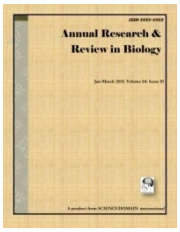

Annual Research & Review in Biology
4(19): 2920-2930, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Isolation and Identification of Cellulolytic and Xylanolytic Bacteria from Huancarhuaz Hot Spring, Peru

Carmen Tamariz-Angeles[1], Percy Olivera-Gonzales[1], Gretty K. Villena[2] and Marcel Gutiérrez-Correa[2*]

[1]Laboratory of Biology, Faculty of Science, Santiago Antúnez de Mayolo National University, Huaraz, Peru.
[2]Laboratory of Mycology and Biotechnology, Universidad Nacional Agraria La Molina, Av La Molina s/n, Lima 12, Peru.

Authors' contributions

This work was carried out in collaboration between all authors. Authors CTA, GKV and MGC conceived and designed the study. Authors CTA and GKV designed the experiments. Author CTA performed the experiments and wrote the first draft of the manuscript. Author POG performed the samplings and the statistical analysis. Authors GKV and MGC reviewed the first draft of the manuscript and made the final manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 6th April 2014
Accepted 28th April 2014
Published 15th May 2014

ABSTRACT

Aims: To isolate and characterize lignocellulase producing thermophilic bacteria from a Peruvian hot spring.
Study Design: Combined sediment and water samples from the hot spring were subjected to direct plating, *in situ* baiting and *ex situ* enrichment. Endoglucanase and xylanase producing bacterial colonies were isolated and characterized.
Place and Duration of Study: Samples were taken from the Huancarhuaz hot spring, Peru (8º56'31.86"S, 77º47'00.53"W) in August 2010 and processed during 2011-2013.
Methodology: Samples were subjected to three isolation methods and bacterial colonies with different color, size and appearance, were isolated, purified by streaking several times and conserved in Tryptic Soy Agar slants at 4ºC. The agar staining method was used to isolate enzyme-producing strains which were then identified by 16S rRNA sequencing and further studied for endoglucanase and xylanase production.

**Corresponding author: Email: mgclmb@lamolina.edu.pe;*

Results: From 19 bacterial isolates only eight were selected for further study as they showed clearing activities on both carboxymethyl cellulose and xylan agar plates. By using 16S rRNA gene phylogenetic analysis, seven isolates were identified as *Bacillus licheniformis* and one as *Cohnella laeviribosi* which was the best xylanase and endoglucanase producer. Maximum endoglucanase activity produced by *C. laeviribosi* EHB4 was obtained at pH 6.0 and at 60ºC and only 50% of its activity was lost at 90ºC for 1h indicating that this enzyme is particularly thermostable.
Conclusion: This is the first report on the production of endoglucanase by *C. laeviribosi*. These findings indicate that Peruvian hot springs are good sources of thermophilic cellulase-producing bacteria and that *C. laeviribosi* EHB4 may contribute to the development of biomass bioconversion processes.

Keywords: Bacillus; cohnella; endoglucanase; peruvian hot springs; thermophiles; xylanase.

1. INTRODUCTION

The high demand for energy is a matter of concern today and this will increase in the coming decades as population grows. Therefore, searching for additional and renewable energy sources will continue since there are expectations that renewable resources will be able to play a significant role satisfying this future energy demand [1]. Also, this has fueled research worldwide for the development of technologies for an efficient use of them. Among renewable resources for energy, biomass which amounts to about 200×10^9 tons per year is one of the most abundant and inexpensive feedstock that can be converted into liquid biofuels and other valuable industrial compounds [2,3]. It is considered that 250 to 500 EJ of biomass energy could be produced by agriculture while still feeding a growing world population [4]. However, the bioconversion of biomass to liquid fuel like ethanol is difficult due to the complex structure and composition of this substrate [5].

The major component of lignocellulosic biomass is cellulose, followed by hemicellulose and lignin. Cellulose and hemicellulose are macromolecules constructed from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. Lignin is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall [6]. The rigid and complex molecular polymeric structure of cellulosic biomass makes lignocellulose highly resistant to chemical attack, solubilization and bioconversion. For the conversion of the biomass into biofuels, it is required to overcome this recalcitrance by using either physical or chemical pretreatment procedures which break down the lignocellulosic structures and thereby enhance either the enzymatic or chemical accessibility [7,8,9]. Accessible cellulosic carbohydrates are hydrolyzed to fermentable sugars and then converted to ethanol by either bacteria or yeasts. The widely accepted mechanism for enzymatic cellulose hydrolysis involves the synergistic activity of endoglucanase, exoglucanase or cellobiohydrolase, and β-glucosidase [10,11]. Additionally, xylanases and other enzymes are also required for hemicellulose hydrolysis [12]. Despite the efforts of many laboratories, the high cost of enzyme production is still the bottleneck in the production of cellulosic ethanol. Therefore, the search for new both cellulase-producing microorganisms and cellulase-encoding genes from several environments will continue for longer time.

Thermotolerant enzymes are of high interest for many enzyme-catalyzed industrial processes which has driven microbial surveys in high-temperature environments like hot springs [13]. Although there are many reports on microbial diversity of hot springs in several

parts of the world, studies on Peruvian hot springs are lacking. In this sense, the objective of this research was to isolate xylanase and cellulase producing bacteria present in the Huancarhuaz hot spring which is located in the central Andean mountains of Peru. As far as we know this is the first report describing bacterial diversity of Peruvian hot springs.

2. MATERIALS AND METHODS

2.1 Sample Collection

Combined sediment and water samples were collected from the Huancarhuaz hot spring in sterile wide-mouthed glass bottles and transported cooled to the laboratory for analysis in the same day. Huancarhuaz hot spring is located in the province of Huaylas, Ancash, Peru (8º56'31.86"S, 77º47'00.53"W). Temperature of 70ºC and pH of 6.5 were registered at the moment of sampling.

2.2 Isolation Procedures

Three isolation methods were used in this study: direct plating, *in situ* baiting and *ex situ* enrichment. In all cases, the agar dilution method was used on a basal salt medium (BSM) containing per liter: 11.7g $(NH_4)_2SO_4$, 3.7g KH_2PO_4, 0.6g $MgSO_4.7H_2O$, 0.8g $CaCl_2.2H_2O$, 0.5g yeast extract, 0.5g peptone, 0.5g $FeSO_4.7H_2O$, 0.16g $MnSO_4.H_2O$, 0.14g $ZnSO_4.7H_2O$, 0.37g $CoCl.6H_2O$. When required, BSM was supplemented with either 10g glucose (agar dilution method) or other carbon sources (qualitative selection) and 15 g agar for plating. The pH was adjusted to 6.5.

For *in situ* baiting, the method of Kublanov et al. [14] was followed by using either 50mg NaOH-pretreated sugar cane bagasse, a 2x2cm piece of Whatman Nº1 filter paper or 50mg beechwood xylan. Baits were kept submerged in the hot spring for 14 days and they were transported cooled to the laboratory for immediate analysis by the agar dilution method.

For the *ex situ* enrichment, 250ml flasks containing 25ml hot spring water and sediment were amended with 25ml basal salt medium and either 0.5g NaOH-pretreated sugar cane bagasse, Whatman Nº1 filter paper or beechwood xylan. They were incubated at 50ºC for 14 days in static state before the isolation by the agar dilution method.

The colonies with different color, size and appearance, were isolated, purified by streaking several times and conserved in Tryptic Soy Agar (TSA, Merck KGaA, Darmstadt, Germany) slants at 4ºC.

2.3 Qualitative Selection of Cellulolytic and Xylanolytic Bacteria

Bacterial isolates were selected for their hydrolytic capacity by the plate staining method [15]. The isolated strains were grown in Tryptic Soy Broth (TSB, Merck) at 50ºC for 20h, then 5μl were inoculated on plates of BSM supplemented with 15g/l agar and either 10g/l medium viscosity carboxymethyl cellulose sodium salt (CMC, Sigma-Aldrich, St. Louis, MO) or 10g/l beechwood xylan (Sigma-Aldrich). Plates were incubated at 50ºC for 5 days. They were stained with 0.1% Congo red (Sigma-Aldrich) for 10 min and washed three times with 1M NaCl. Bacterial isolates showing a clear zone around the colony were selected and maintained on TSA slants at 4ºC.

2.4 DNA Extraction and 16S PCR

The genomic DNA of selected strains was extracted using AxyPrep Bacterial Genomic Miniprep Kit (Axygen Scientific Inc., Union City, CA) following the manufacture's protocol. For amplification of the 16S rRNA gene universal primers set 27F (5'- AGAGTTTGATCCTGGCTAAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') according Reinsenbach et al. [16] were used. The PCR reaction mixture was heated at 94°C for 5min, followed by 20 cycles of denaturation at 94°C for 45 s, annealing at 55ºC for 60 s, and extension at 72ºC for 60s with a final extension step at 72°C for 5 min. The PCR products were sequenced by a commercial company (Macrogen Inc., Seoul, Korea) using universal primers: 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3´). The sequences were analyzed and edited using Chromas Lite version 2.01 software (Technelysium Pty Ltd., South Brisbane, Australia) and CAP3 Sequence Assemble Program [17]. Nucleotide sequences were compared to database of NCBI GenBank (The National Center for Biotechnology Information U. S. http://www.ncbi.nlm.nih.gov/) using BLASTN. Nucleotide sequences were aligned using the multiple alignment program CLUSTAL X version 2.0 [18] and the phylogenetic analysis was carried out with MEGA5 Program [19] using Neighbor-Joining method with 1000 bootstraps and Kimura 2-parameters method. The nucleotide sequences obtained have been deposited in the GenBank database (accession numbers KF911390 to KF911397).

2.5 Enzyme Determinations

For enzyme determinations selected strains were grown in shaken flasks containing 50ml Luria broth supplemented with either 1% CMC or 1% beechwood xylan for 20h at 50ºC and 180rpm. Each flask was inoculated with a 5% of a 0.1 O.D. 8h liquid culture of the selected strain. Three replicates were used in each case. After incubation, liquid cultures were centrifuged at 10,595g and the supernatants were filtered through 0.22μm membranes for enzyme determinations.

Endoglucanase and xylanase were determined in duplicate according to the 96-μl microplate-based method described by King et al. [20] by using either 1% CMC or 1% beechwood xylan in 0.05M phosphate buffer at pH 6.0 as substrate, respectively. One enzyme unit (U) is defined as the amount of enzyme that releases 1μmol product per minute of either glucose or xylose equivalents.

Protein concentration was determined according to the method of Bradford [21], using bovine serum albumin as standard.

2.6 Statistical Analysis

Data were analyzed by SPSS (Version 20.1) software (IBM, New York, NY). Analysis of variance (ANOVA) by the General Linear Models procedure and Duncan's multiple range tests were used to find significant differences between treatments.

3. RESULTS AND DISCUSSION

Hot springs are interesting environments from which bacteria having thermotolerant enzymes can be isolated. A hot spring in the central Andean mountains of Peru was screened for cellulolytic bacteria by using direct plating, *in situ* baiting and *ex situ* enrichment

being the latter the most yielding (Table 1). In both *in situ* baiting and *ex situ* enrichment methods sugar cane bagasse was a better substrate as higher number of bacterial isolates were recovered from it though other substrates have been used elsewhere [14]. It is possible that the complex composition of sugar cane bagasse favored the growth of more bacterial strains as this substrate has been found to be good for cellulase production [22]. From 19 bacterial isolates only eight were selected for further study as they showed clearing activities on both carboxymethyl cellulose and xylan agar plates (Table 2).

All bacterial isolates were gram-positive spore-forming bacilli growing well at temperatures between 35ºC to 50ºC, except strain EHB4 that had poor growth. The identification of these strains was performed by 16S gene analysis. The products of PCR amplified 16S rDNA from all bacterial strains were about 1500bp which corresponds to the size of 16S rDNA gene. For sequence analysis, gene sequence identity was performed by considering a size of 1534bp. BLASTN analysis gave 99% or more identity in all cases. Multiple alignment and phylogenetic analysis showed that seven strains can be assigned to *Bacillus licheniformis* (EHB1, EHB2, EHB3, EHC2, EHC3, IHB1 and IHB2) and one to *Cohnella laeviribosi* (EHB4) both with 100% of boostraps (Fig. 1). Several *Bacillus* species have been isolated from hot springs and other unusual environments all over the World and they may be important for industrial purposes [13,23]. *Bacillus licheniformis* is present in many hot spring samples having interesting capabilities like the production of hydrolytic enzymes, biosurfactans and other industrial useful products [24,25,26,27]. On the other hand, *Cohnella laeviribosi* was isolated from a hot spring of a volcanic area in Indonesia as an endospore-forming rod bacterium that can use L-ribose as the sole carbon source and with an interesting D-Lysose (L-ribose) isomerase activity [28,29]. Although the production of hydrolytic enzymes has been reported for some *Cohnella* species, there are only few reports for *C. laeviribosi* [30,31].

Table 1. Bacterial isolates from Huancarhuaz hot spring, Peru

Method	Enrichment Substrate	Number of isolates	Hydrolytic strains
Direct plating	None	2	-
Ex situ enrichment	Sugar cane bagasse	6	EHB1, EHB2, EHB3, EHB4
	Filter paper	0	-
	Microgranular cellulose	3	EHC2, EHC3
	Beechwood xylan	3	-
In situ baiting	Sugar cane bagasse	4	IHB1, IHB2
	Filter paper	1	-
	FP + microgranular cellulose	0	-
	Beechwood xylan	0	-

In order to determine the hydrolase production capacity of the 8 selected strains, shaken liquid cultures were carried out with either carboxymethyl cellulose or xylan as carbon sources for 20h at 50ºC. All 7 *B. licheniformis* strains grew well on both carbon sources but *C. laeviribosi* EHB4 showed slow growth on the same media. All strains had xylanase activity on both media being higher for *B. licheniformis* strains on carboxymethyl cellulose while for *C. laeviribosi* EHB4 slightly higher xylanase activity was observed on xylan medium. On the other hand, endoglucanase activity was better produced on xylan medium but it was always lesser than xylanase activity (Fig. 3). Total cellulase activity as measured by the filter paper method was very low in all cases as it was also found in other *Bacillus* species [32,33]. Interestingly, the strain *C. laeviribosi* EHB4 produced not only higher xylanase activity but also higher endoglucanase activity having the highest both volumetric

productivity and specific activity of all the strain tested (Table 3). Although *C. laeviribosi* produces xylanases, endoglucanase activity had not been reported yet [30,31]. This endoglucanase activity may be due to neither an unspecific xylanase nor a bi-functional enzyme because its activity level depends on the carbon source as shown in Fig. 2. Therefore, this the first report on the production of endoglucanase for *C. laeviribosi*.

Table 2. Qualitative enzyme screening of selected bacterial isolates from Huancarhuaz hot spring, Peru

Strain	Endoglucanase (mm)	Xylanase (mm)
Bacillus licheniformis EHB1	14.00±1.41	1.75±0.35
Bacillus licheniformis EHB2	14.50±0.71	1.75±0.35
Bacillus licheniformis EHB3	19.5±0.71	2.00±0.00
Bacillus licheniformis EHC2	8.00±1.41	2.00±0.00
Bacillus licheniformis EHC3	8.50±0.71	2.00±0.00
Bacillus licheniformis IHB1	6.00±1.41	1.75±0.35
Bacillus licheniformis IHB2	6.50±0.71	2.00±0.00
Cohnella laeviribosi EHB4	2.00±0.00	2.25±0.35

Values represent mean of four replicates ± SD of the clear zones around colonies

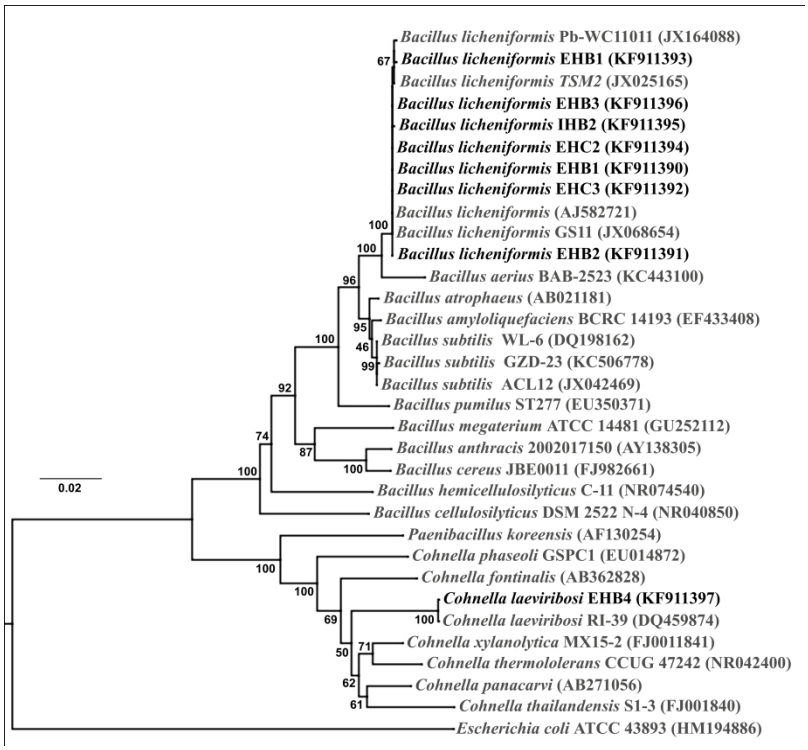

Fig. 1. Comparative sequence analysis of 16S rRNA genes of strains selected for hydrolytic activity on CMC and/or Xylan (EHB1, EHB2, EHB3, EHB4, EHC2, EHC3, IHB1, and IHB2) and related representative species from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) using the Neighbor-joining method. *Escherichia coli* ATTC43893 was used as outgroup to root the tree. GeneBank numbers are indicated in brackets. The Bar represents 2 substitutions per 100 nucleotide positions

Shaken liquid cultures of *C. laeviribosi* EHB4 on xylan were further conducted to study some characteristics of its hydrolase activity. Maximum xylanase activity of cell-free extracts was found at pH 7.0 (Fig. 3a). Xylanase activity was slightly better at 65ºC although there was not a significant difference in activity between 60º to 70ºC (Fig. 3b). However, 70% of its xylanase activity was lost at 60ºC for 1h but it remained with this activity up to 80ºC (Fig 3c). These results are consistent with the characteristics of the extracellular xylanase *eXylC* of *C. laeviribosi* HY-21 but, as expected, different from the intracellular xylanase *iXylC* of the same strain [30]. Although cellulase activity has been reported for other *Cohnella* species like *C. cellulosilytica* [34], *C. laeviribosi* has not been reported as a cellulase-producing species. Interestingly, *C. laeviribosi* EHB4 produced higher levels of endoglucanase in xylan liquid cultures than the other *B. licheniformis* strains isolated from the same hot spring (Fig. 2). Maximum endoglucanase activity produced by *C. laeviribosi* EHB4 was obtained at pH 6.0 and at 60ºC (Fig. 3d, e). Moreover, only 50% of the endoglucanase activity was lost when cell-free extracts were incubated without substrate at 90ºC for 1h indicating that this enzyme is particularly thermostable (Fig. 3f). Several endoglucanases have been found in *Paenibacillus* species [35,36,37,38], the closest relative of *Cohnella*, with different degrees of thermal stability either as single units, bi-functional units or multi-enzyme complexes. As stated above it does not seem that both xylanase and endoglucanase activities are due to a bi-functional enzyme and further studies are needed.

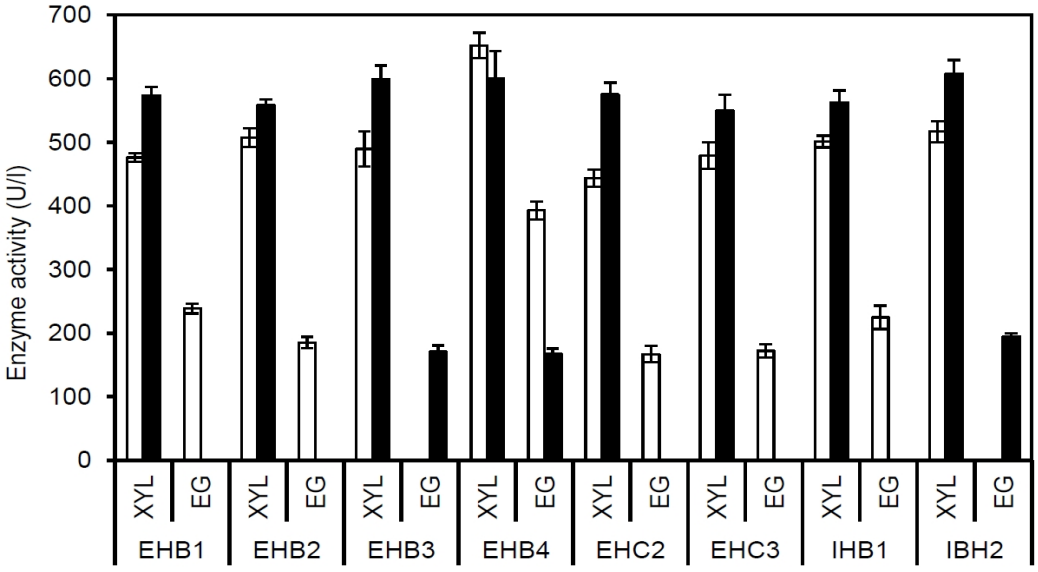

Fig. 2. Endoglucanase (EG) and xylanase (XYL) activities on xylan (white bars) and CMC (black bars) by eight bacterial strains (EHB1, EHB2, EHB3, EHB4, EHC2, EHC3, IHB1, and IHB2) isolated from Huancarhuaz hot spring, Peru. Values represent mean of six replicates ± SD

Table 3. Comparison of some technical parameters for xylanase and endoglucanase production on xylan liquid cultures by eight bacterial strains isolated from Huancarhuaz hot spring, Peru

Strain	XYL	Γ_{XYL}	$Y_{XYL/Pr}$	EG	Γ_{EG}	$Y_{EG/Pr}$
B. licheniformis EHB1	476±7^{d}	23.8±0.3^{d}	8065±116^{e}	238±8^{b}	11.9±0.4^{b}	4040±132^{b}
B. licheniformis EHB2	507±15^{b,c}	25.4±0.7^{c}	8316±239^{e}	185±9^{c}	9.2±0.4^{c}	3030±147^{c}
B. licheniformis EHB3	489±28^{b,c,d}	24.5±1.4^{c,d}	9784 ± 554^{c}	0±0^{e}	0±0^{e}	0±0^{d}
B. licheniformis EHC2	443±14^{e}	22.2±0.7^{d}	10813±334^{b}	167±13^{d}	8.3±0.6^{d}	4067±312^{b}
B. licheniformis EHC3	479± 21^{c,d}	24.0±1.1^{c,d}	9394±411^{c,d}	172±10^{c,d}	8.6±0.5^{c,d}	3369±199^{c}
B. licheniformis IHB1	501±9 ^{b,c,d}	25.1±0.5^{c,d}	9280±169^{c,d}	225±19^{b}	11.2±0.9^{b}	4160±343^{b}
B. licheniformis IHB2	517±17^{b}	25.8±0.8^{b}	8908±285^{d}	0±0^{e}	0±0^{e}	0±0^{d}
C. laeviribosi EHB4	652±14^{a}	32.6±1.0^{a}	14814±455^{a}	393±14^{a}	19.6±0.7^{a}	8921±323^{a}

XYL = xylanase activity (U/l); Γ_{XYL} = Xylanase volumetric productivity (U/l.h); $Y_{XYL/Pr}$ = Xylanase specific activity(U/g protein) EG = endoglucanase activity (U/l); Γ_{EG} = Endoglucanase volumetric productivity (U/l.h); $Y_{EG/Pr}$ = Endoglucanase specific activity (U/g protein).Values represent mean of six repetitions ± SD. Means with the same letter within a column are no significantly different (P<.01)

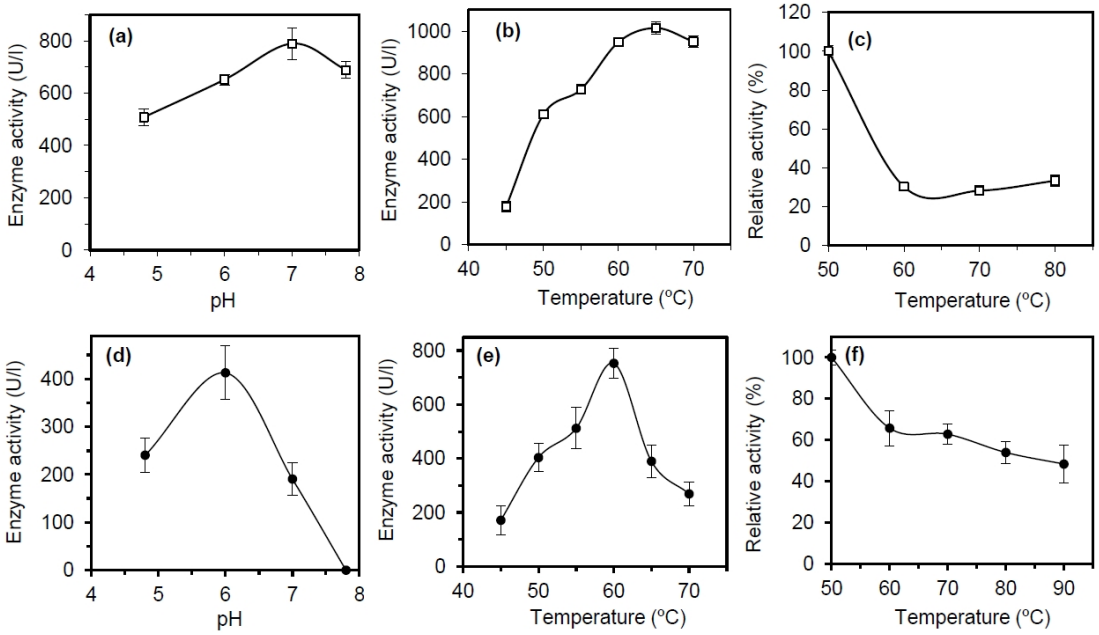

Fig. 3. Characteristics of xylanase (upper panel) and endoglucanase (bottom panel) of *Cohnella laeviribosi* as referred to optimum pH (a, d) and temperature (b, e) on enzyme activity, and thermal stability (c, f). Values represent mean of six replicates ± SD

4. CONCLUSION

In conclusion, we have isolated seven *Bacillus licheniformis* strains and one *Cohnella laeviribosi* strain producing xylanase and endoglucanase from a Peruvian hot spring. Among all the strains isolated in this study, *C. laeviribosi* EHB4 was the best enzyme producer and its endoglucanase activity is the first finding for this species. Endoglucanase activity of

C. laeviribosi EHB4 is maximal at 60ºC and is particularly stable up to 90ºC which is important for various industrial processes.

ACKNOWLEDGEMENTS

This research was supported by a doctoral scholarship to CT-A from the Consejo Nacional de Ciencia y Tecnología (CONCYTEC, Peru) and by grant Nº068-FINCyT-PIBAP-2008 from the National Program of Science and Technology (Peru).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Saxena RC, Adhikari DK, Goyal HB. Biomass-based energy fuel through biochemical routes: A review. Renew Sust Energ Rev. 2009;13(1):167–78.
2. Lin Y, Tanaka S. Ethanol fermentation from biomass resources: Current state and prospects. Appl Microbiol Biotechnol. 2006;69(6):627–42.
3. Menon V, Rao M. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals and biorefinery concept. Prog Energy Combust Sci. 2012;38(4):522-50.
4. National Petroleum Council. Hard Truths: Facing the Hard Truths about Energy. Washington, D.C. National Petroleum Council; 2007.
5. Weber C, Farwick A, Benisch F, Brat D, Dietz H, Subtil T, Boles E. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl Microbiol Biotechnol. 2010;87(4):1303–15.
6. Sánchez C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnol Adv. 2009;27(2):185-94.
7. Kumar R, Singh S, Singh OV. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. J Ind Microbiol Biotechnol. 2008;35(5):377-91.
8. Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F. New improvements for lignocellulosic etanol. Curr Opin Biotechnol. 2009;20(3):372-80.
9. Bainder JB, Raines RT. Fermentable sugars by chemical hydrolysis of biomass. Proc Nat Acad Sci USA. 2010;107(10):4516-21.
10. Zhang Y-HP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol Adv. 2006;24(5):452-81.
11. Wilson DB. Cellulases and biofuels. Curr Opin Biotechnol. 2009;20(3):295-9.
12. Sharma M, Kumar A. Xylanases: An overview. Br Biotechnol J. 2013;3(1):1-28.
13. Mehta D, Satyanarayana T. Diversity of Hot Environments and Thermophilic Microbes. In: Satyanarayana T, Littlechild J, Kawarabayasi Y, editors. Thermophilic Microbes in Environmental and Industrial Biotechnology: Biotechnology of Thermophiles. Dordrecht: Springer; 2013.
14. Kublanov IV, Perevalova AA, Slobodkina GB, Lebedinsky AV, Bidzhieva SK, Kolganova TV, et al. Biodiversity of thermophilic prokaryotes with hydrolytic activities in hot springs of Uzon Caldera, Kamchatka (Russia). Appl Environ Microbiol. 2009;75(1):286–91.
15. Sirisena DM, Maamendra TP. Isolation and characterization of cellulolytic bacteria from decomposing rice straw. J Natn Sci Coun Sri Lanka. 1995;23(1):25-30.

16. Reinsenbach AL, Longnecker K, Kirshtein J. Novel bacterial and archaeal lineages from an *in situ* growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. Appl Environ Microbiol. 2000;66(9):3798–806.
17. Huang X, Madan A. CAP3: A DNA sequence assembly program. Genome Res. 1999;9(8):868-77.
18. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and CLUSTAL X version 2.0. Bioinformatics. 2007;23(21):2947-8.
19. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximu likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-9.
20. King BC, Donnelly MK, Bergstrom GC, Walker LP, Gibson DM. An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts. Biotechnol Bioeng. 2009;102(4):1033-44.
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248–54.
22. Camassola M, Dillon AJP. Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. J Appl Microbiol. 2007;103(6):2196-204.
23. Nogi Y, Takami H, Horikoshi K. Characterization of alkaliphilic *Bacillus* strains used in industry: Proposal of five novel species. Int J Syst Evol Microbiol. 2005;55(6):2309–15.
24. Derekova A, Mandeva R, Kambourova M. Phylogenetic diversity of thermophilic carbohydrate degrading bacilli from Bulgarian hot springs. World J Microbiol Biotechnol. 2008;24(9):1697-702.
25. Lee CC, Kibblewhite-Accinelli RE, Smith MR, Wagschal K, Orts WJ, Wong DWS. Cloning of *Bacillus licheniformis* xylanase gene and characterization of recombinant enzyme. Curr Microbiol. 2008;57(4):301–5.
26. Pakpitcharoen A, Potivejkul K, Kanjanavas P, Areekit S, Chansiri K. Biodiversity of thermotolerant *Bacillus* sp. producing biosurfactants, biocatalysts, and antimicrobial agents. ScienceAsia. 2008;34(4):424–31.
27. Acharya S, Chaudhary A. Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and Bacillus sp. MVS3 isolated from Indian hot springs. Braz Arch Biol Technol. 2012;55(4):497-503.
28. Cho E-A, Lee J-S, Lee K-C, Jung H-C, Pan J-G, Pyun Y-R *Cohnella laeviribosi* sp. nov., isolated from a volcanic pond. Int J Syst Evol Microbiol. 2007;57(12):2902–7.
29. Cho E-A, Lee D-W, Cha Y-H, Lee S-J, Jung H-C, Pan J-G, Pyun Y-R. Characterization of a novel D-Lyxose isomerase from *Cohnella laevoribosii* RI-39 sp. nov. J Bacteriol. 2007;189(5):1655–63.
30. Kim DY, Han MK, Oh H-W, Bae KS, Jeong T-S, Kim SU, et al. Novel intracellular GH10 xylanase from *Cohnella laeviribosi* HY-21: Biocatalytic properties and alterations of substrate specificities by site-directed mutagenesis of Trp residues. Bioresour Technol. 2010;101(22):8814–21.
31. Shiratori H, Tagami Y, Beppu T, Ueda K. *Cohnella fontinalis* sp. nov., A xylanolytic bacterium isolated from fresh water. Int J Syst Evol Microbiol. 2010;60(6):1344–8.
32. Van Dyk JS, Sakka M, Sakka K, Pletschke BI. The cellulolytic and hemi-cellulolytic system of *Bacillus licheniformis* SVD1 and the evidence for production of a large multi-enzyme complex. Enzyme Microb Technol. 2009;45(3):372-8.
33. Rastogi G, Bhalla A, Adhikari A, Bischoff KM, Hughes SR, Christopher LP, Sani RK. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. Bioresour Technol. 2010;101(22):8798–806.

34. Khianngam S, Tanasupawat S, Akaracharanya A, Kim KK, Lee KC, Lee JS. *Cohnella cellulosilytica* sp. nov., isolated from buffalo faeces. Int J Syst Evol Microbiol. 2012;62(8):1921-5.
35. Pason P, Kyu KL, Ratanakhanokchai K. *Paenibacillus curdlanolyticus* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. Appl Environ Microbiol. 2006;72(4):2483-90.
36. Ogawa A, Suzumatsu A, Takizawa S, Kubota H, Sawada K, Hakamada Y, et al. Endoglucanases from *Paenibacillus* spp. form a new clan in glycoside hydrolase family 5. J Biotechnol. 2007;129(3):406-14.
37. Wang C-M, Shyu C-L, Ho S-P, Chiou S-H. Characterization of a novel thermophilic, cellulose-degrading bacterium *Paenibacillus* sp. strain B39. Lett Appl Microbiol. 2008;47(1):46-53.
38. Shi P, Tian J, Yuan T, Liu X, Huang H, Bai Y, et al. *Paenibacillus* sp. strain E18 bifunctional xylanase-glucanase with a single catalytic domain. Appl Environ Microbiol. 2010;76(11):3620-4.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=529&id=32&aid=4579