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Article

Construction of a Metagenomic Library from Compost and Screening of Cellulase- and Xylanase-positive Clones

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Metagenomic library was constructed from compost made with pig manure and mushroom cultural waste using fosmid vector. Composting was carried out using moving roller system with intermittent mixing and forced aeration, and samples were taken from 7th- and 14th-day spots, which represent the mid and late stages of the process, respectively. DNAs of about 40 kb were obtained by preparative electrophoresis on 0.4% low-melting agarose gel to fractionate DNA fragments and to remove humic substances. Total 12,380 fosmid clones were obtained. Restriction analysis of randomly selected clones showed that most clones had different inserts, and average size of inserts was about 35 kb. Two cellulase-positive and five xylanase-positive clones were selected from metagenomic library. Cellulase of clone C1 showed maximal activity at 50°C and pH 6.0, and retained its original activity after 30 min of heat treatment at 60°C. Optimum temperature for xylanases of clones X1, X2, X3, and X4 was 50°C, and that of clone X5 was 55°C. Thermostabilities of xylanases were in the order of X4>X5>X1, X2, and X3. Optimum pH of xylanases of X1, X2, and X3 was 6.0, that of X4 was 5.5, and that of X5 was 5.5~8.0. Xylanase-positive clones could be divided into three groups, X1/X2/X3, X4, and X5, based on influences of temperature and pH on enzyme activity. Sequence analysis of positive subclone of clone C1 showed cellulase, Cel6H, had the highest similarity of 64% to that of *Cellulomonas fimi* (P07984), suggesting cellulase and xylanase from metagenomic library are novel enzymes.

Key words: cellulolytic activity, compost, fosmid, metagenomic library, xylanolytic activity

Diverse microorganisms are found in compost that produce various enzymes such as cellulases, xylanases, lipases, aminopeptidases, and glucosylhydrolases [Godden *et al*., 1983; Tiquia, 2002], but only a small portion of the indigenous microbial species are artificially cultivatable. Therefore, it is necessary to collect all genetic materials using culture-independent approaches to search for valuable genes from the compost microorganisms. Metagenome refers to all genetic materials in an environment sample, consisting of the genomes of many individual organisms [Handelsman *et al*., 1998], and is also referred to as eDNA (environmental DNA) [Brady *et al*., 2002]. Metagenomes from various environments have been studied, but only few were concerned with compost metagenomes [Lämmle *et al*., 2007; Pang *et al*., 2009; Kim *et al*., 2010].

Cellulose and xylan, a highly branched β-1,4-linked polymers of glucose and xylose, respectively, are major components of lignocellulosic materials and can be degraded by many cellulolytic and xylanolytic microorganisms. Complete degradation of lignocellulosic materials requires the actions of several types of enzymes, including endo-β-1,4-glucanase, endo-β-1,4-xylanase, β-glucosidase, and β-xylosidase. With this high content of lignocellulosic materials, compost can be an excellent source for screening cellulolytic and xylanolytic enzymes that degrade lignocellulosic materials to fermentable sugars, which can then be converted into biofuel [Balat and Balat, 2009]. A few metagenome-derived cellulase genes and a xylanase gene were identified recently [Voget *et al*., 2006; Feng *et al*., 2007; Hu *et al*., 2008; Kim *et al*., 2008; Pang *et al*., 2009], but only one cellulase gene has been reported from compost-related samples [Pang *et al*., 2009].

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In the present study, a compost metagenomic library was constructed using a fosmid vector. Subsequently, clones with cellulolytic and xylanolytic activities were isolated, and the influence of temperature and pH on enzyme activities were examined.

Materials and Methods

Preparation of compost metagenome. The raw materials, pig manure and mushroom cultural waste, were mixed and pre-fermented for 1 month, and then processed with a field-scale Hazaka composter, moving the roller with intermittent mixing and forced aeration for 2 weeks [Cho *et al*., 2009]. Samples were taken from the 7th- and 14th-day spots at a depth of 10~20 cm from the surface. Metagenomic DNA was extracted as described previously, with some modifications [Zhou *et al*., 1996; Rondon *et al*., 2000; Kim *et al*., 2010]. Five grams of the sample was added to 13.5 mL of the DNA extraction buffer [100 mM Tris/HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% hexadecyl-methylammonium bromide (CTAB)] and incubated for 1 h at 37°C with rotary agitation at 50 rpm. The mixture was filtered through four layers of cheese cloth. After the addition of proteinase K (100 μg/mL), the filtrate was incubated for 1 h at 37°C with rotary agitation at 120 rpm. The mixture was further incubated for 2 h in a water bath at 65°C with intermittent mixing every 20 min after the addition of 1.5 mL of 20% SDS solution. After centrifugation for 10 min at 7,600×g, the supernatant was mixed with an equal volume of phenol/chloroform/isoamylalcohol solution (25:24:1, v/v), agitated for 30 min, and centrifuged for 10 min at 7,600×g to collect the supernatant. After repeating the extraction step with chloroform/isoamylalcohol solution (24:1, v/v), the primary metagenomic DNA preparation was obtained by adding 0.6 volume of isopropanol, followed by precipitating DNA for 1 h at room temperature, centrifuging the solution for 20 min at 7,600×g, washing the precipitate with cold 70% ethanol, and finally dissolving the precipitate in 500 μL sterile distilled water.

The primary DNA preparation was electrophoresed for over 15 h at 25 V on a 0.4% low melting point agarose gel (16×15 cm, Sigma, St. Louis, MO) overlaid on a 1% agarose gel using λ DNA and λ/*Hin*dIII (Fermentas, Burlington, Ontario) as size markers [Lim, 2004]. The portion of gel corresponding to DNA with size over 35 kb was divided into four fractions by horizontally slicing the gel into pieces 2 mm in width. DNA was extracted from each fraction using GELase (Epicentre, Madison, WI) according to the manufacturer's instructions and analyzed on a 0.4% agarose gel.

Construction of the compost metagenomic library. Construction of the metagenomic library, i.e., DNA end-repairing, ligation of the blunt-end DNA and pCC2FOS™ vector, *in vitro* packaging, and infection of *E. coli* EPI300™-T1^{R} was carried out using the CopyControl™ Fosmid Library Production Kit (Epicentre) according to the manufacturer's instructions. Transformants were grown for 12 h at 37°C on LB agar plates containing 12.5 μg/mL chloramphenicol. Twelve randomly selected transformants were grown in an amplification medium containing the CopyControl Induction Solution (Epicentre). Fosmids of the transformants were extracted, and the quality of the library was tested by identifying the presence of the vector and the insertion of DNA [Kim *et al*., 2010].

Selection of cellulase- and xylanase-positive clones. The fosmid transformants were grown for 12 h on LB agar plates containing 0.5% carboxymethyl-cellulose (CMC, Sigma, St. Louis, MO) and 0.008% trypan blue (LBCT), and the colonies with a weak blue zone were selected as cellulase-positive clones; xylanase-positive clones were selected on the plates containing 0.5% xylan (LBX). Fosmids of the clones were prepared and analyzed for the insert DNA. The nucleotide sequences of the insert DNA were determined by SolGent (Daejeon, Korea). The nucleotide sequence of *cel*6H has been deposited in the GenBank under the accession number HQ143745.

Determination of enzyme activity. Enzyme activities of the transformants were determined using crude extracts. The transformants were grown in 5 mL of LB broth containing 12.5 μg/mL chloramphenicol for 12 h at 37°C with rotary agitation at 200 rpm, transferred to 45 mL of the amplification medium containing the CopyControl Induction Solution, grown for 5 h at 37°C with rotary agitation at 300 rpm, and then harvested by centrifugation for 10 min at 7,600×g. The cells were dispersed in 50 mM of sodium citrate (pH 5.5) buffer, sonicated as previously described [Shin *et al*., 2002], then centrifuged for 20 min at 15,000×g, with the supernatants used as the crude enzyme preparations. Cellulase activity was determined by measuring the amount of reducing sugar released from 0.5% (w/v) CMC as the substrate [Yang *et al*., 2004]. Xylanase activity was assayed using 0.5% (w/v) oat spelt xylan, as described previously [Shin *et al*., 2002]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under these conditions. Protein concentration was determined by the method of Lowry *et al*. [1951].

Characterization of enzymes. Optimum temperature of the enzyme was determined by measuring the enzyme activities at designated temperatures. Thermostability of the enzyme was determined by preincubating the enzyme

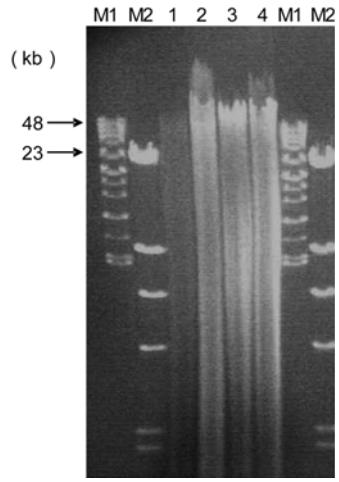

Fig. 1. Gel electrophoresis of the isolated compost DNA. The DNA preparation was electrophoresed for over 15 h at 25 V on a 0.4% low-melting point agarose gel (16 ×15 cm), which was overlaid on a 1% agarose gel. Lanes M1 and M2, λ DNA and λ/*Hin*dIII, respectively, as size markers; lanes 1 and 2, DNAs extracted from the compost sample from the 7th day spot; lanes 3 and 4, DNA extracted from the compost sample of the 14th-day spot.

for up to 30 min in the absence of the substrate at designated temperatures, prior to the activity assay. For the determination of optimum pH, enzyme activities were observed in 50 mM sodium citrate buffer at designated pHs.

Results and Discussion

Extraction of metagenome from compost. Compost samples were collected from the 7th and 14th day spots, which represent the mid and the late stages of the composting process, respectively. The primary metagenomic DNA preparations contained DNA larger than 35 kb (Fig. 1). The concentration of the DNA preparation from the 14th day spot was higher than that from the 7th day spot. However, the preparations were dark brown and were not proper for restriction enzyme digestion or other enzyme reactions (data not shown). Appearance of this color was likely due to the presence of humic substances or other inhibitors, as in the case of soil or forest soil metagenomes [Lim *et al*., 2005; Lämmle *et al*., 2007]. Humic substances are known to inhibit enzymatic manipulations and to decrease transformation efficiency [Tebbe and Vahjen, 1993]. Because humic substances are derived from the degradation of lignin and other dead plant matters, compost may contain more humic substances than soil. These undesirable substances could be removed by preparative electrophoresis on a 0.4% low-melting agarose gel. Several purification procedures for the removal of undesirable substances have been described, however, these methods were time-consuming and laborious, required multiple steps, or resulted in significant loss or degradation of DNAs [Steffan *et al*., 1988; Zhou *et al*., 1996; Miller *et al*., 1999]. The DNAs purified by one step size exclusion chromatography was not only pure enough to be digested by restriction enzymes, but also allowed the construction of libraries with small DNA inserts [Lämmle *et al*., 2007]. For the construction of a metagenomic library with the fosmid vector pCC2FOS, the size of DNA should be about 40 kb. The preparative electrophoresis on a 0.4% low-melting agarose gel in the present study was convenient and effective in removing undesirable substances, and the DNAs obtained were large enough for the construction of a library with a fosmid vector. After the electrophoresis, DNAs of about 40 kb were extracted from the sliced gel and used for the library construction.

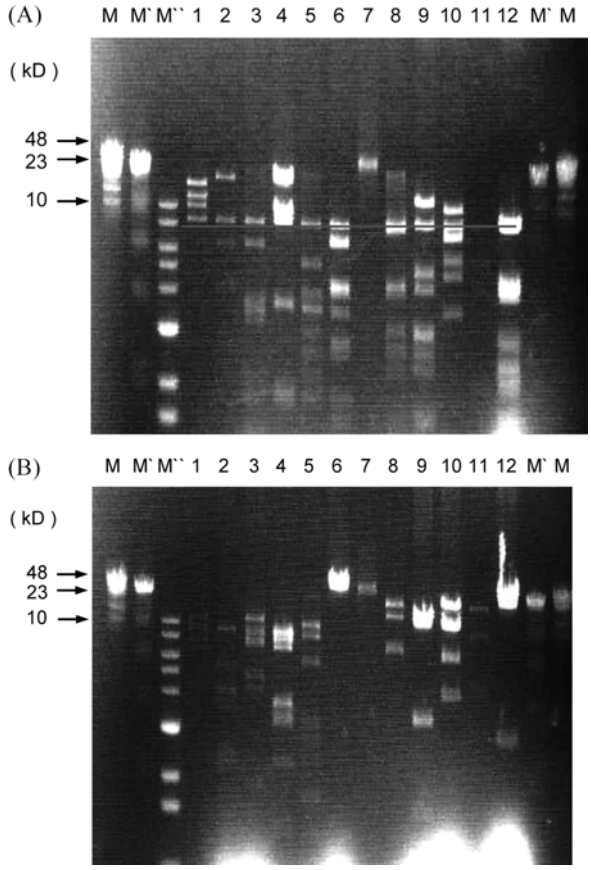

Fig. 2. Restriction enzyme digestion patterns of clones from compost metagenomic library. Fosmids were purified from the 12 randomly selected clones and digested with *Bam*HI (A) and *Eco*RI (B). Line in panel (A) represents the fosmid vector. Lanes M1, M2, and M3: λ DNA, λ/*Hin*dIII, and DNA ladder, respectively, as size markers.

Construction of the compost metagenomic library. After end-repairing the DNAs to make blunt ends, the

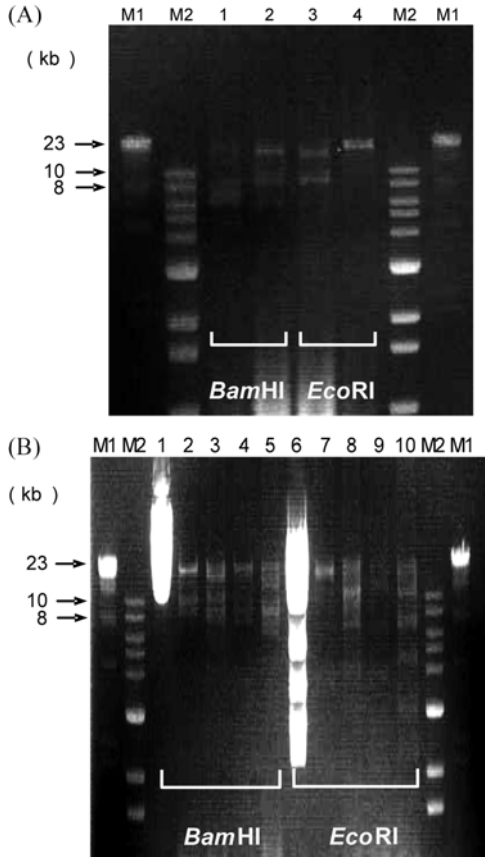

Fig. 3. Restriction enzyme digestion patterns of the cellulase-positive (A) and xylanase-positive (B) clones selected from the metagenomic library. Fosmids from the positive clones were digested with *Bam*HI and *Eco*RI. Lanes M1 and M2, λ DNA and DNA ladders, respectively, as size markers.

fractionated DNA fragments were transformed to *Escherichia coli* using the fosmid pCC2FOS™ vector. A total of 12,380 clones were obtained on plates containing chloramphenicol. The restriction patterns of the fosmids of 12 randomly selected transformants with *Bam*HI and *Eco*RI revealed that the average size of the inserted DNAs was about 35 kb and that most of the clones had different inserts (Fig. 2), suggesting that the high diversity of the DNA inserts are reflected in the library.

Selection of cellulase- and xylanase-positive clones. With the high content of lignocellulosic materials and the diversity of indigenous microorganisms, compost can serve as an excellent source of cellulolytic and xylanolytic enzymes that degrade lignocellulosic materials into fermentable sugars. Two cellulase-positive (C1 and C2) and five xylanase-positive clones (X1 to X5) were selected from the metagenomic library of 12,380 clones, using LBCT and LBX selective plates, respectively. Restriction analysis of the fosmids of the clones showed that they had inserted DNAs with different sizes and restriction patterns, indicating that the clones were different from each other (Fig. 3). The selection rates of the cellulase- and xylanase-positive clones were approximately 1/6,200 and 1/2,500, higher than the selection rates of cellulase-positive clones from cosmid libraries, which were approximately 1/25,000 [Pang *et al*., 2009] and 1/70,000, respectively [Kim *et al*., 2008]. The nature of the compost sample, i.e. the use of mushroom cultural waste as a raw material, could be one of the reasons for the higher rate.

Table 1. CMCase and xylanase activities of the selected metagenome clones

Clone	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Comparative activity* (U/mg)
C1	4.5	2.92	1.54
C2	0	5.58	0
X1	1.4	4.93	0.27
X2	13.1	5.13	2.55
X3	3.0	2.56	1.17
X4	0.2	10.47	0.02
X5	4.1	3.71	1.11

Experiments were carried out in duplicate, and the numerals are mean values.
*These values indicate the relative activities of the positive clones because the values were obtained semiquantitatively from the total activities and the total protein amounts in the cells.

Properties of the enzymes. Only one of the two cellulase-positive clones on the plate showed enzyme activity in LB broth (Table 1). Though the production of proteins by clone C2 was normal, no detectable amount of cellulase activity was produced in the liquid culture. The instability of the introduced plasmid could have rendered the clone to lose the ability to produce cellulase, and/or the clone might not have the full structural gene, thus, the proteolytic digestion of the immature truncated enzyme could have caused the loss of the enzyme activity. The cellulase of the clone C1 exerted its maximal activity at 50°C (Fig. 4A), and lost none of its original activity after 30 min of heat treatment at 60°C (Fig. 5A). The optimum pH for the cellulase activity was 6.0 (Fig. 6A).

Comparative enzyme activities were calculated from total enzyme activities and total protein concentrations in the transformant cells to compare the enzyme activities of the transformants, because the degree of protein expression and/or the specific activity of the target enzyme could be different from each other. Among the five xylanase-positive clones, the clone X2 produced the highest enzyme

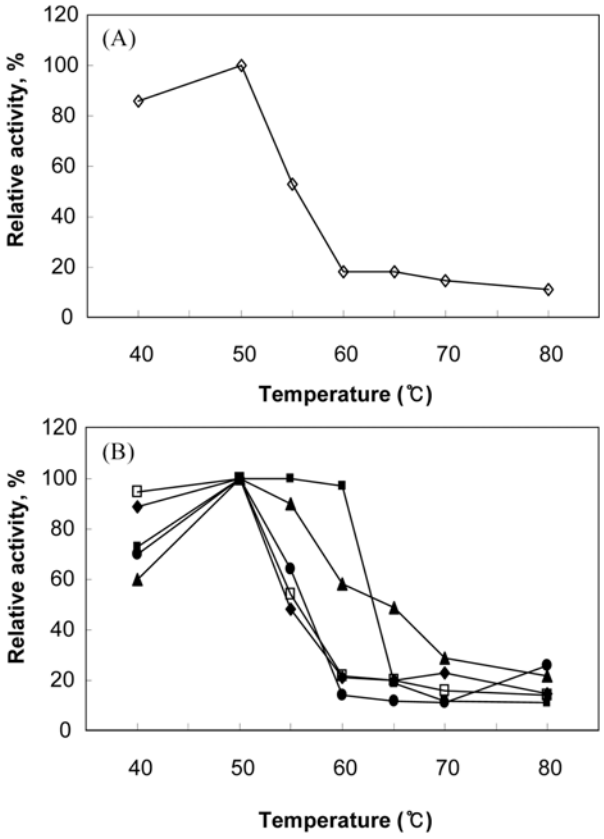

Fig. 4. Effect of temperature on cellulase (A) and xylanase (B) activities of positive clones. Enzyme activity was measured at designated temperatures under the standard condition. ◇, C1; ●, X1; □, X2; ◆, X3; ▲, X4; ■, X5.

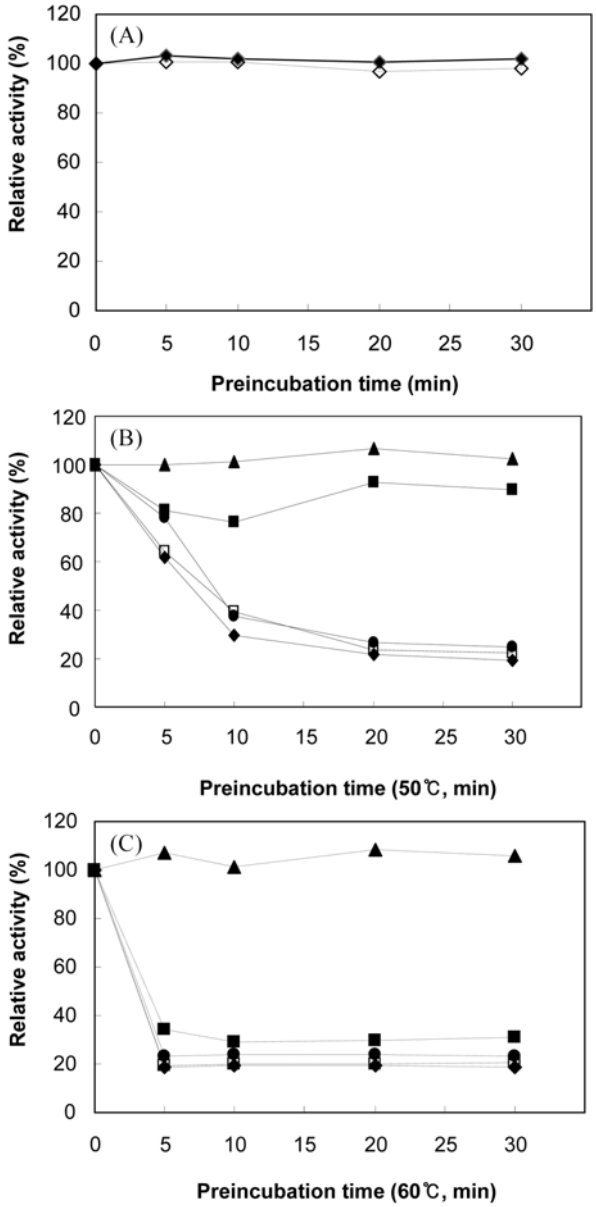

Fig. 5. Thermostability of the cellulase (A) and xylanase (B and C) activities of the positive clones at 50 (__) and 60 (…). Thermostability of the enzyme was determined by preincubating the enzyme in the absence of the substrate, at designated temperatures, prior to the activity assay. ◇, C1; ●, X1; □, X2; ◆, X3; ▲, X4; ■, X5.

activity, i.e., 13.1 U/mL of crude enzyme solution and 2.55 U/mg of protein (Table 1). Optimum temperature for the xylanases of clones X1, X2, X3, and X4 was 50°C, and that of clone X5 was slightly higher at 55°C (Fig. 4B). The xylanase of clone X4 was found to be thermostable and lost none of its original activity after heat treatment for 30 min at 60°C (Fig. 5B and 5C). The xylanase of clone X5 was less thermostable than that of clone X4, but was more thermostable than those of clones X1, X2, and X3 (Fig. 5B and 5C). The optimum pH of the xylanases of clones X1, X2, and X3 was 6.0, and that of clone X4 was 5.5 (Fig. 6B). The xylanase of clone X5 showed a rather broad optimum pH, 5.5~8.0 (Fig. 6B). Based on the influence of temperature and pH on enzyme activity, the xylanase-positive clones could be divided into three groups: X1/X2/X3, X4, and X5.

Sequence analysis of the inserted DNA fragments. A cellulase gene was subcloned from the fosmid DNA of clone C1 by the shotgun method. The sequence analysis of YJ-C227, a positive subclone of clone C1, showed that the cellulase belongs to the glycoside hydrolase Family 6, with the highest similarity of 64% to the cellulase of *Cellulomonas fimi* (P07984) (Fig. 7). The results suggested that the cellulase of C1 might be different from the other reported cellulases. The end sequence analysis of the inserted DNA fragment of the clone X1 revealed a 16S rRNA gene that showed 86% similarity to the *Enterococcus faecalis* strain SFL (AY850358) and an uncultured bacterium clone Lan-37 (Accession No. AY741387) (data not shown).

The results of the present study indicate that the cellulase and xylanase from the metagenomic library

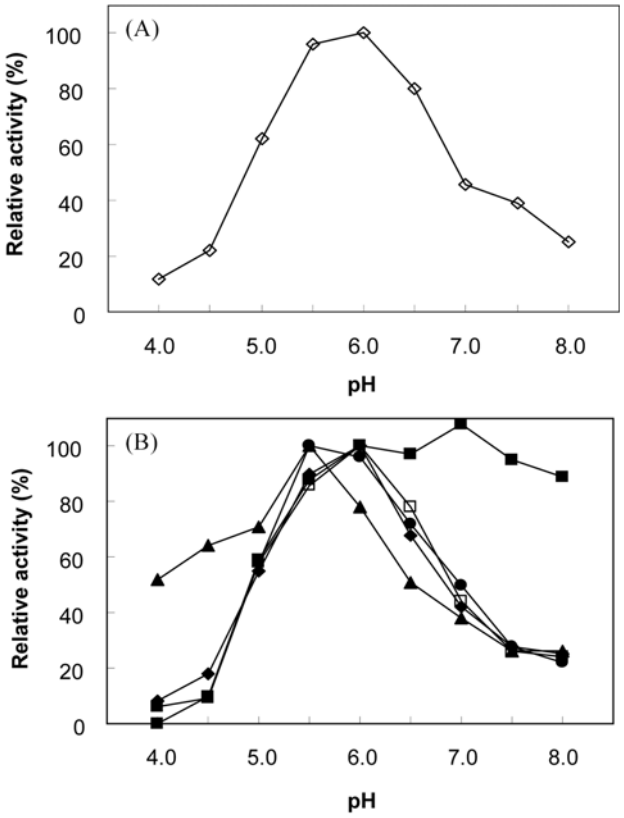

Fig. 6. Effects of pH on cellulase (A) and xylanase (B) activities of positive clones in sodium citrate buffer. Enzyme activity was observed in 50 mM sodium citrate buffer at designated pHs. ◇, C1; ●, X1; □, X2; ◆, X3; ▲, X4; ■, X5.

could be novel enzymes. Further studies are needed to reveal the exact nature of the enzymes screened from the compost metagenomic library.

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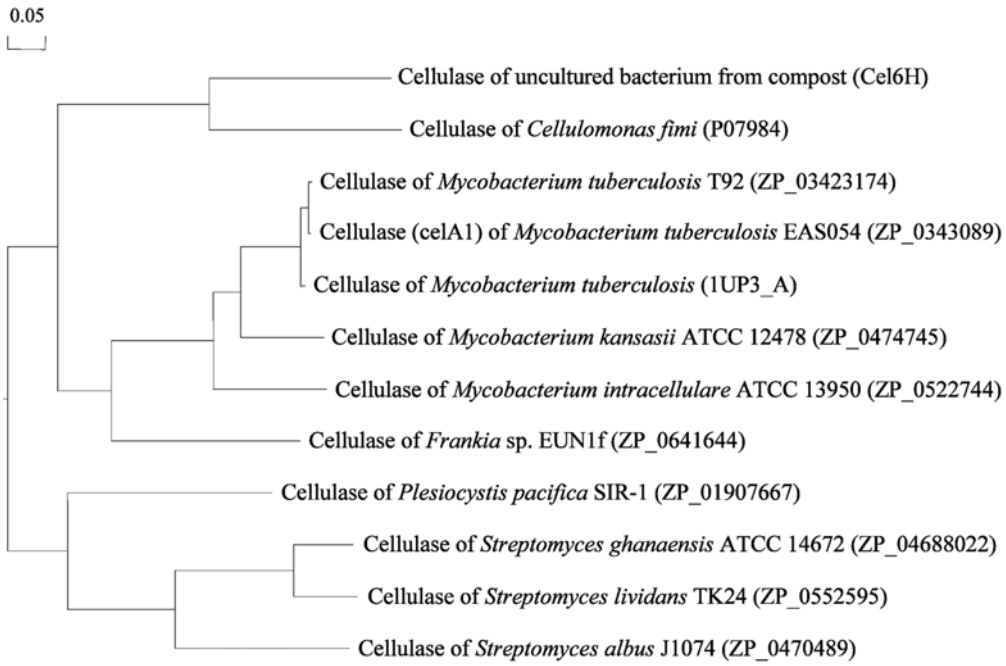

Fig. 7. Phylogenetic tree showing evolutionary relatedness and levels of homology among cellulolytic enzymes.

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