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Research article

A natural consortium of thermophilic bacteria from Huancarhuaz hot spring (Ancash-Peru) for promising lignocellulose bioconversion

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ABSTRACT

The lignocellulose bioconversion process is an eco-friendly and green-economy alternative technology that allows the reduction of pollution and global warming, so it is necessary for thermophilic and thermostable hydrolytic enzymes from natural sources. This research aimed to isolate cellulolytic and xylanolytic microbial consortia from Huancarhuaz hot spring (Peru) from sludge or in situ baiting cultured with or without sugarcane bagasse. According to the hydrolytic activities consortium T4 from in situ baiting was selected. It was cultivated in submerged fermentation at 65 °C, pH 6.5 for eight days using LB supplemented with sugar cane bagasse (SCB), pine wood sawdust (PWS), CMC, xylan of birchwood, or micro granular cellulose. Crude extract of culture supplemented with SCB (T4B) showed better endoglucanase and xylanase activities with higher activities at 75 °C and pH 6. In these conditions, cellulase activity was kept up to 57% after 1 h of incubation, while xylanase activity was up to 63% after 72 h. Furthermore, this crude extract released reduced sugars from pretreated SCB and PWS. According to metagenomic analysis of 16S rDNA, *Geobacillus* was the predominant genus. It was found thermostable genes: a type of endoglucanase (GH5), an endo-xylanase (GH10), and alkali xylanase (GH10) previously reported in *Geobacillus* sp. strains. Finally, Huancarhuaz hot spring harbors a genetic microbial diversity for lignocellulosic waste bioconversion in high temperatures, and the T4B consortium will be a promising source of novel extreme condition stable enzymes for the saccharification process.

1. Introduction

Lignocellulosic biomass (LCB) is an abundant and renewable source of carbohydrates useful for microbial bioconversion to produce second-generation biofuels, bioproducts, or chemicals [1–3]. Although lignocellulose is the most abundant and cheap raw material, its chemical composition, consisting of hydrophobic fibers of cellulose cross-linked with hemicellulose, lignin, and other polymers, makes it recalcitrant and represents a challenge for biodegradation [3,4]. Thus, enzymatic approaches, such as cellulases and xylanases are

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used for LCB degradation. This process is environmentally friendly and feasible due to its high specificity, not toxic, and no substrate loss [5,6].

Cellulose and hemicellulose are the first and second most abundant polymers on Earth, respectively, they are products of the photosynthesis process. Cellulose, a linear polymer of β-glucose, is broken down into smaller sugars such as cello-oligosaccharides, cellobiose, and glucose by synergic action of cellulases, which according to cleavage allocation are classified as exoglucanase or cellobiohydrolase, endoglucanase, and β-glucosidase ([7,8]; Liu et al., 2021b). Hemicellulose, with xylan as a primary component, is a heterogeneous polymer [9]. It is degraded by synergic action of diverse enzymes, such as endo-β-1,4 xylanases, 1,4-β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases, feruloyl esterases, mannan endo-1,4-β-mannanases, β-1,4-mannosidases, and arabinan endo-1,5-α-L- arabinosidases, which act on specific substrate and chemical-bond [10,11]. LCB bioconversion in nature and artificial biorefineries requires these hydrolytic enzymes, but it is found that some oxidative enzymes and accessory proteins could be involved in this complex process named saccharification [5,12–14]. Due that a single microorganism probably does not have all these enzymes and proteins, microbial consortia from different environments or laboratory combinations have been studied to achieve an optimal cocktail of enzymes to improve LCB saccharification [12,15–17]. Consequently, microorganisms with the ability to break down lignocellulose in the environment can be utilized to improve the conversion of lignocellulosic biomass in various industries, including biofuels, composting, paper, food, pharmaceutical, etc. [6,18,19]. So, thermostable and pH-stable enzymes from thermophilic microorganisms are more suitable than mesophilic enzymes because they thrive in harsh conditions by their ability to withstand harsh protein-denaturing, also, high temperature increases reaction rates, reduces reaction time, and decreases the risk of contamination [1,5,6]. In this sense, cellulases and xylanases from thermophilic bacteria could be used to facilitate the development of low-cost and highly efficient processes for the conversion of lignocellulosic biomass [10]. This offers a solution to meet the growing demand for biofuel, provide bioproducts for industrial applications, and can help in the reduction of CO_2 emissions caused by the burning of lignocellulose waste and the use of fossil fuels [2,16,20].

Thermophile microorganisms can be found in diverse habitats, but extremophile microorganisms from hot springs have shown promising applications in biocatalysts and biotechnology [21]. Some thermophile and hyperthermophile bacteria from hot springs have been studied worldwide due to their ability to produce a variety range of useful substances, including extracellular polymeric substances (EPS), biohydrogen, hydrolytic enzymes, bioremediation enzymes, antibiotics, plant growth-promoting substances, and heavy metal-tolerant compounds [22–27]. However, microbial biodiversity in Peruvian hot springs has not been extensively studied, thus their potential for LCB bioconversion at high temperatures remains unclear. In previous research, only a few thermotolerant cellulolytic and xylanolytic bacteria, mainly *Bacillus* strains, were isolated from Huancarhuaz and Chancos hot springs [28,29]. These bacteria were cultured at 50 °C, which may have hindered the growth of thermophile bacteria, leading to poor microbial diversity of axenic cultures. This research aimed to culture bacterial consortia at 65 °C through direct and in situ enrichment. The goal was to select a thermophilic bacteria consortium cultured with LCB waste that produced a thermostable enzyme cocktail. Hydrolytic activities, molecular taxonomy, and gene mining assessments were performed. These results will support future proteomic and metagenomic research.

2. Materials and methods

2.1. Pretreatment of natural substrates

Sugar cane bagasse (SCB) and pine wood sawdust (PWS) were washed with tap water, and dried for 48-h at room temperature, followed by 4 h at 65 °C. One part of each sample was pretreated with NaOH-thermal methodology described by Tamariz-Angeles et al. [29]. All substrates (pretreated and non-pretreated) were milled and sifted with ASTM N°50 mesh (300 μm).

2.2. Sampling, isolation, and selection of the bacterial consortium

Consortia were sampled from Huancarhuaz hot spring (Huaylas province, Ancash department from Peru, GPS references: latitude −8.9422, longitude −77.7833, and altitude 2758 m). The temperature and pH during the sampling were 70 ± 0.2 °C and pH 7.0 ± 0.2, respectively. Two methodologies for sampling were performed: (i) baiting (B) for in situ enrichment and (ii) sludge (S) for direct sampling. In the first case, four baits containing 1 g of pre-treated SCB [29] were left in the hot spring for 21 days. Next, these baits and sludge (300 ml) were collected, stored separately, and transported to the laboratory into sterile thermos. Each type of sample was homogenized in sterile condition and was inoculated (1 ml of sludge or 1 g approx. baiting) in 50 ml of Basal Salt Medium (BSM, per liter: 11.7 g (NH4)2SO4, 3.7 g KH2PO4, 0.6 g MgSO4.7H2O, 0.8 g CaCl2.2H2O, 0.5 g yeast extract, 0.5 g peptone, 0.5 g FeSO4.7H2O, 0.16 g MnSO4.H2O, 0.14 g ZnSO4.7H2O, 0.37 g CoCl.6H2O) or Luria Broth (LB), which were supplemented with 1% of pre-treated SCB [29]. The samples were incubated for seven days at 65 °C. After, 1 ml was sub-cultured in the respective fresh culture media supplemented with pre-treated SCB (1%), it was repeated three times to obtain established microbial consortia.

The last cultures (2 ml) were centrifuged at 10595 g × 2 min. This supernatant was filtered through a syringe Millipore filter (Ø 0.22 μm) to obtain a free-microorganism crude enzymatic extract (CEE). Their endoglucanase and xylanase activities were evaluated on Agar - Agar (1.5%) supplemented with carboxymethylcellulose (CMC) or xylan of birchwood (0.3%) adjusted at pH 6.5 or 7.8, following Congo Red methodology [29], a semi-quantitative test. Three replicates and negative control with BSM or LB were performed, and hydrolysis was conducted for 4 h at 65 °C. After, coloring with Congo Red (0.1%), the clear zone diameters (hydrolysis halo) were measured to select a better consortium.

2.3. Enzyme production in different carbohydrate sources

According to previous findings, the fresh culture of a selected consortium (250 μl at 1.80 DO_{620nm}) was inoculated into 25 ml of Luria broth (1/5) adjusted at pH 6.5 and supplemented with natural and commercial carbohydrate sources (1%). It included pretreated SCB, pretreated PWS, CMC, xylan of birchwood, or micro granular cellulose. The cultures were incubated at 65 °C without or with shaking (180 rpm) for eight days and sampled at 48-h intervals. The crude enzymatic extracts (CEEs) were evaluated for endoglucanase and xylanase activities at 65 °C, pH 6.5 or 7.8 with Rojo Congo methodology (described previously). Based on these results, CEEs with better performance were selected to quantify their enzymatic activity at 65 °C and pH 6.5 for 2 h following microplate format and 3,5-Dinitrosalicylic Acid (DNS) methodology [28,30]. Each sample had three replicates and was measured twice in the microplate spectrophotometer (Epoch, Biotech, USA). Glucose or xylose was used as standard reducing sugar for cellulase or xylanase activity, respectively, and enzymatic activity per CEE volume was calculated considering that one enzyme unit (U) releases 1 μmol of reducing sugar (glucose or xylose) per minute at specific conditions [28].

2.4. Optimal temperature and pH, and thermal stability

Based on the previous findings, endoglucanase and xylanase activities of CEE with better enzymatic profile were assessed under different temperatures (45–90 °C, 5 °C intervals, at pH 6.5) and pHs (5.0–8.0, 0.5 intervals, at 65 °C) of hydrolytic conditions. Optimal temperature and pH were selected for the thermostability test. Thus, CEE was incubated at 75 °C for 1–12 h (1-h intervals) or 6–72 h (6-h intervals) for endoglucanase or xylanase activity, respectively. Residual activity was measured at optimal temperature and pH determined previously. Hydrolysis activities were quantified by the DNS methodology described in the preceding section (*2.2*).

2.5. Saccharification assay

The saccharification assays were performed in two steps: (i) CEE preparation and (ii) saccharification.

2.5.1. Production, semi-purification, and dilution of CEE

The selected consortium was grown by submerged fermentation (Smf) in the selected carbohydrate source and culture medium. First, the culture (900 ml) was centrifuged at 10 595 g and then filtered through a Millipore membrane (Ø 0.22 mm) and kept in an ice bath. The CEE was concentrated and semi-purified through the Amicon®Ultra-15 (Millipore) Kit following the manufacturer's protocol. Immediately, the total cellulase activity (PFase) was measured according to King et al. [30] where a filter paper disk (7 mm) was the substrate, and the hydrolysis was carried out for 20 h at optimal temperature and pH previously determined. Based on PFase activity, the concentrated-CEE was diluted with phosphate buffer (50 mM, optimal pH selected) at 40, 60, and 80 U ml^{-1} PFase activities. Additionally, the endoglucanase and xylanase activities of each dilution were also measured.

2.5.2. Enzymatic hydrolysis of SCB and PWS

Pretreated and non-pretreated substrates (SCB and PWS) were sieved through mesh ASTM N°100 (150 μm), and saccharification was performed following Kismurtono and Sutikno [31] methodology with some modifications. Each substrate (0.4 g) was mixed with 10 ml of diluted CEE (40, 60, and 80 Uml^{-1} PFase) with three replicates. The process was running at the optimal temperature and pH previously determined also shaking at 180 rpm for 120 h. Endoglucanase and xylanase activities were evaluated at 12-h intervals, and the released reducing sugar was measured by the DNS methodology described by Tamariz-Angeles et al. [28].

2.6. Genetic diversity

2.6.1. Genomic DNA extraction

The genomic DNA of the selected consortium (2 ml) was extracted with ZR soil microbial DNA miniprep Kit (Zymo Research) following the manufacturer's manual.

2.6.2. Consortium composition

The metagenomic 16S rDNA technique was applied to determine the microbial composition. The total amount of genomic DNA was quantified using Qubit™ dsDNA HS assay kit. The library preparation and sequencing were carried out by Novogen Co. following standard protocol. First, 16S rDNA amplicons were evaluated using capillary electrophoresis (Agilent 5400). Next, V3–V4 library targeting with 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers was prepared following the Paired-end method and sequenced by Next Generation Sequencing (NGS) of Illumina technology.

Bioinformatic analysis was carried out in Laboratorio de Micología y Biotecnología (LMB)- Universidad Nacional Agraria La Molina (UNALM). All reads were processed using FastQC software (v.0.12.1), edited and assembled with QIIME2 (v.2023.5.0), and cleaned with ASV. Taxonomy classification was determined using SILVA database (v.138_99_16S) and abundance in each taxa level was calculated.

2.6.3. Cellulase and xylanase genes mining

It was determined the presence of some endoglucanases and xylanases genes based on 16S rDNA metagenomic analysis findings. PCR with specific primer sets was performed with PCR Master mix containing a final concentration of buffer 1X, dNTP 0.2 mmol,

Table 1
Endoglucanase and xylanase semi-quantitative assay of bacteria consortia isolated from Huancarhuaz hot spring.

		Code	Endoglucanase (mm)		Xylanase (mm)	
pH			6.5	7.8	6.5	7.8
Sludge	BSM	T1	–	–	–	–
	LB	T2	2.0 ± 0.4^{d}	2.0 ± 0.4^{d}	–	–
Baiting	BSM	T3	–	3.0 ± 0.4^{c}	–	–
	LB	T4	12.0 ± 0.4^{a}	6.0 ± 0.4^{b}	9.0 ± 0.4^{A}	7.0 ± 0.4^{B}

Values are means of four replicates ± SD of clear hydrolysis halos. Means with a different letter exhibit significant differences according to the Duncan test ($p < 0.05$). LB, Luria Broth; BSM, basal salt medium; (−), negative result.

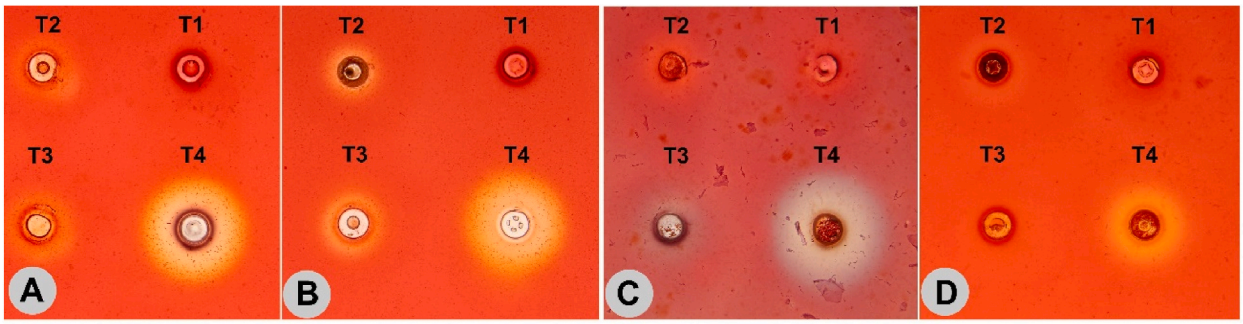

Fig. 1. Enzymatic hydrolysis halos of four microbial consortia. (A–B), endoglucanase activity at pH 6.5 and 7.8, respectively. (C–D), xylanase activity at pH 6.5 and 7.8, respectively.

forward primer 0.1 μmol, reverse primer 0.1 μmol, LongAmp® Hot Start *Taq* DNA Polymerase 0.1 U, and DNA template 0.1 ng. The thermal protocol was performed as initial denaturation at 98 °C × 5 min, followed by 30 cycles: denaturation at 95 °C × 30 s, annealing temperature according to primer sets x 1 min, extension at 65 °C × 1 min, and final extension at 65 °C × 10 min. The amplicons were visualized using agarose gel electrophoresis (1% w/v). Positive results were defined by similar amplicon sizes with references.

2.7. Statistic analyses

All samples were evaluated using three replicates, which were measured twice. It was calculated their mean and standard deviation. The mean comparison was performed with ANOVA and Duncan's test ($p < 0.5$).

3. Results and discussion

3.1. Isolation and selection of the bacterial consortium

The process of isolation microorganisms from environmental samples can be a challenging task due to unknown cultural and nutritional conditions. However, traditional methods using enrichments have been shown to improve the isolation of specific microorganisms, e.g., *in-situ* baiting and *ex-situ* enrichment with SCB increased successfully the number of cellulolytic and xylanolytic thermotolerant bacteria isolated from Huancarhuaz and Chancos hot springs [28,29]. Similarly, in this research, thermophilic bacterial consortium T4 (SCB baiting followed by enriched LB + SCB) showed better endoglucanase and xylanase activity at 6.5 and 7.8 pH. In contrast, T2 (sludge, enriched LB + SCB) and T3 (SCB baiting, enriched BSM + SCB) only exhibited endoglucanase activity, while T1 (sludge, BMS + SCB) did not exhibit any tested activities (Table 1 and Fig. 1A–D). These results suggest that SCB baiting, and enrichment have a positive impact on the selection of consortia with better lignocellulolytic activities.

Besides, it was observed that cellulase and xylanase production was influenced by nitrogen sources [22,32]. Therefore, during the enrichment process, the culture medium (LB or BMS) was assessed. T2 and T4, cultured with tryptone and yeast extract (organic nitrogen source), exhibited better hydrolytic activity than T1 and T3, which were cultured with BSM containing ammonium sulfate as the main nitrogen source (Table 1). Tamariz-Angeles et al. [28] found that *Bacillus subtilis* DCH4 cultured with peptone – during agriculture waste saccharification – showed better endoglucanase and xylanase activity in comparison with ammonium nitrate. Similarly, *Streptomyces* sp. MS-S2 produced more xylanase and cellulase when cultured with yeast extract or tryptone than ammonium sulfate, urea, or sodium nitrate [33]. Sepahy et al. [34] found that ammonium sulfate inhibited xylanase production of *B. mojavensis* AG137 during agriculture waste fermentation.

Table 2
Semi-quantitative hydrolytic activities of consortium T4 from Huancarhuaz hot spring cultured with different substrates.

Enzyme	Substrate	Culture condition	Clear zone or halo (mm)			
			48 h	96 h	144 h	192 h
Endoglucanase	pt-SCB	sh	–	4.5 ± 0.6^{d}	6.5 ± 0.4^{b}	7.1 ± 0.3^{a}
		st	–	–	–	2.0 ± 0.0^{f}
	pt-PWS	sh	–	3.1 ± 0.3^{e}	5.9 ± 0.3^{c}	5.8 ± 0.5^{c}
		st	–	–	1.0 ± 0.0^{g}	1.0 ± 0.0^{g}
Xylanase	Xylan	sh	–	–	3.8 ± 0.5^{g}	5.8 ± 0.5^{e}
		st	–	3.5 ± 0.6^{g}	3.5 ± 0.6^{g}	4.5 ± 0.6^{f}
	pt-SCB	sh	–	11.3 ± 0.5^{b}	13.0 ± 0.0^{a}	8.8 ± 0.5^{d}
		st	–	–	–	–
	pt-PWS	sh	–	$9.0 \pm 0.8^{c,d}$	$9.0 \pm 0.8^{c,d}$	9.3 ± 0.5^{c}
		st	–	–	–	–

(−), negative. Values correspond mean of four replicates ± standard deviation of halos less well diameter. pt-SCB, pre-treated sugar cane bagasse; pt-PWS, pre-treated pine wood sawdust; sh, shaking; st, static. Means with a different letter in each column have significant differences according to the Duncan test ($p < 0.05$).

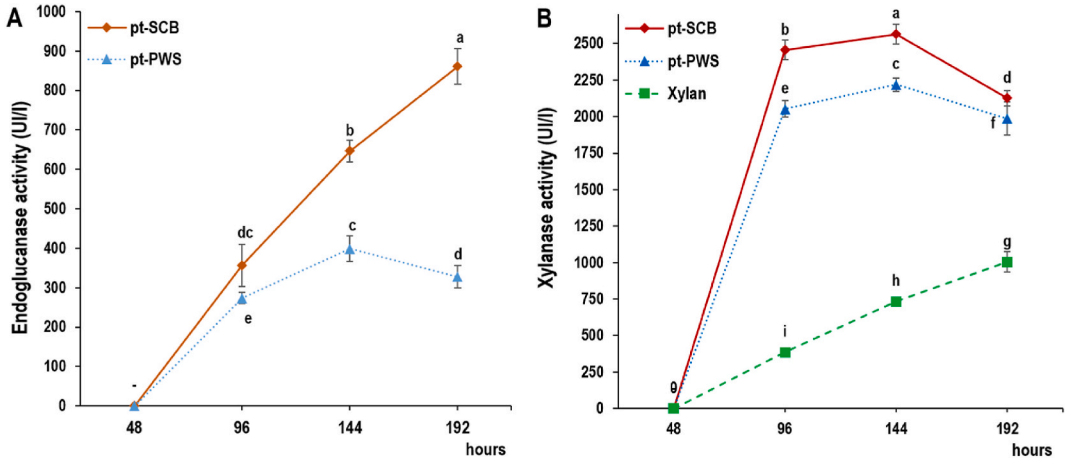

Fig. 2. Endoglucanase and xylanase activities of crude enzyme extract (CEE) from T4 consortium cultured with natural and commercial substrates. A, endoglucanase, and B, xylanase. pt-SCB, pretreated sugar cane bagasse; pt-PWS, pretreated pine wood sawdust. Values are means of six replicates ± DS. According to the Duncan test ($p < 0.05$), means with the same letter are significantly different.

3.2. Cellulase and xylanase production in natural and commercial substrates

Bala and Singh [6] reported that carbohydrate substrates have effects on cellulase and xylanase production and activities. In this sense, cellulase and xylanase production under different substrates (CMC, micro-granular cellulose, xylan of beechwood, pt-SCB, and pt-PWS) were performed by choosing the T4 consortium. Semi-quantitative results showed that static cultures had weak or negative hydrolytic activity, while shaken cultures exhibited higher enzymatic activity (Table 2). It could reveal the presence of aerobic microorganisms in this consortium; also shaking increases contact between microorganisms and substrate improving enzyme production [35].

Moreover, the enzymatic kinetic curves demonstrated that CEEs prepared with natural substrates (SCB, PWS) exhibited better endoglucanase and xylanase activities than CEEs from commercial substrates (Fig. 2A and B). Specifically, CEE-pt-SCB showed high endoglucase activity (860.8 U l^{-1} at 8 days) and xylanase activity (2564.9 U l^{-1} at 6 days) followed by CEE-pt-PWS (398 ± 45 U l^{-1} and 2218 ± 42 U l^{-1}, respectively), compared with CEE-xylan-birchwood (xylanase = 1005 ± 68 U l^{-1}). These results are in concordance with Liu et al. [36], who identified the substrate as a main factor affecting hydrolytic enzyme yield.

3.3. Optimal pH and temperature, and thermal stability

Based on previous findings, T4 was cultured with pretreated-SCB (T4B) for 6 days to determine: optimal pH and temperature, and thermal stability of its CEE. Typically, thermophile cellulases and xylanases exhibited optimal pH ranges between 4.5 and 9.0, while the optimal temperature ranges are between 50 and 80 °C [6]. The CEE-T4B achieved its highest endoglucanase and xylanase activities at pH 6, endoglucanase remained 67.3 ± 3.7 % of its residual activity at pH 5 (Fig. 3A), while xylanase activity was stable through pH 5–8 with residual activities above 85% at this range (Fig. 3B). Furthermore, both enzymatic activities showed two peaks between 45

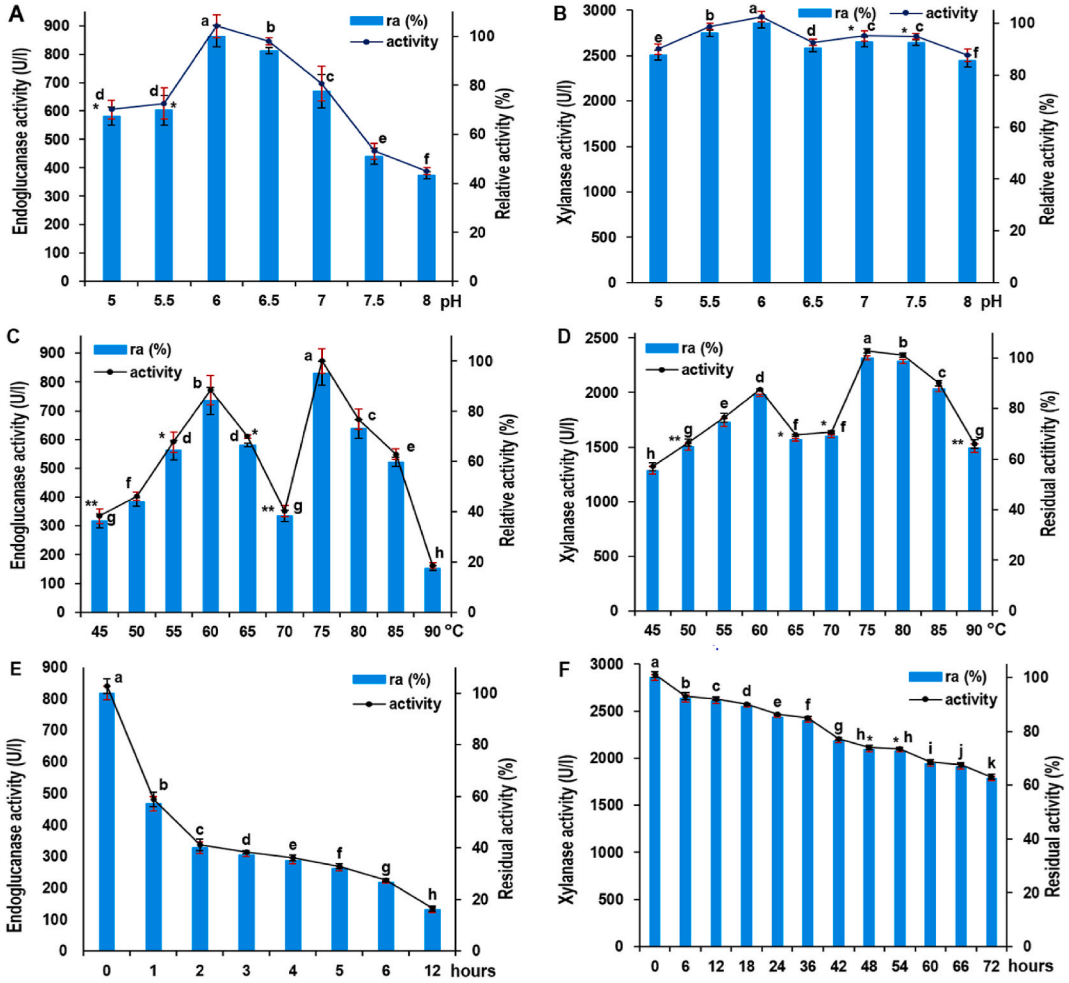

Fig. 3. Temperature, pH, and thermal stability of crude enzyme extract. A and B, endoglucanase and xylanase activities at different pH, respectively, at 75 °C. C and D, endoglucanase and xylanase activities at different temperatures, respectively, at pH 6.5. E and F, endoglucanase and xylanase thermostability, respectively, at pH 6 and 75 °C. ra, residual activity. Values are means of six replicates ± DS and the mean with aster did not have a significant difference according to the Duncan test ($p < 0.05$).

and 90 °C, one at 60 and other at 75 °C. For endoglucanase, they were 734 ± 48 Ul^{-1} (60 °C) and 830 ± 41 Ul^{-1} (75 °C), which remained 89 ± 6 and 100 ± 5 % of relative endoglucanase activity (Fig. 3C). Similarly, for xylanase they were 2026 ± 12 Ul^{-1} (60 °C) and 2379 ± 41 Ul^{-1} (75 °C) corresponding to 85 ± 1 and 100 ± 5% of relative activity (Fig. 3D). Variety of lignocellulolytic enzymes was described [36], and due that T4 is a bacterial consortium these hydrolytic peaks could be associated to its bacterial diversity.

Thermostable enzymes with optimum temperatures above 50 °C can be used for lignocellulose bioconversion via simultaneous saccharification and fermentation even with untreated substrate [10]. However, high-temperature bioconversion is limited or negatively affected by protein denaturalization [37]. T4 consortium exhibited thermophile endoglucanase and xylanase evidenced by its optimal temperature; it is because highly thermostable proteins of microorganisms from extreme habitats have adapted to these conditions [5]. In this sense, the thermostability at 75 °C was assessed considering like hydrolysis condition: 75 °C and pH 6 (optimal for both activities). It was found that T4B-endoglucanase activity remained 57% after 60 min of incubation (Fig. 3E). *Conhella laeviribosi* EHB4, a thermophile bacteria isolated from Huancarhuaz hot spring, maintained 60% of residual endoglucanase activity after substrate-free incubation at 70 °C for 60 min [29]. Furthermore, xylanases with longer enzymatic activity are highly valued for the LCB process [38]. T4B-xylanases exhibited high thermostability retaining 90, 75, and 62% of residual activity after 6, 42, and 72 h of incubation at 75 °C, respectively (Fig. 3F). Similarly, some studies have reported some high thermostable xylanases from thermophilic bacteria, such as, *Geobacillus* sp. WSUCF1 xylanase retained 50% of its activity after being incubated at 60 °C for 18 days and 70 °C for 12 days [38]. An endo-β-1,4-xylanase from *Geobacillus* sp. WBI maintained 100% of its activity after incubation at 65 °C and pH 10 for 1 h, while 75% at 65 °C and pH 11 [39]. *G. thermodenitrificans* JK1 free-cellulase xylanase retained 84% of residual activity after incubation at 55 °C for 60 min [40], and its high homolog enzyme of *G. thermodenitrificans* AK43 kept up more than 90% activity after being incubated at 70 °C for 200 min [41]. Besides, function-structural analysis of diverse glucosidases determined that enzyme stability primarily is related to the type of protein fold [42], also Ajeje et al. [5] found that even minor modifications in xylanase

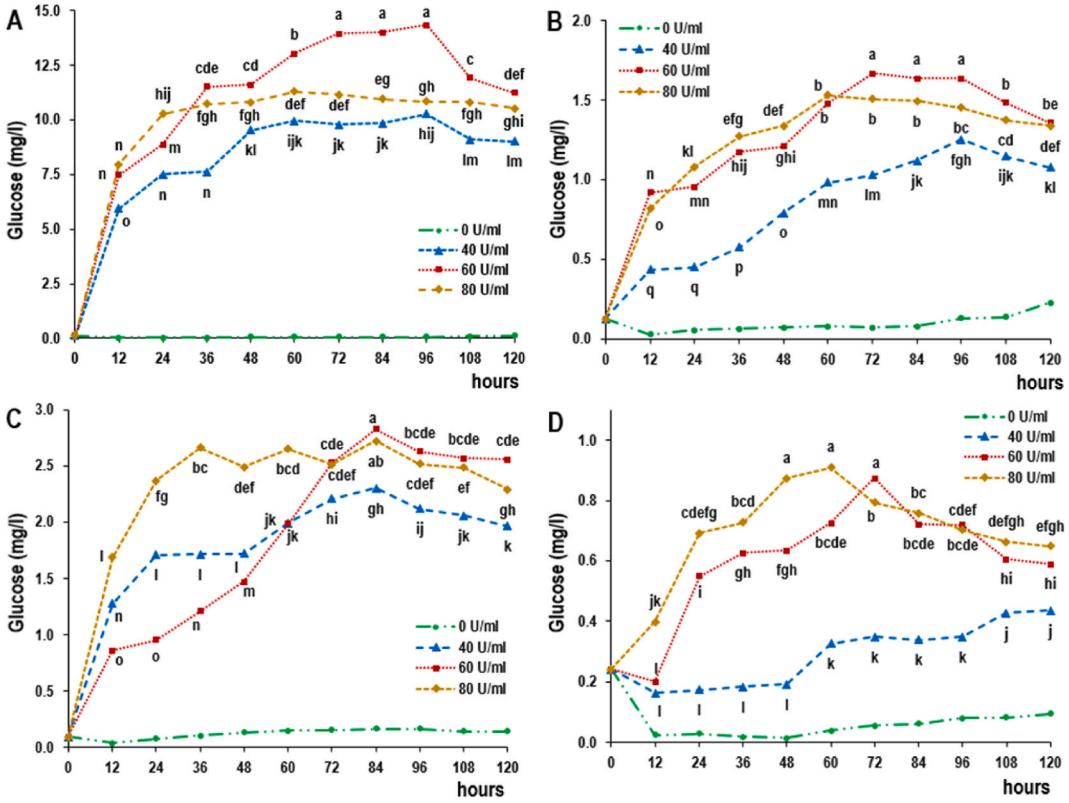

Fig. 4. Saccharification of sugar cane bagasse (SCB) and pine wood (PWS) using crude enzyme extracts of T4 cultivated with pt-SCB. A and C, pretreated SCB and PWS, respectively. B and D, SCB and PWS without pretreating, respectively. 0, control without enzyme; 40, 60, 80 U ml^{-1} of FPase of dilution. Values are means of six replicates ± DS and the means with the same letter did not have significant differences according to the Duncan test ($p < 0.05$).

Table 3
Enzymatic activities of crude enzyme extract used for saccharification.

PFase (Uml^{-1})	Endoglucanase (Uml^{-1})	Xylanase (Uml^{-1})
40.0 ± 2.0	205.0 ± 5.2	1120.0 ± 15.5
60.0 ± 3.1	307.5 ± 7.8	1680.0 ± 23.3
80.0 ± 4.1	410.0 ± 10.8	2240.0 ± 32.2

Values are the mean of six replicates ± DS.

sequence alignment by mutagenesis could enhance their temperature stability.

3.4. Saccharification assay

Renewable sources of fermentable carbohydrates – including agricultural, forestry, and office waste – can be used to produce lignocellulolytic enzymes suitable for biotechnological applications, such as biofuel production, through enzymatic degradation or saccharification. In this study, SCB and PWS pretreated and non-pretreated were incubated with CEE-T4B at 75 °C and pH 6, and released sugar was measured as LCB bioconversion evidence (Fig. 4A–D). Three different CEE-T4B dilutions were assessed (Table 3), which 60 and 80 U ml^{-1} PFase dilutions released more reduced sugar. The pretreated substrates were better hydrolyzed than non-pretreated, also PWS was scarcely attacked probably by their recalcitrant composition and structure. Similarly, Normark et al. [43] found that pretreated Scot pine was more susceptible to enzymatic saccharification than non-pretreated; because pretreatment increases the availability of cellulose and hemicellulose to enzymatic hydrolysis by removing other recalcitrant molecules [44]. Despite CEE-T4B saccharified SCB and PWS, compared to enzyme extracts from some thermophile *Bacillus* species [16] the released sugars were lower may be due to numerous factors, such as enzyme concentration, enzyme cocktail composition, substrate composition, particle size, physical conditions, etc., need to be optimized ([45,46]; [47]). Furthermore, thermophilic microorganisms frequently yield fewer hydrolytic enzymes compared to mesophiles [6]. In this sense, T4B hydrolytic traits suggest continuing with genetic mining because thermophile microorganisms from hot springs can survive and grow in extreme conditions due to their adapted and highly

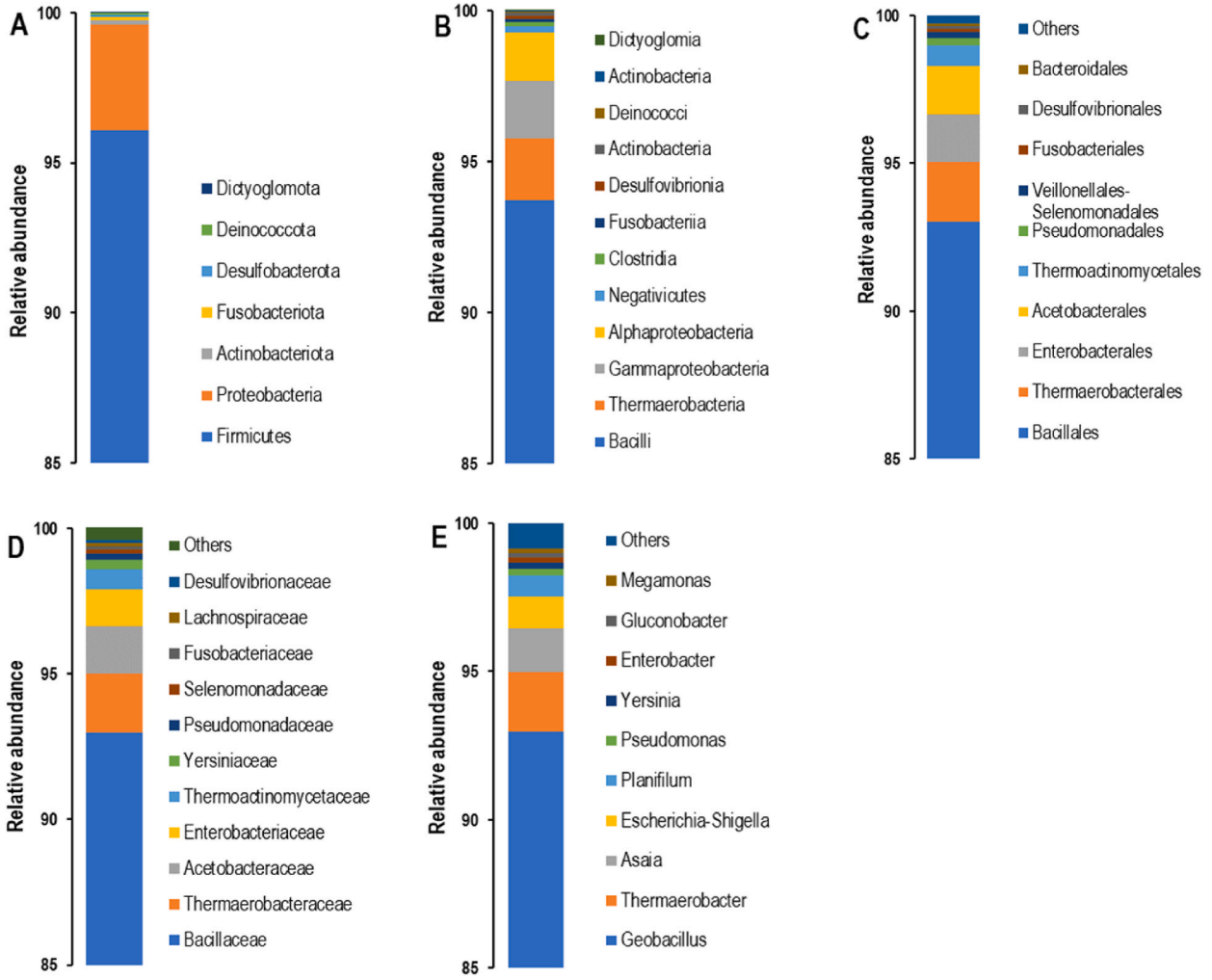

Fig. 5. Taxonomy of OTUs from metagenomic analysis of consortium T4B. **A**, Phylum. **B**, Class. **C**, Order. **D.** Family. **E,** Genus. 10 most abundant of each level taxa.

active enzymes, being important and suitable sources of industrial enzymes for biotechnological application [21,48,49].

3.5. Genetic diversity

Metagenomics studies frequently have three major purposes, which include understanding microbial communities, harnessing the functional potential of microbiota, and discovering novel genes [49]. The microbial taxonomy of T4B, metagenomic 16S rDNA analysis reached 146976 free-chimera sequences or amplicon sequence variants (ASV) with an efficiency rate of 79.41 % (Table S2). These edited sequences were grouped into 74 operational taxonomic units (OTUs) belonging to seven phyla, where Firmicutes was predominant accounting for 96.1% of the total sequences (Fig. 5A). Bacilli class (93.7%), Bacillaceales (93.0%), Bacillaceae family (93.0%), and *Geobacillus* genus (93.0%) were the most abundant in each taxon (Fig. 5B–E). Similarly, the metagenome of culturable microbial communities from two Indian hot springs (54–65 °C) also presented 96% of Firmicutes [49]. In contrast, Proteobacteria was the most abundant phylum in samples of hot spring water [21,48], which means culturable and non-culturable bacteria composition. These results suggest that cultivation may stimulate the growth of Firmicutes rather than Proteobacteria. So, sampling direct, enriched, and/or cultivable microorganisms metagenome analysis will be performed according to the aim research; considering also that microbial presence is frequently poor in extreme habitats [21]. Additionally, metagenomic studies have shown many non-cultivated bacteria and archaebacteria known as “microbial dark matter”[50], and T4B metagenome showed a weak alignment with non-cultivated bacteria (0.9%). This could suggest that non-cultivated microorganisms may be grown in consortium platforms improving their isolation.

Moreover, in concordance with T4B metagenome analysis, *Geobacillus* was the most abundant genus in this consortium. Some cellulolytic and xylanolytic thermophile strains belonging to this genus have been isolated from hot habitats ([11,29,38]; Irfan et al.,

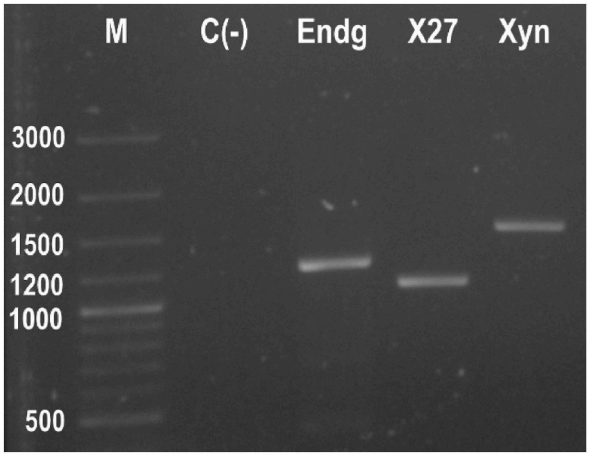

Fig. 6. Cellulase and xylanase gene mining. Endg, Endoglucanase; ×27, free-cellulase xylanase; Xyn, alkali endoglucanase. M, 100bp Plus DNA Ladder (Thermo Scientific™) and C (−), negative control. Agarose gel (1%) with ethidium bromide. The original picture gel is in Fig. S1.

2016; [39,51]). In this sense, the presence of cellulase and xylanase genes reported for some *Geobacillus* strains were prospecting with specific primers, and three amplicons with long near-to reference genes were found (Fig. 6, Table S1, Fig. S1). They were (i) endoglucanase gene (approx. 1300 pb) corresponding to a novel GH5 endoglucanase from *G. thermodenitrificans* [52], (ii) free-cellulase xylanase (×27) gene with approx. 1200 pb reported for *G. thermodenitrificans* [40], and (iii) alkali-thermophile endo-β-1,4-xylanase (Xyn) gen with approx. 1500 pb from *Geobacillus* sp. WB1[39].

4. Conclusion

Huancarhuaz hot spring is an interesting source of thermophile and lignocellulolytic bacteria consortium, where its isolation and selection were improved by *in-situ* baiting and *ex-situ* enrichment, both using sugar cane bagasse. The crude enzymatic extract showed a thermophile cellulase and xylanase profile with LCB saccharification potential. Metagenome taxonomy of cultivated consortium T4B determined that *Geobacillus* genus was predominant, which contains diverse strains reported as thermophile cellulolytic and xylanolytic microorganisms promising to LBC, too. Furthermore, three interesting genes of cellulase and xylanase were detected supporting the hydrolytic activities found previously. Then, research with metagenomic and heterologous platforms to isolate, characterize, and improve the production of these suitable enzymes is recommended due to their high stability under extreme conditions.

CRediT authorship contribution statement

Alberto Castañeda-Barreto: Methodology, Investigation, Formal analysis, Conceptualization. **Percy Olivera-Gonzales:** Supervision, Methodology, Conceptualization. **Carmen Tamariz-Angeles:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27272.

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