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An Improved Protocol for DNA Extraction from Alkaline Soil and Sediment Samples for Constructing Metagenomic Libraries

Digvijay Verma · T. Satyanarayana

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Abstract An improved single-step protocol has been developed for extracting pure community humic substance-free DNA from alkaline soils and sediments. The method is based on direct cell lysis in the presence of powdered activated charcoal and polyvinylpolypyrrolidone followed by precipitation with polyethyleneglycol and isopropanol. The strategy allows simultaneous isolation and purification of DNA while minimizing the loss of DNA with respect to other available protocols for metagenomic DNA extraction. Moreover, the purity levels are significant, which are difficult to attain with any of the methods reported in the literature for DNA extraction from soils. The DNA thus extracted was free from humic substances and, therefore, could be processed for restriction digestion, PCR amplification as well as for the construction of metagenomic libraries.

Keywords Humic substances · Metagenomic DNA · Polyvinylpolypyrrolidone · Polyethylene glycol 8000 · Powdered activated charcoal

Introduction

The phenomenon of "great plate anomaly" employs traditional methods that allow a mere 0.01% to 0.1% of microbial diversity to grow on plates [1, 2]. More than 99% are, therefore, left out of the microbiologists' access. Several methods have been developed to touch the huge proportion of $4–6\times10^{30}$, an estimate of different prokaryotic cells in the environment [3]. Due to improper synchronization of various significant physiochemical parameters such as temperature, pH, O_2 concentration, salinity, and redox potential, the huge figure of prokaryotic cells are almost inaccessible till date. The concept of direct cloning of community DNA helps in overcoming this problem, and thus, the era of metagenomics began [4]. Since then, the culture-dependent approaches have been budging to an uncultured approach (metagenomics). This innovative technology has unlocked the new possibilities for facilitating unprecedented analyses of genome heterogeneity and the

D. Verma · T. Satyanarayana (✉)
Department of Microbiology, University of Delhi South Campus, New Delhi 110 021, India
e-mail: tsnarayana@gmail.com

evolution in the microbial world and almost changed the scenario of microbes from those that flourished on petri dishes. The advances are being made to establish this technology involving three major steps: isolation of metagenomic DNA, library construction, and screening of transformants. The isolation of community DNA is a very decisive step that demands superior quality, non-sheared and high molecular weight DNA because further steps of metagenomics decidedly rely on it. Metagenomic libraries have been exploited for studying microbial diversity as well as to access the functional genes for useful microbial products [5, 6]. High molecular weight and non-sheared DNA is mandatory for constructing libraries especially in lambda phage, cosmid, fosmid, and BAC vectors that demand big inserts up to 40 to 60 kb, which is beneficial in studying the entire metabolic pathways and operons. Fosmid-, cosmid-, and BAC-based libraries are more prone to get big fragments of DNA, and thus, success rate is high with these vectors. Mac Neil et al. [7] constructed a BAC-based metagenomic library in *Escherichia coli* and obtained several small molecules, which showed resemblance with indirubin. Such heterologous cloning has also been exploited for accessing novel genes encoding enzymes, vitamins, and other microbial products from various environmental samples through activity as well as sequence-driven approaches of metagenomics [8–11]. The problem with soil metagenomics is due to humic substances, which co-precipitate with DNA and interfere with its downstream processing [12]. Humic substances chelate Mg^{2+} ions during PCR, and therefore, indirectly affect the activity of Taq DNA polymerase [13]. The presence of minute quantities of humic compounds significantly affects restriction digestion, amplification, and transformation efficiencies by binding with the enzymes used at various steps [14, 15]. Several methods have been developed for extracting metagenomic DNA from a variety of soil samples [13, 16–22]. These methods, however, require further purification of the DNA to cope with the problems associated with polyphenolics and humic substances that lead to the loss of DNA.

The very low level of microbial biomass is the characteristic of alkaline soils/sediments that results in low DNA yield, which is further reduced by the additional purification steps. In this investigation, an improved single-step protocol has, therefore, been developed for the simultaneous extraction and purification of community DNA from various alkaline soils and sediments. This protocol aids in overcoming the problems associated with DNA extraction from alkaline soil/sediment samples.

Materials and Methods

Collection of Alkaline Soil/Sediment Samples

Alkaline soil and sediment samples were collected from different regions of India [Century Paper Mills, Lal Kuan (Uttaranchal), Lonar Lake and Dhule (Maharastra), Sambhar Lake and Pachpadra salt lakes (Rajasthan), and Tarna, Basti (Uttar Pradesh)] in sterile polyethylene bags/tubes and stored at 4 °C. The pH values of the soil and sediment samples were in alkaline range (7.6–11).

Protocol for Optimization of Humic Acid-Free DNA from Alkaline Soils

Soil (1 g) was suspended with 0.4 g of activated charcoal [23] and 20 μL of proteinase K (10 mg mL^{-1}) in 2 mL of modified extraction buffer [*N*,*N*,*N*,*N*-cetyltrimethylammonium bromide 1%, polyvinylpolypyrrolidone (PVPP) 2%, 1.5 M NaCl, 100 mM EDTA, 0.1 M

TE buffer (pH 8.0), 0.1 M sodium phosphate buffer (pH 8.0), and 100 μL RNase A] [24] in 20-mL centrifuge tubes to homogenize the sample and was incubated at 37 °C for 15 min in an incubator shaker at 200 rpm. Subsequently, 200 μL of 10% SDS was added to the homogenate and was kept at 60 °C for 2 h with intermittent shaking. DNA was precipitated by adding 1.0 mL PEG 8000 (30% in 1.6 M NaCl) and left at room temperature for an hour [25]. The precipitated DNA was collected by centrifugation at 8,000×g at 4 °C. The supernatant was discarded, and the pellet was dissolved in 1 mL of TE buffer (pH 8.0) and then100 μL of 5 M potassium acetate was added and incubated at 4 °C for 15 min. The supernatant was collected after centrifugation at 8,000×g and treated with equal volumes of phenol/chloroform (1:1) followed by chloroform/isoamyl alcohol (24:1) at 8,000×g for 15 min. The aqueous layer was transferred to another Eppendorf and treated with 0.7 volume of isopropanol for 1 h at room temperature. The DNA was sedimented by centrifugation at 8,000×g for 20 min at 4 °C. The pellet was washed with 1 mL of 70% (*v*/*v*) ethanol and dried at room temperature. The dried pellet was dissolved in 100 μL of sterile Milli Q water. The DNA was also extracted without the addition of activated charcoal as control. Finally, metagenomic DNA was quantitated, visualized, and analyzed by restriction digestion and PCR amplification.

Commercial Kits

Alternatively, DNA was also extracted from the alkaline soil samples by using different commercial kits (UltraClean™, PowerSoil™ [Mo Bio Laboratories Inc., Carlsbad, CA, USA], Nucleospin kit [Macherey-Nagal, Germany], and Zymo soil DNA isolation kit [CA, USA]). The DNA was finally suspended in 100 μL of sterile Milli Q water for further analysis.

Comparison of Yield and Purity of Crude DNA

The soil DNA from Pantnagar and Lonar soil samples were also extracted by various manual methods as well as commercially available soil DNA isolation kits according to the manufacturer's protocols and compared in terms of yield and purity with the metagenome isolated using the protocol developed in this investigation.

Validation of Metagenome Obtained Through Developed Protocol

Restriction Digestion

To further validate the purity of the extracted metagenomes, the DNA was subjected to restriction digestion. The DNA was digested with Sau3AI (New England Biolabs). One microgram of metagenomic DNA in 20 μL of reaction mixture was treated with 1 U of Sau3AI and incubated at 37 °C for 10 min. The reaction was terminated at 80 °C for 20 min, and the digested DNA was fractionated on 1.2% (*w*/*v*) agarose gel.

PCR Amplification of Microbial Population

Attempts have been made to amplify the consensus regions of bacterial-, archeal-, and fungal-specific regions by using the respective sets of primers (Table 1). The reactions were carried out in 50-μL reaction mixtures in a Thermal Cycler (Bio-Rad, USA). The optimized PCR conditions were: for bacterial 16S rDNA, initial denaturation of 3 min at 94 °C followed by 30 cycles of 30 s at 93 °C, 60 s at 55 °C, and 90 s at 72 °C; archaeal 16S

Table 1 The primer sets used in this investigation

Type	Primer sequences	References
Bacterial 16S rDNA	F 5′ AGAGTTTGTCCTGGCTCA 3′ R 5′ GCTCGTTGCGGGACTTAACC 3′	[2]
Archaeal 16S rDNA	F 5′ GGCCCTAYGGGGYGCASCAGG 3′ R 5′ GTGTGTGCAAGGAGCAGGGAC 3′	[46]
Fungal ITS	ITS1 5′ TCCGTAGGTGAACCTGCGG 3′ ITS4 5′TCCTCCGCTTATTGATATGC 3	[47]

rDNA, 5 min at 95 °C, 35 cycles of 50 s at 94 °C, 60 s at 62 °C, and 60 s at 72 °C; fungal-specific ITS regions, 3 min at 95 °C, 30 cycles of 60 s at 94 °C, 56 °C at 45 s, and 50 s at 72 °C. The final extension time was 7 min at 72 °C in all PCR runs. DNA amplifications were visualized on 1.2% (*w*/*v*) agarose gels.

Construction of Metagenomic Library

Metagenomic DNA (1 μg) obtained by the protocol was partially digested with Sau3AI. The DNA fractions of 3–8 kb were fractionated by 1% (*w*/*v*) agarose gel electrophoresis. The digested metagenomes were ligated into the BamHI digested p18GFP vector. The ligation products were then transformed into *E. coli* DH10B, and the transformants were grown on LB–RBB xylan–amp plates.

Effect of Storage on Soil/Sediment DNA Extracts

An attempt was made to study the effect of storage of DNA extracts on DNA yield and purity. The DNA extracts were centrifuged, and the supernatants were dispensed into 2-mL Eppendorf tubes and stored at −20 °C for a month. DNA precipitation and its quantification were carried out at 1-week intervals.

Results and Discussion

Protocol Optimization to Obtain Humic Acid-Free DNA from Alkaline Soils/Sediments

A majority of the available protocols for soil DNA isolation insist on two steps: extraction of DNA (first step) followed by purification (second step). The purification of DNA from any environmental sample is mandatory for downstream processing. Conventionally, impure DNA is purified either by silica-based columns or by cation or anion exchangers that lead to a significant loss of DNA [17–19, 21–23]. Three different protocols have been combined in this investigation for developing the protocol. The very first strategy was adapted from Zhou et al. [24], which was based on the direct lysis of microbiota present in a soil sample that allowed the recovery of ~92–99% of DNA from an environmental sample. Most of the available methods for metagenomic DNA isolation [13, 17, 26, 27] revolve around this protocol. However, the method developed by Zhou et al. (1996) does not meet the purity requisites as purification ratios like A_{260}/A_{230} (for humic substances, >2.0) and A_{260}/A_{280} (for proteins, >1.7) do not suit downstream processing [28]. Therefore, the

protocol was modified to overcome the purity constraints of the metagenomic DNA by incorporating powdered activated charcoal (PAC) and PVPP that remove humic substances significantly [29]. Activated charcoal, bone char, and PAC have been extensively used for the removal of humic acid, lignin sulfonate, tannic acid, arabic gum polyphenolic compounds, many biodegradable/non-biodegradable colored compounds, and heavy metals [30–34]. As a second strategy, the concentration of PAC was optimized to lower the intense brown color of the slurry. PAC, known as activated carbon or activated coal, is a form of carbon that has been processed to make it extremely porous, and thus, has a vast surface area and pore volume that allows the adsorption of various humic substances [30, 33]. The use of 0.4 and 0.2 g of PAC per gram of soil resulted in high-purity ratios as compared to the control (without PAC); this suggested its significant role in the removal of humic substances (Table 2). Although, Desai and Madamwar [23] used PAC to extract the inhibitor-free metagenome from polluted sediments, the extracted DNA needed further treatment with amberlite IRA-400 resins (anion exchanger) to obtain metal ion- and organic inhibitor-free metagenome. The precipitation of the metagenomic DNA by using 30% PEG 8000 was the third approach. PEG has been used for the precipitation of soil metagenome [35, 36], since isopropanol or ethanol favors precipitation of DNA along with humic substances, while PEG does not co-precipitate humic substances [14, 37]. Similarly, La Montagne et al. [38] reported the use of 10% PEG 8000 instead of isopropanol that resulted in a fourfold reduction in humic substances without decreasing DNA yields. At this stage, the metagenomic DNA becomes almost free from humic substances, which was finally precipitated with isopropanol after routine phenol–chloroform–isoamyl alcohol treatment. As PEG is supposed to be an interfering agent in PCR reactions, a second phase of DNA precipitation was carried out. DNA was quantitated after PEG as well as isopropanol precipitations. There were no significant differences observed in the level of DNA yield in both of the precipitation methods. The soil metagenome extracted by the protocol developed in this investigation was quantitated with the help of Nanodrop 1000 spectrophotometer in terms of two different absorption ratios A_{260}/A_{230} (DNA/humic acid) and A_{260}/A_{280} (DNA/protein). The metagenome obtained through the combined strategy was of high molecular weight and non-sheared with high-purity ratios (A_{260}/A_{280}=1.72) and (A_{260}/A_{230}=1.66), and (A_{260}/A_{280}=1.82) and (A_{260}/A_{230}=1.96) for protein and humic acid-free DNA from Pantnagar and Lonar soil samples, respectively (Fig. 1). The method was further used for the extraction of metagenomic DNA from various alkaline soil and sediment samples. All the samples exhibited purity with acceptable absorbance ratios, and thus, confirm the reproducibility of the protocol (Table 3).

Table 2 Optimization of PAC concentration in the extraction of DNA

PAC concentration (g)	Pantnagar soil		Lonar soil	
	A_{260}/A_{280}	A_{260}/A_{230}	A_{260}/A_{280}	A_{260}/A_{230}
0	1.21±0.025	0.82±0.015	1.34±0.020	0.92±0.025
0.1	1.32±0.015	0.93±0.020	1.56±0.019	1.67±0.035
0.2	1.49±0.020	0.98±0.020	1.82±0.024	1.96±0.024
0.3	1.70±0.026	1.61±0.024	1.84±0.023	1.95±0.022
0.4	1.72±0.025	1.66±0.020	1.82±0.021	1.92±0.019

Mean value of triplicate data±standard deviation

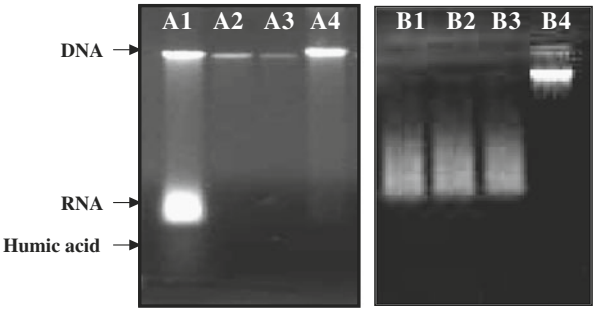

Fig. 1 Photograph of agarose (0.8%, *w*/*v*) gel electrophoresis. *Lane A1*, metagenomic DNA extracted without using PAC showing RNA and humic substances as inhibitors; *lanes A2*, *A3*, and *A4* showed DNA extracted from Lonar (*L1* and *L2*) and Pantnagar soils by using the protocol developed in this investigation. In addition, *lane B1*, *B2*, and *B3* showed successful digestion of these respective soils, which further validate the purity of DNA while *lane B4* showed undigested DNA (isolated without using PAC)

Comparison of Yield and Purity of DNA

The absorbance ratios of the extracted DNA have been compared with other manual as well as commercial methods (Table 4). The protocol of Wechter et al. [39] relies on indirect lysis, where the cells were first separated from the soil samples and then exposed to cell lysis. This is a way to avoid and cope with the humic substances by separating the cells from any environmental samples, but the extracted DNA corresponds to merely 25% to 30% of the total number of bacteria present in the soil [40]. Therefore, a large number of different bacterial colonies that strongly adhere to soil particles do not come in the extracted metagenome. The protocols developed by Agarwal et al. [41] and Desai and Madamwar [23] are modified versions of Zhou et al. [24] and are based on DNA extraction followed by purification either by Sephadex G-200 or a cation exchanger like Amberlite resins. Both these methods reported a significant loss of DNA. The soil metagenome obtained according to Yamamoto et al. [42] was based on repeated freezing and thawing of the soil suspension. In this method, the cells were exposed to physical shock, which does not assure the complete lysis of all the cells present in an environmental sample. While the kits are based on bead beating method followed by DNA elution from spin columns, Yeates et al. [37] had also achieved acceptable purity ratios through bead beating method, but this method is prone to cause shearing of the DNA, and the metagenome obtained is not the ideal representative of the community DNA [25, 43]. Furthermore, the DNA yield is proportional to the beating time that enhances shearing, and therefore, one has to compromise with getting the non-sheared stretches of metagenome. The commercial kits did not result in

Table 3 Extraction of soil DNA from alkaline soils by the protocol developed in this investigation

Place of collection	A_{260}/A_{280}	A_{260}/A_{230}	DNA yield [μg g^{-1} dry weight of soil]
Lonar lake (Buldana, Maharashtra)	1.82±0.019	1.96±0.021	21.5±1.364
Sindkheda (Dhule, Maharastra)	1.79±0.021	1.67±0.018	11.0±1.214
Tarna (Basti, Uttar Pradesh)	1.71±0.020	1.91±0.022	09.8±0.983
Pachpadra Salt Lake (Rajasthan)	1.83±0.024	1.69±0.020	07.5±1.212
Sambhar Salt Lake (Rajasthan)	1.80±0.021	1.66±0.018	08.3±0.567

Mean value of triplicate data±standard deviation

Table 4 Comparison of DNA extraction from alkaline soil of Pantnagar by various methods

Method	A_{260}/A_{280}	A_{260}/A_{230}	DNA yield [μg g^{-1} dry weight of soil]
Zhou et al. (1996) [24]	1.21±0.020	0.82±0.030	12±2.233
Wechter et al. (2003) [39]	1.66±0.021	1.33±0.025	6.7±1.181
Desai and Madamwar (2006) [23]	1.62±0.023	1.23±0.021	9.6±1.272
Agarwal et al. (2001) [41]	1.49±0.019	1.16±0.029	9.8±1.331
Yamamoto et al. (1998)	1.39±0.021	1.22±0.019	9.0±0.533
MN kit, Germany	1.40±0.022	1.29±0.026	5.2±2.452
Mo Bio kit, CA, USA	1.56±0.023	1.41±0.027	6.3±1.323
Zymo soil DNA kit, CA, USA	1.52±0.034	1.49±0.022	7.8±0.982
Protocol developed	1.72±0.021	1.66±0.019	10.5±1.321

Mean value of triplicate data±standard deviation

satisfactory DNA yield and absorbance ratios of protein as well as humic substances (Table 4). In addition, the DNA yields were also not satisfactory as compared to other manual methods and those using commercial kits. The protocol developed in this investigation allowed the extraction of protein and humic acid-free DNA, and thus resulted in high absorbance ratios of Pantnagar (A_{260}/A_{230}=1.66) and Lonar (A_{260}/A_{230}=1.96) soils, indicating that it is almost free from humic substances (Table 4); these are much better than those obtained employing other methods.

Validation of Metagenome Obtained by Developed Protocol

Restriction Digestion and PCR Amplification

The satisfactory quality of the metagenomic DNA extracted from various soil samples was further confirmed by restriction digestion, amplification of specific regions of the

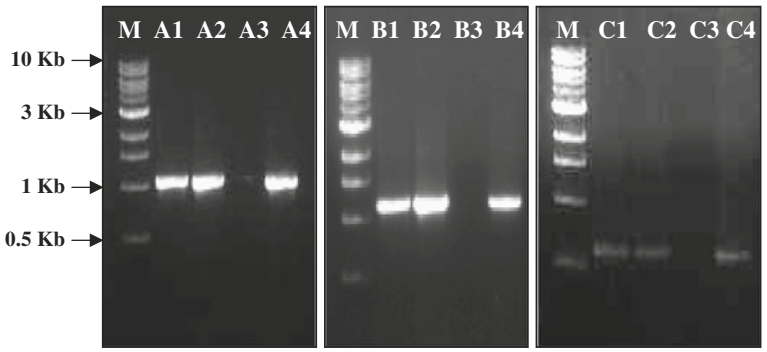

Fig. 2 Photograph showing agarose (1.2%, *w/v*) gel electrophoresis of the PCR amplicons from metagenomic DNA extracted using the developed protocol. *M* indicates 1 kb of DNA ladder (NEB). *Lanes A1*, *A2*, *B1*, *B2*, *C1*, and *C2* show amplicons of archaeal-, bacterial-specific 16S rDNA and fungal-specific ITS regions from Pantnagar and Lonar metagenomes, respectively. *Lanes A3*, *B3*, and *C3* did not show any amplification of the microbial communities (unsuccessful PCR amplifications of archaeal-, bacterial-, and fungal-specific regions using the metagenome extracted without incorporation of PAC), while *lanes A4*, *B4*, and *C4* showed amplifications of *Picrophilus torridus*, *Bacillus acidicola*, and *Pichia anomala* as the respective positive controls

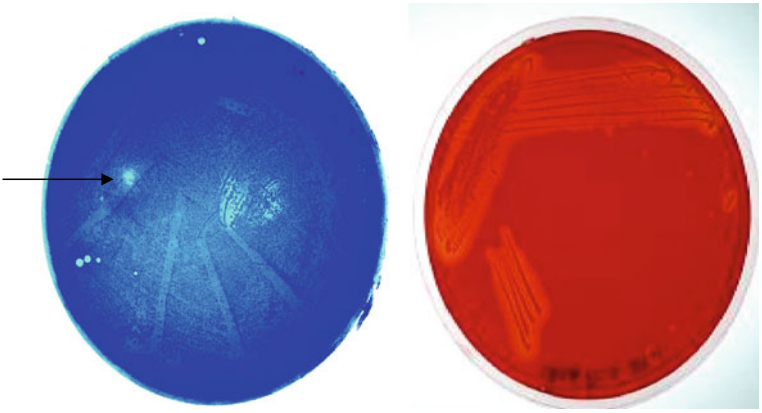

Fig. 3 Metagenomic library constructed from the DNA extracted by the protocol described in this investigation. (**a**) LB–RBB xylan–amp plate with a positive clone showing zone of xylan hydrolysis (*arrow*), (**b**) the clone was streaked on an LB–xylan plate, allowed to grow at 37 °C, and was flooded with Congo red; the clear zone of xylan hydrolysis around the colonies indicates xylanase secretion

genomes of various microorganisms as well as by constructing a metagenomic library. The metagenomic DNA isolated from different soil samples could be digested with Sau3AI within 5 min (Fig. 1). In addition, bacterial- and archaeal-specific 16S rDNA as well as fungal-specific ITS regions were nicely amplified from the different metagenomic DNA samples (Fig. 2). Similarly, restriction digestion and PCR amplification have been used to validate the purity of the metagenomic DNA extracted from the environmental samples [17, 44]. The extracted DNA is an ideal representative of the microbial community of the environment, as it contained signature sequences of bacteria, archaea, and eukarya. Since the extraction of the metagenome is meant for recovering xylanase-encoding genes in this investigation, the metagenomic library was plated out on the LB–RBB xylan–amp plate. A total of ~8,000 clones were obtained, and this further validates the purity of the metagenome. The screening of the metagenomic library also permitted the selection of a positive clone for xylanase (Fig. 3). The clone has been shown to produce xylanase extracellularly as well as intracellularly, which was confirmed by using quantitative xylanase assay [45], and the enzyme is active at alkaline pH and elevated temperatures.

Table 5 DNA yield after preservation at −20 °C

Week	DNA yield [μg g^{-1} dry weight of soil]	
	Pantnagar soil	Lonar soil
1	10.5±1.321	21.50±1.364
2	10.0±1.127	20.78±1.211
3	9.88±1.060	20.47±0.500
4	9.50±1.443	19.00±0.763

Mean value of triplicate data±standard deviation

Effect of Storage of Soil/Sediment DNA Extracts

The DNA extracts could be preserved for a month at −20 °C prior to PEG precipitation and could be used whenever required. The purity of DNA remained unchanged, although a slight reduction in DNA yield from soil/sediment from Pantnagar and Lonar Lake was recorded (Table 5).

Conclusions

The lysis of microbial cells in the presence of PAC and PEG significantly reduced co-precipitation of the humic substances along with DNA, and thus eliminated the need for purification subsequently, and thus, minimizing the loss of DNA. Since the DNA extracted from the alkaline soils and sediments is free from humic substances and proteins, we have been able to use it for constructing metagenomic libraries meant for selecting clones that exhibit xylanolytic activity.

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