Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
ISSN: 1807-863X
actabiol@uem.br
Universidade Estadual de Maringá
Brasil

Rodrigues, Isabela da Silva Vasconcelos; Silva e Silva, Carolina Góes; da Silva, Rafael Salomão; Dolabella, Silvio Santana; Fernandes, Marcelo Ferreira; Fernandes, Roberta Pereira Miranda

Screening of bacterial extracellular xylanase producers with potential for cellulose pulp biobleaching

Acta Scientiarum. Biological Sciences, vol. 41, 2019
Universidade Estadual de Maringá
Brasil

DOI: https://doi.org/10.4025/actascibiolsci.v41i1.42101

Available in: https://www.redalyc.org/articulo.oa?id=187160125017

Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v41i1.42101

Screening of bacterial extracellular xylanase producers with potential for cellulose pulp biobleaching

Isabela da Silva Vasconcelos Rodrigues[1], Carolina Góes Silva e Silva[1], Rafael Salomão da Silva[1], Silvio Santana Dolabella[2], Marcelo Ferreira Fernandes[3] and Roberta Pereira Miranda Fernandes[1*]

[1]Laboratório de Enzimologia, Departamento de Fisiologia, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n, 49100-000, São Cristóvão, Sergipe, Brazil. [2]Departamento de Morfologia, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brazil. [3]Empresa Brasileira de Pesquisa Agropecuária, Aracaju, Sergipe, Brazil. *Author for correspondence. E-mail: robertafernandes@ufs.br

ABSTRACT. In this study, two hundred fifty-seven bacterial isolates from a suppressive soil library were screened to study their secretion of alkali-thermostable xylanases for potential use in cellulose pulp biobleaching. Xylanase activity was evaluated in solid and liquid media using xylan as the carbon source. Isolates were initially evaluated for the degradation of xylan in solid media by the congo red test. Selected strains were evaluated in liquid media for enzymatic activity and determination of total protein concentration using a crude protein extract (CPE). An isolate identified as *Bacillus* species TC-DT13 produced the highest amount of xylanase (1808 U mL^{-1}). The isolate was active and stable at 70°C and pH 9.0, conditions which are necessary for the paper industry. This isolate can grow and produce xylanase in medium containing wheat fiber as a substrate. The CPE of this isolate was used in preliminary testing on cellulose pulp bleaching; enzyme treatment of the pulp resulted in a 5% increase of whiteness.

Keywords: alkali–thermostable enzyme; soil bacteria; biochemical characterization; *Bacillus* spp.

Received on March 20, 2018.
Accepted on February 2, 2019.

Introduction

Cellulose and hemicellulose are carbohydrate polymers and serve as the two major components of lignocellulosic biomass, which is generated through photosynthesis. Cellulose and hemicellulose are located in the cell walls of plants and interlaced through covalent bonds with a non-carbohydrate polymer named lignin (Pérez, Muñoz-Dourado, Rubia, & Martínez, 2002). Xylan is the largest component of hemicellulose and is the second most abundant polysaccharide in the cell walls of terrestrial plants (Thomas, Joseph, Arumugam, & Pandey, 2013a).

A number of enzymes act on the hemicellulose synergistically, leading to complete hydrolysis. Among these, the endo-β-1,4-D-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) are the major enzymes responsible for the hydrolysis of xylan (Juturu & Wu, 2012).

A variety of microorganisms including bacteria, fungi, yeasts and actinomycetes can produce xylan-degrading enzymes. While fungi generally produce a large amount of xylanase, these enzymes produced by bacteria are more stable. Soil bacterial isolates have been investigated due to their potential for xylanase production (Whang et al., 2010).

Xylanases have attracted attention due to their potential applications in various industries, including paper, textiles, and food (Kumar, Dangi, & Shukla, 2018). The interest in xylanase and its application in the paper industry has increased over the years (Thomas, Raveendran, Parameswaran, & Pandey, 2015). In the pulp and paper industries, the use of cellulase-free xylanase for bleaching purposes is better for the environment, as conventional chemical bleaching uses chlorine-based chemicals that are toxic (Saleem, Khurshid, & Ahmed, 2018). The use of xylanase also makes the process more economical and produces fewer toxins compared to conventional chemical bleaching (Beg, Kapoor, Mahajan, & Hoondal, 2001). Xylanases used for this process must be highly active at alkaline pH, and to date only a few enzymes have been reported that meet this standard (Poorna & Prema, 2006). The production of cellulase-free xylanase is very important for application of these enzymes in the paper industry (Yeasmin et al., 2011) because the presence of cellulase may destroy cellulose fibers present in the pulp.

Because xylanase production in bacteria is inducible, the bacteria must be grown in the presence of xylan. The use of pure xylan in growth medium is expensive and is not economically viable in industrial enzymes production. It is therefore necessary to explore less expensive xylan sources for cost-effective production. Agricultural residues may be an acceptable alternative as they are readily available at low cost and can be used to produce enzymes on an industrial scale. These byproducts are not only economically feasible but also provide environmental advantages (Bajaj & Wani 2011).

Widespread biotechnological use of xylanases has prompted researchers to see new sources of these enzymes. In this study, a collection of soil bacteria was screened to identify a producer of high levels of alkali-thermostable xylanase that would serve as a quality source of crude protein extract for use in cellulose pulp biobleaching.

Material and methods

Selection and media composition

For microbial selection, two hundred fifty-seven soil bacterial isolates belonging to the collection of the Soil Microbiology Laboratory (*Embrapa Tabuleiros Costeiros*) were reactivated in yeast mannitol (YMA) media consisting of K_2HPO_4 0.2 g L^{-1}, $MgS0_4$ 0.2 g L^{-1}, mannitol 10.0 g L^{-1}, and yeast extract 0.3 g L^{-1} NaCl 0.05 g L^{-1} and incubated at 32°C at 150 RPM until reaching an optical density (OD) of 0.5. After reaching OD 0.5, an aliquot of 10 µL was inoculated into MSS agar (NH_4NO_3 1%, KH_2PO_4 0.5%, $MgSO_4.7H_2O$ 0.1%, $CaCl_2.2H_2O$ 0.01%, NaCl 0.01% and $MnSO_4.H2O$ 0.01%, agar 2.0% and xylan 0.5%). Agar plates were incubated at 30°C (± 2°C) for seven days. Congo Red 0.1% was added after incubation on the surface of the medium; formation of clear halos was measured to analyze xylanase activity (Sharma & Bajaj, 2005). Isolates that showed an increased degradation of the xylan were selected for further study. The second selection step involved measurement of halos at 1, 2, 3, 5 and 7 days of incubation. Two perpendicular measurements were made for each halo.

Genetic identification of bacteria

Taxonomic affiliations of the bacterial isolates were determined using partial sequences of the 16S rRNA gene. DNA extraction and amplification by PCR using primers 27F and 1488R were performed as previously described (Aderibigbe, Visessanguan, Sumpavapol, & Kongtong, 2011). PCR products were purified using the Wizard® Gel kit PCR Clean-Up System and sent for sequencing at the *Universidade Federal de Pernambuco* – LABCEN/UFPE. The partial 16S rRNA gene sequences were compared to the sequences of bacteria in the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTN program (Altschul et al., 1997).

Enzyme production

The bacterial isolates were inoculated into liquid basal medium composed of beef extract 0.3%, NaCl 0.5%, KNO_3 0.2%, K_2HPO_4 0.1%, $MgSO_4.7H_2O$ 0.05% and xylan 1% as the carbon source (Adhyaru, Bhatt, & Modi, 2013). Isolated colonies were inoculated into 50 mL Erlenmeyer flasks containing 10 mL liquid basal medium with xylan 1.0% and incubated at 32°C/150 RPM, in a shaker Marconi Model MA420, until cultures reached an OD 0.5. Two hundred microliters of this culture were inoculated into flasks containing 20 mL of basal medium with 1.0% xylan and incubated at 32°C/150 RPM for 48 hours. During the incubation period, samples were taken periodically at 6, 12, 24, and 48 hours. Cultures were centrifuged at 14,000 g for 10 min. at 4°C to separate the cells; CPE was maintained at -20°C until the xylanase activity and total protein were analyzed.

Enzyme assay and determination of total protein concentration

The enzymatic activity of the CPE was determined according the 3,5-dinitrosalisylic acid (DNS) method (Miller, 1959). Briefly, reducing sugars formed during incubation of the CPE in the presence of xylan were measured. The reaction mixture containing 90 µL of 100 mM acetate buffer with xylan 1% (pH 6.0) and 10 µL of CPE was incubated at 50°C for 15 min. The reaction was stopped with addition of DNS and heated at 100°C for 5 min. The reaction was quantified colorimetrically (λ 540 nm) using 96-well plates. One unit (U) of xylanase activity was defined as the amount of enzyme required to release 1.0 micromol of xylose per minute. Glucose was used as the standard.

The content of total protein of the CPE was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Enzyme characterization

Optimum temperature, pH and stability

The optimum temperature for xylanase activity of *Bacillus* isolate TC-DT13 was determined by performing the enzymatic assays at a range of temperatures beginning at 20°C and increasing to 80°C, in 10°C increments). The optimal pH was assessed using a pH range of 3.0 to 8.5 (McIlvaine buffer) and pH 9.0 to 10.0 (borate buffer). Enzyme thermostability and pH stability were analyzed by incubating the CPE for 1, 2, 3, and 4 hours at 70°C or in glycine-NaOH buffer (pH 9.0), respectively. Residual xylanolytic activity was determined by DNS as previously described in the enzyme assay section.

Effect of metal ions and EDTA on the activity of xylanase

To investigate the effects of metal ions in the xylanolytic activity, solutions of 10 mM of NH_4Cl, $MgCl_2$, Co^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} or ethylenediaminetetraacetic acid (EDTA) were added. Activity in the absence of metal ions or EDTA was designated as 100%. Residual xylanolytic activity was determined by DNS as previously described in the enzyme assay section.

Demonstration of xylanase induction

To determine whether expression of xylanase was induced or constitutive, the bacterial isolate selected as the major producer of this enzyme was grown in basal medium with xylan or glucose and maltose for 48 hours at 30°C and 150 RPM. Cultures were centrifuged to separate the cells at 14,000 g 10 $min.^{-1}$ $4°C^{-1}$ and the crude protein extract (CPE) maintained at -20°C until the xylanase activity could be analyzed.

Xylanase production using agroindustrial residues

The CPE was obtained as described above by replacing the xylan in the culture medium with agricultural residues: wheat bran, rice bran, oat bran and soy extract. The xylanase activity in the CPE was determined by the DNS method as described in the enzyme assay section.

Cellulase activity

The cellulase activity in the CPE was determined by the method of Schinner and von Mersi (1990), which replaced the xylan with CM-cellulose disodium (7 g L^{-1} acetate buffer 2M, pH 5.5). Samples were read and the standard curve was determined by the DNS (Miller) method as previously described in the enzyme assay section. All experimental work was performed in triplicate and the results are expressed as the mean values.

Biobleaching of paper pulp

Sample cellulose pulp

Unbleached pulp used in bleaching experiments had a Kappa of 17.8, viscosity of 1201 dm^3 kg^{-1} and whiteness of 38% ISO and was obtained as a courtesy of *Indústria Fibria de Aracruz - ES*.

Physical characteristics of the pulp

After the bleaching process, physical-chemical tests were carried out to evaluate brightness (% ISO) using the Kappa or micro-kappa number of the pulp. It is known that the brightness analysis involves numerically quantifying the level of whiteness of the pulp, Kappa or micro-kappa number quantifies the residual lignin present in the pulp.

Color removal of the cellulose pulp

The crude protein extract was used in the bleaching experiments of cellulose pulp using the method of Khandeparkar and Bhosle (2007). CPE buffered in 50 mM glycine-NaOH (pH 9.0) was used at a concentration of 40 U of xylanase per gram of oven-dried pulp. Samples of dried pulp were mixed with diluted enzyme and then incubated at 70°C for 3 hours. The pulp treated with 50 mM glycine-NaOH (pH 9.0) buffer (0 U enzyme) was used as a control. After incubation samples were washed with distilled water, and the absorbance of the filtrate was determined by spectrophotometry at wavelengths ranging from 200 to 400 nm.

Enzymatic pretreatment

A total of 40 U of enzyme was mixed with cellulose pulp at 6% (p v^{-1}) in a polyethylene bag and submerged in water bath at 70°C for 2 hours. After enzyme treatment, cellulose pulp was washed with tap water. Controls were prepared as described above but without the addition of enzyme.

Chemical bleaching

After enzymatic pretreatment, the cellulose pulp was exposed to hypochlorite followed by treatment with hydrogen peroxide. In the first stage of bleaching, the pulp (6% p v^{-1}) was treated with 7% NaOCl and incubated for 1 hour at 70°C. After incubation the pulp was washed with distilled water and treated with 1% hydrogen peroxide and incubated for 1 hour at 70°C. Pulp without enzymatic pretreatment was used as control. The bleached pulp and controls were analyzed to determine the Kappa number, brightness and viscosity at *Industria Fibria de Aracruz - ES*.

Results and discussion

Screening of xylanolitic bacteria

Out of the 257 bacteria isolated from soil, 112 (44%) showed xylanolytic activity on solid media. Among the bacterial strains with qualitative xylanolytic activity, 19 strains (8%) were selected because they displayed larger xylan degradation halos around their colonies when compared with other isolates from the same library (Figure 1).

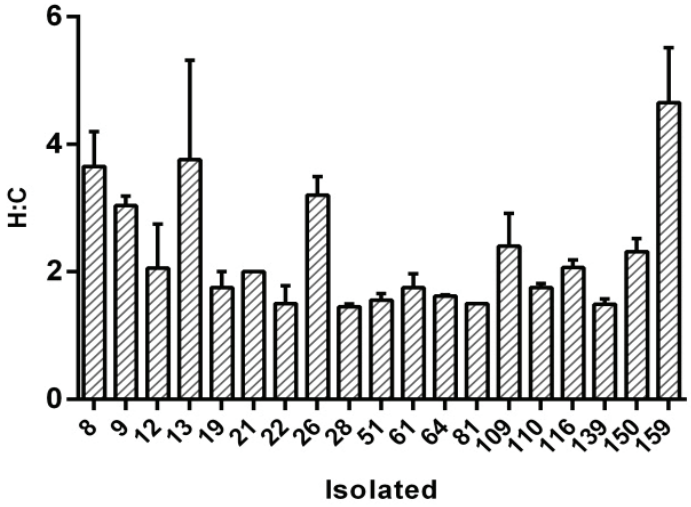

Figure 1. Xylanase activity of bacteria isolates expressed as the ratio of the halo diameters to colony size (cm) (H:C) 7 days after inoculation. Enzyme activity was detected in MSS medium with mineral brine with xylan as sole carbon source. Error bars represent the standard deviation. Experiments were performed in triplicate.

Xylanase production in liquid medium using xylan as the carbon source is shown in Table 1. Most of the isolates showed xylanolytic activity of approximately 15 U mL^{-1}. During screening we identified a very promising strain TC-DT13, which had the highest level of xylanase production approximately 1800 U mL^{-1}. A second isolate TC-DT159 showed similar xylanolytic activity to TC-DT 13 in solid media (Figure 1) did not presented high CPE activity. It may be due to enzyme stability and also by the time of production and activity that in solid media was 7 days and in liquid media 48 hours for enzyme production and 15 min. for the enzymatic assay. Compared to strains described in the literature, this microorganism produced a higher level of xylanase. Duarte, Pellegrino, Portugal, Ponezi, and Franco (2000) reported high activity for four strains of *B. pumilus*, which demonstrated xylanolytic activity of 328, 131, 90 and 167 U mL^{-1}, respectively. In another study, *Bacillus subtilis* BE-91 (Liu et al., 2011) was shown to yield 408 U mL^{-1}, while *Streptomyces* species (Thomas, Sindhu, & Pandey, 2013b) yielded approximately 200 U mL^{-1} of xylanase. Since the isolate TC-DT13 was the best xylanase producer in the present screen, it was chosen for further study. Its capacity to produce xylanases with high activity indicates this isolate could be used to produce xylanases on an industrial scale.

Table 1. Xylanolytic activity of crude protein extract (CPE) and determination of total protein in bacterial strains isolated from soil in their best fermentation times. The experiments were performed in triplicate.

Strain	Xylanolytic activity (U mL^{-1})	Total protein (µg mL^{-1})	Specific activity (µmols $min.^{-1}$ ug of total $protein^{-1}$)	Fermentation time
8	10.18 ± 0.4	67.07 ± 0.73	0.15	48h
9	18.14 ± 7.7	19.93 ± 1.9	0.91	48h
12	40.60 ± 1.4	35.61 ± 0.71	1.14	24h
13	1808.19 ± 49	94.15 ± 4.24	19.21	48h
19	14.96 ± 0.7	75.94 ± 0.7	0.20	48h
21	23.52 ± 0.3	52.56 ± 0.68	0.45	48h
22	16.15 ± 0.9	53.89 ± 0.9	0.30	48h
26	4.79 ± 0.5	46.38 ± 1.5	0.10	48h
28	15.78 ± 1.1	24.98 ± 0.2	0.63	48h
51	17.38 ± 0.8	49.67 ± 3.4	0.35	48h
61	17.46 ± 1.1	38.73 ± 1.5	0.45	48h
64	3.96 ± 0.4	58.1 ± 1.6	0.07	48h
81	16.92 ± 0.5	36.88 ± 0.2	0.46	24h
109	12.75 ± 0.4	42.14 ± 1.9	0.30	48h
110	39.16 ± 1.4	38.41 ± 1.8	1.02	24h
116	108.55 ± 1.5	42.16 ± 2.4	2.57	24h
139	27.48 ± 1.6	55.95 ± 1.1	0.49	48h
150	17.21 ± 1.1	75.76 ± 1.4	0.23	48h
159	6.21 ± 0.9	58.8 ± 1.4	0.11	48h

Genetic identification of bacteria

The identification of sequences and alignments were performed with the GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLASTN program. The 19 isolates selected as the best producers of xylanase were determined to be in the family Bacillaceae (99%) and Paenibacillaceae (1%). The TC-DT 13 strain, which produced the largest amount of xylanase, had high sequence similarities (99%) with two species from the genus *Bacillus*, including *Bacillus pumilus* and *Bacillus safensis* (Table 2).

Table 2. Taxonomic affiliation of bacterial isolates obtained by comparative analyzes of sequences of 16S rRNA gene.

Isolate	Maximum sequence similarity *	Similarity (%)	Affiliate family
8	*Paenibacillus*	99	Paenibacillaceae
9	*Bacillus SP*	99	Bacillaceae
12	*Bacillus subitilis/ Bacillus anyloliquefaciens*	99	Bacillaceae
13	*Bacillus pumilus/ Bacillus safensis*	99	Bacillaceae
19	*Bacillus SP*	99	Bacillaceae
21	*Bacillus subitilis*	99	Bacillaceae
22	*Bacillus SP*	99	Bacillaceae
26	*Bacillus subitilis*	99	Bacillaceae
28	*Bacillus SP*	99	Bacillaceae
51	*Bacillus SP*	99	Bacillaceae
61	*Bacillus SP*	99	Bacillaceae
64	*Bacillus SP*	99	Bacillaceae
81	*Bacillus SP*	99	Bacillaceae
109	*Bacillus SP*	99	Bacillaceae
110	*Bacillus SP*	99	Bacillaceae
116	*Bacillus cereus*	99	Bacillaceae
139	*Bacillus SP*	99	Bacillaceae
150	*Bacillus SP*	99	Bacillaceae
159	*Bacillus SP*	99	Bacillaceae

*Similarity to NCBI data sequencing.

Enzyme characterization

Optimum temperature and stability at 70°C

CPE containing xylanases presented optimal activity at 60°C (Figure 2A) and was stable at 70°C for up to four hours of incubation (Figure 2B). According to these results, xylanase in the crude protein extract of *Bacillus* TC-DT13 can be classified as thermophilic and thermostable.

Regarding enzyme activity, it was observed that xylanases activity remained high until 80°C, with exception of 70°C. In this temperature the activity was about 60% lower than in optimal temperature. This is possible due xylanases isoenzymes present in the CPE that have lower activity at this temperature. Although the activity was lower at 70°C, this activity remained when the CPE was incubated for until 4 hours at this temperature (Figure 2B). Few studies have reported xylanases with activity and stability at high temperatures (Colinns, Gerday, & Feller, 2005). During biobleaching xylanases must be stable at high temperatures and pH (Bocchini, Damiano, Gomes, & Silva, 2003). Most industrial biobleaching processes use temperature as high as 70°C for 4h. Therefore, this xylanase has a suitable profile for application in the paper industry.

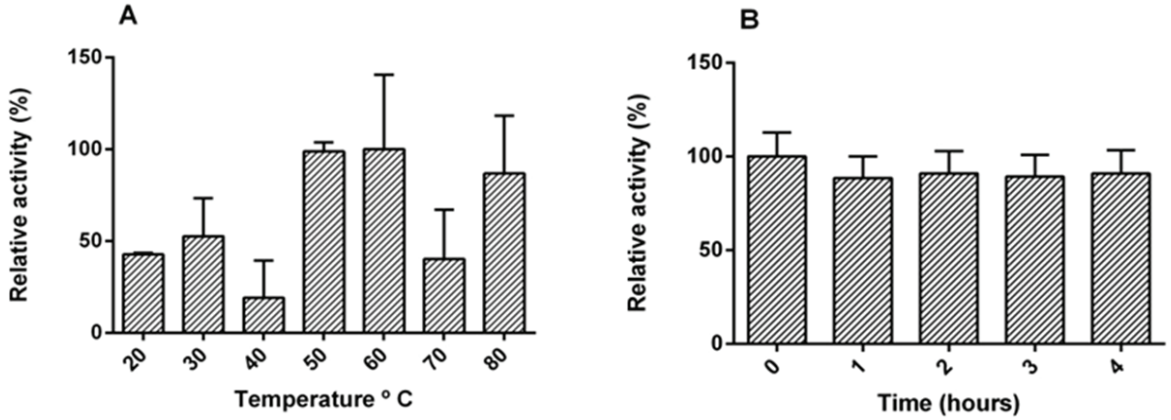

Figure 2. Effect of temperature on activity (A) and stability at 70ºC (B) on the crude protein extract xylanases produced by *Bacillus* species TC-TD 13 after 48 hours of fermentation in medium with xylan for CPE preparation. Relative activity as 100% was expressed as a percentage of the maximum. The experiments were performed in triplicate.

Optimum pH and stability at pH 9.0

Bacillus isolate TC-DT13 CPE showed no xylanase activity in acidic pH (3.2 to 4.4) and exhibited optimal activity at pH 5.6. Enzymatic activity remained stable at approximately 70% of maximum at pH 6.2 to 8.6 (Figure 3A). The observed pH-dependent variations may be due to the presence of several xylan-degrading isoenzymes present in these CPE. Occurrence of multiple xylanases in microorganisms has been reported. *Aspergillus niger* produced five types of endo-xylanase, and *Bacillus subtilis* can secrete three types of xylanase (XynA, XynC and XynD) (Juturu & Wu, 2012; Ko, Han, Shin, Choi, & Song, 2012). The stability of xylanase in CPE was obtained with the incubation in glycine-NaOH buffer (pH 9.0). In the first two hours of incubation, 40% relative activity was observed, and the activity obtained was 80% after three hours of incubation (Figure 3B). Although the TC-DT13 xylanases activity was optimum at pH 5.6, this enzyme has industrial potential due to its stability at pH 9.0 after three hours of incubation.

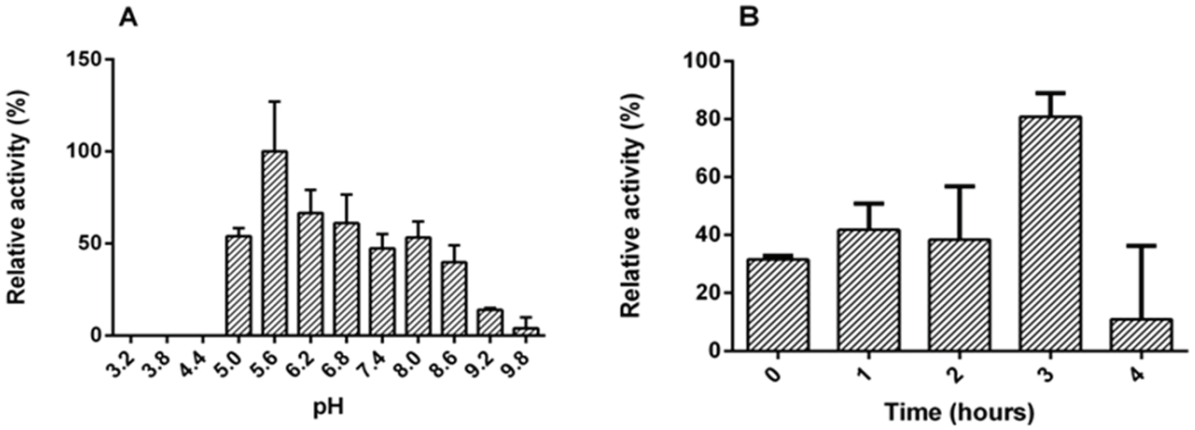

Figure 3. Effect of pH on activity (a) and stability at pH 9.0 (b) of the xylanases from the crude protein extract produced by *Bacillus* sp TC-TD 13 13 after 48 hours of fermentation in medium with xylan for CPE preparation. For stability assays, after incubation xylanase activity was determined at 50ºC and pH 6.0. The maximum activity reaction was considered as 100%. The experiments were performed in triplicate.

Effect of metal ions on the activity

The effects of metal ions on the activity of xylanase were studied using a reaction at 50°C, pH 7.0 for 15 minutes (Table 3). Xylanolytic activity was completely inhibited in the presence 10 mM Cu^{2+} and was decreased to 60% of maximum in the presence of Zn^{2+}. Other metal ions and EDTA had little effect on the enzymatic activity. Inhibition by Cu^{2+} has been previously observed (Yin, Lin, Chiang, & Jiang, 2010; Xu, Liu, Huo, & Dai, 2016) and is possibly due to non-competitive inhibition, where the ion binds to a functional or prosthetic group in the enzyme structure. This data is important in determining which types of ions may or may not be included for industrial processes.

Table 3. Influence of metal ion and reagents for the enzymatic activity of the xylanase from *Bacillus* species CT-DT13.

Additive	Relative activity (%)
Control	100 ± 0.5
Cl-	106 ± 0.2
Mg2+	82 ± 0.2
Co2+	78 ± 0.4
EDTA	77 ± 0.6
Fe2+	76.7 ± 0.5
Zn2+	41 ± 0.8
Cu2+	4 ± 0.1

The relative activities were calculated by taking the activity in the absence of the metals to 100%. The enzyme assay was conducted in triplicate.

Effect of agroindustry wastes on xylanase production by *Bacillus* isolate TC-DT13

The ability of *Bacillus* species isolate TC-DT13 to produce extracellular xylanase was evaluated using different organic residues as carbon sources, such as soybean extract, wheat bran, oat bran, and rice bran; xylan was used as a control (Figure 4). Due to the high costs of associated with cultivation of xylanase-producing organisms, alternatives substrates are of particular interest. Agricultural wastes are low-cost substrates and are often investigated for this purpose. Maximal production of xylanase in CPE was obtained in medium containing agricultural waste of wheat bran 1% (650 U mL^{-1}), followed by oat bran (218 U mL^{-1}). *Bacillus* species isolate TC-DT13 thus can utilize less expensive substrates which induces the production of xylanases. Compared to other studies using this waste, such as those by Tarayre et al. (2013), Guan, Zhao, Zhao, Wang, Huo, Cui, and Jiang (2016) and Ping et al. (2018), the results obtained in this study demonstrated higher xylanase activity at the maximum xylanolitic levels identified in prior the previous studies were 44.3 U mL^{-1}, 52.94 U mL^{-1} and 57.25 U mL^{-1}, respectively, using wheat bran.

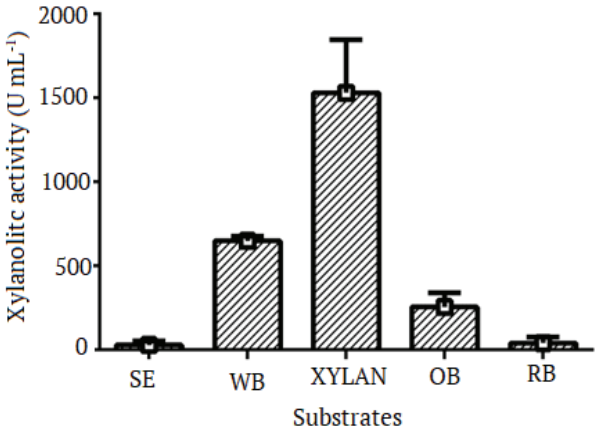

Figure 4. Xylanolytic activity of crude protein extracts from *Bacillus* spTC-DT13 using different substrates such as agricultural residues Soy extract (SE), wheat bran (WB), oat bran (OB) and rice bran (RB), and xylan after 48 hours of fermentation. The experiments were performed in triplicate.

Demonstration of xylanase induction and evaluation of the cellulase activity

CPE produced from media containing glucose and maltose demonstrated about 0.4% activity when compared to CPE produced from xylan containing medium (100% activity). This indicates that xilanase

production in *Bacillus* species TC-DT13 is inducible. Significant proportion of previously-identified microbial xylanases has been inducible in nature (Bajaj, Sharma, & Sharma, 2011).

Bacillus species TC-DT13 CPE showed no cellulase activity, suggesting it can be successfully used in the treatment of cellulose pulp.

Biobleaching of paper pulp

Enzyme pulp pretreatment

According to Khandeparkar and Bhosle (2007), a peak at 280 nm in the ultraviolet spectrum is an indication of lignin release after the enzymatic treatment. In our study, when the pulp was treated with 40 U g^{-1} of dry pulp, it exhibited an absorbance of approximately 0.4 at 280 nm, while the control displayed an absorbance of almost 0.250 nm.

Kappa number of pulp

The Kappa number for untreated pulp was 18.3. After the treatment with TC-DT13 CPE derived xylanases the Kappa number decreased to 17.5, indicating a 5% reduction of residual lignin. This reduction occurred during first enzyme treatment bleaching stage.

In a subsequent experiment, when enzymatically treated pulp was treated with 7% NaOCl, its micro-kappa number was 2.62 and after treatment with 1% H_2O_2 micro-kappa number was reduced further by 1.42, suggesting a reduction of 50% (Table 4).

Table 4. Effect of xylanase treatment on kappa number and brightness of cellulose pulp.

Bleaching state	Brightness (%ISO)	Micro Kappa	Kappa
Untreated	36.4		18.3
X	36.3		17.5
C	84.7	2.20	
CX	84.6	2.62	
CH	82.2	1.38	
CHX	87.5	1.42	

X: Xilanase treatment with 40 U g^{-1} of moisture free pulp (70ºC for 2h; pH 9.0), C: Chlorine treatment with 7% NaOCl (70ºC for 1h), CX: Chlorine treatment with 7% NaOCl on enzyme treated pulp (70°C for 1h), CH: Pulp treatment with 7% NaOCl (70°C for 1h) followed by treatment with 1% H_2O_2 no enzyme (70°C for 1h) CHX: Pulp treated enzymatically followed by treatment with 7% NaOCl (70°C for 1h) and further with 1% H_2O_2.

Pulp brightness

Pulp treated with 40 U of xylanase showed an increase in brightness after each bleaching stage. In the last stage of bleaching, enzyme-pretreated pulp demonstrated a 5% increase in whiteness compared with untreated pulp.

Purified xylanase produced from *Bacillus* and *Streptomyces* species has been previously used to increase paper pulp brightness by 2.5 to 6.5% (Bim & Franco, 2000). The 5% increase observed with crude enzyme in this study indicates that xylanases produced by *Bacillus* species isolate TC-DT13 can be used for industrial applications.

Conclusion

Soil has been proven to contain bacterial isolates that produce xylanolytic enzymes. In this study a *Bacillus* isolate was identified that produced higher amounts of extracellular xylanases. Xylanases produced by *Bacillus* species isolate TC-DT13 have potential for use in industrial biobleaching processes due to their stability and activity at high temperature and basic pH. The crude protein extract from this isolate is also free of cellulase and enhanced the brightness of cellulose pulp by 5.0% compared to untreated pulp.

Acknowledgements

This study was financed in part by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* – Brasil (CNPq), the *Fundação de Apoio à Pesquisa e a Inovação Tecnológica do Estado de Sergipe* (Fapitec/SE) - Brasil, the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - Brasil (CAPES - Finance Code 001), and the *Financiadora de Estudos e Projetos* - Brasil (FINEP).

References

Aderibigbe, E. Y., Visessanguan, W., Sumpavapol, P., & Kongtong, K. (2011). Sourcing starter cultures for *Parkia biglobosa* fermentation I: phylogenic grouping of *Bacillus* species from commercial 'iru' samples. *International Journal for Biotechnology and Molecular Biology Research, 2*(7), 121-127. Recovered from http://www.academicjournals.org/ app/webroot/article/article1382012555_Aderibigbe%20et%20al%20pdf.pdf

Adhyaru, D. N., Bhatt, N. S., & Modi, H. A. (2013). Enhanced production of cellulose-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. *Biocatalysis and Agricultural Biotechnology, 3*(2), 182-190. doi:10.1016/j.bcab.2013.10.003

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402. doi: 10.1093/nar/25.17.3389

Bajaj, B. K., & Wani, M. A. (2011). Enhanced phytase production from *Nocardia* sp. MB 36 using agro-residues as substrates: potential application for animal feed production. *Engineering in Life Science, 11*(6), 620-628. doi: 10.1002/elsc.201100039

Bajaj, B. K., Sharma, M., & Sharma, S. (2011). Alkalistable endo-β-1, 4-xylanase production from a newly isolated alkalitolerant *Penicillium* sp. SS1 using agro-residues. *3 Biotech, 1*(2), 83-90. doi: 10.1007/s13205-011-0009-5

Beg, Q. K., Kapoor, M., Mahajan, L., & Hoondal, G. S. (2001). Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology, 56*(3-4), 326-338. doi 10.1007/s00253 0100704

Bim, A., & Franco, T. (2000). Extraction in aqueous two phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching. *Journal of chromatography. B, Biomedical Sciences and Applications, 743*(1-2), 349-356. doi: 10.1016/S0378-4347(00)00223-1

Bocchini, D. A., Damiano, V. B., Gomes, E., & Silva, R. (2003). Effect of *Bacillus circulans* D1 thermostable xylanase on biobleaching of eucalyptus kraft pulp. *Applied Biochemistry and Biotechnology, 106*(1-3), 393-401. doi: 10.1385/ABAB:106:1-3:393

Bradford, M. M. (1976). A Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254. doi: 10.1016/0003-2697(76)90527-3

Colinns, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews, 29*(1), 3-23. doi: 10.1016/j.femsre.2004.06.005

Duarte, M. C. T., Pellegrino, A. C. A., Portugal, E. P., Ponezi, A. N., & Franco, T. T. (2000) Characterization of alkaline xylanases from *Bacillus pumilus*. *Brazilian Journal of Microbiology, 31*(2), 90-94. doi: 10.1590/S1517-83822000000200005

Guan, G-Q., Zhao, P-X., Zhao, J., Wang, M-J., Huo, S-H., Cui, F-J., & Jiang, J-X. (2016). Production and partial characterization of an alkaline xylanase from a novel fungus *Cladosporium oxysporum*. *BioMed Research International, 2016*(4575024), 1-7. doi: 10.1155/2016/4575024

Juturu, V., & Wu, J. C. (2012). Microbial xylanases: Engineering, production and industrial applications. *Biotechnology Advances, 30*(6), 1219-1227. doi: 10.1016/j.biotechadv.2011.11.006

Khandeparkar, R., & Bhosle, N. B. (2007). Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of kraft pulp. *Bioresource Technology, 98*(4), 897-903. doi: 10.1016/j.biortech.2006.02.037

Kumar, V., Dangi, A. K., & Shukla, P. (2018). Engineering thermostable microbial xylanases toward its industrial applications. *Molecular Biotechnology, 60*(3), 226-235. doi: 10.1007/s12033-018-0059

Ko, K. C., Han, Y., Shin, B. S., Choi, J. H., & Song, J.J. (2012). A Rapid and simple method for preparing an insoluble substrate for screening of microbial xylanase. *Applied Biochemistry Biotechnology, 167*(4), 677-684. doi: 10.1007/s12010-012-9722-8

Liu, Z., Daí, X., Zhang, J., Xu, J., Duan, S., Zheng, K., ... Shi, J. (2011). Screening of xylanase high-producing strain and its rapid separation and purification. *Annals of Microbiology, 61*(4), 901-906. doi: 10.1007/s13213-011-0212-y

Miller, G. L. (1959). Use of dinitrosalicilic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426-428. doi: 10.1021/ac60147a030

Pérez, J., Muñoz-Dourado, J., Rubia, T. de la, & Martínez, E. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology, 5*(2), 53-63. doi: 10.1007/s10123-002-0062-3

Ping, L., Wang, M., Yuan, X., Cui, F., Huang, D., Sun, W., ... Wang, H. (2018) Production and characterization of a novel acidophilic and thermostable xylanase from *Thermoascus aurantiacu*. *International Journal of Biological Macromolecules, 109*(1), 1270-1279. doi: 10.1016/j.ijbiomac.2017.11.130

Poorna, C. A., & Prema, P. (2006). Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. *Biochemical Engineering Journal, 32*(2), 106-112. doi: 10.1016/j.bej.2006.09.016

Saleem, R., Khurshid, M., & Ahmed, S. (2018). Laccases, Manganese peroxidases and xylanases used for the bio-bleaching of paper pulp: an environmental friendly approach. *Protein & Peptide Letters, 25*(2), 180-186. doi: 10.2174/0929866525666180122100133

Schinner, F., & Von Mersi, W. (1990). Xylanase, CM-celulase and invertase activity in soil: An improved method. *Soil Biology and Biochemistry, 22*(4), 511-515. doi: 10.1016/0038-0717(90)90187-5

Sharma, P., & Bajaj, B. K. (2005). Production and partial characterization of alkali-tolerant xylanase from an alkalophilic *Streptomyces* sp. CD3. *Journal of Scientific & Industrial Research, 64*(1), 688-697. Recovered from http://nopr.niscair.res.in/bitstream/123456789/5117/1/JSIR%2064(9)%20688-697.pdf

Tarayre, C., Brognaux, A., Brasseur, C., Bauwens, J., Millet, C., Mattéotti, C., ... Thonart, P. (2013). Isolation and cultivation of xylanolytic *Bacillus subtilis* extracted from the gut of the termite *Reticuliter messantonensis*. *Applied Biochemistry Biotechnology, 171*(1), 225-245. doi: 10.1007/s11356-014-3681-2

Thomas, L., Sindhu, R., & Pandey, A. (2013b). Identification and characterization of a highly alkaline and thermotolerant novel xylanase from *Streptomyces* sp. *Biology Section Cellular and Molecular Biology*, *68*(6), 1022-1027. doi: 10.2478/s11756-013-0248-5

Thomas, L., Joseph, A., Arumugam, M., & Pandey, A. (2013a). Production, purification, characterization and over-expression of xylanases from actinomycetes. *Indian Journal Experimental Biology, 51*(11), 875-884. PMID: 24416921

Thomas, J., Raveendran, S., Parameswaran, B., & Pandey, A. (2015). Production of an alkaline xylanase from recombinant *Kluyveromyces lactis* (KY1) by submerged fermentation and its application in bio-bleaching. *Biochemical Engineering Journal, 102*(1), 24-30. doi: 10.1016/j.bej.2015.02.008

Whang, G., Wang, Y., Yang, P., Luo, H., Huang, H., Shi, P., ... Yao, B. (2010). Molecular detection and diversity of xylanase genes in alpine tundra soil. *Applied Microbiology and Biotechnology, 87*(4), 1383-1393. doi: 10.1007/s00253-010-2564-9

Xu, X., Liu, M., Huo, W., & Dai, X. (2016). Obtaining a mutant *Bacillus amyloliquefaciens* xylanase A with improved catalytic activity by directed evolution. *Enzyme and Microbial Technology, 86*(1), 59-66. doi: 10.1016/j.enzmictec.2016.02.001

Yeasmin, S., Kim, C. H., Park, H. J., Sheikh, M. I., Lee, J. Y., Kim, J. W., ... Kim, S. H. (2011). Cell surface display of cellulase activity-free xylanase enzyme on *Saccharomyces cerevisiae* EBY100. *Applied Biochemistry Biotechnology, 164*(3), 294-304. doi: 10.1007/s12010-010-9135-5

Yin, L. J., Lin, H. H., Chiang, Y. I., & Jiang, S. T. (2010). Bioproperties and purification of xylanase from *Bacillus* sp. YJ6. *Journal of Agricultural and Food Chemistry, 58*(1), 557-562. doi: 10.1021/jf902777r