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New source of cellulase production using a metagenomic technique

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Abstract

The cellulase enzymes with high effectiveness under conditions agreeable to the industrial processes necessities are one of the keys for the successful development of chemical and drug synthesis. The soil metagenome is an affluent source for the discovery of new natural products. The objective of the current study was to identify the isolated functional gene(s) of the cellulase enzyme by using metagenomics. The plan of work composed of collection of different soil samples, isolation of total DNA, fragmentation, cloning, and expression of the isolated gene(s) in the suitable host microorganism. The total genomic DNA was extracted using a kit (QIAGEN), and then digested by different restriction enzymes BamHI. The digested fragments ranging from ~300-5000 bp were ligated, cloned into pUC19 vector, and then transformed into *Escherichia coli* DH5α. The resulting clones were screened as cellulase producers using a qualitative method. The positive clones which showed hydrolysis on the plate were screened once more in Luria-Bertani (LB) medium. The plasmids were isolated and then tested using universal primer (M13), to detect the fragment size and sequence for the Polymerase Chain Reaction (PCR) products. This study establishes an effortless and professional method for cloning of recent cellulase genes through ecological metagenomes. In the outlook, the metagenomic guide approachs may be functional to the elevated selection of novel cellulase from the environment.

Keywords: Cellulase, Metagenome, Qualitative estimation, Plasmid, Cloning

1. Introduction

Functional genomics is being used to search for specific activities of an organism without the need for culturing this organism that harbor the pathways involved (Gillespie *et al*., 2002). In a previous study, Metzker, (2010) reported that parallel sequencing techniques known as next-generation sequencing (NGS) have been recognized as an innovative tool for handling large amounts of sequence data, compared to conventional sequencing methods over the past decade. Currently, the environmental metagenomic\

meta-transcriptomic studies as NGS generate huge sequence data sets that can also be provided for the release of pipelines such as MEGAN-SEED and KEGG (Kyoto Encyclopaedia for Genes and Genomes) (Mitra *et al*., 2011). MEGAN allows the functional analysis of metagenomic and meta-transcriptomic datasets using the SEED classification, based on the given BLAST file. Most of the microorganisms responsible for nutrients cycling in the environment have to be cultivated, and this could include those species responsible for the degradation of cellulose. It has recently been reported by Fernández-Arrojo *et al*., (2010); Ferrer *et al*., (2016) that the discovery of new enzymes through metagenomics has enormous potential to obtain a wide range of useful biocatalysts. The cellulase enzymes are well known at the level of the protein sequencing; however, the variants of genes from the environmental DNA are difficult to amplify. It is possible to identify novel cellulase genes free of DNA amplification, by following a direct metagenome sequencing approach to provide genes that can be cloned, expressed and classified prior to future use. All these are possible without the need for any data on the organisms from which they originated (David *et al*., 2012). The vital goal is to develop a method for the industrial and economic transformation of the lignocellulosic biomass into biofuel molecules. Recently, Rameshwar *et al*., (2018) reported that as cellulose is the most abundant biopolymer and also represents the photo synthetically permanent form of carbon, the most essential step for the improvement of the biofuel manufacture progress is through the efficient hydrolysis of cellulose. The hydrolysis of cellulose takes place through the action of cellulases enzymes which consist of; endoglucanases, exoglucanases and β-glucosidases enzymes. According to Lynd *et al*., (2002), the Endoglucanases (EC 3.2.1.4) randomly hydrolyse the internal bonds in the chain of the cellobiose or cello-oligosaccharides. Several previous studies of Xia *et al*., (2013); Xia *et al*., (2014) focused on the annotation of the carbohydrate-active enzymes in association with cellulose degradation using the metagenome. According to Angenent *et al*., (2004); Goacher *et al*., (2014); Huang *et al*., (2014), the biodegradation of microbial cellulose by cellulosomes and cellulases is a major source of flow of carbon from fixed carbon sinks to atmospheric CO_2, and acts as an essential function in some industrial biological processes. β-Glucosidase is an important enzyme for the full conversion of the cellulosic biomass into glucose. Moreover, Coenen *et al*., (1995); Zhang *et al*., (1996); Swiegers *et al*., (2005); Kim *et al*., (2007); Lu *et al*., (2013) added that the β-glucosidase enzyme is concerned with the production of many other biological, catalytic and artificial molecules such as; nutrient supplements, flavour precursors, alkyl or oligo-saccharide synthesis, pharmaceuticals and feed additives. The aim of this study was to isolate functional gene(s) of the cellulase enzyme by using metagenomics technique.

2. Material and methods

2.1. Sample collection and preparation

This screening study was conducted during 2018. A total of 12 soil samples were collected from different locations of Alexandria governorate, Egypt, and were used for isolation of the DNA.

2.2. Preparation of the chromosomal DNA

The genomic DNA was isolated by using a specific kit for DNA isolation from soil (QIAGEN).

2.3. DNA restriction digestion

The chromosomal DNA was digested in 20 µl reaction volumes, then 2 µl of the enzyme buffer and 1-2 units of the restriction enzyme were added. The DNA digestions with the restriction enzymes were carried out under the reaction conditions specific for each enzyme, as suggested by the manufacturers (Fermentas). Different restriction enzymes were used including; HindIII, BamHI, EcoRI and SalI.

2.4. DNA ligation

A ligation was made into 20 µl reaction volume, 2 µl of T4 DNA ligase buffer, 1 unit of T4-DNA ligase

enzyme, the digested DNA was inserted, and then a vector (4:1) was added. The reaction was performed at 16°C overnight. The PUC 19 vector was used as the screening and expressed vector in reference to Sambrook *et al*., (1989).

2.5. Preparation and transformation of the competent cells

An isolate of *E. coli* DH5α was prepared according to Sambrook *et al*., (1989). Approximately, 100 ml of LB was inoculated with 100 µl of an overnight culture of E. coli DH5α, and then incubated at 37°C with shaking (170 rpm) till the OD (600 nm) become 0.6-0.7. The flask was ice chilled and divided into 50 ml portions. The cells were collected with centrifugation at 4000 rpm, and then re-suspended in TSS solution (10 g/ l polyethylene glycol (PEG 6000), 50 g/ l (v/v) dimethylsulfoxid (DMSO), 50 mM $MgCl_2.6H_2O$). This cell suspension was dispensed into sterile Eppendorf tube (200 µl aliquots), which was frozen immediately at -80°C. The frozen aliquots of the competent cells were allowed to thaw on ice. After that, the DNA (ligation mixture) was added to the tube, and then incubated for 20 min. in ice. The tube was heat shocked at 42°C for 60 s, about 800 µl of LB medium was added, and then the tube was incubated for 1 h at 37°C, with continuous shaking. Aliquots of 200 µl were spread on the selective LB plates containing; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal), and Isopropyl β- d-1-thiogalactopyranoside (IPTG).

2.6. Preparation of the plasmid-DNA from *E. coli*

Mini-plasmid extraction was done using GEBRI kit as follows: cells (1.5 ml) from overnight culture of *E. coli* were collected by centrifugation at 7000 rpm, suspended in 300 µl of solution I, and then lysed by the addition of 300 µl solution II with mild shaking. About 300 µl of solution III was added to precipitate the protein and the genomic DNA, followed by centrifugation at 13000 rpm for 10 min. The supernatant that contained the plasmid DNA was precipitated with isopropanol, washed with 70% (v/v) ethanol, and then re-suspended in 30 µl of dist. water.

2.7. Screening and measurement of the enzyme activity

Pure clones were screened for cellulase activity in nutrient agar (NA) medium supplemented with the substrate. For qualitative estimation of the cellulase enzyme, the substrate plate method was used. About 0.2 % (w/v) of the substrate (carboxy methyl cellulose) was added into LB medium. The substrate was solubilized, 20 g/ l agar was added, autoclaved, poured in Petri plates, inoculated with the desired bacteria, and then incubated at 28°C for 48 h. The appearance of a transparent halo zone around the bacterial colonies after addition of the indicator (0.2 % w/v Congo red) and washing with 1 M sodium chloride indicated production of the cellulase enzyme.

2.8. Sequencing of the DNA

The DNA fragments containing genes which confer the cellulolytic activity from the positive clone were analysed using a universal primer: M13 F: AGGCCCTGCACCTGAAG, and M13 R: TCAGCGCCTGGTACC according to Soliman *et al*., (2007).

3. Results

The incidence of the positive cellulase clones obtained from the collected soil samples using the metagenomic technique, was evaluated through the isolation of DNA. The DNA purity noticed is 1.8 with concentration of 1298 ng/ µl. Results after ligation reveled that about 96 clones are recovered from the digested DNA using the Bam HI, 84 clones are selected from the DNA digested with HindIII, 110 clones are selected from the DNA digested with EcoRI, and finally about 49 clones are selected from DNA digested with SalI.

3.1. Screening and selection of the cellulase positive clones

In a screening program for the isolation of cellulase enzymes producing clones, about 339 clones are obtained from the different DNA, which are ligated to puc19 digested with the same enzyme. The qualitative screening of the cellulase was performed on carboxy methyl cellulose agar (CMC) plates at 37°C (Fig. 1). Results revealed that 13 clones among all the tested clones showed variation in their hydrolytic potential that are expressed through zone diameter in mm, however only 5 clones exhibit a promising result concerning the specific activity. The quantitative estimation of cellulase by the selected clones demonstrates that the 5 clones (1, 2, 3, 4 and 5) give (4, 12, 15, 2 and 11 U/ min/ ml), respectively.

3.2. Extraction of the plasmid

Plasmid extraction of the selected cellulase clones is clear in Fig. (2), where the plasmid concentration and purification were estimated using a Nano Drop 2000 UV Visible Spectrophotometer. Results in Fig. (2) demonstrate that the plasmid concentration is different among the 5 clones (1300-1890 ng/ µl), with purity values that range from 1.8- 2.

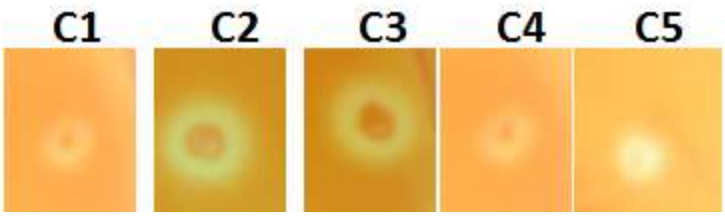

Fig. 1: The five positive cellulase clones obtained on CMC agar plates through qualitative screening. The 5 clones (C1, 2, 3, 4 and 5) give (4, 12, 15, 2 and 11 U/ min/ ml), respectively.

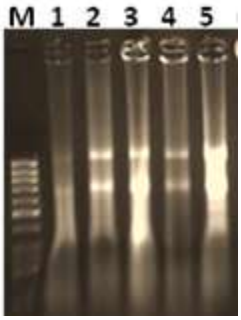

Fig. 2: Agarose gel electrophoresis of the plasmid extracted from the 5 positive cellulase clones where; M: 10 kb DNA marker

3.3. Molecular phylogeny of the selected clones

The PCR was applied to the selected clones coded as; 1, 2, 3, 4 and 5. The molecular identification of the promising clone 1 (selected from DNA digested with Bam HI), clone 2 (selected from DNA digested with HindIII), clone 3 (selected from DNA digested with EcoRI), clones 4 and 5 (selected from DNA digested with SalI), were identified using the M13 universal primer. The products of the PCR analysed by 1% agarose gel are shown in Fig. (3). The identification and classification of the cellulase gene were carried out by comparing its sequence with the other sequences present in the GenBank data base.

3.4. Phylogenetic analysis of the cloned fragment

For construction of the phylogenetic tree, multiple sequence alignments were performed using Clustal W version 1.83 with default parameters. On the basis of results of the multiple sequence alignments, a phylogenetic tree is constructed for the cellulase gene, by applying the maximum-likelihood method implemented in the Tree-Puzzle software (version 5.2) as shown in Fig. (4).

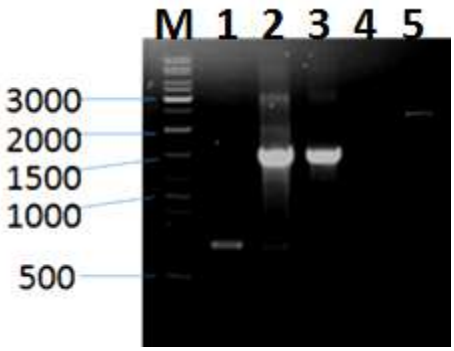

Fig. 3: Agarose gel electrophoresis of the amplified PCR fragment of the M13 gene of the 5 positive cellulose clones. Where; M: 10 kb DNA marker.

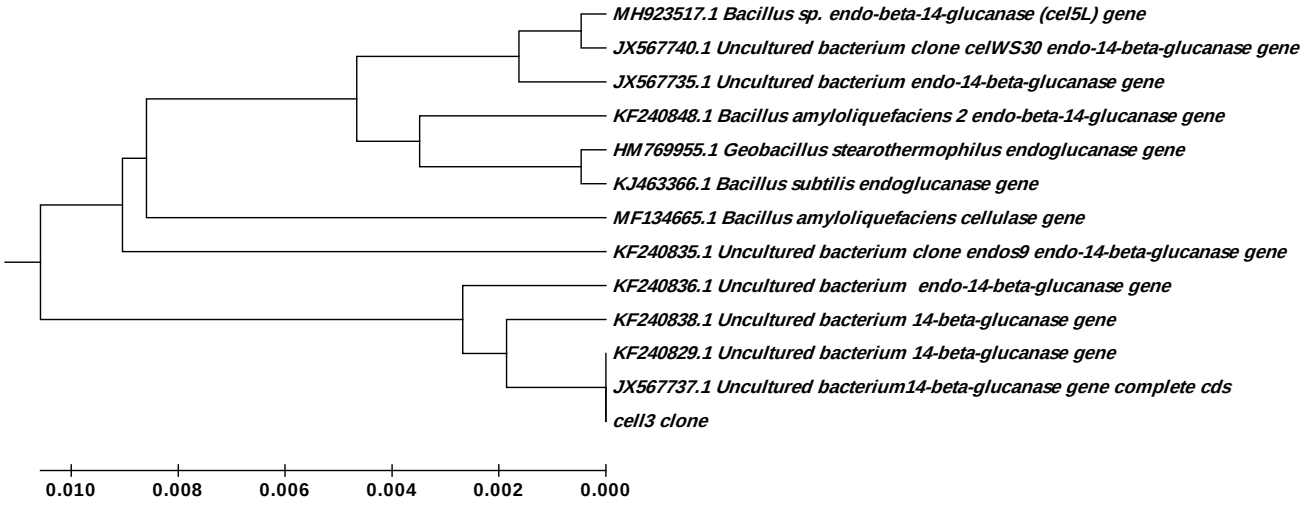

Fig. 4: The phylogenetic tree of the cell3 gene of clone 3 compared with different glucanase genes from the GenBank database. This dendrogram is generated using the Mega 5 Software.

4. Discussion

Some cellulases genes are obtained by functional screening through the metagenome technique. Several previous studies of Uchiyama and Miyazaki, (2009); Ko *et al*., (2013); Mewis *et al*., (2013); Yan *et al*., (2013) reported that the effectiveness of this strategy for discovering new cellulases from the environmental metagenomes, which can hardly meet the growing industrial demands. The hydrolysis of cellulose takes place through the action of cellulases enzymes which consist of; endoglucanases, exoglucanases and β-glucosidases enzymes. According to Lynd *et al*., (2002), the Endoglucanases (EC 3.2.1.4) randomly hydrolyse the internal bonds in the chain of the cellobiose or cello-oligosaccharides. Previous studies of Xia *et al*., (2013); Xia *et al*., (2014) focused on the annotation of the carbohydrate-active enzymes in association with cellulose degradation using the metagenome. Through the protein engineering activities, a metagenome derived from β-glucosidase was also investigated. Yang *et al*., (2013) cloned, characterized the pulp sewage β-glucosidase, and identified the amino acids involved in substrate binding and in catalysis, by using the site-directed mutagenesis.

In the current study, the expression of the cellulase (cell 3) gene was carried out in *E. coli* DH5α. A good intracellular expression however is obtained after cellulase induction using the IPTG. This is in accordance with the previous studies of Amaki *et al*., (1992); Tulin *et al*., (1993), who expressed the same enzyme in *Bcaillus brevis*. The cellulolytic microorganisms cause significant cellulose hydrolysis, but after hydrolysis diversion towards different metabolic pathways gives mixed gaseous acidogenic fermentation products (Lynd *et al*., 2002; Demain *et al*., 2005; Ganesh and Sang, 2012). Similar characterization of the bacterial endoglucanase has been reported (Park, 2001); however, limited characterization of the cellulase enzyme has been carried out.

Conclusion

The metagenomic technique is used to detect new protein engineering activities. Thus, new cellulases can be produced by using this metagenome analysis.

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Conflict of interest

The authors declare that they have no conflict of interests

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