Minufiya J. Agric. Res. Vol.38 No. 5: 1089 - 1097 (2013) "http://www.mujar.net"

CLONING AND SEQUENCE OF CELLULASE – ENCODING GENE (Cel An5) FROM *BACILLUS SUBTILIS* STRAIN AN5 ISOLATED FROM EL-BAGOUR MENOUFYIA EGYPT

Amira I. Nofal and A. M. Ibrahim
Gen. Eng. and Biotech. Research Inst., Menoufiya University., Sadat City

(Received : Aug. 26 , 2013)

ABSTRACT: *In the present work, one bacterial strain was isolated from soil in El-Bagour, Egypt and characterized according to Bergey's manual, then the sequence of 16srRNA was determined. The isolated strain (Bacillus subtilis AN5) has been found to be obligate aerobic, spore forming, Gram positive, rod shaped, and catalase producer, in addition to the ability of digestion of cellulose, hemi-cellulose, amylose and other forms of carbon source.*
Two primers were used in PCR reaction to get the carboxy methyl cellulose (CMC) digesting cellulose enzyme, which was found to be 1.6 K.bp and was then cloned using TA vector. The enzyme activity of was determined through the analysis of hollow zone around the Escherichia coli (E-coli) bacteria that was cultured on agar medium containing CMC.
From sequence analysis, cel A belongs to glycosyl hydrolase family 5 and exhibit high similarity to cellulases from Bacillus sp. AENR19 Bacillus subtilis strain CJ-H-TSA1 and Bacillus subtilis strain VB18.
In addition, the cellulase gene in the nucleotide sequence was about 1000 bp encoding 511 amino acid residues producing proteins with molecular weight, 55 Dalton.
On the other side, the optimum PH of the enzyme was 7 and 37^{o} For the optimum temperature.

Key words: *Cellulase cloning, Bacillus subtilis -AN5 PCR*

INTRODUCTION

Biomass energy is a promising option of renewable energy but the feedstock used for producing biomass energy should come from non-food biomass or agricultural waste to avoid the competition with food source and arable land (Hui *et al.,* 2010). This cellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts (Sadhu1 S. 2013). Hence, lignocellulosic materials are utilized as feedstock for the production of liquid (i.e., ethanol, butanol) or gas (i.e., hydrogen or methane) fuels (Yu and Zhang, 2003; Lo *et al.,* 2008a,b). Agricultural wastes such as wheat straw, biogases, rice straw and napiergrass were successfully converted to ethanol and hydrogen after hydrolysis (Lever *et al.,* 2010; Lo *et al.,* 2008b, 2009). Complete hydrolysis of the enzyme requires synergistic action of 3 types of enzymes, namely, cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase), and beta-glycosidase (Sonia , *et al,* 2013).

Cellulase is one kind of biocatalysts composed of endo-b-1,4-Dglucanase, exo-b-1,4-D-glucanase and b-1,4-D-glucosidase, which were used to convert cellulose fibers to soluble sugar for follow up biofuels production by bacteria or yeast (Shanmughapriya *et al.,* 2010). The endo-b-1,4-D-glucanase reacted on the amorphous part of cellulose, causing fragmentation of cellulosic materials. The exo-b-1,4-D-glucanase hydrolyzes crystalline cellulose into simple sugars such as cellobiose and cellodextrin. Finally the cello oligosaccharides were degraded to glucose by b-glucosidase (Li *et al.,* 2009; Soni *et al.,* 2010). Cellulases are often produced from fungal species, such as *Trichoderma sp.* and *Aspergillus sp.* Some bacteria are also effective in producing cellulolytic enzymes, such as *Cellulomonas sp., Bacillus sp.,* and *Clostridium thermocellum* (Suto and Tomita, 2001). *Bacillus sp.* strain able to produce novel and effective cellulase and xylanase enzymes, which were used to hydrolyze agricultural wastes to prepare carbon source for biohydrogen production.

Cellulases have been applied in food, animal feed, textile, biofuel, paper, pulp, and pharmaceutical industries (Kang *et al* , 2007).

Cellulases *are* used in the textile industry for cotton softening and denim finishing; in laundry detergents for color care, cleaning; in the food industry for mashing; in the pulp and paper industries for drainage improvement and fiber modification, and they are even used for pharmaceutical applications (Cherry J. R., 2003) . In nutshell, the cellulose enzymes will be commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes will also grow rapidly. So, cellulose enzyme will be the most stirring technology of future. And continuous research for advances in speckled aspects for cellulose production (such as cost, substrate specificity, and specific activity) are desired to achieve improved techno economic feasibility. The present work was carried out to detect DNA sequence of cellulase encoding gene (Cel An5) from *BACILLUS SUBTILIS* strain AN5.

MATERIALS AND METHODS

1. Isolation of microorganisms producing cellulase enzymes

We isolate microorganisms producing cellulases, El bagour city – El menoufyia soil. The soil was suspended with 0.85% (w/v) NaCl, and the suspension was cultivated at 37°C for 5 days in a solid medium containing 2.0% (w/v) rice hull as the sole carbon source, 0.5% K_2HPO4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.06% $(NH4)_2SO_4$ and 1.5% (w/v) agar. Cultures of isolated microorganisms utilizing rice hull were prepared by transferring cells from the agar plate to 200 ml of neutral agar medium in 500ml Erlenmeyer flasks. The medium used for production of the cellulase contained the following components: 2.0% rice hull, 0.25% yeast extract, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.06% $(NH4)_2SO_4$. The resulting cultures were incubated for 3 days at 37 °C under aerobic conditions. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated from carboxymethylcellulose (CMC) solubilized in 50mM Tris–HCl buVer, pH 8.0 (Bailey *et al.,* 1992). One microorganism was selected for the production of cellulase.

2. Identification of isolates

The isolated target strain was inoculated into LB medium and was cultivated at 37°C and 200 rpm for 24 h. An appropriate amount of cells were taken from the culture and the genomic DNA of the cells was extracted by DNA Purification Kit (Viogene, Taiwan) for the sequence analysis. Amplification of the 16S rRNA gene was conducted by polymerase chain reaction (PCR) using F8 and R1510 primer (Wang *et al.,* 2009b). The PCR products were sequenced (Mission Biotech, Taipei, Taiwan) and the obtained sequence was compared with the database available in NCBI GenBank to determine the identity of the isolated strain.

3. Analyses of 16S rRNA and Sequence and cloning of cellulase encoding gene An5

For the sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (MEGAquick-sp™ Total). Two primers annealing at the 5- and 3- end of the 16S rRNA were ***Cel F*** - GGGGATCCATGATGCGAAGGAGGAAAA GAT

Cell R - GGGTCGACCTAATTTGGTTCTGTTCCCC AA

The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified. The purified products were cloned into (pGEM-T Easy vector (Promega Co., Madison, USA) and subsequently sequenced using GeneJet Plasmid Miniprep Kit- Thermo Scientific). The obtained data are presented in Fig. (2)

Cloning of cellulase gene

DNA digestion

The genomic DNA was digested by using restriction endonulease *Hin*dIII. The reaction buffer was (10mM Tris-HCl, pH 7.5; 10mM $MgCl_2$; 50mM NaCl; 1mM dithioerythritol (DTE) obtained from (Sigma).

Extraction of DNA from agarose gel

Digested DNA was extracted from agarose gel by using QIAquick Gel Extraction Kit (QIAGEN Inc.)

Ligatoin of DNA into psk+

Ligatoin reaction consists of (5x ligation buffer, 2µl; DNA, 4 µl; *p*sk+, 1µl; *E.coli* DNA ligase 0.5 µl; H_2o, 2.5 µl) incubation for 12h at 15°C.

Introducing plasmid DNA into cells

5 µl of ligation mixture was added to 100 µl of competent *E.coli* DH5α, prepared by Calcium chloride protocol (Sambrook, *et al.,* 1989) using heat shock procedure. The transformed cells spread on nutrient agar supplemented with 50 µg/ml ampicillin.

Enzyme activity assay:

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller *et al,* 1959) using glucose (Sigma) as the standard. The composition of DNS reagent was as follows: 2 g sodium hydroxide, 2 g 3,5-dinitrosalicylic acid, 4 g potassium sodium tartrate, 0.4 g phenol and 0.1 g sodium bisulfite in 200 ml dH2O. The assay consisted of 100 µl enzyme preparation with 50 µl of a 2% (w/v) solution of the desired substrate and 250 µl buffer (50 mM potassium phosphate at pH 6.5). Assays were performed at 50°C for 30 min (unless otherwise stated). The color development was performed by adding 150 µl of the assay to 300 µl of DNS reagent after which it was heated at 100°C for 5 min, cooled on ice for 5 min and readings taken at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme within the assay that released 1 µmol of reducing sugar per min under the conditions indicated. Where activity was tested on an insoluble substrate, assay tubes were agitated during the course of the assay to keep the substrate suspended.

4. Determination of Protein concentration:-

Protein concentration was determined by a modified Bradford method (Bradford 1976) and using bovine serum albumin (BSA) as a standard. Bradford reagent (250 µl) was added to 10µl of protein sample for standard measurement of protein. For low protein concentrations, 230 µl of Bradford reagent (Sigma) was added to 25 µl of sample and the protein concentration we determined.

5. Purification of Cellulase

After the broth, incubated with *Bacillus subtilis*, was centrifuged at 8,000 R/min for 20 min and passed through a 0.45 µm membrane to remove cells, the crude cellulase was precipitated by 60-80% saturation of ammonium sulfate. The precipitate was centrifuged at 8,000 R/min for 15 min and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) overnight.

6. SDS–PAGE and zymography analysis

The SDS– PAGE analysis was conducted using a 12% polyacrylamide gel to determine the molecular weight of the existing enzymes. For the zymography of endoglucanase,0.5% CMC was used as substrate and the reaction was performed at 37 ^{o}C for 30 min and the clear zones were visualized by 0.1% Congo red solution (Sakamoto and Toyohara, 2009).

7. Influence of Temperature and pH of enzyme activity:-

a) Optimum Temperature:

To 900 µL of 05% CMC in 20 mM phosphate buffer (pH 7.0), 100 µL of appropriate concentration of enzyme were added and incubated at various temperatures (0, 20, 30, 40, 50, 60, 70, 80, 90 ^{o}C) for 30 min. The activity were then measured according to Miller (Miller, 1959).

b) Optimum pH:

To 900 µL of 1.0% CMC in various pHs (pH 3.0-7.0: 50mM citrate buffer; pH 6.0-9.0: 50 mM phosphate buffer; pH 8-11: 50 mM carbonate buffer), 100 µL of cellulase was added and measured the activity according to Miller (Miller, 1959).

RESULTS AND DISCUSSIONS

In this research we were able to detect sequence and clone cellulase encoding gene from *Bacillus subtilis* An5 into E. coli.

The optimal pH for *Bacillus subtilis* An5 *cellulase* was pH 7.0. It was the same as *Bacillus subtilis* KU-1(Zakaria *et al.,* 1998); *Bacillus subtilis* 168, (El-Helow and Khattab, 1996) .but it was lower when compared to pH 7.5 for *Streptomyces ipomoea* (Montiel *et al.,* 2002); and *Vibrio* sp. strain MA-138 (Tamaru *et al.,* 1997); where it was higher when compared to pH 6.0 for Bacillus subtilis WY34 (Jiang *et al.,* 2006); *Enterococcus casselIflavus* (Oda *et al.,* 1993);*Caldocellum saccharolyticum* (Luthi *et al.,* 1991); and pH 6.5 for *Vibrio* sp. strain MA-138 (Tamaru *et al.,* 1995); and it was inside the range of *Bacillus stearothermophilus* that not vary between pH 5.5 and 7.5. (Talbot and Sygusch 1990).

The highest activity for *cellulase* was determined at 37°C the same as *Bacillus subtilis* 168 (El-Helow and Khattab 1996). While it was lower than *Vibrio* sp. strain MA-138, 40°C (Tamaru *et al.,* 1995); *Vibrio* sp. strain MA-138 , 45°C (Tamaru *et al.,* 1997).

Bacillus subtilis An5 *cellulase* was strongly inhibited by BA2+ on the other hand it was activated slightly by Co^{2+} the same as *Enterococcus casselIflavus* (Oda *et al.,* (1993), and strongly activated by Mn2+. Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller *et al,* 1959) using glucose (Sigma) as the standard. Highest activity of cellilase has been detected in OD 250 as 90%con. (4.2 ul). Protein concentration came to be around 55 dalton .

Analyses of 16S rRNA and cellulase A (cel A) gene sequences :-

Two primers annealing at the 5- and 3-end of the 16S rRNA were :-

Cel F –

GGGGATCCATGATGCGAAGGAGGAAAA GAT

Cell R –

GGGTCGACCTAATTTGGTTCTGTTCC CCAA (Chun and Bae, 2000). PCRamplification was performed in a final reaction volume of 100 ml,The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified.

The SDS– PAGE analysis was conducted using a 12% polyacrylamide gel to determine the molecular weight of the cellulase enzyme , it about:

Length = 499 amino acids

Molecular Weight = 55263.08 Daltons

1. Bacterial Isolation and identification of the isolated bacterial strains

Soil samples were obtained from El-bagour to isolate bacterial strains exhibiting high cellulolytic activities using CMC-amended BHM agar plates. Nearly 30 pure strains were isolated from the soil samples. Among them, the strain with the highest CMC hydrolysis activity (based on clear zone observation after Congo red staining) was chosen as the target strain (named strain AN 5) for further evaluation of its potential in mass production of cellulases. The result of the 16S rRNA gene phylogenic tree indicates that strain AN5 was belonged to the Bacillus sp. and highly similar to *bacillus sutablise* (99.9% sequence similarity). Thus, the strain was identified as bacillus sp. AN5 , whose 16S rRNA sequence was registered at NCBI Gene Bank with an accession number of JQ746509. isolates were morphologically characterized and biochemically tested for their activities.

The positive 11 isolates were recognized by the formation of clear zone around colonies due to degradations (hydrolysis) of

LBG at a red background. The bacterial isolates were picked from the master plate to freshly prepared plates and retested three times more. Diameter of hallo zones were measured at 9 cm Petri dish (Fig. 1), three isolates named An5, An17 and An19 were found to be the most active isolates their diameter of hydrolyzed areas were 2.0, 1.8 and 1.7 cm respectively. *Bacillus subtilis* strain AN5 identified according to the methods in *Bergey's Manual of Systematic Bacteriology* and also 16srRNA gene was sequenced and deposited to the GenBank under accession number JQ746509. *Bacillus subtilis* strain AN5 was a facultative anaerobic, mesophilic, spore-forming, gram-positive, motile, rod-shaped organism and produced catalase.

It is clear from Fig. (1) that the studied isolate having a high level of CMCase that is able to hydrolyze CMC giving rise to the clear area around the growing colonies .

2. Protein standard curve

Protein concentration was determined about 1800 dalton by a modified Bradford method (Bradford 1976).

3. PCR reaction :

PCR reaction, (M 1kb ladder fermentas :
Lane 1 PCR product of 16s/An5
Lane 2 PCR product of cel An5, give 3 bands (about 2.5 kb , 1.2kb and 170bp).

Fig. (2) it is clear from this Figure that product of 16 S rRNA in lane 1. Where as lane 2 is showing the PCR product of cel AN 5. There were three bands at molecular weight of 2.5, 1.2 Kb and 170 bp.

4. SDS–PAGE and zymography analysis:

Fig. (3) cleary shows the molecular weight of purified col AN5 protein to be 55 dalton comparing to the marker protein. This data is in agreement with what Wang *et al* (2009b) reported on isolated callulase enzyme from microorganisms isolated from different environmental niches

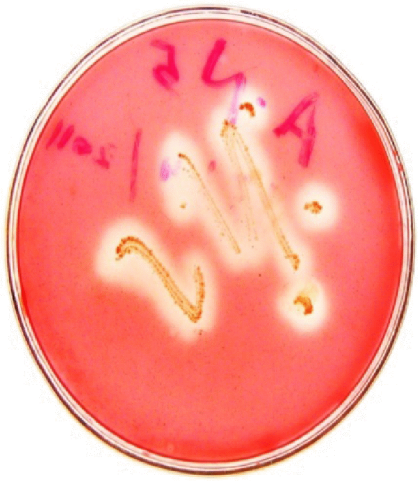

Fig. (1). Bacillus sp. Strain-An5 plated on LBG.

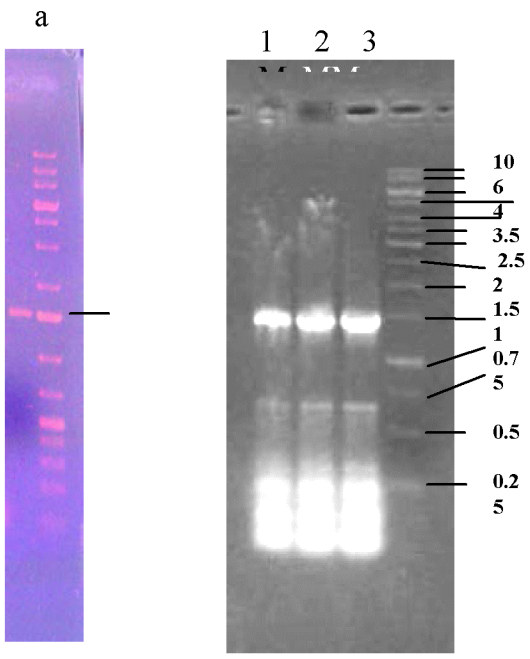

Fig. (2). a- PCR product of 16s rRNA of *Bacillus subtilis* strain-AN5; b - M 1kb ladder DNA, Lane 1,2, and 3 are the PCR product of cel AN5 from *Bacillus subtilis* strain-AN5.

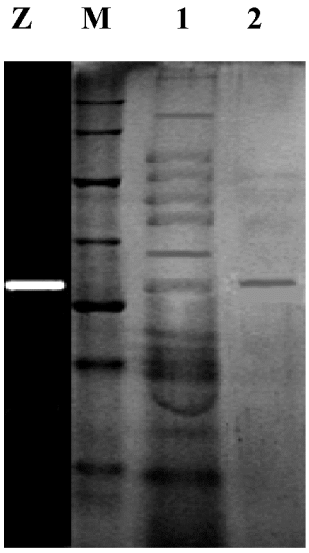

Fig. (3). SDS-PAGE zymogram, M, protein marker sigma KDa (260, 140, 100, 70, 50, 40, 35, 25), lane 1 crude protein, lane 2, partially purified Cel AN5 protein and lane Z , zymogram.

Sequencing of cellulase encoding gene :

The sequence analysis showed that the length of bacterial strain An 5 was about 1500 bp.

Influence of Temperature and pH on enzyme activity

1) Optimum Temperature

The highest activity for cellulase was obtained at 37C .

Fig. (4) clearly shows the cellulase activity at different degrees of temperatures . It is a typical enzyme reaction graph, as there is an increase of activity with increasing temperatures until it reaches the optimum at 37 C. This data is in good agreement with what Sadhu and Kanti (2013) Published.

2) Optimum pH

To 900 µL of 1.0% CMC in various pHs (pH 3.0-7.0: 50mM citrate buffer; pH 6.0-9.0: 50 mM phosphate buffer; pH 8-11: 50 mM carbonate buffer), 100 µL of cellulase was added and measured the activity according to Miller [Miller (1959)].

Fig. (5) presented cellulase activity at various levels of pH. It is clear that the optimum pH came to be 7 .

Whereas pH 8 gave a little inhibition to the enzyme activity . The optimum pH record the enzyme activity to be 90% whereas pH 8 gave 88% . This finding is in agreement with what Sania *et al* (2013) have been Published .

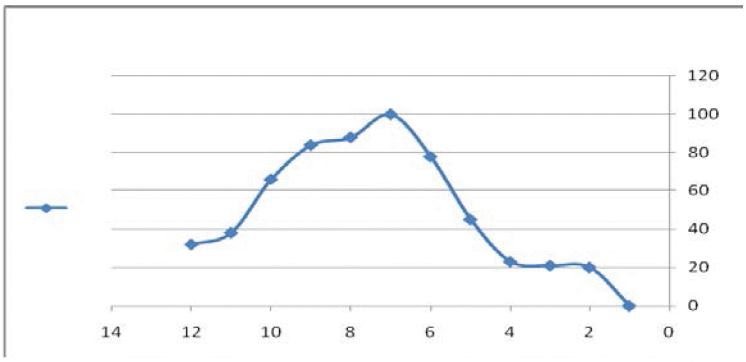

Fig.(4): Enzyme activity at different degrees of temperatures.

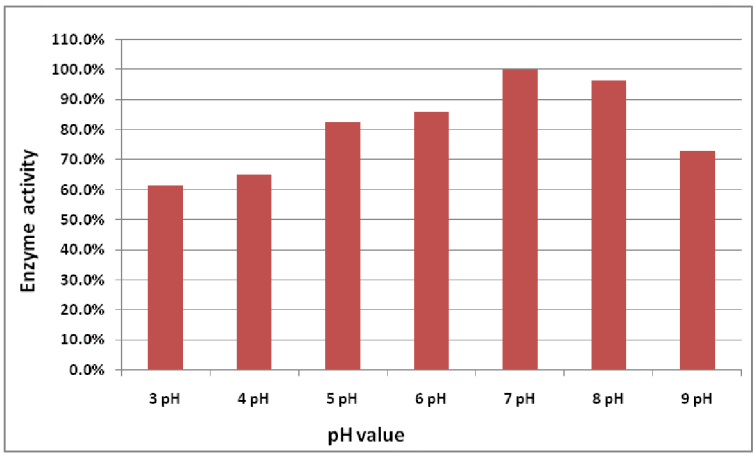

Fig.(5): Enzyme activity at various levels of PH.

REFERENCES

Biely, P., M. Vrsansk, M. Tenkanen and D. Kluepfel (1997). Endo-_-1,4-xylanase families:differences in catalytic properties. J Biotechnol;57:151–66.

Cherry, J. R. and A. L. Fidants (2003). "Directed evolution of industrial enzymes: an update," Current Opinion in Biotechnology. 14: 438–443.

Demain, A.L., M. Newcomb and J.H.D. Wu (2005). Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev. 69: 124–154.

El-Helow, E.R. and A.A.Khattab (1996). The development of a *Bacillus subtilis* 168 culture condition for enhanced and accelerated beta-mannanase production. Acta Microbiol Immunol Hung. 43:289-99.

El-Helow, E.R., S.A. Sabry and A.A. Khattab (1997). Production of β- mannanase by *B. subtilis* from agro-industrial by-products: screening and optimization *Mannanase from wastes.* Antonie van Leeuwenhoek 71: 189–193.

Kang, H. J., K. Uegaki, H. Fukada and K. Ishikawa (2007). Improvement of the enzymatic activity of the hyperthermophilic cellulose from Pyrococcus horikoshii, Extremophiles, 11: 251–256.

Lever, M., G. Ho and R. Cord-Ruwisch (2010). Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation. Bioresour. Technol. 101,7083-7087.

Li, Y., Q. Yin, M. Ding and F. Zhao (2009). Purification, characterization and molecular cloning of a novel endo-b-1,4-glucanase AC-EG65 from the mollusc Ampullaria crossean. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 153:149–156.

Lo, Y.C., M.D. Bai, W.M. Chen and J.S. Chang (2008b). Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. Bioresour. Technol. 99: 8299–8303.

Lo, Y.C., Y.C. Su, C.Y. Chen, W.M. Chen, K.S. Lee and J.S. Chang (2009). Biohydrogen production from cellulosic hydrolysate produced via temperature-shiftenhanced bacterial cellulose hydrolysis. Bioresour. Technol. 100, 5802–580.

Marion, M. B. (1976). A Rapid and Sensitive Method for the Quantitation of Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analyt. Biochem. 72: 248-254.

Sakamoto, K. and H. Toyohara (2009). Molecular cloning of glycoside hydrolase family 45 cellulase genes from brackish water clam Corbicula japonica. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 152, 390–396.

Sadhu1, S. and T. Kanti (2013). Cellulase Production by Bacteria: A Review. British Micro. Research J., 3(3): 235-258.

Sambrook, J., E.F. Fritsch and T. Maniatis (1989). Method in Molecular Cloning: Laboratory Manual. Cold Spring Harbor Laboratory Press, NY.1: 2- 3.

Sonia, S., D. Aparna, B. Lal Gupta and Saksham Gupta (2013). Optimization of Cellulase Production from Bacteria Isolated from Soil. ISRN Biotechnology,Accepted 5- Jan.

Suto, M. and F. Tomita (2001). Induction and catabolite repression mechanisms of cellulase in fungi. J. Biosci. Bioeng. 92 (4): 305–311.

Subramaniyan, S. and P. Prema (2000). Cellulase-free xylanases from Bacillus and other microorganisms. FEMS Microbiol. Lett. 183: 1–7.

Tamaru, Y., T. Araki, T. Morishita, T. Kimura, K. Sakka and K. Ohmiya (1997). Cloning, DNA Sequencing, and Expression of the *β*-1,4-Mannanase Gene from a Marine Bacterium, *Vibrio sp*. Strain MA-138. J. Fermen. Bioengin., 83:201-205.

Wang, F., F. Li, G. Chen and W. Liu (2009b). Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. Microbiol. Res. 164, 650–657.

Yu, Z. and H. Zhang (2003). Pretreatments of cellulose pyrolysate for ethanol production by Saccharomyces cerevisiae, Pichia sp. YZ-1 and

Zymomonas mobilis. Biomass Bioenergy 24, 257–262.
Zakaria, M.M., S. Yamamoto and T. Yagi (1998). Purification and characterization of an endo-1,4-β-mannanase from *Bacillus subtilis* KU-1. FEMS Microbiol. Lett. 158:25-31.

كلونة ومعرفة تتابع القواعد النيتروجينية للجين المنتج لانزيم السيلولاز من بكتيريا معزولة من الباجور - المنوفية - مصر

أميرة إبراهيم أحمد نوفل ، عاطف محمد إبراهيم

معهد الهندسة الوراثية والتكنولوجيا الحيوية – السادات – المنوفية

الملخص العربي

فى هذا العمل تم عزل وتوصيف سلالة بكتيرية من أرض مصرية (مركز الباجور– مصر) طبقا للخطوات المتعارف عليها (Bergey's Manual) بالإضافة إلى تحديد تواليات القواعد النيتروجينية لجين ال 16srRNA.

واتضح أن العزلة البكتيرية (الباسلس ستلس AN5) لاهوائية إختيارية, مكونة للجراثيم, موجبة لصبغة الجرام, متحركة, عصوية الشكل وتكون الكاتاليز بالإضافة لقدرتها على تكسير السليللوز ,2/1 سليللوز , الأميلوز وأشكال أخرى من مصادر الكربون.

تم استخدام 2بادىء لتحضير DNA بتفاعل البلمرة المتسلسل لجين إنزيم السليللوز المحلل للكربوكسى ميثيل سليللوز (CMC) حيث حصلنا على 1.6 كيلو زوج من القواعد وتم تحميلها على أحد النواقل (TA vector).

ثم تم تحديد نشاط إنزيم السليللولاز وذلك من خلال تحليل الهالات المتكونة حول بكتيريا القولون (E-coli) النامية على بيئة محتوية على الكربوكسى ميثيل سليللوز.

وقد اتضح أن الجين المدروس يتماثل مع *Bacillus* sp. AENR19 *Bacillus subtilis* strain CJ-H-TSA1 and *Bacillus subtilis* strain VB18.

بالإضافة أنه قد وجد أ، طول الجين حوالى 1500 زوج من القواعد ويعبر عن 511 حمض أمينى مكونا بروتين له وزن جزيئى (55 دالتون).

كما اتضح أن درجة الحمض المثلى للإنزيم 7 ودرجة الحرارة المثلى 37 درجة مئوية.