism and enzymatic activity has been frequently reported, to our knowledge, detailed descriptions of the mechanisms of action are very

scarce for most metals on extracellular hydrolytic enzymes [25] involved in the decomposition of plant polymers, such as cellulose and lignin. Although metal inhibition might result from partial interference between the active center of these enzymes and their substrates, the action of Barium could be explained by its higher reactivity than other metals [26]. This could contribute to enzyme activation by facilitating ionic interactions between enzyme and polymer substrate, providing stronger stabilization of negative charges than other metals [26], and so enhancing the catalytic activity of extracellular hydrolytic enzymes decomposing plant residues. The potential relevance of Barium on the activity of extracellular enzymes directly involved in plant biomass degradation has been barely considered in previous literature.

The biotechnological industry using microorganisms and enzymes for the processing of plant materials and biomass is currently searching for increasingly efficient enzymes. Advances on strategies to improve activity of novel and previously used enzymes would nicely complement the objective of reaching the highest rates and most complete decompositions of plant materials. Some examples have been presented in this study. Hydrolytic reactions by newly discovered enzymes resulted in increased activities of these extracellular hydrolytic enzymes by up to 80 percentage points of initial activity when supplemented with Barium salts. These data represent a good example showing potential for the optimization of enzymatic and microbial resources aimed to plant biomass degradation.

4.   Conclusion

Improving the efficiency of hydrolytic enzymes is a major step in the development of cost effective and sustainable use of plant biomass as resources in biotechnological transformations. This study used culture-independent methods to retrieve environmental gene sequences from thermophilic bacteria which were cloned and characterized. We highlight the observation that Ba^{2+} supplementation significantly enhanced the activity of extracellular hydrolytic enzymes (i.e., cellulase and esterase).

Acknowledgements

Conflict of Interest

All authors declare no conflicts of interest in this paper.

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