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CLONING AND SEQUENCING OF CELLULASE-ENCODING GENE (*cel 2*) FROM LOCALLY ISOLATED *BACILLUS SP.* STRAIN A4

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ABSTRACT

A Bacillus bacterium, *Bacillus sp.* strain A-4 was isolated from the Egyptian soil and identified according to the methods in *Bergey's Manual of Systematic Bacteriology.* The bacterial isolate, *Bacillus* sp. strain A-4 was a facultative anaerobic, mesophilic, spore-forming, Gram-positive, motile, rod-shaped organism and produced catalase. *Bacillus sp.* strain A-4 can degrade cellulose, hemicellulose, amylose and other forms of carbon sources. A DNA genomic library constructed from *Bacillus sp.* Strain A-4 and screened for cellulase (CMCase) activity. Recombinant β-glycosyl hydrolase activity was detected on the basis of the clearing of hallos around *Escherichia coli* colons grown on a substrate containing carboxymethylcellulose (CMC). The nucleotide sequence of the cellulase gene (*cel 2*), corresponded to complete open reading frame 1167 bp that codes for a 389 amino acid and a protein with a molecular mass of 4249Da. From sequence analysis, *cel 2* belongs to glycosyl hydrolase family 5 and exhibit high similarity to *engD* and *engO* from *Clostridium cellulovorans* and *celB* from *Ruminococcus albus*. The crude enzyme preparation from *Bacillus sp.* A-4 exhibits activity over a broad range of pH5 to 6 and has good stability across temperatures 50°C and pH6.

INTRODUCTION

Cellulose is the most abundant and only renewable biomass in the biosphere resources available in large quantities which need to be properly utilized to meet our needs of energy, chemicals, food and feed as a long-term solution. It is expected that such products can solve the problems of food, energy and the environment Yoshihiro *et al.*, (2001). A wide range of some 100 species of bacteria and fungi are naturally capable of degrading cellulose through the concerted action of several enzymes that collectively are referred to as cellulase. These enzymes are involved in the degradation of crystalline cellulose into glucose, which could easily be converted to many chemicals (Virendra and Ghose 1981; Amano and Kanda 2002).

Soil bacteria such as many members of the family *Bacillaceae* are able to use a wide variety of carbohydrates, among them several glucans such as cellulose, the most abundant carbohydrate in nature; β-1,3-glucan (laminarin) and β-1,3-1,4glucan (lichenan). *Bacillus* species is well known for its ability to produce one or more of a variety of extracellular enzymes capable of hydrolyzing poly-saccharides, proteins, nucleic acids, lipids

and antibiotics (Glick and Pasternak 2003; Ara *et al.* 2007).

Exocellular β-glucanases and β-xylanases are produced by many members of the family *Bacillaceae* (Priest, 1977). They are increasingly becoming subjects for study because of their potential use in brewing industriy (Enari and Markkanen, 1975), in the bioconversion of agri-cultural wastes to more useful products, such as single- cell proteins, fuels, and chemical feed stocks (Ryu and Mandels, 1980; Sandhu and Kennedy, 1984). The mechanism by which these enzymes are transported out of the cell is of interest in itself and could have practical application in industry when efficient export of cloned gene products is introduced into biological systems (Sanchez *et al*., 2005; Li *et al*., 2006). Several *Bacillus sp* strains which produce endo β-1, 4-glucanases or celluloilytic endoglucanases were identified, and their genomes have been used as sources for cloning of the respective genes (Bahri and Numan 2001; Li *et al*., 2006). β-glucosidase such as lichenan and its hydrolysis products could be considered as alternative carbon sources utilized under conditions of glucose limitation. Steinmet, 1993; lee and Kim, 1999 studied cloning and expression of an endo-β1,4-D-glucanase in *Bacillus cellulyticus*. Extensive studies on proteins (such as cellulase, protease and amylase) secreted by *Bacillus species* (Dhillon *et al.,* 1985) showed that the following *Bacillus species* produce cellulase: *Bacillus cereus* (Thayer 1978) *Bacillus licheniformis* (Dhillon *et al.,* 1985), *Bacillus subtilis* (Robson and Chanbliss,1984) and *Bacillus polymyxa* (Fogarty *et al.,* (1974).

The present study was undertaken to search for new strains of *Bacillus sp* strain A4 in the Egyptian ecosystems with high cellulase activity. Soil samples from Menofiya governorate, were collected the isolation and the identi-fication of *Bacillus sp* was made on the bases of its physiological and morphological features. Clone of a novel cellulase gene (*cel 2*) from *Bacillus sp.* strain A4 transform in *E. coli* DH5α and sequencing.

MATERIALS AND METHODS

Materials

Bacterial strain: Morphological properties and taxonomic characteristics of the *Bacillus sp.* strain A4 were studied according to the methods in *Bergey's Manual of Systematic Bacteriology* (Sneath *et al.*, 1986) and *E.coli* DH5α bacteria were used.

Methods

Isolation of local bacterial: The bacterial isolate used in this study was isolated from several samples of soil rich in cellulose agriculture wastes from local area of Menofiya-Egypt according to Chartchai *et al.,* (1998).

Enzyme assays determination: Colonies were picked according to their strong growth, different morphology and streaked on culture media plus CMC as a carbon source plates. The selected cultured in nutrient agar media. The bacterial colonies were grown overnight on LB medium, overlaid with 0.2% carboxymetlylcellulose (CMC) and incubated for 24 hours. CMC-containing plates (as mentioned bellow) were stained with Congo Red (0.1%). After two to three washes with I M NaCl, indicated cellulase activity according to Glick and Pasternak (2003).

Isolation of genomic DNA: Genomic DNA isolation from *Bacillus sp.* strain A4 using Large- and small-scale plasmid purification kit (Promega).

Enzymes and vector used:he restriction enzyme (*Hind*III, *Eco*RI, *Bam*HI, *Kp*nI, *Pst*; *Sal*I) were purchased from New England Biolabs, Gibco and Manheim Boehringer**.** Volumes used of restriction enzyme were (7Unit/µl), RNase enzyme (100µg/ml), restriction enzyme buffer 3 µl, genomic DNA (10ng/µl), deionized water 20µl. The digestion was carried out at 37 ˚C for different times 15, 30, 45, 60, 75, 90 min.

Constructs of cellulase gene: The pBlue Script SK+ (Stratagene inco.,CA, USA) was linearized (50 µg/µl) by incubation for 1 hour at 37˚C with *Hind*III enzyme / 1µL (7unit/µl) and dephosphorylated with Calf intestine phosphatase (Cip) at a final concentration of 0.25U/µg DNA. *Bacillus sp* DNA was partially digested with *Hind*III for 1 hour at 37˚C and fractionated by 0.7% agarose gel electrophoresis (w/v) in TAE buffer. Fragments of 1.3 kb size range were recovered by elution of DNA from agarose gel (using High pure PCR product, purification Kit, Roche, Cat No.1723676).

The ligation: The ligation of *Bacillus sp.* strain A4 1.3 kb DNA fragments cellulase gene to linearized vector was carried out at 16˚C overnight. The reaction was content vector pBlueScript SK+ 1µl, Ligase enzyme (T_4) (3 Unit/µl) 4µl, DNA (insert) 4µl, T_4 DNA ligase buffer (5X) 1µl. The mixture was incubated at 16˚C overnight. The ligation mixture of recombinant plasmid was introduced into *E.coli* DH5α as the recipient strains for the recombinant plasmids (Roche Applied Science, Promega) by heat shock according to Maniates *et al*., 1989. Transformed cells were selected grown on Luria-Bertani medium media with ampicillin (100ug/ml) and overlaid onto a plate containing 0.2% CMC, then incubated at 37^{o}C for additional 24 hours. Transformed colonies were screened for carboxymethylcellulase (CMCas) activity.

DNA sequence determination:Positive clones were sequenced at South Korea (Macrogen Co., Korea) by the dedoxy-nucleotide chain termination method using T7 (TAATACGACTCACTATAGG) forward and T3 (AATTAACCCTCACTAA AGGG) reverse primers sequencing by automated sequence machine of Sanger *et al*., 1997. The nucleotide DNA sequence was analysed by GENETYX software (software development Co. ltd., Tokyo, Japan) and Homology searches in GenBank were performed with BLAST program (Kenji *et al.*, 1997).

Restriction maps of cellulase gene: Restriction enzymes *(Hind*III, *EcoR*I, *EcoR*II, and *Fok*1) were used in positive clones as specified. Restriction maps were constructed from single and multi-restriction digests of plasmids expressing CMCase activity.

Purification of recombinant Cel2: *E. coli* harboring pcel 2 were cultured to early stationary phase at 37^{o}C with vigorous shaking. The cells were collected by centrifugation, and the periplasmic fraction was prepared by the osmotic shock method (Manoil and Beckeith. 1986)**.** The chromatography of the recombinant cel 2 was performed with a column 2.5 X 40 cm. The periplasmic fraction was applied to sephadex-G100 equilibrated with 10mM Tris-HCl (pH 7.0). After being washed with 3 bed volumes of the same buffer, the column was eluted at a flow rate of 60 ml/h. The active fractions were collected and concentrated by saturated ammonium sulfate. After centrifugation, the precipitate was dissolved in 3ml 10mM Tris-Cl (pH7.0). The concentrated enzyme was dialysed against the same buffer and used as purified enzyme.

Determination of cellulase activity:

Cellulase (CMCase) activity was determined from *Bacillus sp.* strain A-4 according to (Bayer and Lamed 1986). The amount of the reducing sugar (glucose) was estimated from standard curve of known glucose concentrations (Boraston *et al*., 2004). One unit (IU) of cellulase activity was defined as the amount of enzyme catalyzing the production of 1µmol of reducing sugar equivalent per min under the conditions used. The enzyme activity was determined at different pH values (pH 4, pH5,and pH7), and at different metal ions Fe SO_4-$5H_2O$, $CuSO_4$-$5H_2O$ and $MnSO_4$-H_2O concentration (50 mM) of each at pH 7 and 37˚C for one hour.

Gel electrophoresis analysis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used purification of recombinant *cel2* performed on a 10% polyacrylamide gel by the method of Laemmli (1970) and Bradford (1976). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. The molecular weight standards were used from a high-molecular-weight (Fermentas). After SDS-PAGE, the gels were renatured in a renaturation buffer (100 mM succinic acid, 10 mM $CaCl_2$, 1 mM dithiothreitol, pH 6.3) for 2 h at 25°C with gentle shaking. The renatured gel was then incubated in a fresh renaturation buffer for 1 h at 37°C with gentle shaking. The clearing zones corresponding to enzyme activities were visualized with 0.3% (wt/vol) Congo red (stained for 10 min and destained with 1 M NaCl solution). Mass spectrometric analysis was performed to identify the *engO* on cellulosome, and recombinant proteins were separated by SDS-PAGE as described previously according to Hansmeier, *et al*., (2004)

RESULTS

Isolation and characterization of *Bacillus* sp. strain A-4: In the present work several isolates of bacteria representing local of Menofiya Govrnerate, Egypt were studied to determine their cellulase activities. This identification was based on the ability of bacteria to degrade carboxymetlylcellulose (CMC) into glucose and selected only one isolate used in the present study. Identification and classification of *Bacillus sp.* Strain A4 is a rather tedious task. Previous attempts were mainly based a facultative anaerobic, spore-forming, gram-positive, motile,rod-shaped organism and produced cellulase. Thus, this bacterium was identified as belonging to the genus *Bacillus* according to *Bergey's Manual of Systematic Bacteriolog.*

Screening the ability of bacterial isolates to degrade cellulose: The colonies were overlaid with LB medium containing 0.2% carboxymethyl cellulose (CMC) a soluble derivative of cellulose. The petri plates were incubated at 37˚C for 24 hours. During this time, the CMC molecules that are present in the immediate vicinity of a colony that both synthesizes and secretes cellulase were partially digested. The digested regions of the CMC were visualized first by flooding the Petri plate with a solution of the dye Congo Red. Showed CMC degradation activity of a clear halo surrounded cellulytic bacterial colony that cellulase was encountered in the *Bacillus* sp. strain A-4 isolate more than other isolates.

Isolation of cellulase (*cel2*) gene from *Bacillus sp* chromosomal DNA: In order

to isolate cellulase (*cel2*) gene from *Bacillus sp.* strain A4, genomic DNA was digested by different restriction enzymes (*Hind*III, *Eco*RI, *Bam*HI, *Kp*nI, *Pst*; *Sal*I). Shows was digested genomic DNA, only *Hind*III restriction enzyme clearly identified the 1.3 kb DNA fragments cellulase (*cel2*) gene and the basted digested at 90 minutes in figure (1).

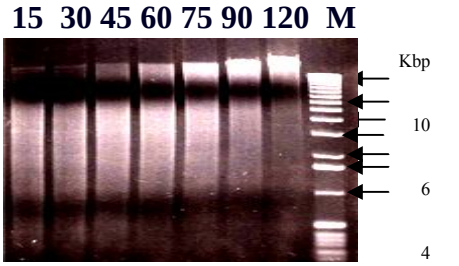

Figure-1: Agarose gel electrophc
a- gDNA digestion with *Hind*III at different times (minutes) and molecular marker (M).

Congo Red screening of recombinant clones expressing *Bacillus sp.* strain A4 cellulase gene: Approximately 1000 *E. coli* transformants from a *Bacillus* sp. strain A-4 were screened for cellulase activity. Detection of positive transformants was based on clearing of halo formation around transformed colonies that grew on agar-CMC plates (Fig. 2). Three positive clones were isolated for further characterization.

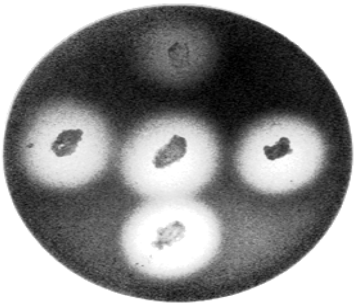

Figure-2: CMCase activity of *E. coli* carrying pcel 2 grown on CMC-nutrient agar plate.

Cloning of the cellulase 2 gene and restriction map: Cloning of the 1.3kb DNA fragment insert into pBluescript SK+ and 2 subclones pcel 2L and pcel 2R. The restriction map of positive transformed clones was confirmed using *Eco*RI, in which the expected band sizes were detected as illustrated in figure-3. It was shown that digesting the plasmid from *E.coli* DH5α positive colonies (4.3 kb) with *Eco*RI, resulted in the production of two fragments with molecular weights about 3 kb vector and 1.3 kb insert fragment. The clones had the same size of genomic DNA insert of about 1.3 Kb. Appropriate restriction enzymes (*Hind*III, *Eco*RI, *Eco*RII, and *Fok*I.) were used for *pcel 2* gene maping (Figure, 4 and 5). Transformants carrying pcel 2L and pcel 2R didn't exhibited cellulase activities by plate assays and all of them mailed to South Korea (Macrogen, Inco.) for sequencing.

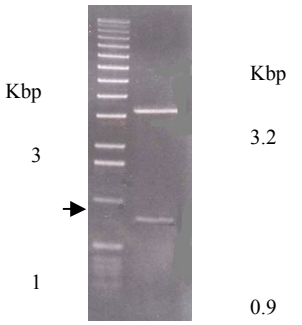

Figure-3: The recombinant plasmid digested with *Eco*RI.

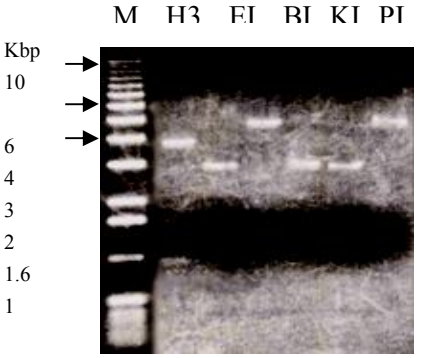

Figure-4: single and double cut of cloned gene (cel 2), with different restriction enzymes digestion, M, 1kb ladder; H3, *Hind*III; EI, *Eco*RI; BI, *Bam*HI; KI, *Kpn*I; PI, *Pst*I; SI, *Sal*I.

Figure-5: Endonuclease restriction map of cel 2 from *Bacillus spp.*The restriction enzymes, H3, *Hind*III; EI, *Eco*RI; EII, *Eco*RII; FI,*Fok*I.

Nucleotide sequence of *cel 2* gene: The nucleotide sequence of the DNA insert in pcel 2 revealed an open reading frame containing the structural region of the cellulase gene (*cel 2*). The relative position and direction of transcription are indicated by arrow under *pcel 2* in Fig. (6).The complete nucleotide sequence of the region containing the *cel 2* and the sequence of its predicted translational product *cel 2*, is shown in Fig. (6). An alignment between the DNA sequences showed that *cel 2* of *Bacillus sp.* Strain A-4 has 99% and 87% similarity with *engD* and *engO* from *Clostridium cellulovorans* and *celB* from *Rumino-coccus albus,* respectively.

A putative ribosome-binding site (RBS) AGGAGAA, bold-faced was found 17 nucleotides upstream from the initiation codon of *cel 2* in Fig. (6). The ATG codon at position 114 was probably the translation initiation codon for *cel 2*. The *cel 2* enzyme, encoded from ATG initiation codon to the ochre termination codon TAG at position 1882, had 389 amino acids (Fig. 6), an estimated molecular mass of 42KDa, and a PI 5.35.

Amino acid sequence: The amino acid sequence derived from the open reading frame of (Cel 2) was analyzed. Table (1) show that the hydrophobic residues represent 192 amino acid (49.36%) number of alanin (A) is 35 (9 %), val (v) is 29 (7.46%) are used as the most popular hydrophobic amino acid. In addition, they were followed by a stretch of hydrophilic amino acid residues which represent by 105 amino acid (26.99 %), the most hydrophilic amino acids are Asp (D) is 26 (6.68%) and Lys (K) and the neutral amino acids are 92 aa.

The codon usage: The codon utilization of *cel 2* gene shows a 202 for A or T at the wobble position as has been observed for genes from *Clostridium* genus. The percentage was codon 51.93% in table (2). An amino acid alignment of *cel 2*, with other family 5 cellulases demonstrated a high degree of homology. There were nearly 71.2% and 70.4% identity between *cel 2* of this work and cellulase *celB* from *Ruminococcus albus* and engD from *Clostridium cellulovorans*, respectively (Fig. 7).

SDS-PAGE and zymogram: The protein compositions of the native *Bacillus* sp. Strain A4 and positive clone

crude enzyme preparation were analyzed by SDS-PAGE. The crude enzyme preparation showed at least 12 proteins with molecular masses in the range of 116 to 18.4kDa (Fig. 8). Among them, were eight dands molecular size of 66.4 to 18.4kDa. Figure (8A) Coomassie staining part, lane M, Protein Marker, lane 1, crude proteins, and lane 2, partially Purified protein and Figure (8B) Zymogram part, lane 1, crude proteins, and lane 2, partially Purified protein. The molecular mass of the interest protein was estimated to be 42KDa (Fig. 8).

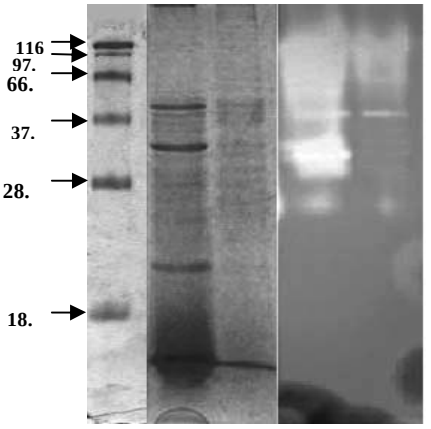

Figure -8: (**A**) Coomassie staining part, lane M, Protein Molecular weight Marker, lane 1, crude proteins of the native bacterium, and lane 2, partially Purified recombinant protein and (**B**) Zymogram part, lane 1, crude proteins, and lane 2, partially Purified protein.

The analyzed the recombinant cel2 protein by zymograms and compared it with the native crude enzyme protein cel2. The cel2 gave one major band in the zymogram with CMC, and the molecular mass of the enzyme was estimated to be 42 kDa (Fig. 8B, lane 2), which is similar in size to that of the mature native cel2 deduced from the nucleotide sequence (42.49 kDa). A band with an apparent molecular mass of 42 kDa was detected in the pcel2 proteins purified from *Bacillus sp.* Strain A4 (Fig. 8A and 8B, lanes 2). The size of the protein was in good agreement with that of the full-length cel2 produced by recombinant *E. coli* and the size calculated from the deduced amino acid sequence.

The crude enzyme preparations from *Bacillus sp.* Strain A4 were examined. Cel2 showed strong activity toward carboxymethyl cellulose (CMC), and hemicellulose (locust bean gum LBG) but showed no activity towards xylan. The optimum pH for activity was found to be pH 5 to 6 when the enzyme activity was assayed at 37°C in sodium acetate buffer solutions at various pHs (data not shown). The optimum temperature for activity was found to be 40 to 50°C at pH 6 (data not shown). The values were 145 μmol min^{-1} mg^{-1} and 17.2mg/ml for CMC and hemicelluloses.

DISCUSSION

This paper presents isolation and characterization of new cellulolytic bacterium, *Bacillus* sp. Strain A-4 from the Egyptian soil 2005. *Bacillus* sp. is rod shaped, mesophilic,Gram-positive, spore forming, catalase positive and can hydrolyses cellulose, hemicellulose, amylose and other forms of carbon sources. The cellulolytic enzyme system of *Bacillus sp.* A-4 was found to be associated with cells from the early exponential growth phase to the late stationary growth phase. Detection of *Bacillus sp* was based on clearing of halo formation around *Bacillus sp* that grew on agar-CMC plates. Several authors have used the standard procedures, as

those used in the present study, to isolate and identify *Bacillus spp* bacteria from soil or other resources and also to test its ability to degrade microcrystalline Avicel cellulose and acid- swallen cellulose. Chartchi *et al.* (1998) isolated *Bacillus subtilis* 5H from the soil in swallen cellulose. Chartchi *et al.* (1998) isolated *Bacillus subtilis* 5H from the soil in Chiang Mai Thailand. Singh *et al.* (2004) isolated a novel strain of *Bacillus sphaericus* JSI producing thermostable alkaline CMCase, endo 1,4- glucanase from soil. Grant *et al.* (2004) collected several samples from sediments and surrounding Soda Soils (SS) and extremely saline and alkaline lakes of the Wadi-El Natrun in the Western Desert of Egypt. Clones derived from lake sediments (LS) most closely matched *Clostridium spp, Natronoincola histidinovorans, Halocella, cellulolytica, Bacillus spp*, *Xanthomonas campestrvs* and *Fibrobacter succinogenes*. Recently, Shimei *et al.* (2005) isolated and identified two strains of *Bacillus subtilis* M_4A and JM_4B from soil and showed to produced antimicrobial peptides. Also Sumitomo *et al*. (2007) isolated and identified *Paenibacillus sp* from a soil sample that was producing α 1,3-glucanase. Glick and Pasternak (2003) showed that the clear halos around colonies are indicative for β-glucanase activity in *Clostridium*, *Thermoanaera-bacter*, *Thermomonos- pora*, *Erwinia*, *Pseudomona, Cellvibrio*, *Ruminococcus*, *Fibrobacter* and *Bacillus sp*.

Cloning, sequencing analysis and expression of a novel cellulase 2 (*cel2*) encoding gene and expressed in *E.coli* DH5α. Detection of cellulose activity from positive colonies was based on clearing of halo formation around transformed colonies that grew on agar-CMC plates. These results are in agreement with Neil *et al.,* (1984) found that growth of *E.coli* strains carrying PEC1,PEC2 or PEC3 on minimal agar containing CMC., followed by Congo Red stain, characterized to degrade CMC by a large halo around the clones. Mackay *et al.,* (1986) cloned *Bacillus subtilis* (strain PAP115) 3 kb *pst1* fragment which contains an endo-β-1-4-gluconase gene inserted in the vector M13 and transformed in to *E. coli.* Marek *et al.,* (1987) cloned *Clostridium thermocellum* 2kb BamHI and SalI fragment which contains endo β 1,4-gluconase gene and were inserted in plasmid pBR322 and transformed to *E.coli*. Detected was enzyme activity of the large halo produced on LB plates overlaid with CMC agar and stained with Cango Red in transformed colonies. Lee and Kim (1999) cloned *Bacillus cellulyties* K-12 Avicelase of an endo-β-1,4-D-gluconase gene into *E. coli* by using the vector pT7T3u19 and *HindIII* libraries of the chromosomal inserts. Sanchez *et al.,* (2005) cloned *E. coli* alkaline cellulose gene (*CELB1*) that was present in a 2.5kb *Hind*III fragment from the alkalophilic *Bacillus sp*.

The nucleotide sequence of the DNA insert in *pcel 2* revealed an open reading frame containing the structural region of the cellulase gene (*cel 2*). The *cel 2* enzyme, encoded from ATG initiation codon to the ochre termination codon TAG at position 1882, had 389 amino acids an estimated molecular mass of 42KDa, and a PI 5.35. An amino acid alignment of *cel 2*, with other family 5 cellulases demonstrated a high degree of homology. There were nearly 71.2% with cellulose *celB* from *Ruminococcus albus* (Ohmiya *et al*., 1989) and 70.4% cellulase *cel* from *Clostridium cellulo-vorans* (Doi, *et al*., 1998) identity

between *cel 2* of this work.). An alignment between the DNA nucleotide sequences showed that *cel 2* of *Bacillus sp.* Strain A-4 has 99% and 87% similarity with *engD* and *engO* from *Clostridium cellulovorans* (Hamamoto, *et al*.1990 and Han, *et al*., 2005) and *celB* from *Ruminococcus albus (*Ohmiya, *et al.,* 1989) respectively.

The amino acid sequence derived from the open reading frame of (*cel 2*) was analyzed. show that the hydrophobic residues represent 192 amino acid (49.36%) number of alanin (A) is 35 (9 %), val (v) is 29 (7.46%) are used as the most popular hydrophobic amino acid. In addition, they were followed by a stretch of hydrophilic amino acid residues which represent by 105 amino acid (26.99 %), the most hydrophilic amino acids are Asp (D) is 26 (6.68%) and Lys (K) and the neutral amino acids are 92 aa. The purified recombinant Cel2 had a molecular mass 42 KDa after migration on SDS-PAGE (10%), this is in agreement with the molecular weight deduced from the amino acids. Sanchez *et al.,* (2005) sequenced a cellulase gene (*Cel B1*) from the alkalophilic *Bacillus sp.* and cloned in *E. coli* which involved an open- reading frame encoding 389 amino acids. Results permitted to include it in family 5 of the glycosyl hydrolases. Sumitomo *et al.* (2007) determined nucleotide sequence of the alpha-1,3-glucanase gene from *Paenibacillus* . It consisted of 3651 bP open reading frame that encoded a predicted 1217 amino acid polypeptide including a 43 amino acid signal peptide. The mature enzyme showed similarity to mutanases RMI of *Bacillus sp.* Strain RMI and *Bacillus circufans* KA-304 showed 65.6% and 62.7% identify, respectively. The size of the protein was in good agreement with that of the full-length cel2 produced by recombinant *E. coli* and the size calculated from the deduced amino acid sequence. The mass spectrometry technique was also used for the identification of proteins separated by SDS-PAGE (Bequin, 1983; Hansmeier, *et al*., 2004).

The crude enzyme preparation from *Bacillus sp.* A-4 exhibits activity over a broad range of pH5 to 6 and has good stability across temperatures 50°C and pH6. It is capable of degrading cellulose, and hemicellulose (locust bean gum LBG).

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81    AAGAGGAGAAAGGAGAAAGAGAAACTTTAAGGTAATGTCTACTGCTACAAATGAAGTTGTTTATACAGGTAACATCGAAG   160

      G  C  R  Y  N  E  A  A  G  E  I  N  R  F  G  D  F  S  S  F  T  T  P  G  T  Y  K
161   GGTGTAGATACAATGAAGCAGCTGGTGAAATAAATAGATTTGGAGATTTTTCTTCTTTTACAACACCAGGAACTTATAAG   240

      I  Q  T  D  S  L  G  S  S  Y  D  F  T  I  S  E  D  I  Y  N  N  V  F  K  D  A  V
241    ATTCAAACAGATAGCTTAGGAAGTTCATATGATTTTACTATATCAGAAGATATTTATAATAATGTATTTAAGGATGCTGT   320

         R  F  F  Y  M  Q  R  C  G  Q  E  L  P  A  I  Y  A  G  K  W  A  H  P  A  C  H
321    GAGATTTTTCTATATGCAAAGATGTGGACAAGAGCTTCCAGCAATATACGCTGGTAAATGGGCACATCCTGCTTGTCATA   400

      T  E  L  A  R  I  Y  G  T  D  K  K  I  D  V  S  G  G  W  H  D  A  G  D  Y  G  R
401    CAGAGCTTGCTAGAATCTATGGAACAGATAAAAAAATTGACGTATCTGGAGGTTGGCATGATGCTGGTGACTATGGCCGT   480

      Y  V  V  A  T  S  K  A  V  A  D  L  L  S  A  Y  N  D  N  K  A  A  F  G  D  D  F
481    TATGTAGTTGCTACTTCAAAAGCTGTTGCTGACTTATTATCAGCATATAATGATAATAAAGCTGCCTTTGGAGATGATTT   560

         N  I  P  E  S  G  N  G  V  S  D  V  L  D  E  V  K  Y  Q  L  Q  W  L  L  K  M
561    TAATATACCTGAAAGTGGAAATGGAGTTTCCGATGTTTTAGATGAAGTAAAATATCAATTACAGTGGCTATTAAAAATGC   640

      Q  E  P  T  S  G  G  V  Y  H  K  E  L  C  A  G  F  P  G  H  Y  A  T  I  L  K  K
641    AAGAGCCAACAAGTGGTGGAGTTTATCATAAGGAACTTTGTGCTGGATTCCCTGGACACTATGCCACAATTTTGAAAAAG   720
```

```
       D  A  L  I  V  S  P  I  S  T  T  A  T  A  D  F  A  A  V  M  A  M  G  Y  E  N  F
721    GATGCACTTATAGTATCACCTATTTCTACAACTGCAACAGCAGATTTTGCAGCAGTTATGGCAATGGGCTATGAAAACTT   800

         K  D  I  D  A  E  L  A  Q  K  C  L  A  A  G  E  L  A  W  S  Y  L  E  K  T  P
801    TAAGGATATAGATGCAGAACTTGCTCAAAAATGTTTAGCAGCAGGAGAATTAGCTTGGAGCTACCTTGAAAAAACACCAA   880

       T  I  P  F  T  N  P  K  G  I  S  T  G  E  Y  G  D  G  Y  D  G  D  E  R  Y  W  A
881    CTATTCCGTTTACAAATCCAAAAGGTATTTCAACAGGAGAATATGGTGACGGTTATGATGGTGATGAAAGATATTGGGCA   960

       A  A  E  L  L  K  A  T  G  N  S  K  Y  D  D  A  F  K  K  M  L  N  L  K  F  E  N
961    GCCGCTGAATTGCTTAAGGCTACAGGTAATAGTAAATACGATGATGCCTTTAAGAAAATGCTTAACTTAAAATTTGAAAA   1040

       G  Y  G  W  A  S  V  G  H  Y  G  N  K  A  Y  L  T  A  K  A  A  D  Q  N  V  A
1041   TGGTTATGGTTGGGCTAGCGTAGGTCATTATGGAAACAAAGCATATTTAACAGCAAAAGCTGCTGACCAAAATGTAGCTG   1120

       G  N  I  R  N  H  V  L  N  E  A  Q  Q  I  V  S  L  A  K  N  D  G  Y  N  I  S  N
1121   GTAACATTAGAAACCACGTTTTAAACGAAGCACAACAAATAGTGTCTTTAGCTAAAAATGATGGGTATAACATTTCCAAT   1200

         G  T  N  Y  Y  W  G  S  N  M  N  V  T  N  N  A  I  L  L  A  E  A  Y  K  L  S  P
1201   GGAACAAATTACTATTGGGGCAGCAATATGAATGTAACTAATAATGCTATATTACTAGCAGAAGCATATAAGCTTAGTCC   1280
         * stop codon
1281   TTAGCCAGAATATTTAGAATATGCTAAAGAACATATCAAT   1320
```

Figure-6: Nucleotide and deduced amino acid sequences of the *pcel 2* gene and flanking sequences. The deduced amino acid sequence of the *pcel 2* is shown as 1-letter symbols below the nucleotide sequence, and amino acid residues are numbered beginning with the first methionine. The translation termination codon is denoted by an asterisk. Numbers to the right and left of the sequence correspond to nucleotides and amino acids, respectively.

Table -1: Amino acid composition (hydrophobic, neutral and hydrophilic residues) of pcel 2 from *Bacillus sp.* **aa.** Amino acid; **T.** total number

Type of residues	Name of aa	No. of aa	% of each	T_{No}	$T_{\%}$
Hydrophobic residues	Gly(G)	22	5.66	192	49.36
	Leu(L)	24	6.17		
	Phe(F)	19	4.88		
	Ala(A)	35	9.00		
	Ile(I)	23	5.91		
	Trp(W)	8	2.06		
	Val(V)	29	7.46	92	23.65
Neutral residues	Met(M)	11	2.83		
	Pro(P)	21	5.40		
Hydrophilic residues	Ser(S)	12	5.66	105	26.99
	Gln(Q)	7	1.80		
	Thr(T)	35	9.00		
	Cys(C)	2	0.51		
	Asn(N)	36	9.25		
	Asp(D)	26	6.68		
	His(H)	7	1.80		
	Glu(E)	17	4.37		
	Arg(R)	12	3.08		
	Lys(K)	23	5.91		
	Tyr(Y)	20	5.14		

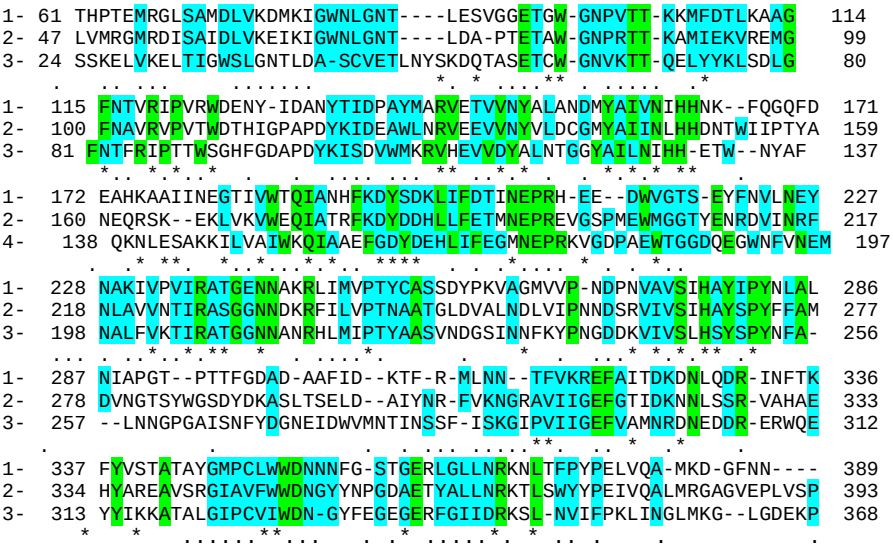

Figure-7: Alignment of the primary structure of, 1- cellulase *cel2* from *Bacillus sp.* with those of other enzymes from family 5 of glycosyl hydrolases; 2- *celE* from *Clostridium thermocellum*, 3- *CelB* from *Ruminococcus albus*. Only regions of high-level similarity are shown. Identical amino acids conserved in all of the proteins are shaded. The positions with identical amino acids which occur in all sequences are marked with a small star.

Table-2: Codon usage in the *cel 2* of *Bacillus sp.*

Codon	aa.	No of time used	Code	aa.	No of time used
TTT	Phe	4	TAT	Tyr	7
TTC	Phe	10	TAC	Tyr	5
TTA	Leu	17	CAT	His	5
TTG	Leu	20	CAC	His	6
CTT	Leu	12	CAA	Gln	16
CTC	Leu	4	CAG	Gln	19
CTA	Leu	12	AAT	Asn	13
CTG	Leu	13	AAC	Asn	6
ATT	Ile	11	AAA	Lys	14
ATC	Ile	5	AAG	Lys	12
ATA	Ile	18	GAT	Asp	5
ATG	Met	18	GAC	Asp	4
GTT	Val	2	GAA	Glu	5
GTC	Val	2	GAG	Glu	3
GTA	Val	8	TGG	Trp	6
GTG	Val	6	CGT	Arg	1
TCT	Ser	1	CGC	Arg	0
TCC	Ser	3	CGA	Arg	0
TCA	Ser	3	CGG	Arg	0
TCG	Ser	0	AGT	Ser	0

CCT	Pro	3	AGC	Ser	1
CCC	Pro	0	AGA	Arg	7
CCA	Pro	3	AGG	Arg	4
CCG	Pro	0	GGT	Gly	3
ACT	Thr	9	GGC	Gly	0
ACC	Thr	3	GGA	Gly	3
ACA	Thr	12	GGG	Gly	5
ACG	Thr	6	TGT	Cys	4
GCT	Ala	4	TGC	Cys	0
GCC	Ala	0			
GCA	Ala	0			
GCG	Ala	3			

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