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EVALUATION OF PROTEOLYTIC *Bacillus* spp. ISOLATED FROM SOIL AND CHARACTERISTION OF THEIR GROWTH AND ACTIVITY OF PROTEASES

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ABSTRACT: The aim of this study was to isolate, screen and optimize proteolytic bacteria from different soil samples. Soil samples collected from El-Sharkia Governorate and Wadi Abu Sobeira, Aswan Governorate were screened for proteolytic bacteria. The later soil was characterized by saline soil. Proteolytic bacteria from each soil was studied by cultivating in a protease production medium (PPM), low in carbon content. Substrate specificity of the isolated proteolytic bacteria was assayed by adding seven nitrogen sources and six carbon sources. Each one was added to PPM compared to the control (peptone and glucose), respectively. Four bacterial isolates were selected and they exhibited excellent ability to hydrolyze high-molecular weight proteins (skim milk, gelatin and casein). These isolates showed extracellular protease activity on skim milk agar plates (20 mm) after 24 hr., at 37°C. These isolates were identified as *Bacillus cereus* strain A1(4), *B. toyonensis* A3(14), *B. cereus* strain A4(26) and *B. cereus* strain A8(60) based on partial sequencing of 16S rDNA. Protease production by these strains [A1 (4), A3 (14), A4 (26) and A8(60)] compared to *B. subtilis* cultivate in PPM reached a maximum at 24 and 48 hr., with values ranged between 81.83 and 76.16 U/ml. Starch and mannitol were the best substrates for enzyme production, while glycerol could not influence production of protease. Among various organic nitrogen sources, beef extract, soybean and skim milk, which are, commercial, were found as the best substrate for bacterial growth and enzyme production, while $NaNO_3$, KNO_3 and $(NH4)_2SO_4$ could not affect production of protease or bacterial growth. This enzyme showed high capability for removing proteins and stain spots from cloths.

Key words: Proteolytic bacteria, *Bacillus*, soil, protease, enzyme, carbon and niteogen sources.

INTRODUCTION

Proteases are one of the most important industrial enzymes and are used in a variety of industrial applications, accounting for more than 65% of the total industrial enzyme market (Banik and Prakash, 2004). Microbial proteases are preferred to enzymes from plant and animal sources, since they possess almost all the characteristics desired for biotechnological applications and in numerous industries (Gulrajani *et al.*, 2000; Gupta *et al.*, 2002; Najafi *et al.*, 2005; Prakash *et al.*, 2005; Rao and Narasu, 2007), and they are important tools in medical and pharmaceutical processes (Bhaskar *et al.*, 2007). These proteases are the single class of enzymes widely used in detergents, pharmaceuticals, leather and the food and agriculture industries (Azura *et al.*, 2009). Proteases are important components of biopharmaceutical products such as contact lens cleaners and enzymatic debriders (Anwar and Saleemuddin, 2000; Sjodahl *et al.*, 2002). Proteases catalyze or hydrolyze protein and therefore play a vital role in various industrial applications (Li *et al.*, 2004; Schallmey *et al.*, 2004; Nijland and Kuipers, 2008). Conversion of wastes into useful biomass by microorganisms and their enzymes is a new trend, and new protease-producing microorganisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme (Rathakrishnan *et al.*,

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2012). In food processing industry the proteolytic activity of microorganisms is associated with a wide variety of processes such as fermented foods, manufacture of protein hydrolyzates, food industry like meat tenderizing, cheese flavour development, treatment of flour in the manufacture of baked goods, improvement of dough texture, flavour and colour in cookies and so forth (Adinarayana and Ellaiah, 2002; Adinarayana *et al.*, 2003; Casaburi *et al.*, 2008), oil extraction (Kashyap *et al.*, 2007), bakery (Lauer *et al.*, 2000), clarification of juices (Dawes *et al.*, 1994). Microbes represent an excellent source of proteases due to their broad biochemical diversity (Godfrey and West, 1996), their rapid growth, the limited space and the low cost that growing substrates required for their cultivation (Gupta *et al.*, 2002). Furthermore, many *Bacillus* spp. secrete large amounts of proteases than that required for their physiological activities. The cost of enzyme production is a major obstacle in its successful industrial application (Oskouie *et al.*, 2008), so it should be produced in high yields in a low-cost medium.

Extremophilic microorganisms are adapted to surviving in ecological niches such as high temperatures in volcanic springs, at low temperatures in polar regions, at high pressure in the deep sea, at very low and high pH values (pH 0 ± 3 or pH 10 ± 12), or at very high salt concentrations (5±30%). These microorganisms produce novel organic compounds and stable biocatalysts that function under these conditions comparable to those prevailing in many industrial processes (Sondes *et al.*, 2016; Mechri *et al.*, 2017). Their ability to propagate under the conditions where other organisms either cannot grow or grow little, microorganisms living in extreme environments, termed extremophiles, have always gained a significant attention from scientists. In particular, they are a source of several enzymes with unconventional biochemical and molecular characteristics and unique metabolic capabilities are the major points of attractions in biotechnological applications (Robertson *et al.*, 1996). Thus, as per earlier report, search for new robust biocatalysts producing microbes from extreme must be an endless task (Godfrey and West, 1996). The enzymes isolated from those microorganisms have been reported to be active not only at high temperatures but also in the presence of organic solvents and detergents (van den Burg, 2003). For these reasons the aims of the present study were to isolate and characterize extracellular producing bacteria from one of those environmental niches (high saline soil), which have not been studied in details, is Wadi Abu Sobeira located at Aswan in northern Egypt. This kind of soil is a good source for extremophiles microorganisms (van den Burg, 2003). These bacteria could be promising potential for biotechnological and industrial applications. Therefore, the second aim of this study was to focus on optimizing the production of extracellular protease by testing various nutritional factors.

MATERIALS AND METHODS

Soil Samples

Soil samples were collected from different locations in Wadi Abu Sobeira which is located about 30 km northern of Aswan city. Wadi Abu Sobeira soil samples (n=12) which is one of several wadies in the southern extremities of the Eastern Desert embedded in the Nubian sandstone formation of the region. It extends along an east west axis for about 60 km, and its mouth on the Nile. Also, soil samples were collected from El-Sharkia Governorate (n=3) from three different regions (Zagazig, Hyaheia and Balbis).

Isolation of Proteolytic Bacteria

Isolating of bacteria was estimated by the dilution plate technique (Page *et al.*, 1982). Subsequent decimal dilutions were prepared in sterile buffer peptone water (BPW) and analyzed by duplicate by plate count method. The following microbial analyses were carried out: The compositions and preparation of media (g/l) were as follows: beef extract 5, peptone 10, NaCl 5, distilled water 1000 ml, agar 15, pH 7.2. The bacterium-mixing plate method was used and the 10^{-3}~10^{-7} dilutions of soil sample were used as an inoculums. The colonies were counted after incubation at 30 ˚C for 3 days for counting total viable count. The skim milk agar (g/l) (skim milk 28, casein 5, yeast extract 2.5, glucose 1, distilled water 1000 ml agar 15, pH

7.2) was used for isolating and counting proteolytic bacteria. The selected colonies after purification, *via* sequential steps of plating on nutrient agar. Purified cultures were characterized for *Bacillus* spp. with standard description of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974; Holt *et al.*, 1993).

Screening of Proteolytic Bacteria and Preliminary Assay for their Proteolytic Activities

Screening procedures based on agar plate assays have been in use for long, aiming at detection of extracellular proteolytic activity in microorganisms (Safarík and Safaríková, 1994). A total of 111 isolates of *Bacillus* spp. were screened for protease production by using casein agar (0.5% casein in nutrient agar), gelatin agar (0.5% gelatin in nutrient agar) and skim milk agar (1% skim milk in nutrient agar) at 37°C for 24–72 hr., (Adinarayana *et al.*, 2003). Proteolytic activities of *Bacillus* spp. were detected on the basis of appearance of clear zones around the bacterial colonies that revealed the capability of bacteria for producing protease. The protease positive colonies obtained and the most potent four isolates were selected for quantitative test of protease. The isolates were subcultured onto nutrient agar slants, grown at 37°C for 24 hr., maintained at 4°C, and subcultured at four-week intervals.

Screening for Best Growth and Protease Producers

Among the 20 isolates obtained in this screening effort, only 4 isolates exhibited proteolytic activity-measured as clearance extent on nutrient agar + 1% (*W/V*) skim milk. The isolates with the highest protease activity- *i.e.* those leading to the largest clearance ratios ([clearance extent] = [digestion diameter]−[colony diameter]), of at least 10 mm by 1 day and at least 25 mm by 5 days, were selected for a more refined measurement of enzyme activity. Upon this isolation, the selected isolates were further cultured in nutrient broth (NB) for additional estimation of protease activity in liquid medium, using casein as substrate. The cell-free supernatants (from overnight culture characterized by an OD_{600} of 0.6) were used for colorimetric assessment of extracellular protease activity released during 24, 48 and 72h – as described elsewhere (Queiroga *et al.*, 2007). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein so as to produce an absorbance change equivalent to that of 1.0 µmol of tyrosine per min, at pH 7.5 and 37°C.

Identification of Proteolytic Bacteria

After screening of bacilli isolates, the highest proteolytic for enzyme activity. The isolates were identified as genus and species in Hospitals Clinical Pathology Department, Zagazig University by using matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-TOF-MS). Genetically analysis was also done for four isolates. Four isolates were identified in Sigma Scientific Services Company, Giza, Egypt using 16S rRNA according to the methods of Maniatis *et al.* (1989), using the GeneJet genomic DNA purification Kit (Thermo K0721). Molecular sequencing of the DNA fragment containing the 16S intragenic spacer corresponding to the conserved region of 16S rRNA. The amplification of the16S rRNA was performed through PCR technique, using Taq DNA polymerase, genomic DNA as a template, and 3′forward and 5′ reverse universal primers. The primers used have nucleotide sequence as: (3 f: 5′-AGAGTTTGATCCTGGC-3′, 5 r: 5′-TACCTTGTTACGACTT-3′). The results of the 16S rDNA nucleotide sequence of isolate has been deposited in GenBank and aligned with the 16S rRNA sequences available in nucleotide database in NCBI, (National Center for Biotechnology Information, Available at: (https: //www.ncbi.nlm.nih.gov/nuccore/NR_121761.1), using BLAST software, (Basic Local Alignment Search Tool) (Lyon *et al.*, 2000). Agarose gel electrophoresis was performed according to standard protocols using 1×TAE buffer (Sambrook and Russell, 2001), and the DNA fragments were isolated from gels using the GFXTM PCR DNA and Gel Band Purification Kit (from GE Healthcare, Buckinghamshire, UK). Purified DNA fragments were finally sequenced and computer-assisted sequence analysis was performed using Vector NTI Advance TM 10 Software (from Invitrogen, Carlsbad CA, USA), and comparison of the sequences obtained was carried out using the BLAST software (from NCBI).

Protease Production

Two cultures media (M1 and M2) were used in this work for protease production. The first medium (M1) contained (g/l of distilled water): glucose 1.0, peptone 10.0, yeast extract 0.2, $MgSO_4$ 0.1, K_2HPO_4 0.5 and $CaCl_2$ 0.1 (Qadar *et al.,* 2009) and the second medium (M2) contained (g/l of distilled water): glucose 10.0, peptone 5.0 yeast extract 3.0, $MgCl_2$ 0.2, $CaCl_2$ 0.4 (pH 7.0) (Sangeetha *et al.,* 2007). Glucose was sterilized separately and aseptically added to the flasks containing the liquid medium, after cooling. The cultures were cultivated in Luria-Bertani (LB: 10 g/ liter tryptone, 5 g/l yeast extract, 5 g/l NaCl) broth medium (Miller, 1972) for 18 hr. Then, overnight cultures with optical density 600 (OD_{600}=0.3) were inoculated at 1% in the above enzyme production media (150 ml in 500 ml Erlenmeyer flasks) and incubated at 37°C for 24, 48 and 72 hr., in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for determination of proteolytic activity. Bacterial biomass was determined by measuring optical density at OD_{600} nm. Capabilities of protease production of *Bacillus* strains (5 individuals, four local strains and external one) were examined. These strains were selected for further studies to evaluate the activity of the best strain for enhancing the protease production.

Protease Assay

Total protease activity was measured using a casein substrate by a modification of the Anson Method (Keay and Wildi, 1970). One ml of the culture supernatant was mixed with 1 ml 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10 min at 37°C. The reaction was stopped by adding 2 ml 0.4 M Trichloroacetic acid (TCA). After 30 min stand at room temperature, the precipitate was removed by centrifugation (6000 rpm, 10 min) and the colour developed was measured at 660 nm. A standard curve was generated using solutions of 0–60 µg/ml tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/ml tyrosine under the experimental conditions used. The estimations were based on a tyrosine calibration curve.

Effect of Nutrients Conditions on Bacterial Growth and Enzyme Activity

The effects of some nutrients such as carbon and nitrogen sources on enzyme production and bacterial growth by different *Bacillus* strains were investigated. Glucose (0.1% *W/V*) was replaced in the production medium (M1) with starch, sucrose, mannitol, glycerol, fructose and maltose (Qadar *et al.,* 2009). Different organic and inorganic nitrogen sources including triptone, beef extracts, commercial soybean powder, commercial skim milk powder, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3were tested in the production medium (M1) (Fulzele *et al.,* 2011). These nitrogen sources were used to replace the peptone and yeast extract which were the original nitrogen source in the growth medium (Qadar *et al.,* 2009). In briefly, the cultures (5 individuals) were cultivated in LB broth medium for 18 hr. Then, overnight cultures with optical density 600 (OD_{600}=0.3) were inoculated at 1% in the above enzyme production medium (150 ml in 500 ml Erlenmeyer flasks) and incubated at 37 °C for 24, 48 and 72 hr., in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for determination of proteolytic activity. Bacterial biomass was determined by measuring optical density at OD_{600} nm. Capabilities of protease production of *Bacillus* strains (5 individuals) were examined.

Application of Alkaline Protease in Removing the Blood Stains

The cell-free supernatants (from overnight culture characterized by an OD_{600} of 0.8 on protease producing medium) were used for removing the blood stains. The application of alkaline protease in removing the blood stains was carried out according to the method of Najafi *et al.* (2005) with slight modification. A clean piece of pure white cotton cloth was soaked in animal blood for 15 min and then, allowed to dry at 80°C for 5 min in hot air oven. The dried cloth was cut into equal sizes (4 × 4 cm) and incubated with cell-free supernatants at 50 °C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2 min without scrubbing and then, dried in open air.

The same procedure was done with the control without an enzyme exposure. The experiment was carried out three times and the ability of the tested enzyme for removing the blood stain was recorded and photographed.

Statistical Analysis

The one-way Analysis of Variance (ANOVA) test was used to assess the statistical significance of differences in enzyme production and bacterial growth for each strain between those on the control compared to those in different carbon or nitrogen sources. This was achieved using the Statistical Package for Social Sciences (SPSS v.22) with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The soil samples from Wadi Abu Sobeira were black to brownish in color, clay to sandy and sticky. The data indicated a considerable variability in physical and chemical properties of the investigated soils, being site dependent to a great extent. Quoting illustrative figures is pertinent, the soil clay, silt and sand content ranged from (0.07% to 70.93), (0.04% to 38.82) and (3.13% to 93.55), respectively. The salinity in terms of electrical conductivity and Na^{+2} ranges from 0.72 to 59.53 dS m^{-1} and 3.46 to 360.3 soluble ions mmol/l, respectively. The pH level and total $CaCO_3$ in the soil samples ranged between 6.96 to 8.01 and 0.09 to 10.31%, respectively (Table 1). The groups of bacteria that can grow at very high salt concentrations (5 ± 30%) or under alkaline conditions in the presence of NaCl are referred to as halotolerant alkaliphiles and haloalkaliphiles. The dual extremity of these extremophiles, high salt and alkaline concentration make them attractive strains for exploration of novel alkaline proteases for biotechnological potential (Li *et al.,* 2004; Schallmey *et al.,* 2004; Nijland and Kuipers, 2008). One of those environmental niches, which have not been studied in details, is Wadi Abu Sobeira located in northern Aswan, Egypt. Considering the above mentioned facts, the present study focused on isolation of new alkaline protease producing bacteria from Wadi Abu Sobeira, and optimization of the enzyme production by investigation of the effect of various environmental parameters.

Isolation and Identification of Protease Producing Bacteria

Members of the genus *Bacillus* spp. were widely used in industry in the large-scale production of enzymes, such as proteases. Of particular industrial importance are proteases with activity at alkaline pH and high temperature (De Boer *et al.,* 1994). In total, 111 bacteria isolates were isolated from Wadi Abu Sobeira soil (89 isolates) and El-Sharkia soil (22 isolates), 73 isolates showed protease activity, non-proteolytic (n=38), and high proteolytic (n=20) on skim milk agar (Fig. 1). Among the proteolytic bacteria 18 isolates from Aswan and two isolates from El-Sharkia showed the largest zone of hydrolysis (≥ 17 mm) (Fig. 2). These isolates [A1 (2 and 4), A2(13), A3(15, 17, 18 and 19), A4(24, 26, 27 and 29), A5(33), A6(39), A7 (52 and 53), A8(60), A9(65), A10(74), Z13 (92) and B15(105)] exhibited the highest proteolytic activity with a clear zone after 24, 48 and 72 hr. Among the isolates, there are four isolates caused the largest zone of hydrolysis (19-20 mm) (Fig. 2). These isolates were identified based on morphological and biochemical characteristics as *Bacillus* spp.. Also, 4 isolates were identified and confirmed at Faculty of Medicine, Zagazig University, Egypt by using MALDI-TOF-MS, as *Bacillus cereus*. Based on these results, four isolates A1 (4), A2 (13), A4 (26) and A8 (60) were selected for identification by 16S rRNA and evaluated for protease production under different conditions in subsequent experiments compared to *Bacillus subtilis* which was obtained from Agricultural Microbiology Department, Faculty Agriculture, Zagazig University, Egypt and characterized by its proteolytic activity. Fig. 3 compares clear zones of proteolytic *B. cereus* (A1 (4), A4 (26), A8 (60)) and *B. toyonisis* A2(13) compared to *B.subtilis* on growing on different media (skim milk agar, casein agar and gelatin agar). All the strains showed positive results for enzyme activity.

These four isolates were selected for molecular identification and could be assigned to the *Bacillus cereus*, based on genetic similarity of their partial 16S rDNA gene sequence. The BLAST search of 16S rDNA gene sequence against sequences in nucleotide

Table 1. Some physical and chemical properties of Wadi Abu Sobeira soils, Aswan

Sample No.	Code of samples	Total $CaCO_3$ (%)	pH	EC (dS/m)	Water Soluble ions mmol/l							
					Ca^{++}	Mg^{++}	Na^{+}	K^{+}	$CO_3^{=}$	HCO_3^{-}	Cl^{-}	$SO_4^{=}$
1	**A1**	0.09	7.25	1.52	4.00	5.00	5.54	0.46	0.0	2.00	6.50	6.50
2	**A2**	6.78	7.34	5.34	11.00	7.75	34.60	0.75	0.0	1.00	41.50	11.60
3	**A3**	5.94	7.68	1.54	7.00	4.00	3.59	0.51	0.0	3.50	8.00	3.60
4	**A4**	1.82	7.95	0.72	2.00	1.00	3.81	0.49	0.0	2.50	3.00	1.81
5	**A5**	0.73	7.78	0.96	3.00	2.00	3.78	0.62	0.0	3.00	5.00	1.40
6	**A6**	2.91	7.74	1.34	4.00	4.30	4.20	0.50	0.0	3.00	6.50	3.50
7	**A7**	0.60	7.06	16.98	65.00	17.40	82.70	4.60	0.0	2.50	152.60	14.60
8	**A8**	10.31	6.96	59.53	196.00	26.0	360.3	12.70	0.0	4.00	568.8	22.20
9	**A9**	0.53	7.94	1.38	4.50	4.60	3.74	0.56	0.0	4.50	6.00	2.90
10	**A10**	0.35	7.98	0.92	2.80	2.00	3.83	0.37	1.00	3.00	4.90	0.10
11	**A11**	2.58	7.82	1.04	3.00	10.00	3.70	0.50	0.50	4.30	4.50	1.60
12	**A12**	2.28	8.01	0.68	1.90	0.90	3.46	0.24	0.0	3.50	2.00	1.00

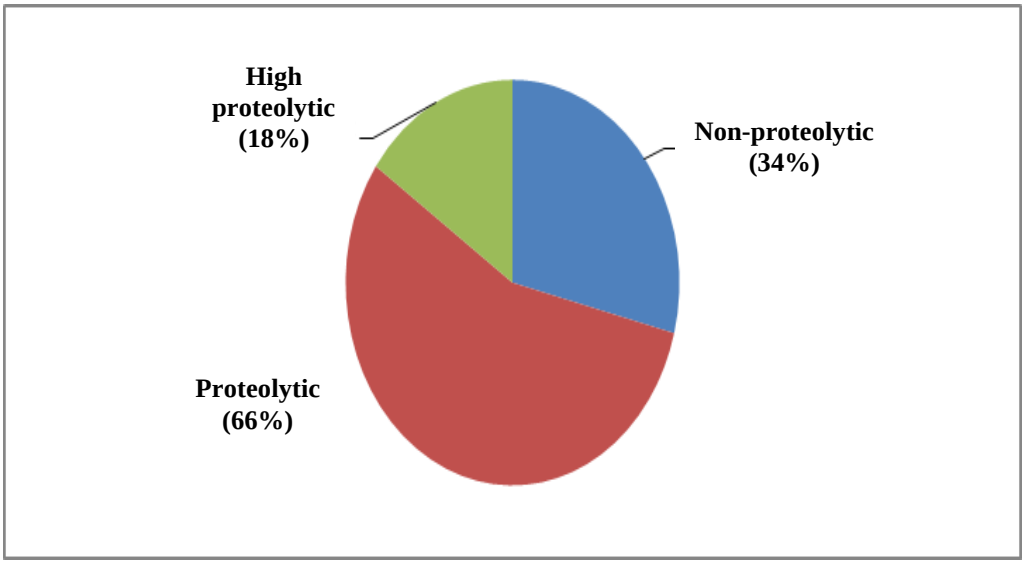

Fig. 1. Isolates (n=111) from the soil bacterial population, fraction of non-proteolytic (NP: n=38), proteolytic (P: n=73) from them high proteolytic (n=20)

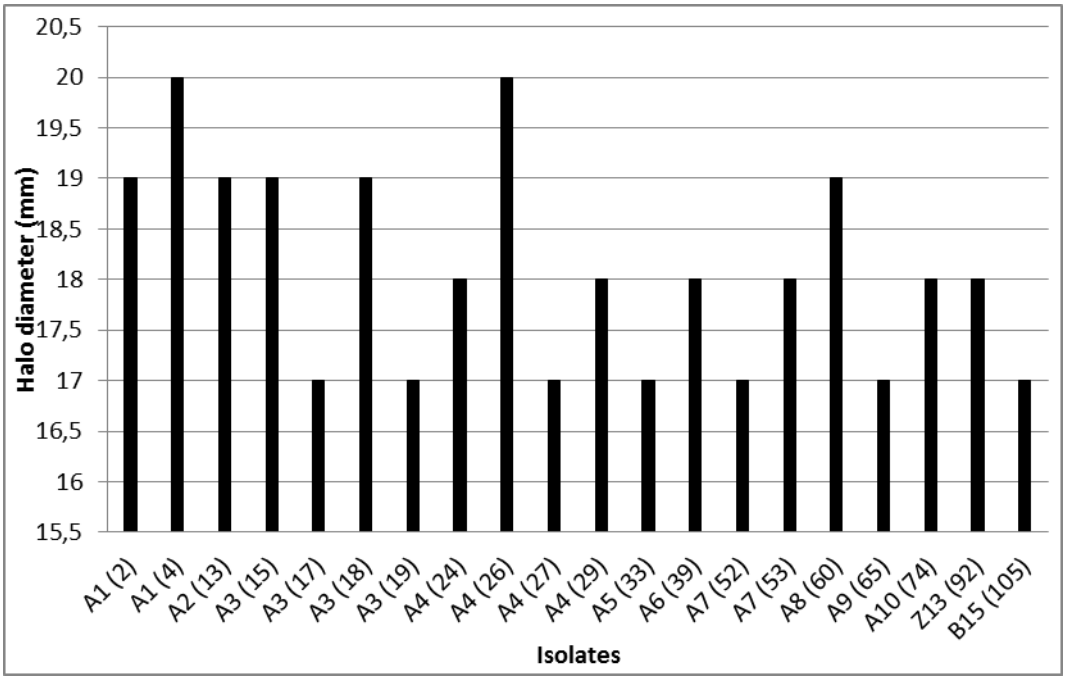

Fig. 2. Proteolytic activity (halo diameter (mm)) of the proteolytic bacteria isolated from soil-using skim milk agar, incubated for one day at 30°C

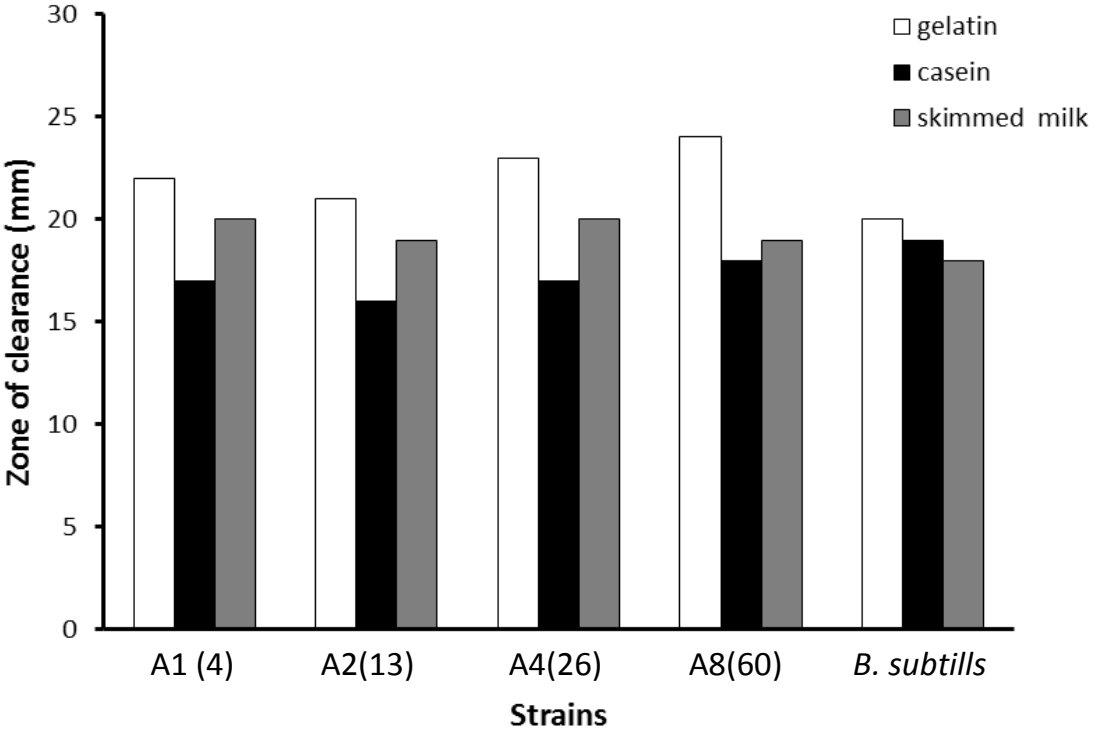

Fig. 3. Proteolytic activity of *Bacillus* spp. on gelatin, casein and skimmed milk

database has shown 98% homology with *Bacillus cereus* A4 (26) and A8(60) meanwhile A1(4) and A2 (13) isolates were identified as *Bacillus cereus* (89%) and *Bacillus toyonisis* (86%), respectively (Table 2). Based on the sequence similarity results of four strains are likely to be one of these strains (A2 13) is novel species as they *Bacillus toyonisis* differ with the nearest phylogenetic neighbour at the 16S rDNA gene sequence.

The polyphasic taxonomic study presented by Jiménez *et al.* (2013) provides sufficient discriminative parameters to classify *B. cereus* BCT-7112T as a new species for which the name *Bacillus toyonensis* sp. nov. is proposed, with BCT-7112T (=CECT 876T; =NCIMB 14858T) being designated as the type strain. The sequences associated with these strains were found in GenBank with the session number: *Bacillus cereus* A1(4) (NR_074540.1); *Bacillus toyonensis* A2 (13) (ACCESSION NR_ 121761); *Bacillus cereus* A4(26) (NR_115714.1) and *Bacillus cereus* A8(60) (NR_114582.1) as shown in Table 2.

Effects of Culture Conditions on the Growth and Enzyme Activity of Four Isolated *Bacillus* strains and *B. subtilis*

Effect of different media composition on protease production

The previous studies have shown that culture media composition influences the expression of extracellular proteases (Nicodeme *et al.,* 2005; Sangeetha *et al.*, 2007; Wang *et al.,* 2008; Qadar *et al.,* 2009). Proteases produced from microorganisms are constitutive or partially inducible in nature and, under most culture conditions, *Bacillus* species produce extracellular proteases during post-exponential and stationary phases. Extracellular protease production in microorganisms is also strongly influenced by media components, *e.g.* variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose (Beg and Gupta, 2003). Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other physical factors, such as aeration, and temperature, also affect the amount of protease produced (Hameed *et al.,* 1999; Puri *et al.,* 2002). For this reason this work proposed the use of two media: the first medium M1 (Qadar *et al.,* 2009) and the second medium (M2) (Sangeetha *et al.,* 2007). The media included yeast extract as unique source of both, vitamins and nucleic acids. The first medium was limited in energy source from sugars. This poor growing condition would be expected to enhance the microbial extracellular proteases production. Protease producing bacteria in these media were observed after 24, 48 and 72 hr., at 30°C. The results showed that the maximal protease activity was obtained when the M1 was used (Table 3). The bacterial growth and protease production by these strains (A1(4), A2 (13), A4(26) and A8(60)) compared to *B. subtilis* cultivate in protease production medium (PPM) reached a maximum at 24 and 48 hr., with levels of 1.90 and 1.86 OD_{600} and 81.83 and 76.13 U/ml, respectively. This M1 was chosen as the medium on which to grow the bacteria for bacterial growth and examination of the proteolytic specificity of bacteria. This medium showed high significantly ($P < 0.05$) in enzyme activity in case of all strains used at 24 or 48 hr. The series of experiments was carried out to study the effect of different factors on the protease production medium (M1). Tryptone, beef extract, soybean powder, skim milk, $NaNo_3$, $(NH_4)_2SO_4$ and KNo_3 were used as nitrogen sources instead of peptone in the media of growing *Bacillus* spp.

Effect of nitrogen sources on protease production

Results in Table 4 present the effect of the aforementioned nitrogen sources on the growth as well as proteases production by five *Bacillus* spp. The results indicated that the maximum enzyme production and bacterial growth by all *Bacillus* spp. under investigation were enhanced significantly ($P < 0.05$) by addition of nitrogen enriched supplements instead of tryptone, beef extract, soybean and skim milk compared to the control whereas the addition of nitrogen enriched supplements like $NaNO_3$ and $(NH_4)_2SO_4$ resulted in the lower enzyme production significantly ($P > 0.05$) than the control. Among these supplements, the maximum enzyme activities obtained by A1 (4) and A2 (13) isolates were (79.4 and 71.7 U/ml) and (32.6 and 72.3 U/ml) when beef extract and KNO_3 at 24 hrs were used, respectively. Also, the maximum enzyme activities obtained by A1 (4) and A2 (13) isolates were (75.5 and 72.0 U/ml)

Table 2. Identification of the most active four isolates showing proteolytic activity by 16S rDNA

No.	Code of isolates	Name of isolates by MALDI-TOF-MS	Name of isolates by 16S rDNA	Homology	Accession number of strains
1	**A1 (4*)**	*Bacillus cereus*	*Bacillus cereus*	89%	NR_074540.1
2	**A2 (13)**	*Bacillus cereus*	*Bacillus toyonensis*	86%	NR_121761
3	**A4 (26)**	*Bacillus cereus*	*Bacillus cereus*	98%	NR_115714.1
4	**A8 (60)**	*Bacillus cereus*	*Bacillus cereus*	98%	NR_114582.1

*The number between practices is the number of the isolate.

Table 3. Effect of incubation time on protease production by five *Bacillus* strains during their growth in medium (M1) of Qadar *et al.* (2009) and medium (M2) of Sangeetha *et al.* (2007)

Media used	M1		M2	
Time (hr.)	**OD_{600}**	**U/ml**	**OD_{600}**	**U/ml**
		***B. cereus* A1(4)**		
24	1.90^{bc}	81.83^{j}	1.78^{c}	72.25^{h}
48	1.84^{bc}	76.13^{i}	1.76^{c}	72.21^{h}
72	1.82^{bc}	69.82^{bc}	1.71^{abc}	61.11^{a}
		***B. toyonensis* A2(13)**		
24	1.54^{ab}	72.28^{f}	1.43^{abc}	63.21^{c}
48	1.58^{ab}	72.91^{g}	1.41^{abc}	63.23^{c}
72	1.43^{a}	71.68^{e}	1.45^{abc}	64.26^{d}
		***B. cereus* A4(26)**		
24	1.57^{ab}	69.58^{b}	1.35^{ab}	61.23^{a}
48	1.65^{abc}	70.15^{cd}	1.54^{abc}	65.34^{e}
72	1.63^{abc}	70.45^{d}	1.34^{a}	62.34^{b}
		***B. cereus* A8(60)**		
24	1.76^{abc}	69.55^{b}	1.63^{abc}	62.36^{b}
48	1.67^{abc}	75.35^{h}	1.43^{abc}	70.12^{g}
72	1.65^{abc}	76.25^{i}	1.36^{abc}	64.23^{d}
		***B. subtilis* (as reference bacteria)**		
24	1.98^{c}	66.34^{a}	1.57^{abc}	61.23^{a}
48	1.86^{bc}	76.16^{i}	1.64^{abc}	65.46^{e}
72	1.87^{bc}	75.35^{h}	1.74^{bc}	66.45^{f}

Means in the same row, with different superscript, differ significantly ($P< 0.05$).

Table 4. Effect of nitrogen source on *Bacillus* growth and protease activity. The culture density ($OD6_{00}$) and extracellular protease activity (U/ml) were determined during 24, 48 and 72 hr., incubation at 37°C and at initial pH 8

Nitrogen sources	Control		Tryptone		Beef extract		Soybean		Skim milk		$NaNO_3$		$(NH4)_2SO_4$		KNO_3	
Time (hr.)	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml
								***B. cereus* A1(4)**								
24	1.3^{a}	56.0^{h}	1.5^{bcde}	73.1^{i}	1.8^{ab}	79.4^{i}	2.1^{b}	66.2^{f}	1.7^{ef}	56.0^{c}	0.74^{ab}	39.5^{c}	0.9^{cdef}	38.9^{e}	0.5^{bcd}	32.6^{c}
48	1.2^{a}	55.1^{fg}	1.6^{de}	70.1^{g}	1.7^{ab}	75.5e	1.9^{ab}	65.3e	1.4abcd	42.8a	0.68 a	35.8b	0.9bcde	35.6d	0.6bcd	32.0 b
72	1.4^{a}	54.8^{f}	1.2^{ab}	55.7^{a}	1.6^{ab}	72.2a	2.0^{ab}	59.6a	1.5abcde	46.1b	0.86 ab	36.2b	0.8abcd	32.6c	0.2 a	30.5 a
								***B. toyonensis* A2(13)**								
24	1.3 a	54.8 f	1.4abcde	57.6c	1.7 ab	71.7c	1.8 a	65.5e	1.6cdef	63.3e	1.20bcd	44.6e	0.9cde	30.7a	1.5 f	72.3 l
48	1.2 a	55.4 g	1.3 abc	56.2b	1.6 ab	72.0cd	1.9 ab	63.7c	1.7 def	68.0i	1.3 def	49.1i	1.2ef	51.6j	1.5f	72.9m
72	1.4 a	54.2 e	1.5bcde	58.5d	1.6ab	71.7c	2.0 ab	60.7b	1.4abcd	60.0d	0.7 a	50.0j	0.7 abc	43.4f	1.4f	71.7 k
								***B. cereus* A4(26)**								
24	1.3 a	57.8i	1.6 bcde	75.8k	1.5ab	69.0 b	1.9 ab	88.5 j	1.3abc	67.5h	1.2cde	39.7c	0.5 a	30.7a	0.6bcd	47.3 i
48	1.2 a	52.4c	1.8de	82.1n	1.5 a	66.3 a	1.9 ab	88.9k	1.4abcd	72.5k	1.3cdef	42.9d	0.8abcd	31.5b	0.8 de	53.5 j
72	1.2 a	50.6a	1.4 abcd	70.8h	1.6 ab	69.0b	2.2 ab	64.3d	1.2a	63.7f	1.13 bc	45.3f	0.57 ab	46.4h	0.2ab	35.0de
								***B. cereus* A8(60)**								
24	1.3a	54.8f	1.6bcde	74.7j	1.8 ab	78.9 h	1.8ab	83.9 g	1.5 abcd	73.6l	1.5cdef	46.3g	1.4 fg	49.0i	0.4abc	41.2 h
48	1.2 a	51.2b	1.8 e	83.1o	1.8 ab	77.7 f	2.0ab	84.3 h	1.2ab	66.3g	1.5def	45.1f	1.1 def	46.2h	0.4abc	40.1g
72	1.2 a	52.4c	1.7 de	80.7m	1.7ab	78.4 g	1.9 ab	87.9 i	1.5bcdef	75.3m	1.6 ef	31.2a	1.0 cde	43.9g	0.2a	35.3 e
								B. subtilis								
24	1.3a	57.5i	1.1 a	62.7f	1.9b	82.2 k	2.1 ab	88.5 j	1.6bcdef	77.0n	1.3cdef	52.2k	0.9bcde	30.9a	0.3 ab	37.8 f
48	1.2 a	52.7cd	1.7de	79.4l	1.9 ab	81.3j	2.0 ab	88.9 k	1.4 abcd	71.0j	1.6ef	47.4h	1.4fg	31.8b	0.7cde	34.9 d
72	1.2 a	53.0d	1.4abcd	60.5e	1.9 ab	89.7l	2.0 ab	89.2 k	1.9f	85.9o	1.6f	46.1g	1.7g	30.8	1.0e	34.8 d

Means in the same row with different superscript, differ significantly (P< 0.05).

and (32.0 and 72.9 U/ml) when beef extract and KNO_3 at 48 hrs were used, respectively. However, the maximum enzyme activity was significantly ($P < 0.05$) by *B.cereus* A4 (26), *B.cereus* A8 (60) and *B. subtilis* namely (88.9, 84.3 and 88.9 U/ml) as influenced by soybean application after 48 hr., compared to the control, respectively (Table 4).

The production of protease was probably enhanced due to the high protein and amino acid components in the beef extract, tryptone and soybean. Also, growth curve determination of the degraded bacteria *Bacillus* strains, (*B. cereus*) A1 (4), (*B. toyninisis)* A2 (13), (*B. cereus*) A4 (26), (*B. cereus*) A8 (60) and (*B. subtilis)* and the protease production were maximum at 24, 48 and 72 hr., of incubation, respectively. It can be observed that there was a slow and steady growth of *B. cereus* A8 (60) and *B. subtilis* strains in the nutrient medium indicating the lag phase *i.e.* time required by the organism to adapt itself to the nutrient medium. There was an increase in protease production as it seen with the growth of *B. cereus* A1(4) and *B. toyonenisis* A2(13) isolates growth after 24 hr., and the protease production maximized at 24 hr., indicating maximum protease production in the late exponential phase. Beyond 24 hr., both the protease activity as well as the bacterial growth remained slowly decreased and became constant. Thus, it could be concluded that during

fermentation, production of protease by these isolates started in the early lag phase and reached a peak in the late exponential phase. In addition the obtained results revealed that soybean and skim milk powder, which were commercial products, led to the highest protease production and bacterial growth as well. So the production cost of the enzyme could be lessened by using soybean or skim milk powder in the growth medium of the organism. This finding was in similar to the finding of Shafee *et al.* (2005) who found that beef extract was the best substrate for protease production. In an another report which was similar to these results was obtained by Uyar *et al.* (2011) who found that skim milk had a significant effect on the production of the extracellular protease. Utilization of soybean also showed a better growth with all strains tested than with the other nitrogen sources used. These results suggested that the optimum conditions for protease production were necessarily the same as the best conditions for growth. This observation was in agreement with the previous study done by Camila *et al.* (2007). Another investigation presented by Asokan and Jayanthi (2010) revealed different results; since the optimum incubation time for enzyme production found was 96 hr. In other investigation a maximum activity of protease was attained after 48 hr., of fermentation, after which the activity started to decline (Khan *et al.,* 2011).

Effect of carbon source on protease production

Table 5 show the results of the production media of proteases with various carbon sources. The results indicated that the maximum enzyme production and bacterial growth were enhanced by the addition of carbon enriched supplements instead of starch, fructose, sucrose, mannitol, maltose, glucose and glycerol. Among these supplements, the maximum enzyme activities obtained by [A1(4), A2(13), A4(26) and A8(60) strains and *B. subtilis*] were ranged from 78.0 to 80.7 U/ml, 80.8 to 87.0 U/ml, 73.3 to 84.9 U/ml, 72.2 to 84.2 U/ml and 52.2 to 82.7 U/ml when each carbon source such as starch, sucrose, mannitol, fructose and maltose was used, respectively (Table 5). Similar findings were reported (Sen and Saytyanarayana, 1993; Pastor *et al.,* 2001; Santhi, 2014), where starch gave a maximum protease production. The other reports showed that the maximum protease production was obtained by *Bacillus* sp. strain CR-179 in liquid medium containing starch (1%) and corn steep liquor (0.4%) as a carbon source (Sepahy and Jabalameli, 2011). The formation of protease by *Bacillus*-A4 (26) isolate started from early stationary phase and reached a maximum in 24 hr., when starch or mannitol was used, with the levels of 84.9 and 86.8 U/ml, respectively. The reductions were observed after 72 hr., being 81.1 and 81.7 U/ml, respectively. Also, the formation of protease by *Bacillus*-A2 (13) strain started from early stationary phase and reached a maximum in 24 hr., when mannitol or maltose was used, with a value of 86.8 or 85.2 U/ml then these values began to slow down at 72 hr., to reach 85.4 and 77.6 U/ml, respectively. Regarding the effect of carbon source on bacterial growth, the same trend was also observed with the aforementioned strains. *Bacillus* spp. are spore-forming bacteria; thus during sporulation and also germination, it increases protease activity (Cihangir and Ve Aksoz, 1998). A4 (26) isolate and *B. subtilis* showed more production in protease during the late exponential phase, with the levels of 73.9 and 79.9 U/ml at 24 hr., then these values were raised at 72 hr., to reach 76.4 and 82.3 U/ml when maltose was used, respectively (Table 5). Scientists acclaimed that during sporulation and germination, hydrolyzed proteins were used to compose proteins for endospores or vegetative cells (Prestidge *et al.,* 1971; James and Mandelstam, 1985). This process requires an increase of protease production during the late exponential phase (Ward, 1985). Using of cost-effective growth medium for the production of alkaline proteases from an alkalophilic *Bacillus* spp. is especially important (Joo *et al.,* 2002). Therefore, there are urgent need to find new strains of bacteria with the ability to produce proteolytic enzymes with novel properties and the development of low-cost media. *Bacillus* strains (A1 (4), A2 (13), A4(26) and A8 (60) were capable of using a wide range of carbon sources, but production of protease varied according to each carbon source. From these results, it could be concluded that starch or mannitol was the best substrate for enzyme production, followed by sucrose or maltose or fructose while glucose or glycerol was less induces for enzyme production. Moderate to a good value of protease activity was produced in the presence of maltose or

Table 5. Effect of carbon source on *Bacillus* growth and protease activity. The culture density ($OD6_{00}$) and extracellular protease activity (U/ml) were determined during 24, 48 and 72 hr., incubation at 37°C and at initial pH 8

Carbon source	Control		Starch		Sucrose		Mannitol		Glycerol		Fructose		Maltose	
Time (hr.)	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml
***B. cereus* A1(4)**														
24	1.7a	75.3g	1.7cd	80.7 i	1.7d	78.8 m	1.8ab	78.6c	1.7e	29.4a	1.6 a	79.8 ij	1.6ab	78.0f
48	1.7a	75.5 g	1.7cd	79.2 h	1.6cd	79.8 n	1.7ab	81.3e	1.5de	29.5a	1.6a	77.8 h	1.6ab	77.1 d
72	1.7a	75.6g	1.6 bcd	77.2 f	1.6bcd	76.5k	1.7ab	77.9b	1.2 abcd	30.8d	1.5a	73.7d	1.5ab	76.8 c
***B. toyonensis* A2(13)**														
24	1.6a	72.6 e	1.8cd	80.8 i	1.3abcd	57.6f	2.0 b	86.8 j	0.8 a	30.3 c	1.7 a	82.1k	1.7ab	85.2 j
48	1.56 a	69.9 ab	1.6 cd	77.8 g	1.7 d	55.3 e	1.9 b	87.0j	1.7 e	31.3 e	1.6 a	80.1j	1.5ab	85.3 j
72	1.56 a	70.2 bc	1.6 bcd	76.6 e	1.2 ab	53.4 d	1.8 ab	85.4 i	1.1 abc	29.9 b	1.6a	76.8g	1.3a	77.6 e
***B. cereus* A4(26)**														
24	1.6 a	72.5 e	1.8d	84.9l	1.6 bcd	76.8 kl	1.9 ab	84.9 h	1.3 bcd	29.3a	1.5 a	73.3 d	1.5 ab	73.9 a
48	1.6 a	69.7 a	1.8cd	83.0 k	1.5 abcd	75.0 j	1.8 ab	82.6 f	1.4cde	31.2e	1.4 a	72.5 c	1.6 ab	76.3 b
72	1.6 a	69.8a	1.7 cd	81.1 j	1.4abcd	76.9 l	1.7ab	81.7 e	1.2bcd	29.3a	1.4 a	71.1 b	1.6 ab	76.4b
***B. cereus* A8(60)**														
24	1.6a	73.8f	1.4 abc	70.9 b	1.4 abcd	72.2 i	1.8 ab	84.2 g	1.4cde	41.9 h	1.5 a	75.7e	1.7 ab	79.3 g
48	1.6a	70.5c	1.2ab	66.2 a	1.2abc	68.0 h	1.8 ab	82.7 f	1.4cde	40.4 f	1.6 a	76.1f	1.7 ab	80.0 h
72	1.6a	71.1d	1.1a	74.7 d	1.2 a	64.3 g	1.6 ab	79.5 d	1.4 bcde	40.1 f	1.4a	70.7 a	1.5 ab	76.4 b
B. subtilis														
24	1.6a	72.3 e	1.5bcd	74.3 c	1.4abcd	52.2 c	1.5 a	75.5 a	1.0 ab	41.1 g	1.6 a	77.0g	1.7 ab	79.9 h
48	1.6a	69.6a	1.5bcd	74.5 cd	1.5abcd	51.3 b	1.8ab	82.7 f	1.4cde	41.1 g	1.6 a	79.6 i	1.6 ab	79.3 g
72	1.6 a	69.6 a	1.6cd	78.0 g	1.4abcd	42.1a	1.8ab	82.7 f	1.5de	44.3 i	1.7 a	77.0 g	1.7 b	82.3 i

Means in the same row with different superscript, differ significantly ($P< 0.05$).

fructose. This result was in agreement with previous report which showed that starch or maltose caused a high level of enzyme expression in *Bacillus* species (Shafee *et al.*, 2005; Bhatiya and Jadeja, 2010). Utilization of maltose was also shown to result in better protease activity than consuming fructose with all strains tested except with A1(4) which showed better results in protease activity with fructose than with maltose. Utilization of mannitol and starch, also showed higher growth than consuming other carbon sources used with isolates A1(4), A2(13) and A4(26). It could be suggested that the optimum conditions for protease production were necessarily the same as the optimum conditions for the bacterial growth. This observation was in agreement with the previous study done by Camila *et al.* (2007), who showed that starch was the best carbon source for both growth and protease production.

Application of Alkaline Protease in Removing the Blood Stains

The highest proteolytic strains were applied for removing blood stains from cloths. All strains of *B. cereus* A1(4), *B. toyonensis* A2 (13), *B. cereus* A4(26), *B. cereus* A8(60) and *B.subtilis* displayed growth and zones of clearing on different media (skim milk agar, casein agar and gelatin agar). All strains showed

fast growth and positive results for enzyme activity on different sources of carbon and nitrogen. Thus, in this study cell-free supernatants (from overnight culture characterized by an OD_{600} of 0.8 on protease producing medium) were used for removing the blood stains. As is evident in Fig. 5, the crude protease enzyme removed the blood stain from the fabric significantly, and the stain was intact in the fabric treated with the buffer alone. This enzyme showed high capability for removing proteins and stain from cloths. Similar results has already been obtained from *Bacillus cereus* isolated from marine samples which removed the animal blood stains from the fabric (Abou-Elela *et al.*, 2011). Also, in case of removing blood stain from cloths, it was seen that the protease from *Pseudomonas aeruginosa* PD100 was enable to remove blood stain very easily without addition of any detergent (Najafi *et al.*, 2005). This protease showed high capability for removing proteins and stains from cloths and also it could be used as an alkaline protease in detergent powder or solution. Its ability to act in the presence of solvents and detergents can be exploited for this purpose in future study.

Conclusions

In this work, various bacterial isolates from high saline soil were studied for protease producing activity. Proteolytic activity was measured for the highest enzyme producing strains. Structural, staining and biochemical activity results had revealed that the obtained isolates were *Bacillus* spp., The novel *Bacillus* strains have been then identified by 16S rDNA phylogenetic analysis as *Bacillus cereus* A1(4), *B. cereus* A4(26), *B. cereus* A8(60) and *B.toyonenisis* A2 (13). The higher proteolytic activities were exhibited by isolates *B. cereus* A1(4), and *B. cereus* A4(26). Optimization of medium components and cultural conditions for enhancing a final production of extracellular protease by *B. cereus* (A1 (4), A8(60), A4 (26) and *B.toyonisis* A2 (13) compared to *B. subtilis* were investigated. The most emerging and significant findings were the ability of these *Bacillus* strains to utilize soybean flour or skim milk as a sole nitrogen source and starch or sucrose as a sole carbon source to produce a maximal level of protease after an incubation time for 24 or 48 hr. The crude enzyme showed high capability for removing blood stain from cloths indicating that it could be used in detergent powder or solution. Further research will be needed for improvement these strains, purification of crude protease, determination of encoded gene sequence of protease, and further scaling up using fermenter. The enzymes may serve as a model system and may pave the way for engineering novel ways for eco -friendly industrial applications.

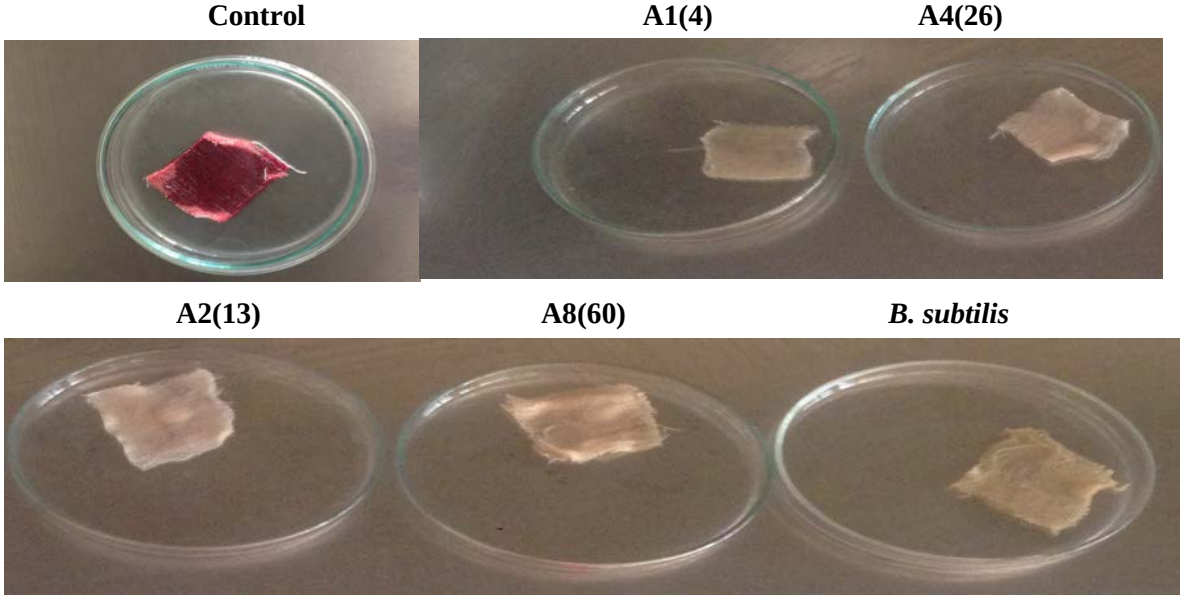

Fig. 5. Removal of blood stains from cotton cloth pieces by crude alkaline protease of isolates A1 (4), A4 (26), A2 (13), A8 (60), *B. subtilis* (incubated at 40°C for 60 min.)

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تقييم بكتريا الباسليس المحللة للبروتين والمعزولة من التربة وخصائص نموها و نشاط انزيمات البروتيازيس

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الهدف من هذه الدراسة هو عزل وانتخاب وتوصيف ومعظمة إنتاج إنزيم البروتيازيس من البكتيريا المحللة للبروتينات والمعزولة من عينات التربة، حيث تم استخدام عينات التربة لعزل البكتيريا المحللة للبروتينات من وأدى أبو سوبيرا الذي يقع على بعد حوالي ٣٠ كم شمال مدينة أسوان ومن محافظة الشرقية، تم دراسة البكتريا المحللة للبروتين من التربة عن طريق الزراعة في وسط إنتاج إنزيمات البروتيازيس المعدل ذات المحتوي المنخفض في الكربون، تم تقييم البكتيريا المعزولة والمحللة للبروتين عن طريق إضافة سبعة مصادر من النيتروجين وستة مصادر من الكربون، كل واحد على حدا بدلا من الجلوكوز والببتون علي التوالي وكانت هذه البكتيريا قادرة على تحلل البروتينات ذات الوزن الجزيئي المرتفع (الحليب الخالي من الدسم، والجيلاتين والكازين)، وأظهرت أربع عزلات فقط (4) B-A1) و (15) B-A3 و B-A4 (26)و (60) A8 نشاط عالي في تحلل البروتينات علي بيئة أجار الحليب الخالي من الدسم (٢٠ ملم)، تم تعريف هذه العزلات على أنها سلالات بكتيريا الباسيليس سيريوس (4) A1 تويوننسيس (15) A3 و سيريوس سلالة (26) A4 وسيريوس سلالة 16S - (60) A8، وقد بلغ إنتاج إنزيم البروتيازيس من هذه السلالات (A1 و A3 و A4 و A8) وكذلك سوبتيليس (Z1) التي تزرع في وسط إنتاج البروتيازيس الحد الأقصى عند ٢٤، ٤٨ ساعة بقيم وصلت في مدى يتراوح من ٨١.٨٣ و ٧٦.١٦ وحدة/مل، وكان النشا والسكروز والمانيتول والفركتوز والمالتوز من أفضل المصادر لإنتاج الإنزيم في حين أن بعض السكريات النقية مثل الجلسرين لا يمكن أن تؤثر على إنتاج البروتيازيس، من بين مختلف مصادر النيتروجين العضوية كان فول الصويا والحليب الخالي من الدسم التجاري أفضل المصادر النيتروجينية بينما كانت KNO_3 و $(NH_4)_2SO_4$ لا يمكنها أن تؤثر على إنتاج البروتيازيس، كما أن الإنزيم أعطى كفاءة عالية في إزالة بقع بروتينية وصبغة الدم من القماش.

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