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Prospecting for Cellulase Enzymes Based on Sequences and Functional Screening from Metagenomic Libraries

C. Wiwat and J. Sillapee*

Department of Microbiology, Faculty of Pharmacy, Mahidol University, Sri-Ayudhaya Rd, Bangkok, 10400, Thailand.

Abstract

Microorganisms dominate the biosphere, yet current methods of culture reveal less than 1% of the microbial diversity. There is a vast amount of information held within the genomes of uncultured microorganisms, and metagenomics is one of the key technologies used to access and investigate this potential. Cellulose is an abundant biopolymer and sustainable bioconversion from agricultural waste to value products such as biofuel. Thus cellulase is attractive for their potential in the biotechnological application. Mangrove soils are in general acidic in nature and extremely salt condition can appear some bacterial strains that can tolerant to salinity condition and also can produce extracellular cellulase enzyme.

In order to exploit the most of microbial diversity resources to find bioactive compounds or genes, mangrove soil metagenomic libraries were constructed. DNA was directly extracted from the selected mangrove soil, Satul province, fragments of the soil DNA were ligated to an expression plasmid pBluescriptII SK(+), transformed to surrogate host *Escherichia coli* DH5α, and the white clones out on LB medium were selected to accumulate libraries. More than 100 positive clones with average insert size of about 1 kb were collected. The libraries were screened for cellulase enzyme producer. Over 20 clones with cellulase producing were found out from these libraries by cellulase screening method using carboxymethyl cellulose as a substrate. The two cellulolytic clones, celP8 and celP24, were shown the best activity after screening. There were chosen for sequencing and the size of the inserted gene of *celP8* and *celP24* are 421 and 127 bp, respectively. Theoretical predicted size of protein of celP8 and celP24 are 15,436 dal and 4,656 dal, respectively. Addition with blasting in GenBank, there are no similar sequences found from all known cellulase genes.

Further characterization, the optimal pH of celP8 and celP24 were presented at pH 10.0 and 11.0, respectively. While the optimal temperature of celP8 and celP24 were 35°C and 25°C, respectively. SDS-PAGE and zymogram analysis demonstrated that celP8 and celP24 had CMCase activity indicating those are endoglucanases. Even their homology searches were no cellulose-binding domain (CBD) or conserved domain of any classes of cellulases. It is suggested that they may be new or undiscovered subclass of cellulolytic enzyme from mangrove soils from the south of Thailand.

Key words: Metagenome, Mangrove soil, Cellulase.

***Corresponding author:** Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. Email: pymwt@mahidol.ac.th

INTRODUCTION

Microorganisms encompass the most life forms in the biosphere. Modern biotechnology can utilize those microorganisms especially bacteria because of their genetic, physiological and metabolic diversity. They can present in every niche on earth hence their evolution given the versatility. The demands of biocatalysts and active compounds are increasing but the known species of bacteria are limited. That reason is for 1% or less of the bacterial diversity in most environmental samples is in unculturable forms[1].

In an effort to explore the biotechnological potential biocatalysts from uncultured microorganisms, cultivation independent metagenome approaches have been widely adopted[23]. Moreover, the soil is often considered to be one of the main reservoirs of microbial diversity on the planet. This diversity could provide a range of information about the origins of microbial functional diversity as well as novel valuable genetic resources. Mangrove and fresh water swamp forest have a unique characteristic with specialized ecosystems. They are waterlogged in nature, so the soil is loosely, aerated, and enrich with the organic matters. The bacterial communities have special adaptations that are suitable to those ecosystems. A mangrove forest at Satul Province, south of Thailand, contains the saline-acidic soil because it receives direct effects from the sea and in general acidic in nature. The characteristics of soil are the biopropecting of the potential candidate enzymes which halo, alkalo or acidotolerant for biotechnological and pharmaceutical applications.

The metagenomic toolbox provides methods for accessing, storing, and analyzing the DNA extracted from the quasi-total microbial community and thus can provide an otherwise hard-to-attain insight into the biology and evolution of environmental microorganisms[6]. Subsequent high-throughput screening of the libraries has yielded novel enzymes with unique biochemical and biophysical characteristics not found for those from cultured microorganisms[3].

The current interest in the production of energy from biomass has provided fresh impetus for research in the area of cellulose degradation[10]. Cellulose, a linear polymer of β-linked glucose molecules, present in plant cell walls, is the most abundant biopolymer. Prior studies for natural cellulose hydrolysis have revealed many cellulolytic microorganisms and their complex cellulases[16,19]. Bacterial and fungal cellulases can be classified into three types: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21)[7]. The two different structural types of cellulase systems in bacteria are designated non-complex and complex. Some anaerobes are known to produce an extracellular multi-enzyme complex called a cellulosome. In contrast, cellulases from the majority of aerobes bind directly to the cellulose. Endoglucanases cleave intramolecular β-1, 4-glucosidic bonds randomly and carboxymethyl cellulose (CMC) is often used for determining endoglucanase activity, called CMCase[16]. Many reports described the isolation and characterization of cellulases from different environmental genomic libraries[4,9,12,19,21,25,26]. However cellulase is very important in agricultural waste treatment and be widely used to produce sustainable bioenergy, the novel cellulases with the potential candidates in biotechnological appilication will be necessary to explore.

Most of the cellulases (1, 4-β-D-glucan glucanohydrolases; EC 3.2.1.4) produced by microorganisms are now known to be active at acidic or neutral pH and usually to be inactive in the alkaline pH range[5].

Despite many recent reports describing the isolation and characterization of enzymes including cellulases from different environmental genomic libraries[8], the biotechnological potential of novel cellulases from uncultured microbial community has been far from fully explored. This research was aimed at discovering new cellulase genes from microorganisms by directly cloning and

activity-based screening the genomic DNA from mangrove soils environmental samples. To our knowledge, this is the first report of a metagenome-derived cellulases isolated from mangrove ecosystems in Thailand.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Escherichia coli DH5α was purchased from Gibco BRL Life Technologies (Eggenstein, Germany). Plasmid vector pBluescriptII SK (+) (Stratagene Amsterdam, NL) and *Bacillus subtilis* ATCC 6633 were used as positive controls during activity-based screening for cellulase. *E. coli* cells were routinely grown in Luria–Bertani (LB) medium at 37°C, supplemented with 100 μgml^{-1} ampicillin (LB_{Amp})

Extraction of DNA from environmental samples

DNA was extracted from mangrove soil samples from Satul province. For construction of the library described in this study, compost was collected from the surface layer (not deeper than 25 cm). Samples were stored at −20°C within 1 h after sampling.

For DNA extraction, a protocol based on the direct lysis method of Zhou et al. (1996)[30] according to Kauffmann et al. (2004)[11] was used. In addition to this protocol, the DNA-containing supernatants obtained after the lysis steps were mixed with hexadecylmethylammonium bromide (CTAB) to complex cell debris like denatured proteins and polysaccharides as well as humic compounds[30]. Briefly, samples of 1 g were homogenised by vortexing in 2.6 ml extraction buffer (100mM Tris–HCl pH 8.0, 100mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5M NaCl, 1% CTAB). Three cycles of freeze–thawing were performed in liquid nitrogen and at 65°C (water bath).

After addition of 50 μl of proteinase K (20 mg ml^{-1}), samples were incubated at 37°C for 30 min with continuous shaking at 120 rpm. Three hundred microliters of 20% (w/v) SDS were added before incubation at 65°C for 2 h with gentle shaking every 15–20 min. The supernatants were collected after centrifugation at 4000 x *g* for 10 min; the pellets were re-extracted by resuspending in 2 ml extraction buffer, incubation at 65°C for 10 min and centrifugation as before. The combined supernatants were mixed with 1/10 volume of 10% (w/v) CTAB and centrifuged again. Following extraction of the resulting supernatant with phenol-chloroform–isoamylalcohol (25:24:1, v/v), DNA of the aqueous phase was precipitated with isopropanol, washed with 70% (v/v) ethanol, dried and resuspended in 100 μl of 10mM Tris–HCl pH 8.5.

Purification of metagenomic DNA

Crude DNA extracts were purified in a single step by size exclusion chromatography with CHROMA SPIN + TE-1000 columns (BD Biosciences Clontech, Heidelberg, Germany), equilibrated in 10 mM Tris–HCl pH 8.5, according to the manufacturer's protocol.

DNA quantification was done by UV spectrophotometry at 260 nm. Generally, DNA fragments were separated by gel electrophoresis on 0.7% agarose.

Construction of metagenomic plasmid library

DNA was partially digested with *MboI* (Promega, Mannheim, Germany) and purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Plasmid vector pBluescriptII SK (+) was digested with *Bam*HI, dephosphorylated with calf intestinal alkaline phosphatase (Promega) and purified via gel elution. The DNA fragments were ligated into pBluescriptII SK (+) at molar ratios of 1:3 to 1:7 (vector to insert) using T4 DNA Ligase (Promega). Two hundred microliters of competent *E. coli* DH5α cells were transformed by 10 μl of ligation mixture. Recombinant clones were selected at 37°C for 24 h on LB_{Amp} agar plates containing 27 μg ml^{-1} isopropyl β-D-thiogalactopyranoside

(IPTG) and 27 μgml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal).

For arraying of single clones, colonies were transferred to agar plates containing LB_{Amp} medium and grown for at least 2 days at 37°C. For storage, this library was replicated plates containing LB_{Amp} after incubation for 2 days at 37°C, stored at −80°C.

Molecular analyses of the cellulase genes

Subcloning was performed to localize the cellulase genes and to shorten the inserts for effective sequencing and biochemical characterization. The smallest fragments expressing cellulase activities were sequenced. Possible open reading frames (ORFs) were identified with the ORF finder at National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Comparisons of sequences with those in the databases were made with BlastN, BlastX and BlastP (BLAST, basic local alignment search tool) at NCBI.

Enzyme activity detection

Cellulase-positive clones were screened by using a colorimetric assay on Congo red indicator plates[22]. Colonies harbouring recombinant DNA were grown on LB agar supplemented with 0.5-1% carboxymethylcellulose (CMC). After an appropriate incubation period at 37°C, the agar medium was flooded with an aqueous solution of Congo red (1 mg ml^{-1} for media containing CMC) for 15 min. The Congo red solution was then poured off, and plates containing CMC were further treated by flooding with 1M NaCl for 15 min. Cellulase-expressing colonies were surrounded by a yellow halo against a red background. The visualized zones of hydrolysis could be stabilized for at least 2 weeks by flooding the agar with 1 M HCl, which changes the dye color to blue and inhibits further enzyme activity.

Gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[14] using 15% gels. Samples were dissolved in a solution containing 2% (wt/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 15 mM Tris-HCl (pH 6.8) and heated in a boiling water bath for 3 min. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250. The molecular mass markers used were the low-molecular weight calibration kit (Pharmacia, Uppsala, Sweden).

Zymogram analysis

SDS-PAGE was performed with the cytoplasmatic extracts of the active subclones harboring the shortest inserts to fractionate the proteins. The gels of 0.02% CMC substrate were then soaked in a refolding buffer (washed with 25% [v/v] of isopropanal for 4 times, left overnight and then 0.05 M citrate buffer pH 4.8 for 4 times) to allow the renaturations of the denatured proteins. For the zymogram analysis of the endoglucanases, the treated gel was incubated at 37°C for 3 h followed by staining and destaining as described above in the cellulase screening.

Enzyme activity assay

Cellulase activity was measured by a DNS method, through determination of reducing sugars liberated[13] 0.9 ml of CMC substrate in 0.05 M citrate buffer pH 4.8 and 0.1 ml of crude enzyme were incubated for 30 minutes at 37°C before adding 1 ml of DNS solution. The treated samples were boiled for 5 min prior to cool down in cold water for color stabilization. The optical density was read at 540 nm against reagent blank by a spectrophotometer (Thermo Spectronic, USA).

Protein determinations

Protein concentrations were determined by using a Bradford method with bovine serum albumin (BSA) as a standard[2].

Characterization of cellulase

1. Effect of pH on cellulase activity

The assay mixture consisted of 100 μl of enzyme solution was mixed with 900 μl of substrate solution buffered to various pH values. The following buffers were used: citrate pH 4.0, 4.5, 5.0, 5.5 and 6.0;

phosphate pH 6.0, 6.5 and 7.0; Tris-HCl pH 7.0, 7.5, 8.0, 8.5 and 9.0 and glycine-NaOH pH 9.0, 9.5, 10.0, 10.5 and 11.0, all at 50 mM. The reaction mixture was incubated at 37°C for 30 min and the glucose released as detected by DNS-reagent was measured at 540 nm. The cellulase activity was expressed as the percentage of relative activity compared with the highest enzyme activity.

2. Effect of temperature on cellulase activity

The assay mixture consisted of 100 µl of enzyme solution was mixed with 900 µl of substrate solution dissolved in 50 mM citratre-phosphate pH 5.5. Activity assays were performed using the DNS-method at 4, 25, 35, 45, 55, 65 and 80°C for 30 min. The enzyme activity was expressed as the percentage of relative activity compared with the highest enzyme activity.

3. Effect of pH on cellulase stability

Stability assays were performed by incubating 100 µl of cellulase in 400 µl of buffers of different pH values (citrate pH 4.0, 4.5, 5.0, 5.5 and 6.0; phosphate pH 6.0, 6.5 and 7.0; Tris-HCl pH 7.0, 7.5, 8.0, 8.5 and 9.0 and glycine-NaOH pH 9.0, 9.5, 10.0, 10.5 and 11.0, all at 50 mM) without substrate at 37°C for 30 min. The residual cellulase activity was measured by DNS-method. The assay mixture was added with 500 µl of substrate solution dissolved in 50 mM citrate-phosphate buffer pH 5.5 and then incubated at 37°C for 30 min.

4. Effect of temperature on cellulase stability

Stability assays were performed by incubating 100 µl of cellulase in 400 µl of 50 mM citrate-phosphate buffer pH 5.5 at 4, 25, 35, 45, 55, 65 and 80°C for 30 min. without substrate and then immediately cooled on ice for 10 min. The residual cellulase activity was measured by DNS method. The reaction was added with 500 µl of substrate solution dissolved in 50 mM citrate-phosphate pH 5.5 and then incubated at 37°C for 30 min.

5. Substrate specificity of cellulase

The celP8 and celP24 were used to study its ability on hydrolyze Carboxymethyl cellulose, Methylcellulose, Ethylcellulsoe, Avicel, Cellulose azure and Hydroxypropyl methyl cellulose. Cellulase activity was measured by a DNS method, through determination of reducing sugars liberated[13]. 0.9 ml of substrate in 0.05 M citrate buffer pH 5.0 and 0.1 ml of crude enzyme were incubated for periods of time (30 min, 12, 24, 48 and 72 h) at 37°C before adding 1 ml of DNS solution. The treated samples were boiled for 5 min prior to cool down in cold water for color stabilization. The optical density was read at 540 nm against reagent blank by a spectrophotometer (Thermo Spectronic, USA).

RESULTS AND DISCUSSIONS

Extraction and purification of environmental DNA

For several years, investigations of microbial diversity were mainly based on isolation and laboratory cultivation of bacteria which lead to underestimation of the true diversity. However, only bacterial isolates for which optimal cultivation criteria have been determined can be recovered by this approach.

Even if several different media and growth conditions are used, many of the native microbes will not grow in the laboratory. It is generally accepted that culture-based methods miss much of the microbial diversity in environmental samples.

The soil contained decomposed organic matters revealing a dark mud texture. The salinity was 15 ppt and the pH was 5.00 (data not shown). The total viable aerobic count was presented about 10^5 CFU/ml. In the presence of 15% NaCl, similar to the salinity of soil, the bacterial count was about 10^6 CFU/ml. The results showed ten times of bacterial count increasing in the presence of salt ir

that the predominant culturable bacterıa ın these mangrove ecosystems are halophilic bacteria which versatile in the conditions of acidic and high salt concentration in nature.

Two current methods used to obtain microbial DNA from soils or sediments

are bacterial cell extraction followed by cell lysis and DNA recovery and direct extraction by alkaline lysis. The direct extraction method produces a better DNA yield than the cell extraction method[28].

Molecular analyses for the study of soil microbial communities often depend on the extraction of DNA directly from soils. Most direct extraction protocols start with relatively harsh cell breakage steps such as bead-beating and freeze-thaw cycles, followed by the addition of detergents and high salt buffers and/or enzymic digestion with lysozyme and proteases. After typical organic extraction and alcohol precipitation, further purification is usually needed to remove inhibitory substances from the extract.

A persistent problem with soil extractions is that DNA tends to adsorb to soil particles giving lower yield. Adding detergents and salts can help to alleviate this problem, although SDS can inhibit PCR if not removed in subsequent steps.

Soils present some of the most difficult challenges to the development of suitable extraction and purification procedures. The complex matrix of soil harbors a variety of substances that inhibit the activity of polymerases and restriction enzymes or interfere with hybridization and detection protocols[17]. In this study we have developed a rapid method for direct extraction of DNA from soil by applying a freeze-thaw approach. This method overcomes the drawbacks of the previous techniques.

Accessing the metagenome by direct cloning depends on efficient recovery of high-quality microbial DNA from the environmental sample. In this study, the direct lysis protocol described by Kauffmann et al. (2004)[11] which reported the DNA can be recovered with high yield and low fragmentation was used for isolation of metagenomic DNA from mangrove soil samples. It was found that DNA recovered was still highly contaminated with humic substances indicated by a brown color of crude DNA extracts. The humic acid materials have similar charge characteristics as of DNA resulting in their co-purification events. Humic compounds strongly constrain DNA cloning, because they interfere with spectrophotometric DNA quantification, inhibit enzymatic manipulations like restriction digestions, ligations and decrease transformation efficiency[20]. The phenolic groups in humic acids denature biological molecules by bonding to amides or are oxidized to form quinine which covalently bonds to DNA[27]. In our s tudies, following cell lysis which is a first purification step of DNA is achieved by organic solvent extraction (phenol and chloroform), followed by ethanol and isopropanol-assisted precipitation[24]. Several purification procedures for removal of these inhibitory substances have been described, which differ with respect to convenience, quality and yield of DNA. Comparing the popular purification method is the use of agarose gel electrophoresis to separate DNA from humic materials. The gel containing polyvinylpolypyrrolidone (PVPP) is incorporated a short distance from the wells of an agarose gel. The DNA is unimpeded upon passage through the PVPP, while humic materials can be trapped. In other cases, the PVPP is included throught out the gel[17]. We found that the recovery yield is very low. A very traditional method, cesium chloride gradients, appears to be very effective. According to Steffan et al.[24] result, it noted that even extensive purification with CsCl gradient centrifugation resulted in DNA loss and did not remove all humic acids which showed the same result in our laboratory. There have been suggestions that differential ethanol precipitations can be used to remove humic materials. It was not found this protocol to be very effective in the variety of soil types as same as for mangrove soil in our laboratory. The DNA losses during extensive purification may compromise the detection of rare DNA sequences.

Finally, we compared the suitability of different methods to sufficiently purified crude DNA to support cloning. Similar to previous study[14], the best results were achieved using size exclusion chromatography with CHROMA SPIN+TE-1000 columns. This procedure resulted in nearly complete removal of all visible contaminants in one-step, without significant negative impact

on DNA quality, i.e. without further fragmentation (Figure 1). The success of the project depends on obtaining DNA extracts that are in sufficient quantity and of sufficient purity to be manipulated using molecular biology protocols.

Most of the DNA fragments extracted from the soils were larger than 23.1 kb, indicating that the present protocol does not cause severe DNA shearing. A total yield of about 12-40 µg of DNA per gram sample (wet weight) was obtained with the applied extraction and purification procedure (Table1).

DNA treated both column and electroelution was sufficiently pure to be digested by restriction enzymes, *Mbo*I, thus allowing the construction of small DNA insert libraries.

Thus, CHROMA SPIN+TE-1000 columns provided a rapid, simple and adequate clean-up of DNA in one single step without further degradation of DNA which depended on the sample, resulting in metagenomic DNA suitable for subsequent cloning and screening the various hydrolytic enzymes.

Construction and characterisation of a metagenomic DNA expression library

To facilitate the expression of inserted genes, the well-established high copy plasmid vector, pBluescriptII SK (+) was selected for library construction. *Mbo*I-digested DNA was ligated into pBluescriptII SK (+), followed by transformation of *E.coli* DH5α. Approximately 100 white colonies were obtained per 0.3-0.4 µg of DNA derived from soil, as extrapolated from the number of colonies achieved after transformation with 1:7, vector to insert molar ratio of ligation mixture. This observed cloning efficiency supports the suitability of the selected purification method for removal of inhibitory substances.

Activity-based screening of the metagenomic library

The mangrove ecosystems are well known for their productive and biodiversity richness. Soil characteristics of mangrove forests at Satul Province, south of Thailand, provide a remarkable environment for diverse bacterial communities with the specialized functional heterogeneity. To study the productivity of the mangrove communities, we screened the expression of targeted genes encoding the hydrolytic enzymes with biotechnological values, cellulase. Congo red staining in conjunction with CM-cellulose medium provides a useful screening for cellulolytic microorganisms. Positive colonies are surrounded by a halo indicating CM-cellulose hydrolysis. Haloes in the Congo red test result only if the substrate is hydrolyzed to oligomers with fewer than 5 sugar units (Neil et al., 1984). The use of Congo red as an indicator for β-D-glucan degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Incubation times are reduced from weeks or months to less than 1 day, and results are generally unambiguous. The method is readily adaptable to the screening or characterization of large numbers of isolates, using replica plating techniques[22].

The libraries were screened for cellulolytic activity using CMC (Table 1). From screening of 100 transformants, about 20 colonies showed cellulolytic activity. Restriction analysis of the recombinant plasmids isolated from CMC hydrolyzing clones revealed insert sizes of 0.7-1.0 kb. We selected the two cellulase producing clones, celP8 and celP24, for further gene analysis and partial characterization of gene encoding proteins.

To identify CMCase activity in gel, SDS-PAGE and zymogram analysis of the *B. subtilis* B6, celP8 and celP24 were performed using the partial purified enzymes by the acetone precipitation (Table 2). Denaturing zymogram analysis was a simple method of characterizing CMCases, but further evaluation of its compatibility with cellulase systems from other sources is needed. The recent demonstration that Congo red shows a strong interaction with polysaccharides containing contiguous β-(1→4)-linked D-glucopyranosyl units and a significant interaction with β-(1→3)-D-glucans and possibly some hemicellulosic galactoglucomannans provides a new basis for the assay of β-D-glucanase activities, using unmodified soluble substrates in an

agar gel plate diffusion system. The potential advantages of this system in enumerating and characterizing cellulolytic microorganisms derive largely from the intense color of the dye-glucan complex, which allows the use of very low substrate concentrations and a corresponding decrease in the time required to detect lower levels of enzyme activity. In addition, the system allows the use of a variety of well-characterized soluble β-D-glucans to differentiate organisms on the basis of the linkages which they are able to degrade[22].

Sequencing-Based screening of the metagenomic library

As the gene size of target hydrolytic enzymes does not exceed 1–2 kb, these relatively small DNA fragments are optimal regarding expression efficiency[15]. To analyze DNA sequence of insert, the plasmid DNA from the positive clones, celP8 and celP24, that containing the inserted DNA was subjected for sequencing. The size of the inserted genes of *celP8* and *celP24,* are 421 and 127 bp, respectively. The homology search was performed using blastN revealed that there was shown no homology to others cellulase genes. On the basis of a comparison of translated sequences, the celP8 and celP24 protein are distinguishable from all known cellulase genes (Table 3).

In this study, the total proteins concentration and CMCase activity of crude enzymes were determined comparing to the positive control *B. subtilis* ATCC 6633 (Table 3). To confirm the cellulose activity of celP8 and celP24, the partial characterization of the cellulases with the cytoplasmic extracts of the active subclones were performed. The enzyme activity was depending on the transcribed, translated and folded correctly protein in the heterologous host including their localization, either intracellular or extracellular. The heterologous expression may be the inclusion bodies or intracellular. To determine whether active cellulases are extracellular or intracellular, the presences of protein in culture supernatant and crude enzyme extracts were tested by adding to wells of agar plate containing CMC. The results showed in Figure 2 and Table 4 indicated that the active cellulases form both celP8 and celP24 are extracellular enzymes. These observation agreed with these cellulase can hydrolyze CMC in agar plate without cell disruption. Similar observations have been described by Kim et al. (2008)[12], they also could detect cellulase activity with no attempt to optimize gene expression.

The results in Figure 3 demonstrated that celP8 and celP24 had a CMCase activity although their homology searches were no carbohydrate binding domain (CBD) or conserved domain of any classes of cellulases. The enzymes have no defined CBD and are thus referred to as non-modular cellulases[16]. Cellulases lacking CBD show reduced activities against insoluble cellulose while retaining the capacity to depolymerise soluble cellulosic substrates.

Our results prove that there are many hitherto unidentified genes encoding various candidate enzymes with the potential of biotechnologically and industrial application with properties reflected their environmental ecosystems. In this presence study, we reported the identification and partial characterization of two novel endoglucanases, celP8 and celP24 from the consortium of microorganisms in mangrove ecosystems by functional screening of the small-inserted metagenomic libraries, about not exceed 1 kb DNA fragment which when expressed in *E. coli*, enabled it to utilize CMC, soluble cellulose as sole carbon source. The results were shown that they had no identities with any sequences from GenBank database. From the results, these enzymes were possessed CMCase or endoglucanase activity from the cellulase assay and zymogram analysis. They may be prospecting for the new and undiscovered subclass of the cellulolytic enzymes indicated the broad evolutionary diversity in these mangrove ecosystems from Satul province, Thailand. In addition, this research will provide the new opportunity for the development of biotechnological industries in Thailand.

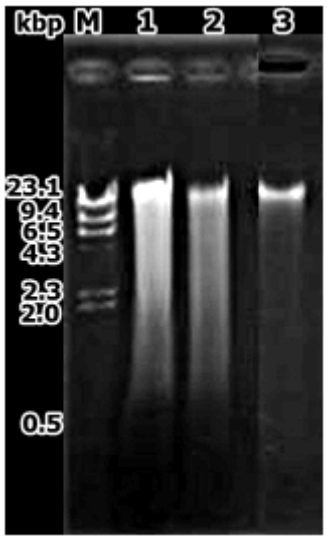

Figure 1. The purification of extracted DNA from mangrove soils with CHROMA SPIN + TE-1000 columns. Lane M: Marker λ/*Hind*III. Lane 1&2: Extracted DNA without purification. Lane 3: After purification, the DNA was shown clearly band with less impurities fragmentation.

Table 1. Metagenomic libraries constructed from different environmental sample.

Metagenomic libraries	Yielded (µg)*	Average insert size (kb)	Hit rates of hitherto enzymes
Mangrove forest soil	12-40	0.7-1	cellulase: 20 in 100

*µg of gDNA obtained from a gram soil (wet weight), extrapolated from the concentration of high purity gDNA after purification

Table 2. Partial purification of celP8 and celP24

Material	Cloned tested	Total protein (mg)	Total activity (U ml^{-1})	Recovery (%)*
Crude extracts	*B. subtilis* ATCC 6633	0.470	0.389	100
	celP8	0.205	0.068	100
	celP24	0.192	0.043	100
Precipitation with acetone	*B. subtilis* ATCC 6633	0.844	0.786	13.5
	celP2#8	0.613	0.762	74.7
	celP24	0.544	0.668	100

* % recovery calculated from total activity

Table 3. Nucleotide sequences analysis of *celP8* and *celP24* genes.

Active clone	Insert size (bp)	Score (Bits)	Identities (%)	Description	ORF[a] (Nt range[b], AA[c])	Most similar protein[d], Score(Bits), %Identities
celP8	421	69.8	73%	*Burkholderia multivorans* ATCC 17616 genomic DNA, complete genome, chromosome 2	7-420, 137	Putative small-conductance mechanosensitive channel transmembrane protein of *Ralstonia metallidurans* CH34, 89.0, 40%
celP24	127	42.8	84%	Zebrafish DNA sequence from clone CH211-151G22 in linkage group 21, complete sequence	20-126, 35	No significant similarity found

[a] ORF, open reading frame
[b] Nucleotide range (Nt range) of predicted ORF within insert

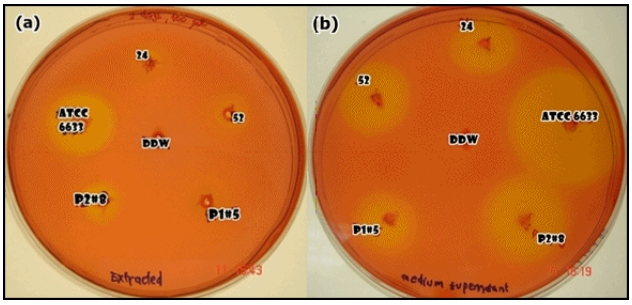

Figure 2. Plate screening confirmation for CMC-hydrolyzing activity from a metagenomic library using a Congo red staining. The positive cellulase activity of *Bacillus subtilis* ATCC 6633 was prepared as a positive control and distilled water as a negative control.
(a) The cloned DNA with cellular enzyme extraction by sonication method. Crude extracts were added to wells of agar plate containing CMC.
(b) Clones with extracellular enzyme produced into medium as supernatant.

Table 4. Hydrolysis zone indicating the cellulase activity. The culture supernatant and crude enzyme extracts were tested by adding to wells of agar plate containing CMC.

Sample tested	**Hydrolysis zone (mm)**	
	Culture supernatant	**Crude extracts**
B. subtilis ATCC 6633	33	26
celP8	20	9
celP24	15	6

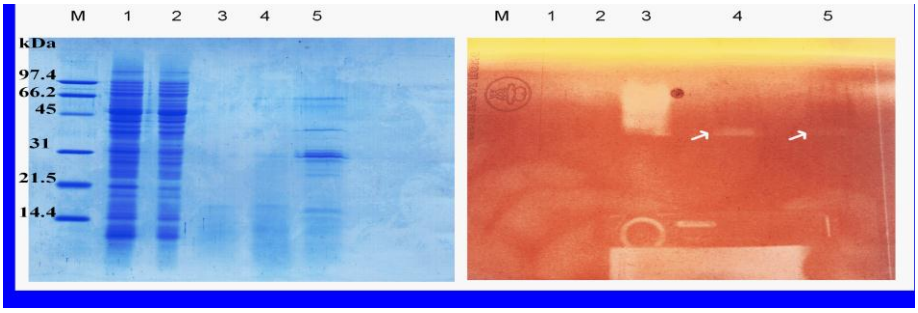

Figure 3. Zymogram of the 2 cloned cellulases in denature polyacrylamide gel elctrophoresis. (a) Staining of proteins in the crude extracts separated on the polyacrylamide gel with Coomassie blue G-250 (b) Carboxymethyl cellulose (CMCase) activities detected on CMC- containing polyacrylamide gel. M, low-molecular weight SDS-PAGE marker: Lane 1, host *E.coli* DH5α: lane 2, *E.coli* DH5α harbouring pBluescript II SK (+) : lane 3, *B. subtilis* ATCC 6633: land 4, celP8: land 5, celP24. The arrows indicate to a zone in which CMC was hydrolyzed by the cellulases.

Enzyme Characterization

Most of the cellulases (1,4-β-D-glucan glucanohydrolases; EC 3.2.1.4) produced by microorganisms are known to be active at acidic or neutral pH and usually to be inactive in the alkaline pH range[5]. The activity curves with respect to pH show for the endoglucanase a pH optimum at pH 10.0 and 11.0 (Figure 4) for celP24 and celP8, respectively. Both cellulase preparations displayed maximal activities around pH 10.0 to 11.0. The activity depended on the pH and the kinds of buffers used. It was observed that glycine-NaOH was higher activities than Tris-HCl at the same pH and Tris-HCl buffer was also higher activities than phosphate buffer. Moreover, the citric buffer gave the lower activities compared to phosphate buffer at the same pH values. They were maximum stable at around pH 6.5 in phosphate buffer and gave the stability in any pH and buffers conditions even in acidic and alkaline conditions (Figure 6). While the control, ATCC 6633, stable at acidic conditions more than alkaline conditions. The stability decreased continuously from acidic to alkaline conditions. celP8 and celP24 endoglucanase preparations displayed a temperature optimum around 35°C and 25°C, respectively and were stable up around 35˚C (Figure 6 and Figure 7) . It should be noted that the useful temperature range of enzymes, at which they are not completely inactivated within a few minutes, is always some 10 to 20˚C lower than the activity maximum.

The K_m and V_{max} values on carboxymethyl cellulose (CMC) substrate were 1.90 mg/ml and 148.2 units/mg protein, respectively.

However, no data were given on the thermal stability and chemoresistance of the heterologous enzymes.

The role of endoglucanases in the hydrolysis of cellulose was studied by analysing the activity of endoglucanases towards different substrates: avicel, cellulose azure, ethylcellulose, methylcellulose and Hydroxypropyl methyl cellulose

The enzyme hydrolyzed CMC and acid-swollen Avicel. They were highly efficient in degrading ethylcellulose (Table 5). They gave the most resulting in highly degrade for all of substrates. The presence of β-1, 4 bonds appears to be required for enzyme action, since laminarin, a predominantly β-1, 3-linked glucan, was not degraded. No significant degradation was observed. The enzyme is therefore considered to be a, β-1, 4-endoglucanase.

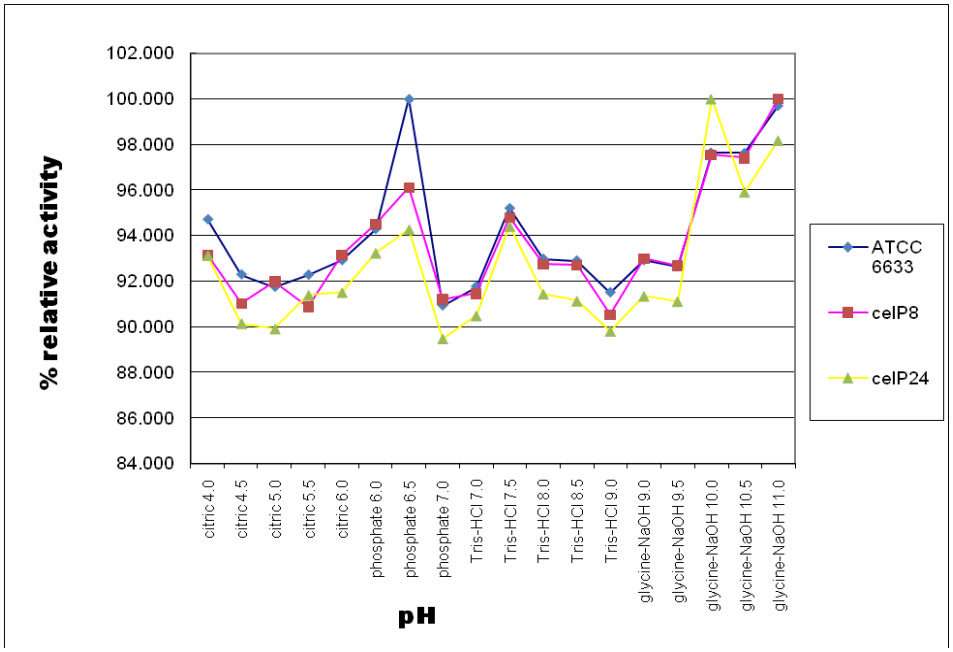

Figure 4. The Effect of pH on the activity of cellulase from cloned DNA compared with control, *Bacillus subtilis* ATCC 6633. Assay conditions: 37˚C, the bufferes used were Citric buffer: pH 4.0, 4.5, 5.0, 5.5 and 6.0; phosphate buffer: pH 6.0, 6.5 and 7.0; Tris-HCl buffer : pH 7.0, 7.5, 8.0, 8.5 and 9.0 and glycine-NaOH buffer: pH 9.0, 9.5, 10.0, 10.5 and 11.0, all at 50 mM. The results were expressed as a percentage of the relative activity of the enzyme.

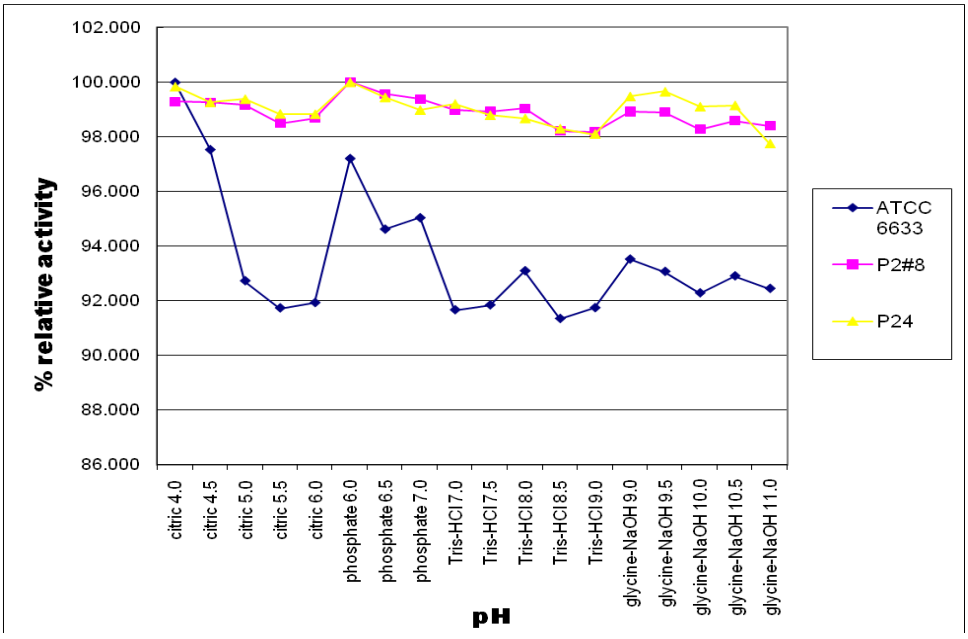

Figure 5. The Effect of pH on the stability of cellulase from cloned DNA compared with control, *Bacillus subtilis* ATCC 6633. Assay conditions: 37˚C, the bufferes used were Citric buffer: pH 4.0, 4.5, 5.0, 5.5 and 6.0; phosphate buffer: pH 6.0, 6.5 and 7.0 ; Tris-HCl buffer : pH 7.0, 7.5, 8.0, 8.5 and 9.0 and glycine-NaOH buffer: pH 9.0, 9.5, 10.0, 10.5 and 11.0, all at 50 mM. The results were expressed as a percentage of the relative activity of the enzyme.

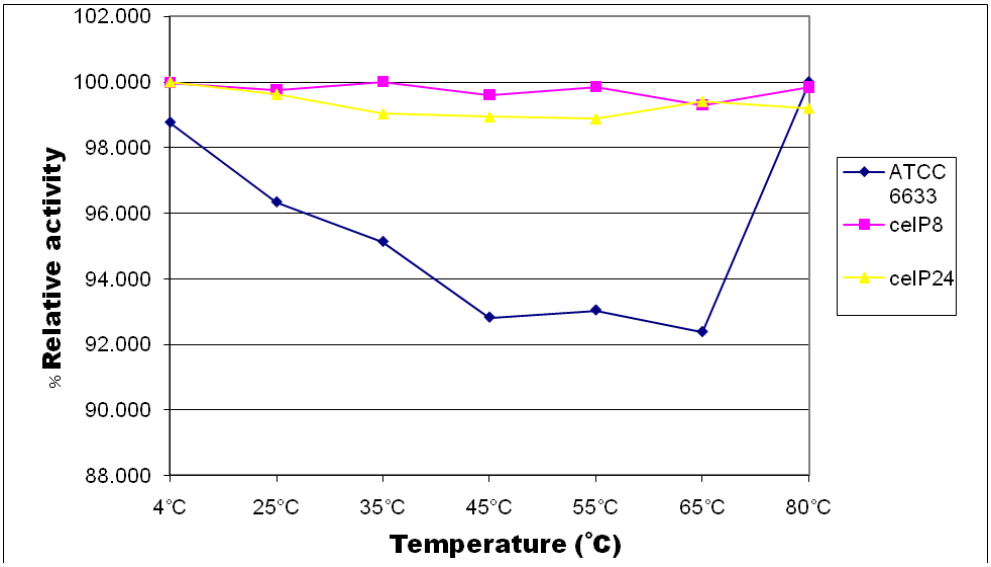

Figure 6. The Effect of temperature on the activity of 2 cellulase actives cloned compared with control, *Bacillus subtilis* ATCC 6633. Assay conditions; 4, 25, 35, 45, 55, 65 and 80˚C of incubation, 50 mM citrate-phosphate buffer pH 5.0. The results were expressed as a percentage of the relative activity of the enzyme.

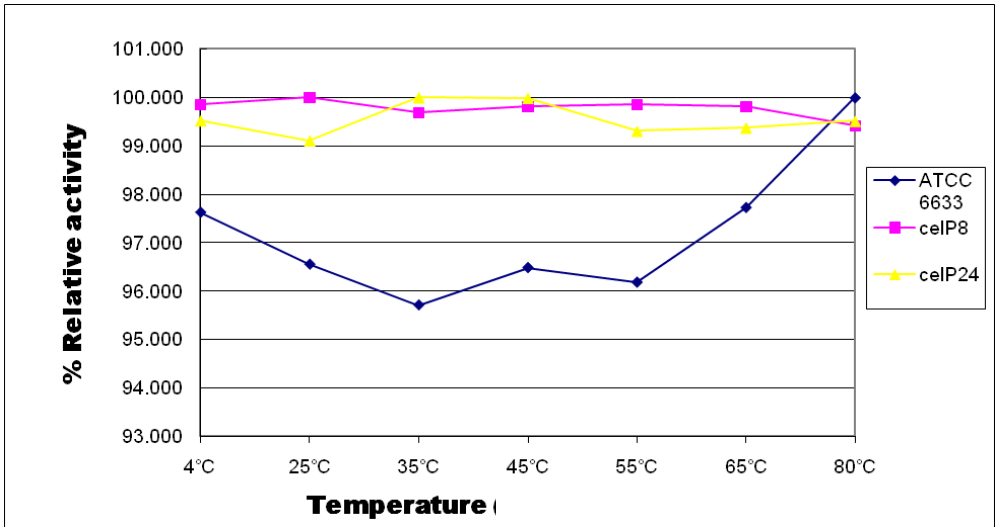

Figure 7. The Effect of temperature on the stability of 2 cellulase actives cloned compared with control, *Bacillus subtilis* ATCC 6633. Assay conditions; 4, 25, 35, 45, 55, 65 and 80˚C of incubation for 30 min, 50 mM citrate-phosphate buffer pH 5.0. The results were expressed as a percentage of the relative activity of the enzyme.

Table 5. Specificity of cellulase of 2 cloned samples from metagenomic in respect of various cellulose substrates.

Substrate	Relative activity (%)	
	celP8	celP24
Avicel	99.49	96.44
Carboxymethyl cellulose	96.76	97.13
Cellulose azure	97.80	95.96
Ethylcellulose	100	100
Hydroxypropylmethyl cellulose	99.29	92.25
Methylcellulose	97.56	96.53

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REFERENCES

1. Amann RI, Ludwig W, Schleifer K-H. Phylogenetic identification and in situ detection of individual microbial cells without culturing. *Microbiol. Rev***.** 1995; 59:143–169.
2. Bradford MM. A Rapid and Sensitive Method for the Quantition of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* 1976; 72:248-252.
3. Cowan SW, Schirmer T, Rummel G, *et al*. Crystal-Structures Explain Functional-Properties of 2 *Escherichia coli* Porins. *Nature*.1992; 358:727-733.
4. Ferrer M, Golyshina OV, Chernikova TN, *et al.* Novel hydrolase diversity retrieved from a metagenomic library of bovine rumen microflora. *Environ Microbiol.* 2005; 7:1996–2010.
5. Fumiyasu F, Toshiaki K, Nobuhiro S, *et al.* The Third Cellulase of Alkaline *Bacillus* sp. strain N-4: Evolutionary Relationships Within the *cel* Gene Family. *Elsevier. Gene.* 1989; 76:289-298.
6. Ginolhac A, Jarrin C, Gillet B. Phylogenetic Analysis of Polyketide Synthase I Domains From Soil Metagenomic Libraries Allows Selection of Promising Clones. *Appl. Environ. Microbiol.* 2004; 70:5522–5527.
7. Han KF and Baker D. Recurring Local Sequence Motifs in Proteins. *J. Mol. Biol.* 1995; 251:176-187.
8. Healy PJ, Harper PA. and Dennis JA. Phenotypic Variation in Bovine a-Mannosidosis. *Res. Vet. Sci*. 1990; 49-82.
9. Healy FG, Ray RM, Aldrich HC, *et al*. Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. *Appl Microbiol Biotechnol*. 1995; 43:667–674.
10. Humphrey AE. Fuel from Biomass. *Department of Energy Meeting*. 1978; 12 -13.
11. Kauffmann M, Schmitt J, Schmid RD. DNA isolation from soil samples for cloning in different hosts. *Appl Microbiol Biotechnol* 2004; 64:665-670.
12. Kim SJ, Lee CM, Han BR, *et al*. Characterization of a gene encoding cellulase from uncultured soil bacteria. *FEMS Microbiol Lett*. 2008; 282:44–51.
13. Krootdilaganandh J. Isolation and Selection of Thermotolerant Bacteria Capable of Producing Cellulase. *Chiang Mai: Chiang Mai University Press*. 2000; 20-21.
14. Lämmle K, Zipper H, Breuer M, *et al.* Identification of novel enzymes with different hydrolytic activities by metagenome expression cloning. *J. Biotechnol*. 2007; 127:575–592.
15. Lorenz P, Liebeton K, Niehaus F, Eck J. Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. *Curr Opin Biotechnol*. 2002; 13:572–577.
16. Lynd LR, Weimer PJ, Willem HZ, *et al*. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev*. 2002; 66:506–577.
17. Mark AS, Sophia YD and Charles FK. Direct Extraction of DNA from Soils for Studies in Microbial Ecology. *Curr. Issues Mol.Biol*. 2003; 6:1-8.
18. Neil RG, Maureen LL, Douglas GK, Robert CM and Anthony JW. Mode of Action and Substrate Specificities of Cellulases from Cloned Bacterial Genes. *The journal of Biological Chemistry*. 1984; 259:10455-10459.
19. Pang H, Zhang P, Duan C-J *et al.* Identification of cellulase genes from the metagenomes of compost soils and functional characterization of one novel endoglucanase. *Curr Microbiol* 2009; 58: 404-408.
20. Rajendhran J and Gunasekaran P. Strategies for accessing soil metagenome for desired applications. *Biotechnol Adv*. 2008; 26: 576–590.
21. Rees HC, Grant S, Jones B, *et al*. Detecting cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries. *Extremophiles.* 2003; 7:415–421.
22. Ronald MT and Peter JW. Use of Congo Red-Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rument. *Applied and Environmental Microbiology.*1982; 777-780.
23. Rondon MR, August PR, Bettermann AD. Cloning the Soil Metagenome: A Strategy for Accessing the Genetic and Functional Diversity of Uncultured Microorganisms. *Appl Environ Microbiol.* 2000; 66:2541–2547.
24. Steffan RJ, Goksoyr J, Bej AK, Atlas RM. Recovery of DNA from soils and sediments. *Appl. Environ. Microbiol*. 1988; 54:2908–2915.

25. Voget S, Steele HL, Streit WR. Characterization of a metagenome-derived halotolerant cellulase. *J Biotechnol.* 2006; 126:26–36.
26. Wang F, Li F, Chen G, Liu W. Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. *Microbiol Res* 2009; 164:650-657.
27. Young CC, Burghoff RL, Keim JG, Minak-Berbero V, Lute JR, Hinton SM. Polyvinylpyrrolidone–agarose gel electrophoresis purification of polymerase chain reaction amplifiable DNA from soils, *Appl. Environ. Microbiol.* 1993; 59: 1972–1974.
28. Yu-Li T and Betty HO. Rapid Method for Direct Extraction of DNA from Soil and Sediments. *Applied and Environmental Microbiology*. 1990; Apr. 1070-1074. Vol. 57 No.4.
28. Zhang YHP and Lynd LR. Cellulose Utilization by Clostridium Thermocellum: Bioenergetics and Hydrolysis Product Assimilation. *Proc. Natl. Acad. Sci. USA*. 2005; 102:7321-7325.
29. Zhou J, Bruins MA and Tiedje JM. DNA Recovery from Soils of Diverse Composition. *Appl Environ Microbiol.* 1996; 62:316–322.