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Purification and characterization of an extracellular cellulase from *Anoxybacillus gonensis* O9 isolated from geothermal area in Turkey

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Abstract

In the present study, cellulase was purified and characterized from *Anoxybacillus gonensis* (Gen bank Number: KM596794) which was isolated and characterized from Agri Diyadin Hot spring. It was found to synthesize cellulase which had a wide range of industrial applications. Twenty four-hour-cultured bacteria induced cellulase production and specific activities during the purification steps were 1.47, 81.06 and 109.4 EU mg^{-1} protein at crude extract, ammonium sulphate precipitated and DEAE-Sephadex purification steps. The highest enzyme activity was observed at 50°C and the optimum range of pH was 3-10. Molecular weight of enzyme was determined approximately 40kDa. The kinetic parameters of cellulase against carboxymethylcellulose (CMC) were 153.4 µmol min^{-1} mg for V_{max} and 0.46mM for K_m. Among effectors of the enzyme, Zn^{2+}, Ca^{2+}, Co^{2+} and EDTA decreased enzyme activity.

Key words

Anoxybacillus gonensis, 16S rRNA sequencing, Cellulase, Characterization

Introduction

Thermophilic organisms exhibit high thermal stability and have resistance towards denaturation impact of acidic and alkaline environments, thus thermophiles are very important in biotechnological field. The most important feature of thermophilic organisms is production of enzymes that catalyze biochemical reactions at higher levels of temperatures and these enzymes are widely used in many industrial areas such as molecular biology, food, petroleum, chemical and paper industry. In recent years, many enzymes from these thermophilic bacteria have been successfully purified, characterized and used in industried applications have been performed. One of the industrially important enzymes is cellulase (Bozoglu *et al*.,2013).

Cellulose is the most abundant organic compound in the biosphere and annual production of cellulose was 10^7 tons in 2005 (Bakare *et al*., 2005). Cellulose structure consists of linear chains of approximately 8.000-12.000 residues of D-glucose units linked with β-1.4 bonds (Martins *et al*., 2011).

Cellulases, which are synthesized by a large diversity of microorganisms including thermophilic bacteria are inducible enzymes and catalyze the conversion of insoluble cellulose to simple water soluble monomers (Kuhad *et al*., 2011). Exoglucanase acts on the ends of cellulose chain and removes cellobiose as end product, thus it is called cellobiohydrolysas or CBH. Endoglucanase randomly attack internal β-1.4 glucan chain and β-glycosidase or cellobiase (BGL) acts specifically on β-cellobiose structure by releasing glucose (Jahangeer *et al*., 2005; Kuhad *et al*., 2011).

Cellulases have a wide range of applications in paper, textile, wine, food processing, animal feed, detergent, olive oil extraction and agricultural industry (Kuhad *et al*., 2011). Among these areas, application in detergent, leather and paper industries demands highly stable enzymes active which are at extreme pH or temperature. Thermostable enzymes have more advantages than non-thermostable enzymes such as resisting higher process temperatures such that enzymes from thermophilic organisms gain importance. On the other hand,

investigation of existing and novel producers would be crux of future researches of industrially important cellulases; however commercial cellulases are being produced (Ibrahim and El-diwany, 2007).

The purpose of the present study was to identify O9 strain, thermophilic bacteria, purification, characterization and examination of the kinetics of extracellular cellulase produced by this strain.

Materials and Methods

Isolation and characterization of isolate : Water sample was taken from Diyadin Hot spring, Agri in Turkey where natural temperature of the resource was approximately 70ºC. The sample was incubated on nutrient agar plates at 55 ºC for 24-48 hrs. One of the different colonies was selected randomly by using microscopy and subcultured after incubation period was completed. Isolated and purified bacterial strain (O9) was stored in nutrient broth (NB) with 15% glycerol at -86 ºC for further studies.

Physico-chemical requirements of thermophilic bacterial growth were determined by incubating 35-80 ºC with 1°C of intervals at pH 4.0-12.0. The effect of NaCl was studied in NB medium containing 2.0, 3.0, 4.0, 5.0, 8.0, 10.0% (w/v) NaCl.

Total genomic DNA was extracted from bacterial sample (O9) using a method previously described by Adiguzel and Atasever (2009). 16S rRNA gene was selectively amplified from purified genomic DNA using oligonucleotide primers designed to anneal to conserved positions at 3' and 5' regions of bacterial 16S rRNA genes. The forwardprimer, UNI16S-L (5'ATTCTAGAGTTTGATCATGGCTC A-3'), corresponding to position 11 to 26 of *Escherichia coli* 16S rRNA and reverse primer, UNI16S-R (5'-ATGGTACCGTGTG ACGGGCGGTGTGTA-3'), corresponding to complement of positions 1411 to 1393 of *E. coli* 16S rRNA with previously described method (Beffa *et al*., 1996) and the PCR product was cloned to pGEM-T vector system (Promega,UK).

DNA sequence of 16S rRNA gene sequence with an applied biosystem model 373A was determined by ABI PRISM cycle sequencing kit (Macrogen, Netherland) after PCR amplification and cloning of 16S rRNA gene of the strain. 1429 nucleotides among 16S rRNA were determined. This sequence was compared with the complementary sequences within Gen Bank by BLAST search (Altschul *et al*.,1990).

Production and purification of cellulase : The test isolate (O9) was cultured in NB. The culture broth was incubated for 24 hr at 55°C at 150 rpm and then broth was centrifuged at 10000 rpm for 10 min to remove the cells. The crude enzyme extracts were used for further purification steps. The first purification step was performed using ammonium sulphate precipitation. Solid $(NH_4)_2SO_4$ was added by gently mixing the crude enzyme extract with 0-20, 20-40, 40-60, 60-80 and 80-100% intervals in ice bath. After mixing, the bulk was centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was poured and the precipitate was dissolved in 20mM sodium citrate buffer (pH 6.0) and dialyzed against same buffer.

Diethylaminoethyl (DEAE) Sephadex ion exchange column (2.5 x 30) was studied for purification of crude cellulase extract after ammonium sulphate precipitation and dialysis. The column was washed with 20mM of sodium citrate buffer (pH 6.0) and equilibrated until elute protein detection failed with same buffer. NaCl gradient from 0 up to 1M was applied to elute proteins attached to the column. The flow rate of collected-5-ml fraction was 10mlmin^{-1}. The absorbance of protein elution was spectrophotometrically measured at 280nm. Cellulase activities of fractions were measured and the active fractions were combined and stored at -18°C untill use.

Protein concentration was determined spectrophotometrically at 595nm using Bovine Serum Albumin (BSA) as standard (Bradford, 1976).

Cellulase activity towards carboxymethyl cellulose (CMC) was measured by incubating crude enzyme extract (0.5ml) and 0.5% (w/v) carboxymethyl cellulose (1ml) in 20mM sodium citrate buffer, pH 6.0 at 50°C for 30 min. The reaction was blocked by addition of 1.5ml of 3,5-dinitrosalicylic acid reagent and heated at 90°C for 5 min in hot water bath. The reaction mixture was cooled down to room temperature and absorbance was read at 540nm against blank. The blank was prepared with distilled water instead of enzyme (Wood and Bhat, 1998). The amount of glucose per ml was estimated from standard curve prepared with known glucose concentration. One unit of cellulase activity was expressed as the amount of protein that liberated reducing sugar equivalent to 1µg of glucose per minute under assay conditions. Specific enzyme activity was expressed as unit of enzyme activity per mg of protein.

Determination of kinetic and physicochemical parameters of cellulase : The impact of Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+} ions and EDTA on cellulase was investigated. All the metal ions were tested in three runs for each concentration, and cellulase activity was measured at different concentrations of the effectors.

0.5 ml of purified enzyme and 1ml of 0.5% CMC in 20mM sodium citrate buffer (pH 6.0) was incubated at various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90°C) for 30 min to determine optimum temperature of purified cellulase.

Purified cellulase was pre-incubated at various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90°C) for 2 hrs for temperature stability and samples were taken at 15 min interval. The residual activity was measured according cellulase activity

assay.

3ml of buffer at various pH values (pH 3.0-4.0: 10mM sodium acetate buffer; pH 5.0, 6.0, 7.0, 10.0: 10mM sodium phosphate buffer; pH 8.0, 9.0: 10mM Tris-HCl buffer) and 3ml of purified cellulase were combined and incubated for 10 hrs. Cellulase activities for pH optimum were measured shortly after combination of purified enzyme and buffers. For pH stability, samples were taken at 1hr intervals during 10hr-incubation and the residual activity was measured.

Specific activity against 0.5% CMC and 0.5% xylan (w/v) in 20mM sodium citrate buffer (pH 6.0), 0.5% starch in hot distilled water and 0.5% phytate were determined to evaluate the substrate specificity of purified cellulose.

SDS-PAGE gel electrophoresis and zymogram analysis : Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed through 10-30% discontinued method (Laemmli, 1970). To the purified enzyme, equal volume of sample buffer (1M Tris-HCl pH 8.0, 10% SDS, glycerol, 5% β-mercaptoethanol) was added and the samples was boiled at 100 ºC for 5 min. Boiled samples and protein standard were then subjected to SDS-PAGE in a gel casting apparatus (Bio-RAD, USA) with execution buffer (0.25M Tris-HCl, 1.92M Glycine, 0.1% SDS (pH 8.3). Electrophoresis was performed at room temperature for 0.5 hr at 60 V and 1hr at 120 V.

Zymogram analysis was slightly similar to SDS-PAGE method. Equal volume of purified enzyme, 0.5% CMC substrate and sample buffer (1M Tris-HCl pH 8.0, 10% SDS, glycerol) were combined and loaded in gel apparatus. The execution buffer and operation conditions were same as SDS-PAGE. The gel for zymogram analysis was taken out after electrophoresis was complete and hold in 2.5% Triton X-100 in 100mM sodium citrate buffer (pH 6.0) to remove SDS. The gel was washed thrice with the same buffer to get rid of SDS completely and incubated in 0.5% CMC substrate solution at 50°C for 30 min.

The gels, after SDS-PAGE and zymogram, were stained according to silver staining protocol. Silver staining was carried out in five steps; fixation (10% acetic acid and 30% ethanol in distilled water), reduction (3.2% (w/v) sodium acetate, 30% (v/v) ethanol, 0.1% (w/v) sodium thiosulphate, 2% (v/v) glutaraldehyde), staining (10% silver nitrate, 0.03% formaldehyde) and washing (1.4% (w/v) sodium carbonate and 0.04% (v/v) formaldehyde).

Results and Discussion

Water sample from Agri Diyadin Hotspring was spread out on nutrient agar plates and incubated. A group of physiological and biochemical tests were performed and it was deduced that 55°C ± 1°C temperature, 6.0-8.0 pH and 2%-7% salt concentration were optimum for bacterial growth. These findings complied with the criteria of thermophilic bacteria which were found at temperature higher than 50°C (Adiguzel *et al*., 2009). Colony development was a determinative issue for isolation and one of the colonies which was chosen randomly from isolation quality was used for cellulase production in NB.

1429 nucleotides of O9 isolate were aligned to compare with the sequences of the related bacteria and 16S rRNA sequence analysis showed that there was a strong similarity (≥98%) between the test strains and representative strains in gene bank of *Anoxybacillus*. A few studies were carried out previously and our data was found compatible with literature (Stackebrandt and Goebel,1994; Sung *et al*., 2002; Belduz *et al*., 2003; Dulger *et al*., 2004; Adiguzel *et al*., 2009). In addition, the Gen Bank accession number of O9 strain was KM596794.

Purification steps were carried out when cellulase production of *A. gonensis* O9 was completed. In the first step, ammonium sulphate precipitation of cellulase was performed with a wide range of saturation (0-100%) after 24 hrs incubation of bacterial growth. Highest precipitation and purification of cellulase was noted at 60-80% saturation point (Andriani *et al*., 2012) with 55 fold (Table 1). In the second step, passage from DEAE-Sephadex column purified the enzyme to 74-fold. Specific activity of purified cellulase increased up to 109.4EU mg^{-1} protein.

The kinetic parameters were identified against different substrates (starch, xylan, CMC and phytate) because cellulases might have catalytic and synergetic activity against different substrates due to their different subunits (Xia *et al*., 2008). As a result; K_m values of cellulase from *A. gonensis* O9 were changeable (Table 2). Same K_m value indicated that cellulase from *A. gonensis* O9 could show affinity against both xylan and CMC because the binding patterns of both xylan and CMC were β-1,4 chemical bonds however these linkages were xylosidic and glycosidic bonds for xylan and CMC, respectively (Kumar*et al*., 2012).

Zn^{2+} and EDTA decreased cellulase activity at 1mM concentration, however, cellulase activity was increased with 5mM of Zn^{2+} and did not change at higher level of EDTA. On the

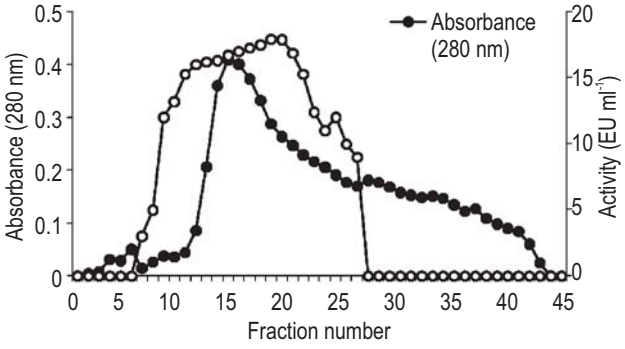

Fig. 1 : Purification of cellulase by ion exchange chromatography using DEAE-Sephadex

Table 1 : The purification process of cellulase from *A. gonensis* O9

Enzyme fraction	Volume ml	Activity EU ml^{-1}	Total activity		Protein (mg ml^{-1})	Specific activity (EU mg^{-1} protein)	Purification fold
			EU	%			
Crude extract	50	49.13	2456.5	-	33.5	1.47	-
$(NH_4)_2SO_4$ precipitation	30	25.13	753.9	30.7	0.31	81.06	55.14
DEAE-Sephadex	25	20.34	508.56	20.7	0.186	109.4	74.42

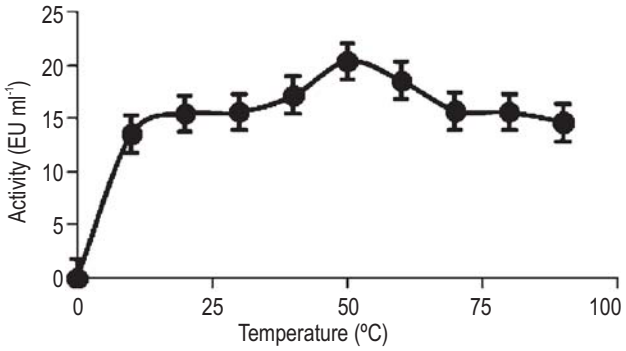

Fig. 2(a) : Effect of temperature on the activity of purified cellulase from *A. gonensis* O9

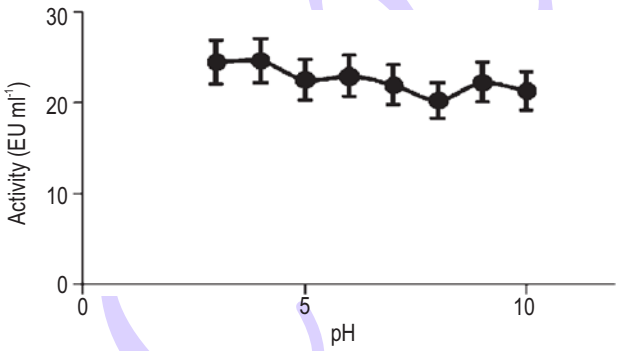

Fig. 2(b) : Effect of pH on the activity of purified cellulase from *A. gonensis* O9

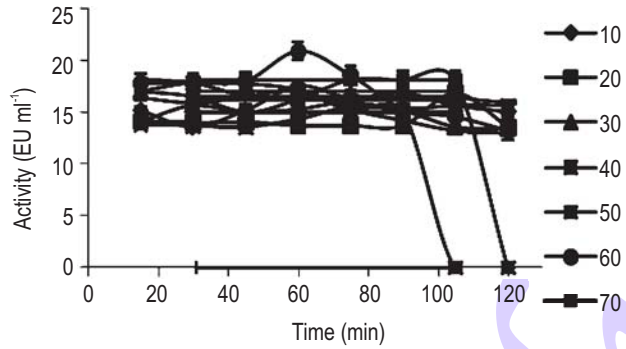

Fig. 2(c) : Temperature stability of purified cellulase from *A. gonensis* O9

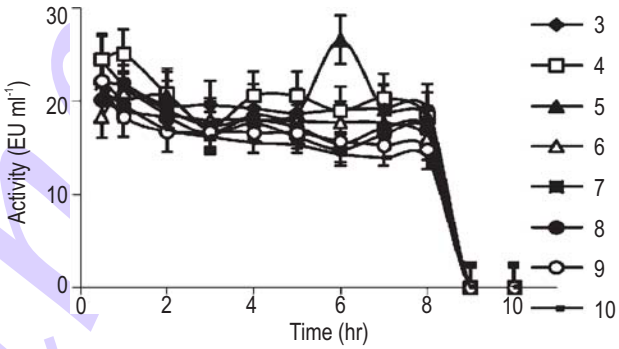

Fig. 2 (d) : pH stability of purified cellulase from *A. gonensis* O9

Table 2 : V_{max} and K_m values for some substrates of cellulase from *A. gonensis* O9

Substrate	V_{max} (μmole min^{-1}mg)	K_m (mM)
Starch	198,8	0,163
Xylan	233,1	0,375
CMC	153,4	0,46
Phytate	265,09	32,26

other hand, 1mM of Ca^{2+} caused 15% loss in enzyme activity of cellulase from *A. gonensis* O9, while Co^{2+} completely inhibited enzyme at any concentration (Table 3). Liang *et al.* (2010) reported that even 1mM of Co^{2+} inhibited cellulase activity and maximum enzyme activity was obtained with 5.5mM of Ca^{2+}. Purified cellulase showed maximum activity at approximately 50 ºC (Fig. 2a, b). The optimum temperature for cellulases from *Bacillus* sp CH43 and *Bacillus* sp HR68 which were reported by Mawadza *et al*. (2000) were 70ºC and 65ºC, respectively.

Optimum pH range for purified cellulase was 3-10, and this wide range might provide few of the industrial applications with high level temperature tolerance. Ibrahim and El-diwany (2007) purified cellulase from *A. flavithermus* at pH 6-9 with optimal pH of 7.5. The purified enzyme kept most of its activity even at higher temperature despite longer incubation periods. Maximum cellulase activity was obtained at 60°C, 60th min of incubation, at 5.0 pH and 6th hour of incubation (Fig. 2c, 2d). The optimum pH of CMCase purified from *Bacillus* and *Geobacillus* sp. was 5.0 (Rastogi *et al*., 2010) and a thermostabile cellulase gene from *Clostridium* was expressed in *E. coli* and showed maximum-stabile activity at 60ºC, even after 1 hr (Peng *et al*., 2011).

10% SDS-PAGE was carried out just after the active fractions from DEAE-Sephadex were combined and SDS was

Table 3 : Effect of some metal ions and chemicals on cellulase activity from *A. gonensis* O9

Chemicals	Concentration (mM)	Relative activity (%)	Concentration (mM)	Relative activity (%)
Control	-	100 ± 0.0	-	100 ± 0.0
$NiCl_2.6H_2O$	1	87.95 ± 1.2	5	94.85 ± 0.2
$ZnCl_2$	1	0	5	177.33 ± 1.3
EDTA	1	0	5	99.16 ± 0.6
$CuCl_2$	1	86.28 ± 2.01	5	202.19 ± 0.8
$Pb(NO_3)_2$	1	89.76 ± 0.4	5	118.03 ± 0.14
$Hg(NO_3)_2.H_2O$	1	148.94 ± 1.3	5	121.44 ± 0.7
$CaCl_2.2H_2O$	1	85.4 ± 0.7	5	0
$MgCl_2.6H_2O$	1	0	5	108.63
$FeCl_2.4H_2O$	1	0	5	245.14
$CoCl_2.6H_2O$	1	0	5	0

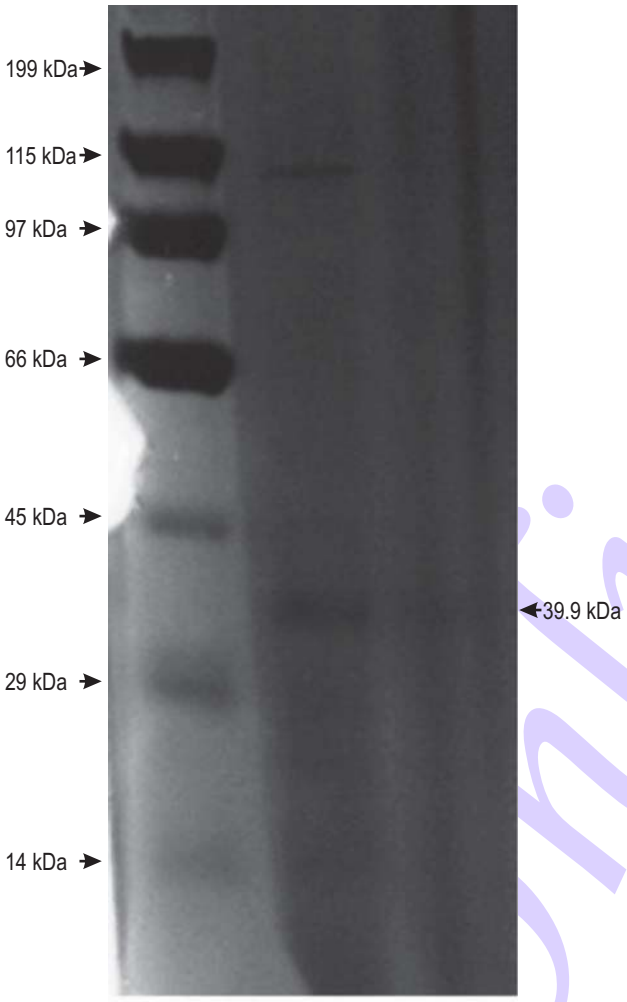

Fig. 3 : Native zymogramof the purified cellulase from *A. gonensis* O9

removed from gel before silver staining.39.9 kDa-single band is shown in Fig.3. Purified cellulase from *Bacillus* sp had 29 kDa single band and cellulase from *Bacillus pumilus* sp had 30-65 kDa molecular weight (Ariffin *et al*., 2006). Cellulase from *B. circulans* had 43 kDa molecular weight (Hakamada *et al*., 2002), and Mawadza *et al*. (2000) carried out cellulase purification from *Bacillus* sp. that had 40 kD molecular weight.

It was concluded that catalytic activities of cellulase purified from *A. gonensis* O9 were rather high, highly stable against temperature, different pH values and resistant to metal ions. Thus, purified cellulase could be suitable in different industrial areas.

Acknowledgments

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References

Adiguzel, G.C. and M. Atasever: Phenotypic and genotypic characterization of lactic acid bacteria isolated from Turkish dry fermented sausage. *Romanian Biotech. Lett.,* **14**, 4130-4138 (2009).

Adiguzel, A., H. Ozkan, O. Baris, K. Inan and M. Gulluce: Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *J. Microbiol. Meth*., **79**, 321-328 (2009).

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman: Basic local alignment search tool. *J. Mol. Biol*., **215**, 403-410 (1990).

Andriani, D., C. Sunwoo, H.W. Ryu, B. Prasetya and D.H. Park: Immobilization of cellulase from newly isolated strain *Bacillus subtilis* TD6 using calcium alginate as a support material. *Bioproc. Biosyst. Eng*., **35**, 29-33 (2012).

Ariffin, H., N. Abdullah, K. Umi, Y. Shirai and M.A. Hassan: Production and characterization by *Bacillus pumilus* EB3. *Int. J. Eng. Technol*., **3**, 47-53(2006).

Bakare, M.K., I.O. Adewale, A. Ajayi and O.O. Shonukan: Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens.Afr. J. Biotechnol*., **4**, 898-904 (2005).

Beffa, T., M. Blanc, P.F. Lyon, G. Vogt, M. Marchiani, J.L. Fischer and M. Aragno: Isolation of *Thermus* strains from hot composts (60 to 80°C). *Appl. Environ. Microbiol*., **62**, 1723-1727 (1996).

Belduz, A.O., S. Dulger and Z. Demirbag: *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. *Int. J. Syst. Evol. Microbiol*., **53**, 1315–1320 (2003).

Bozoglu, C., A. Adiguzel, H. Nadaroglu, D. Yanmis and M. Gulluce: Purification and characterization of laccase from newly isolated thermophilic *Brevibacillus* sp (Z1) and its applications in removal of textile dyes. *Res. J. Biotechnol*., **8**, 56-66 (2013).

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*,**72**, 248-254 (1976).

Dulger, S., Z. Demirbag and A.O. Belduz: *Anoxybacillus ayderensis* sp. nov. and *Anoxybacillus kestanbolensis* sp. nov. *Int. J. Syst. Evol. Microbiol*., **54**, 1499–1503 (2004).

Hakamada, Y., K. Endo, S. Takizawa, T. Kobayashi, T. Shirai, T. Yamane and S. Ito: Enzymatic properties, crystallization and deduced aminoacid sequence of an alkaline endoglucanase from *Bacillus circulans*. *Biochim. Biophys. Acta*, **1570**, 174-180 (2002).

Ibrahim, A.S.S. and A.I. El-diwany: Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. *Aus. J. Basic Appl. Sci.*, **1**, 473-478. (2007).

Jahangeer, S., N. Khan, S. Jahangeer, M. Sohail, S. Shahzad, A. Ahmad and S.A. Khan: Screening and characterization of fungal cellulases isolated from the native environmental source. *Pak. J. Bot.*, **37**, 739 (2005).

Kuhad, R.C., R. Gupta and A. Singh: Microbial cellulases and their industrial applications. *Enzyme Research*, **10**, 1-10 (2011).

Kumar, D., M. Ashfaque, M. Muthukumar, M. Singh and N. Garg: Production and characterization of carboxymethylcellulase from *Paenibacillus polymyxa* using mango peel as substrate. *J. Environ. Biol.*, **33**, 81-84 (2012)

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Natur*e, **227**, 680-685 (1970).

Liang, Y., Z. Feng, J. Yesuf and J. Blackburn: Optimization of growth medium and enzyme assay conditions for crude cellulases produced by a novel thermophilic and cellulolytic bacterium, *Anoxybacillus* sp. 527. *Appl. Biochem. Biotech*.,**160**, 1841-1852 (2010).

Martins, D.A.B., H.F.A. do Prado, R.S.R. Leite, H. Ferreira, M.M.S. Moretti, R. da Silva and E. Gomes: Agroindustrial Wastes as Substrates for Microbial Enzymes Production and Source of Sugar for Bioethanol Production. In:Integrated Waste Management- II (Ed: S. Kumar). InTech., p:322 (2011)

Mawadza, C., R. Hatti-kaul, R. Zvauya and B. Mattiason: Purification and characterization of cellulases produced by *Bacillus* strain. *J. Biotechnol.,* **83**, 177-187 (2000).

Peng, J., W. Wang, Y. Jiang, M. Liu, H. Zhang and W. Shao: Enhanced soluble expression of a thermostable cellulase from *Clostridium thermocellum* in *Escherichia coli*. *Curr. Microbiol.*, **63**, 523-530 (2011).

Rastogi, G., A. Bhalla, A. Adhikari, K.M. Bischoff, S.R. Hughes, L.P. Christopher and R.K.. Sani: Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. *Biores. Technol*., **101**, 8798-8806 (2010).

Stackebrandt, E. and B.M. Goebel: Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.*, **44**, 846-849 (1994).

Sung, M.H., H. Kim, J.W. Bae, S.K .Rhee, C.O. Jeon, K. Kim, J.J. Kim, S.P. Hong, S.G. Lee, J.H. Yoon, Y.H. Park and D.H. Baek: *Geobacillus toebii* sp. nov., a novel thermophilic bacterium isolated from hay compost. *Int. J. Syst. Evol. Microbiol.*, **52**, 2251–2255 (2002).

Wood, T.M. and K.M. Bhat: Methods in Enzymology Cellulose and Hemicellulose. Academic Press.,New York, (1998).

Xia, W., P. Liu and J. Liu: Advance in chitosan hydrolysis by non-specific cellulases. *Biores. Technol.*, **99**, 6751-6762 (2008).