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Isolation and Identification of New Cellulases Producing Thermophilic Bacteria from an Egyptian Hot Spring and Some Properties of the Crude Enzyme

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Abstract: Thermophilic cellulases producing bacteria have been isolated from an Egyptian hot spring by enrichment of the water and soil samples with cellulose for 3 weeks at 70 °C. Three isolates termed EHP1, EHP2 and EHP3 have been isolated. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that strain EFP1 had highest homology (98.5%) with *Anoxybacillus flavithermus,* EFP2 showed 98.5 % similarity with *Geobacillus thermodenitrificans* and EHP3 showed 99.0 % similarities with *Geobacillus stearothermophilus*. Maximal cellulases production by *Anoxybacillus flavithermus* EHP2 was detected at the end of the stationary phase (36 h). The crude cellulase had activity toward avicell, CMC, cellobiose, and xylan, but there was no detectable activity on *p*-nitrophenyl-β-d-glucopyranoside. The rate of CMC degradation was higher than any other substrates used in this study. The optimum temperature and pH for the crude enzyme activity was 75 °C and 7.5, respectively.

Keywords: Cellulases, thermophiles, hot spring, isolation, 16S rDNA

INTRODUCTION

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee, S.M. and Koo, 2001). The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase; endoglucanase (1,4-β-d-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-β-d-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (β-d-glucoside glucohydrolase; EC 3.2.1.21) (Yi, J.C., *et al.,* 1999). The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-glucosidase (Bhat, M.K and S. Bhat, 1997). Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Pola, A., 1998). Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gong, C.S., *et al.,* 1999; Homel, M.E., 1999). Application of enzymes in detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature. The search for extremophilic organisms is one of the means for obtaining enzymes with properties suitable for industrial applications. There are quite a few advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes (Kristjansson, J.K., 1989). The main advantage is that as the temperature of the process is increased, the rate of reaction increases. A 10 ~ increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed (Haki, G.D., and S.K., Rakish, 2003). The thermostable enzymes are also able to tolerate higher temperatures, which give a longer half-life to the enzyme. The use of higher temperatures (above 60 ~ also is inhibitory to microbial growth, decreasing the possibility of microbial contamination. Enzymes from thermophilic organisms have been increasing, partly due to the ability to clone the genes from the thermophiles into mesophilic production strains [Berquist]. One of the natural habitats of the thermophilic bacteria is the hot spring where the temperature of the water is between 70 to 100 °C. A hot

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spring is a spring that is produced by the emergence of geothermal-heated groundwater from the earth's crust (Kauze, T., *et al.,* 2006). There is hot springs all over the earth, on every continent and even under the oceans and seas. Hammam Pharoan is one of the hot springs in Egypt which has been not yet explored in details from the microbiological point of view. The aim of this study was to isolate and identify new cellulases producing bacteria from Egyptian hot spring.

MATERIAL AND METHODS

Enrichment, Isolation and Screening for Cellulases Producing Thermophilic Bacteria:

Water and soil samples were collected in sterile containers from an Egyptian hot spring, Hammam pharoan, in Sinai dessert in the east of Egypt at the red sea beach. The temperature of the water was between 70 °C to 90 °C according to the site of collection. The pH of the water was around 7.5 Two protocols were used for isolation of thermophilic cellulases producers:

Direct Isolation:

The collected soil samples were suspended and serially diluted in sterile distilled water up to 10^{-4}, 100 µl of each dilution and water samples (without dilution) were spread on agar plates and incubated at 70 °C for 1-2 weeks. The plates were stained with Congo red, destained with 1 M NaCl solution, and scored for a clear halo surrounding the colony (Wood. T.M., and K.M. Bhat, 1988).

Enrichment of Thermophilic Cellulases Producers:

For enrichment of the water and soil samples for cellulases producing thermophilic bacteria, 5 gm of sterile CMC and 5 gm of avicel were addede to a soil/water samples, mixed and incubated for 3-4 weeks at 70 °C. The samples were diluted in sterile distiled water, plated on agar medium and incubated at 70 °C for 1-2 weeks. The agar medium contained: Nitrilotriacetic acid (100mg/l), $CaSO_4$ (60 mg/l), $MgSO_4$ (100 mg/l), KNO_3 (103 mg/l), $NaNO_3$ (689 mg/l), Na2HPO4 (111mg/l), $MnSO_4$ (2.2 mg/l), ZnSO4 (0.5 mg/l), $CuSO_4$ (0.016 mg/l), H_3BO_3 (mg/l), Na_2MoO_4 (0.025 mg/l), $CoCl_2$ (0.046 mg/l), $FeCl_3$ (0.28 mg/l), 1 gm/l trypton, 1 gm/l yeast extract, 10 gm/l CMC and agar 25 gm/l.

Analyses of 16S rDNA Gene Sequences:

For the sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Co., Madison, USA). Two primers annealing at the 5′ and 3′ end of the 16S rDNA were 5′ -GAGTTTGATCCTGGCTCAG-3′ (positions 9–27 [*Escherichia coli* 16S rDNA numbering]) and 5′ -AGAAA GGAGG TGATC CAGCC-3′ (positions 1542–1525 [*E. coli* 16S rDNA numbering]), respectively [14]. PCR amplification was performed in a final reaction volume of 100 µl, and the reaction mixture contained each primer at a concentration of 0.5 µM, each deoxynucleoside triphosphate at a concentration of 200 µM, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 U of *Taq* DNA polymerase. The PCR reaction was run for 35 cycles in a DNA thermal cycler (Model No. 9700, Perkin–Elmer Co. Wellesley, USA). The following thermal profile was used for the PCR: denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The final cycle included extension for 10 min at 72 °C to ensure full extension of the products. The amplified PCR products were then analyzed in a 1.0 % (w/v) agarose gel, excised from the gel, and purified. The amplified 16S-rDNA was cloned into pGEM®-T vector (Promega, Madison, USA), and transformed into *Escherichia* coli. Plasmid DNA was isolated from the positive clones using Qiaprep® Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid specific primers USP and RSP and the prokaryote specific forward primers, 16F530, 16F926 and reverse primers 16R519 and 16R907 (Lane, D.G., 1991), were used as infra-red dye labelled dideoxyoligonucleotides. 16S-rDNA was sequenced using the Cycle ReaderTM Auto DNA sequencing kit (MBI Fermentas, St. Leon-Rot, Germany) and LI-COR® automated DNA sequencing machine (MWG-Biotech) according to manufacturer's instructions. The 16S-rDNA gene sequence of the isolates was aligned with reference 16S-rDNA sequences of the European Microbiological Laboratory (EMBL), GenBank (gb, Germany) and the data base of Japan (dbj) using the BLAST algorithm (Altuschul, S.F., *et al.,* 1997) available in NCBI (National Centre for Biotechnology information) in internet.

Production and Some of Crude Cellulases:

The production of enzyme was carried out in the same medium used for isolation without addition of agar. A loop full of culture from agar plate was inoculated into 50 ml-glass tube containing 5 ml of production

medium, and incubated at 180 rpm and 70 °C. This culture was then inoculated into 500 ml capacity Erlenmeyer flask containing 95 ml of the same medium and incubated at 70 °C for 48 h two millilitres aliquots were withdrawn at 2 h interval and were centrifuged at 10 000 g for 10 min at 4 °C. The cells pellets were washed twice with 50 mM Tris–HCl buffer, pH 7.0 and resuspended in 2 ml of the same buffer. Cellulases activity was measured in both cell free supernatant and in the cells to detect the extracellular and the cells bound enzyme activity. Cells and insoluble materials were removed by centrifugation at 10 000 g for 10 min at 4 °C and the cell-free supernatant was filtered through a 0.45-µm pore-size membrane filter and was used as the source of crude cellulases enzyme.

Enzyme Assay:

Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, G.L., 1959), through the determination of the amount of reducing sugars liberated from carboxymethylcellulose (CMC) solubilized in 50 mM Tris–HCl buffer, pH 7.0 (Baily, M.J., *et al.,* 1992). This mixture was incubated for 20 min at 70 °C. For crystalline cellulose substrates, incubation times were extended to 2h h and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical density was measured at 550 nm. The cellulase activity was determined by using a calibration curve for glucose (Sigma–Aldrich, UK). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute. The substrate specificity of the crude enzyme was determined by performing the assay with different substrates; avicel (microcrystalline cellulose), carboxymethyl-cellulose (CMC), cellobiose, β-glucan (β-1,3-1,4-linkage), *p*-nitrophenyl-β-d-glucopyranoside (PNPG) and xylan.

Protein Determination:

Protein concentration was determined according to the method described by Bradford, M.M., (1976). One ml of Bradford reagent was added to 50 µl of sample and the extinction was measured after 5 min at 595 nm. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80, and 100 µg/ ml distilled water. One ml of Bradford reagent was added to 50 µl BSA standard and the extinction was measured after 5 min at 595 nm.

Effect of Temperature and PH on the Activity of Cellulases:

The influence of temperature on the catalytic activity of cellulases was determined by measuring the enzyme activity at temperatures range from 30 °C to 85 °C under the standard assay conditions. The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 5 to 11 at 70 ºC using different suitable buffers, 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate (pH 7.0, 7.5 and 8.0), 50 mM glycine-NaOH buffer (pH 8.5, 9.0, 9.5, 10.0) , respectively

RESULTS AND DISCUSSIONS

Thermophiles are adapted to survive at high temperature in hot springs. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes. The enzymes from thermophiles, hence, are of great interest for industrial applications (Haki, G.D., and S.K. Rakslit, 2003). The total count of thermophilic bacteria in water and soil samples collected from Hammam pharoan hot spring, in eastern Egypt, were $2X10^4$ and $3X10^5$ CFU, respectively. No cellulases producing bacteria were able to be isolated with direct plating method (without pre-enrichment step). Isolation of cellulases producers from soil and water samples that enriched for cellulases producing thermophilic bacteria, by incubating soil and water samples with cellulose for about 4 weeks and incubated at 70 °C, resulted in isolation of 3 different strains showing clear zone after staining with Congo red (Fig. 1). These results clearly indicate the low count of cellulases producers in this hot spring, which is most likely due to the low content of organic material in the water samples of the hot spring and indicated the importance of an enrichment step for the isolation of polysaccharide hydrolyzing enzyme (Kazue, T., *et al.,* 2006).

Identification of the Cellulases Producing Thermophilic Bacteria:

Three strains showing clear zone after staining with Congo red, designed as EHP1, EHP2 and EHP3. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that strain EFP1 had highest homology (98.5 %) with *Anoxybacillus flavithermus;* EFP2 showed 98.5 % similarity with *Geobacillus*

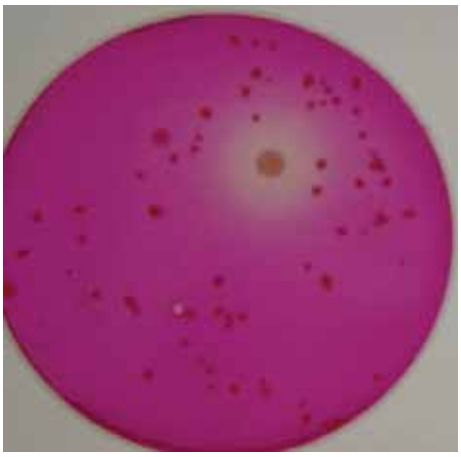

Fig. 1: Isolation of cellulase producing thermophilic bacteria using agar medium containing 1 % (w/v) CMC. Samples were incubated at 70 ºC for 1-2 weeks. The plates were stained with Congo red and destained with 1M NaCl solution. Clear zone indicated the hydrolysis of CMC as a result of cellulases production

thermodenitrificans and EHP3 showed 99.0 % similarities with *Geobacillus stearothermophilus*. *Anoxybacillus flavithermus* and *Geobacillus thermodenitrificans* have been isolated and identified as a new species in 2000 and 2001, respectively (Pikuta, E., *et al.,* 2000; Nazina *et al.,* 2001). To best of our knowledge there is no report about the production of cellulases enzymes by *Anoxybacillus flavithermus* or *Anoxybacillus flavithermus*

Production of Cellulases by Anoxybacillus flavithermus EHP1: The production of enzyme was carried out in the same medium used for isolation without addition of agar as described in the material and methods. The stationary phase of growth was reached after about 12 h. The production of cellulases was detected after 4 h of cultivation and increased during growth and reached maximum level (65.50 U/ml) at the end of the stationary phase, 36 h of growth, and remained constant during prolonged cultivation up to 48 h. The cellulase was completely secreted into the culture medium, that no cell-associated cellulases activity was detected.

Some Properties of the Crude Cellulases of Anoxybacillus flavithermus EHP1: The substrate specificity of the crude cellulase was determined by performing the enzyme assay with different substrates. The crude cellulase degraded avicell, CMC, cellobiose, and xylan, but there was no detectable activity on *p*-nitrophenyl-β-d-glucopyranoside (PNPG) (Table1). The rate of CMC degradation was higher than any other substrates used in this study. Some activity was also noted on β-glucan containing β-1,3-1,4-linkages showing reasonable sensitivity of the β-1,3-linkage to the enzyme. Furthermore, the enzyme exhibited significant activity toward avicell which is in contrast to cellulases from some other thermophilic bacteria that showed very low or no activity against crystalline cellulose (Mawadza, C., *et al.,* 2000). Avicell is a microcrystalline cellulosic material and cellobiose is a disaccharide with β-1,4-linkage that is produced from the partial hydrolysis of cellulose. Xylan is consists mainly of β-1,4-linked xylose unit and hemicellulose xylan belongs to the biopolymers that are most abundant in wood and other plants such as grasses, cereals and herbs (Petzold, K. Schwikai and T. Heinze, 2006). The enzyme showed the capacity to hydrolyse both β-1,4 and β-1,3 glycosidic linkages. The enzyme could not hydrolyze PNPG like other endoglucanases known to date. This pattern of substrates specificity is closed that reported for endoglucanases produced by *Bacillus amyoliquefaciens* DL-3 (Lee, Y., *et al.,* 2007).

Effect of Temperature on the Enzyme Activity:

The effect of temperature on the activity of crude cellulases was determined at various temperatures ranging from 30 °C to 85 °C at pH 7.0 (Fig. 2). The enzyme showed a good activity between 65 °C to 75 °C with maximum activity at 75 °C, The optimum temperature for cellulase activity produced by other thermophilic *Bacillus* sp CH43 and *Bacillus* sp HR68 isolated from another hot spring were 70 °C and 65 °C respectively (Mawadza C., *et al.,* 2000). However it showed abrupt reduction in the activity at 80 °C. Optimum temperature range of cellulases activity of 80-100 °C has been reported for enzymes isolated from several archaebacteria (Barger, J.M. *et al.,* 1989).

Table 1: Activity of the cellulases from *Bacillus Anoxybacillus flavithermus* EHP1

Substrates	Activity (U/ml)	Specific activity (U/mg protein)
CMC	65.56	81.95
Avicell	25.30	31.62
Cellobiose	30.12	37.65
Xylan	11.5	14.37

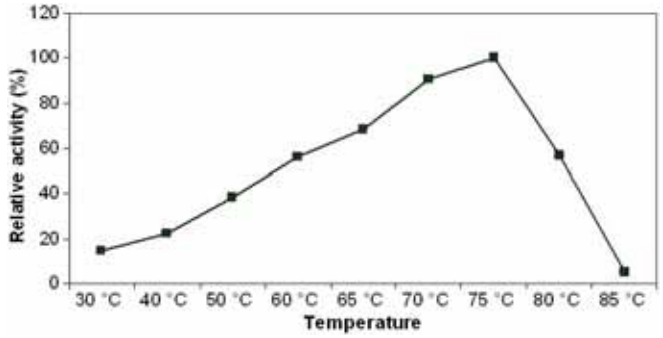

Fig. 2: Effect of temperature on the activity of crude cellulases from Anoxybacillus flavithermus EHP1. The cellulases activity was measured using CMC in 50 mM Tris -HCl buffer, pH 7, at different temperatures. Standard deviations of the relative activities were in the range of 1.0-3.5 %.

Effect of PH on the Cellulaose Activity:

The effect of the pH on the crude cellulase activity of *Anoxybacillus flavithermus* EHP1 was examined at various pHs ranging from pH 3.0 to pH 10.0 as shown in (Fig. 3). The enzyme has a broad range of pH activity (pH 6-9) with optimal pH at 7.5 which is close to the optimum pH value of most Bacillus cellulases (Fukumori, F., *et al.,* 1985). The enzyme had about 50 % activity at pH 9 and about at pH 10. At pH 5, the enzyme expressed over 30 %.

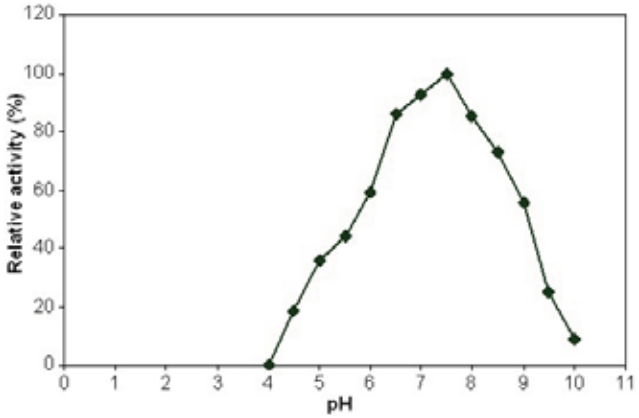

Fig. 3: Effect of pH on the activity of crude cellulases from *Anoxybacillus flavithermus* EHP1. Activity was measured at 70 °C at different pH vales, 50 mM citrate buffer (pH 5.5-7.5), phosphate buffer (pH 6-8), glycine-NaOH buffer (pH 8-10):

The results of this work indicated that the Hammam Pharoan hot springs, in eastern Egypt, are a rich source of many thermophilic bacteria which could be a good source of interested enzymes from the industrial point of view and further studies are recommended on this hot springs including study of microbial biodiversity and the biotechnological potent of the isolated strains.

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