academicJournals

Vol. 16(21), pp. 1210-1220, 24 May, 2017
DOI: 10.5897/AJB2017.15984
Article Number: 0B84F5D64479
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Isolation and characterization of a haloalkaliphilic protease producer bacterium from Wadi Natrun in Egypt

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Received 10 March, 2017; Accepted 28 April, 2017

A Gram-positive, rod-shaped, spore-forming haloalkaliphilic bacterium designated as NA7 was isolated from the surface of a *Helianthemum nummularium* root sample obtained from Wadi Natrun in Egypt**. Sequence analysis of the 16S rRNA gene revealed a *Bacillus haloalkaliphilius* strain as the closest match with 99% identity. In a shake flask culture containing 10% NaCl, adjusted to pH 10 and incubated at 37°C, the isolated strain produced thermostable extracellular alkaline protease with relatively stable maximum activity records (0.610-0.625 TU) within a relatively long stationary phase that exceeded 60 h. A 2-level fractional factorial design (Plackett-Burman) was then applied to screen for nutritional and cultivation factors regulating protease production by the isolate and to appraise their effects. Calculated statistical parameters revealed that NaCl and $MgSO_4$ are the most significant independent variables affecting alkaline protease production by NA7 and suggested a near-optimum culture condition. Verification of this predicted condition resulted in an alkaline protease specific activity record of 509 TU/mg protein with a 1.27 fold increase when compared to the basal medium culture.**

Key words: Alkaline protease, *Bacillus haloalkaliphilus*, Wadi Natrun, haloalkaliphiles.

INTRODUCTION

Bacteria are by far the most popular source of commercial alkaline proteases. Bacterial alkaline proteases are characterized by their broad substrate specificity, high activity at alkaline pH and an optimal temperature around 60°C (Rao et al., 1998). These properties of bacterial alkaline proteases render them suitable for use in silk degumming, tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Rao et al., 1998; Gupta et al., 2002; Prakasham et al., 2006; Mokashe et al., 2015).

Numerous Bacillus strains belonging to *B. licheniformis,*

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B. subtilis, B. amyloliquefaciens, and *B. majovensis* isolated from many different exotic environments have been explored and exploited for alkaline protease production (Gupta et al., 2002). However, thermophilic and alkaliphilic bacilli produce alkaline proteases that can considerably withstand high temperatures, alkaline pH and chemical denaturing agents (Johnvesly and Naik, 2001; Sai-Ut et al., 2015). Although, enzymes such as alkaline proteases, alkaline amylases, and alkaline cellulases expressed by alkaliphilic microorganisms have been extensively investigated and set to be used on industrial scales (Takami et al., 2000; Mokashe et al., 2015), there are no reports describing the expression of alkaline protease by a *Bacillus haloalkaliphilus*.

Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore their genes are a potentially valuable source of information waiting to be explored and exploited by the biotechnologists (Khmelenina et al., 1997; Mokashe et al., 2015). Alkaliphiles consist of two main physiological groups of microorganisms; alkaliphiles and haloalkaliphiles (Khmelenina et al., 1997; Mokashe et al., 2015). Members of the former group require alkaline pH of 9 or more for their growth, while those of the latter require an alkaline pH (pH 9) in addition to high salinity.

In this study, NA7, a Gram-positive, rod-shaped, spore-forming bacterium characterized as a *B. haloalkaliphilius* genotype was isolated from an alkaline desert environment characterized by high concentrations of sodium carbonate in Wadi Natrun, Egypt. The growth of the isolate was investigated with a special respect to its efficiency to produce extracellular alkaline protease in response to variations in fermentation factors affecting enzyme production using 100 mL Erlenmeyer flask cultures.

MATERIALS AND METHODS

Media

All media were sterilized by autoclaving at 121°C for 20 min and the pH was adjusted before sterilization. The following culture media (g/L) were employed throughout the work:

DSM 371 medium specific for isolation of haloalkaliphilies (pH 9.0-9.5)

The DMS 371 medium contained the following: KH_2PO_4, 1.0; KCl, 1.0; NH_4Cl, 1.0; $CaSO_4$. $2H_2O$, 0.24; $MgSO_4.7H_2O$, 0.17; trace element solution SL-10, 1.0 mL; agar, if necessary, 20.0; NaCl, 200.0; Na_2-glutamate, 1.0; yeast extract, 5.0; casamino acids, 5.0; and Na_2CO_3, 5.0 (DSMZ GmbH). The medium pH was adjusted to 6.5 before autoclaving. Na_2CO_3 was sterilized separately from medium, thenceforth it was added after cooling. Agar was heated and dissolved before the addition of sodium chloride.

Trace element solution

The trace element solution contained the following: HCl (25%; 7.7 M), 10 mL; $FeCl_2.4H_2O$, 1.5 g/L; $ZnCl_2$, 0.07 g/L; $MnCl_2.4H_2O$, 0.1 g/L; H_3BO_3, 0.006 g/L; $CoCl_2.6H_2O$, 0.19 g/L; $CuCl_2.2H_2O$, 0.002 g/L; $NiCl_2.6H_2O$, 0.024 g/L; and $Na_2MoO_4.2H_2O$, 0.036 g/L. $FeCl_2$ was dissolved first in HCl, thence forward the other salts were dissolved in water. Finally, both solutions were mixed and the volume was adjusted up to 1000 mL.

Modified nutrient agar medium (pH 9 ± 0.2)

This medium contained the following: Peptone, 5; beef extract, 3; NaCl, 200.0; $MgSO_4$, 2; and agar, 20.

Alkaline agar medium containing skimmed milk (pH 10 ± 0.2)

Following the method described by Ibrahim et al. (2007), this medium was prepared with the following salts and materials: Skimmed milk, 100; yeast extracts, 10; Na_2CO_3, 20; NaCl, 150; and agar, 20.

Medium for production of alkaline proteases (pH 10.5 ± 0.2)

The alkaline protease production medium contained the following: Glucose, 10; peptone, 5; yeast extracts, 5; K_2HPO_4, 1; $MgSO_4$, 0.2; Na_2CO_3, 15; and NaCl, 150. Medium constituents were autoclaved separately and mixed after cooling (Jasvir et al., 1999), with few modifications.

DNA extraction buffer

The Gram-positive bacterial lysis buffer used comprised 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; and 1.2% Triton X-100. Immediately before use, lysozyme was added (20 mg/mL).

Isolation, preservation and maintenance of bacterial isolates

Bacterial isolates were isolated from the roots of the most dominant plants in Wadi Natrun desert *Artemisia monosperma* and *Helianthemum nummularium*. Roots were directly inoculated into flasks containing liquid DSM 371 medium for culture enrichment. This was followed by plating aliquot of 1 mL of enriched liquid medium of samples on DSM 371 and modified nutrient agar media. Bacterial isolates were purified by streak plate technique on DSM 371 solid medium to obtain pure colonies. Isolates were preserved by freezing at -8°C in DSM 371 broth medium containing 10% glycerol (Berardesco et al., 1998). Bacterial isolates were maintained, for up to two weeks, by continuous sub-culturing on DSM 371 medium and keeping in a refrigerator.

Molecular characterization of the experimental isolate

DNA was extracted as described by Sambrook et al. (1989) and the 16S rRNA region was amplified using the bacterio-specific universal primers F27 (5′AGAGTTTGATCCTGGCTCAG3′) and R1492 (5′TACGGYTACCTTGTTACGACTT3′). The polymerase chain reaction (PCR) mixture consisted of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 µL dNTPs and 2.5 units of Taq polymerase in 50 µL of polymerase buffer. The PCR was carried out for 30 cycles in 94°C for 1 min, 55°C for 1 min and 72°C for 2

min. The amplified fragment was prepared for sequencing by purification using QIAquick PCR purification kit (Qiagen). Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger et al. (1977) was carried out using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan). The sequencing reaction was performed with four different fluorescent labels identifying the ddNTPs. These flurophores were excited with two argon lasers at 488 and 514 nm, respectively when the respective bands passed the lasers during the electrophoresis. The specific emissions were detected and the data were collected for analysis (Prober et al., 1987; Freeman et al., 1990). The thermal cycling mixture was as follows: 8 µL of Big Dye terminator mix, 6 µL of the sequencing primer (10 pmol) and 6 µL of the sample (PCR product), then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95ºC, then 49 cycles of 30 s at 95ºC, 10 s at 52ºC and 4 min at 60°C. The products were purified using special column according to the instruction of the manufacturer. The elute was taken and high dye formamide was added (1:1; volume ratio), run at 95°C for 5 min for denaturation and shock on ice. The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment. Molecular phylogeny was performed using Bio Edit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

Detection of extracellular enzymes produced by the bacterial isolates

Qualitative detection of enzymes was carried out by replica plating method described by Lederberg and Lederberg (1952).

Production of alkaline protease

Aliquot of 25 mL of alkaline protease production medium in 125 mL Erlenmeyer flasks was inoculated by a standard inoculum from seed culture and incubated over night at 180 rpm and 37°C. After incubation, 1 mL was aseptically transferred to 100 mL alkaline protease production medium in 250 mL Erlenmeyer flasks and incubated at 37°C. The incubation period was varied depending on the experiment conducted. Cells and insoluble materials were removed by centrifugation at 10,000 × g for 10 min at 25°C. Cell-free supernatant was used as a source of crude alkaline protease (Ibrahim et al., 2007).

Alkaline proteases assay

Aliquot of 150 µL cell-free supernatant was mixed with 400 µL (2.0 %) azo-casein in warm 0.0125 M borax NaOH buffer solution (pH 9.5 at 25°C) and incubated for 60 min at 37°C. The proteolysis was interrupted by adding 1.0 mL cold 10% trichloroacetic acid (TCA) solution. After centrifugation at 20,000 × g for 5 min, a portion of 100 µL NaOH (10 N) was added to 900 µL of supernatant in order to increase the color intensity of the dye. Hence, the absorbance was read at 340 nm (Iversen and Jørgensen, 1995). The protease activity of sample was calculated in terms of tryptic units (TU) using trypsin standard curve. Increasing OD (wave length) by one unit was considered as one activity unit (Chandrashekar and Gujar, 2004). All experiments were performed in duplicates and the averages of results were taken.

Protein determination

Protein concentration was determined according to Lowry et al. (1951).

Effect of temperature and initial pH on *B. haloalkaliphilus* growth and proteases expression

Aliquots of the enzyme production medium adjusted to pH 10 were inoculated and incubated for 4 days at temperatures of 25, 30, 35, 40 and 45°C under standard assay conditions. Bacterial growth was monitored and the specific protease activity (TU/mg protein) was determined at different time intervals. Effect of pH on alkaline proteases activity was determined by measuring enzyme activity at varying pH values ranging from 8 to 12 at 37°C using 50 mM Na_2HPO_4-NaOH buffer.

Elucidation of nutritional factors influencing alkaline proteases production

A screening experiment in which the most important cultivation factors concerning protease expression were elucidated by applying a two-level fractional factorial design, the Plackett-Burman experimental design was used (Plackett and Burman, 1946; Lotfy et al., 2006; Kumari et al., 2015). Firstly, evaluation of the relative importance of various constituents within a complex cultural medium was carried out. This was followed by model validation of the predicted near optimum medium compared to the basal medium and the Plackett-Burman reverse medium.

In this experiment, seven independent variables were screened in eleven combinations organized according to the Plackett-Burman design matrix described in the results section. For each variable, a high (+) and low (-) level was tested. The main effect of each variable was determined with the following equation:

$$E_{xi} = (\Sigma_{pi+} - \Sigma_{pi-}) / N$$

Where E_{xi} is the variable main effect, Σ_{pi+} and Σ_{pi-} are alkaline proteases production responses in trials where the independent variable (X_i) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. All trials were performed in duplicates and the averages of percentages of alkaline proteases production results were treated as the responses. Accordingly, a main effect figure with a positive sign indicates that the high concentration of this variable was nearer to optimum and a negative sign indicates that the low concentration of this variable was nearer to optimum. The factor that had no effect would give a value of zero.

Significant variables were identified by statistical analysis of the Plackett-Burman experimental results. The analysis was performed using the *t*-test function supported by Microsoft Office Excel to determine the statistical significance of the measured response.

Antibiotics sensitivity test

The following antibiotics were used to determine the antibiotic resistance patterns of the selected isolate: Norfloxacin (10 µg), Levofloxacin (5 µg), Ciprofloxacin (5 µg), Vancomycin (30 µg), Augmentin (amoxycillin 20 µg and 10 µg Clavulanic acid), Amikacin (30 µg), Netilmicin (30 µg), Unasyn (10 µg) and Imipenem (10 µg).

RESULTS

Isolation and characterization of a promising haloalkaliphilic protease producer

Thirteen halophilic bacterial phenotypes nominated NA1-

Table 1. Sources of isolation and morphological characteristics of different bacterial isolates.

Isolate	Isolation source	Colony colour	Cell shape	Gram stain	Sporulation
NA1	*Artemisia monosperma* plant roots	Beige	Rods	+ve	Non
NA2	*A. monosperma* plant roots	Deep yellow	Rods	+ve	Non
NA3	*A. monosperma* plant roots	Beige	Rods	+ve	Non
NA4	*Helianthemum nummularium* plant roots	Yellowish beige	Rods	+ve	Non
NA5	*H. nummularium* plant roots	Beige	Rods	+ve	Non
NA6	*H. nummularium* plant roots	Beige	Rods	+ve	Non
NA7	*H. nummularium* plant roots	Beige	Rods	+ve	Spore former
NA8	*H. nummularium* plant roots	Beige	Rods	+ve	Spore former
NA9	*H. nummularium* plant roots	Pale orange	cocci	-ve	Non
NA10	*H. nummularium* plant roots	Beige	Rods	+ve	Non
NA11	*A. monosperma* plant roots	Beige	Rods	+ve	Non
NA12	*A. monosperma* plant roots	Beige	Rods	+ve	Non
NA13	*A. monosperma* plant roots	Deep yellow	Short Rods	+ve	Non

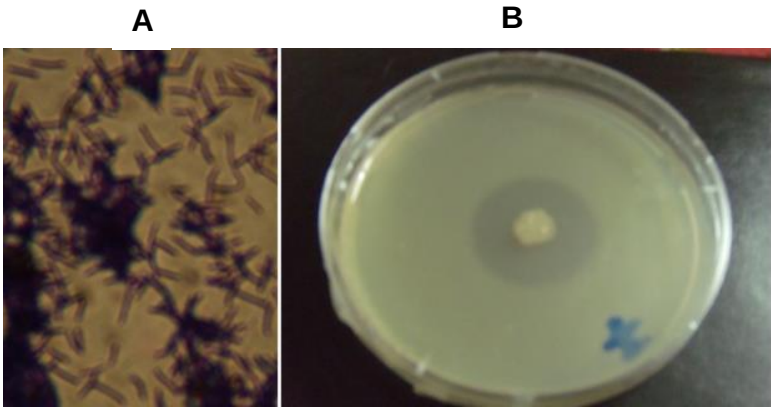

Figure 1. Cells of isolate NA7 under light microscope magnification × 100 (A) and casein hydrolysis by alkaline protease produced by NA7 isolate (B).

NA13 were successfully isolated on DSM 371 and modified nutrient agar from the rhizospheres of the most dominant desert plants in Wadi Natrun; *A. monosperma* and *H. nummularium*. Among the phenotypes isolated from *H. nummularium* plant roots, only one was found to be spherical Gram negative (NA9) and six were Gram positive rod-shaped including two spore formers (NA7 and NA8). On the other hand, all the isolates obtained from *A. monosperma* root samples were characterized as Gram positive non-spore former rods (Table 1).

Examination on 1% skimmed milk agar plates indicated that 11 of the bacterial isolates express extracellular alkaline proteases with maximum clear zone diameters recorded by the spore former isolates NA7 followed by NA9 and NA5 (Figure 1).

Isolate NA7, the most promising extracellular alkaline protease producer, was further characterized. In addition to being Gram positive, rod-shaped and spore former, it showed creamy mucoid colonies with circular margins on the skimmed milk agar plates. The isolate was also subjected to nine different antimicrobial agents; representing five diverse antibiotic groups. As shown in Table 2, NA7 exhibited high sensitivity to netilmycin, moderate sensitivity to norfloxacin and amikacin and low sensitivity to ciprofloxacin and vancomycin. On the other hand, it showed resistances to levofloxacin, unacyn, imipenem and augmentin.

Genotypic characterization on NA7 was carried out by partial sequencing of the purified 16S rDNA fragment and the edited sequence has been deposited in the GenBank under the accession number KT783669. The sequence was then aligned to closely related genotypes through BLAST. It showed 99% identity to the homologous fragments of *B. haloalkaliphilus*. The phylogenetic position of the isolate NA7 among representatives of related bacterial species is shown as a 16S rDNA-based dendogram in Figure 2.

Growth and extracellular alkaline protease activity on the liquid enzyme production medium were monitored at different time intervals during 4 days of shaking (180 rpm)

Table 2. Susceptibility of isolate NA7 to different antibiotics.

Mode of action	Antibiotics group	Antibiotics	Susceptibility
Inhibition of nucleic acid function	Fluoroquinolone	Norfloxacin	++
		Levofloxacin	R
		Ciprofloxacin	+
Inhibition of cell wall synthesis	Glycopeptides	Vancomycin	+
Inhibition of protein synthesis	Aminoglycosides	Netilmycin	+++
		Amikacin	++
Inhibition of cell wall synthesis	Cephalosporins	Imipenem	R
Inhibitor of cell wall synthesis	β-lactams	Unacyn	R
		Augmentin	R

+, Low sensitivity; ++, moderate sensitivity; +++, high sensititvity; R, resistance.

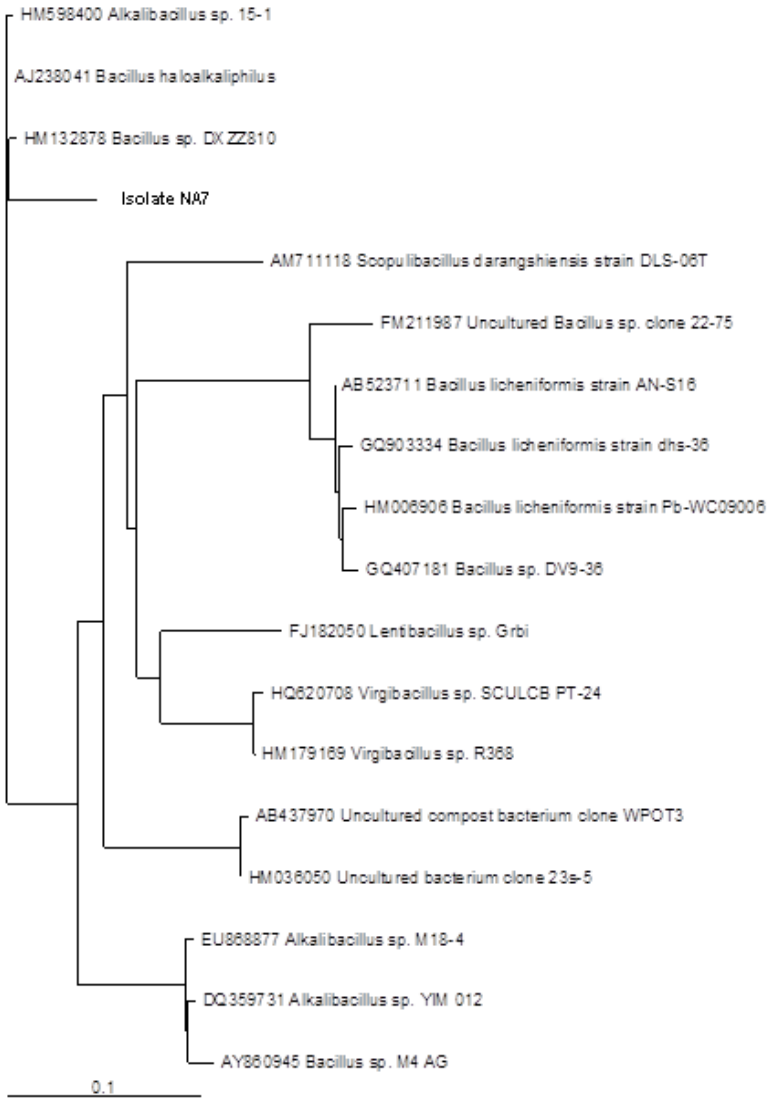

Figure 2. Phylogenetic tree of the isolate NA7 based on partial sequencing of 16S rRNA.

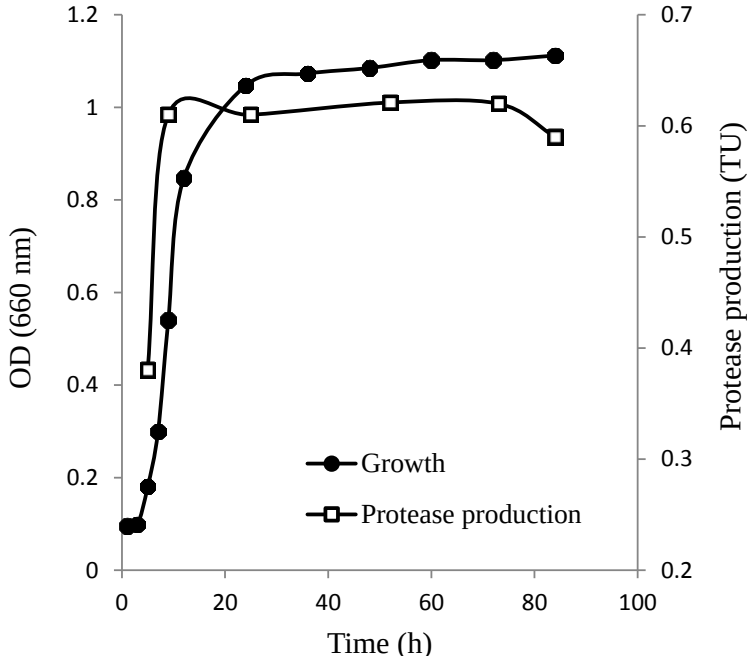

Figure 3. Growth curve and alkaline protease expression by isolate NA7.

at 37°C. The results represented in Figure 3 showed that a lag phase of 3 h was followed by a sharp exponential growth for the next 21 h. The stationary phase was attained after 24 h of incubation. It was also clear that, protease production was dependent on the cell progressive state. It increased markedly during the log phase and reached a maximum of approximately 0.625 TU during the stationary phase. A slight decrease in protease activity was observed after 60 h of almost stable maximal enzyme activity.

Elucidation of fermentation factors affecting protease expression by NA7

Various nutritional and environmental parameters play crucial roles in microbial growth and enzyme productivity. Therefore, studies were carried out on the influences of temperature, pH and medium components on alkaline protease production by NA7. As shown in Figure 4, the specific protease activity reached maximum (357 TU/mg protein) at 40°C after 72 h of incubation which represent approximately 1.27, 1.27, 2.03 and 2.03 folds as that attained at 25, 45, 30 and 35°C, respectively. The effect of initial pH in the range 8 to 12 was examined and the results suggested that pH values lower or higher than 10 were concomitant with lower specific protease activity results and growth rates (data not shown).

The Plackett-Burman design (Plackett and Burman, 1946) was applied to reflect the relative importance of fermentation medium components and their interactions with respect to protease expression by NA7. This includes nutritional variables such as carbon source (glucose), nitrogen sources (yeast extract, peptone, casein and casamino acids), and salts (NaCl and $MgSO_4$). The chosen levels of the 7 variables were presented in Table 3. The design was applied with 11 different trials as shown in Table 4. Other fermentation conditions including pH, 10; inoculum size, 1%; incubation temperature, 40°C and culture volume, 50 mL were kept constant. The specific activity of alkaline proteases was determined after 12 h of incubation and the results ranged between 278 and 465 TU/mg protein (Table 4).

The main effect values calculated for each medium component based on the recorded specific activity results (Table 5) indicate that NaCl followed by $MgSO_4$ concentrations were the most effective independent variables (Figure 5). This conclusion is also supported by the statistical significance levels shown in Table 5. It is also clear that the examined high levels of glucose, yeast extract, peptone, NaCl and $MgSO_4$ affected the response positively. The highest proteolytic specific activity result (465 TU/mg protein) was achieved by the trial number 8 that contains NaCl in a concentration of 20% (W/V) and the lowest record (278 TU/mg protein) was obtained by the trial number 6 which has only 10% NaCl (Table 4). On the contrary, the presence of high levels of casein and casamino acids decreased the ability of the cells to produce the enzyme. Accordingly, it can be predicted that a fermentation formula that contains the low levels of casein and casamino acids together with the examined high concentrations of the other medium components is closer to the optimum condition required for the

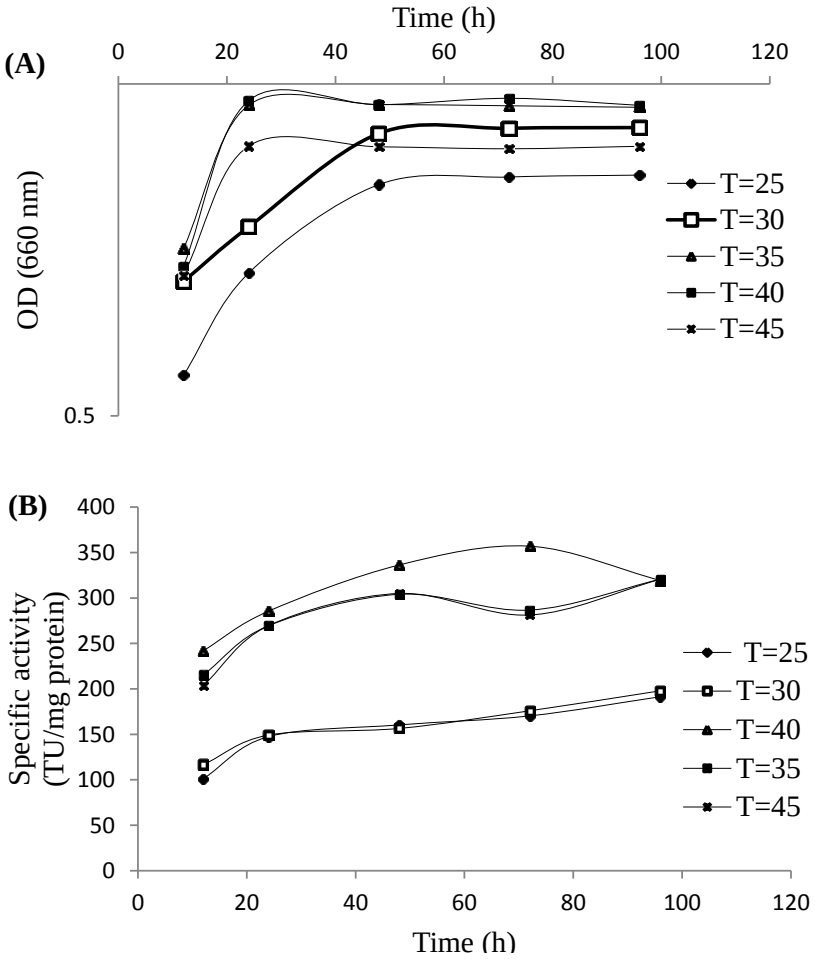

Figure 4. Effect of incubation temperature on isolate NA7 growth (A) and expression of alkaline protease expressed as specific activity (B).

Table 3. Concentration of medium constituents at their low, basal and high levels in the Plackett-Burman experimental design.

Variable	Symbol	Level (g/L)		
		Low level (-1)	Basal medium (0)	High level (+1)
Glucose	G	5	10	15
Yeast extract	Y	3	5	7
Peptone	P	3	5	7
NaCl	Na	100	150	200
Casein	C	0	1	3
Casamino acid	CA	0	1	3
$MgSO_4$	Mg	0	0.2	1

expression of alkaline protease by NA7.

In a verification experiment, the predicted near-optimal condition that contains (g/L): glucose, 15; yeast extract, 7; peptone, 7; NaCl, 200; $MgSO_4$, 1 was compared to controls conditions represented by the basal and the anti-optimized culture media. As shown in Figure 6, the specific activity of alkaline proteases has been raised up to 512 TU/mg protein which represents a 1.27 and 1.39 fold increases when compared to the basal and anti-optimized conditions, respectively.

Table 4. The Plackett-Burman experimental design matrix for 7 variables and 11 trials.

Trial	Independent variable							Specific activity (TU/mg protein)
	G	Y	P	Na	C	CA	Mg	
1	-1	-1	-1	+1	+1	+1	-1	347
2	+1	-1	-1	-1	-1	+1	+1	363
3	-1	+1	-1	-1	+1	-1	+1	338
4	+1	+1	-1	+1	-1	-1	-1	366
5	-1	-1	+1	+1	-1	-1	+1	408
6	+1	-1	+1	-1	+1	-1	-1	278
7	-1	+1	+1	-1	-1	+1	-1	328
8	+1	+1	+1	+1	+1	+1	+1	465
9	0	0	0	0	0	0	0	398
10	0	0	0	0	0	0	0	402
11	0	0	0	0	0	0	0	405

Table 5. Statistical analysis of the Plackett-Burman experimental results.

Variables (g/L)	Specific activity of alkaline protease			
	Main effect	t-value	p-value	Significance levels
Glucose	51	0.30172	0.7779	22.1%
Yeast extract	101	0.61122	0.56348	44%
Peptone	65	0.38638	0.72499	18%
NaCl	279	2.20351	0.07875	93%*
Casein	-37	-0.2181	0.83802	16%
Casamino acid	-113	0.68927	0.51641	48%
MgSO4	225	1.89187	0.11708	90%*

*Significant (≥ 80%, p-value ≤ 0.2).

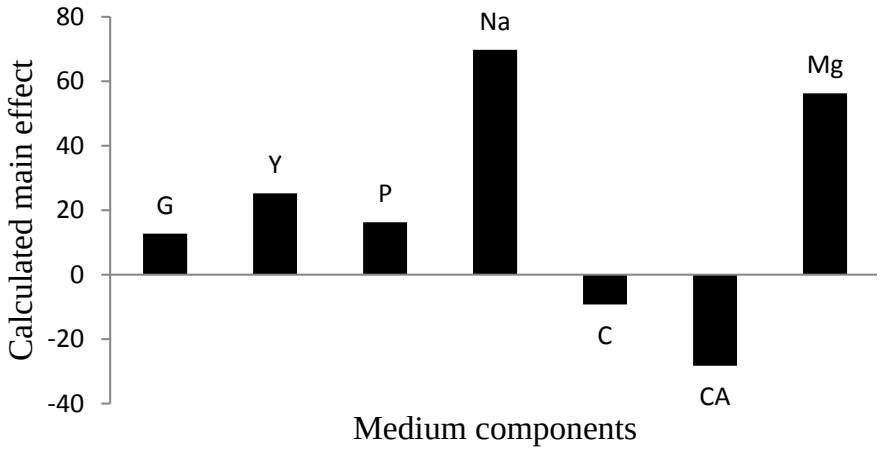

Figure 5. Effect of medium composition on alkaline proteases production by isolate NA7 according to the Plackett-Burman experimental results.

DISCUSSION

Screening for intrinsically stable biocatalysts is a prominent area of research in biocatalysis (Illanes, 1999). Therefore, organisms able to survive and thrive under extreme environmental conditions are promising sources

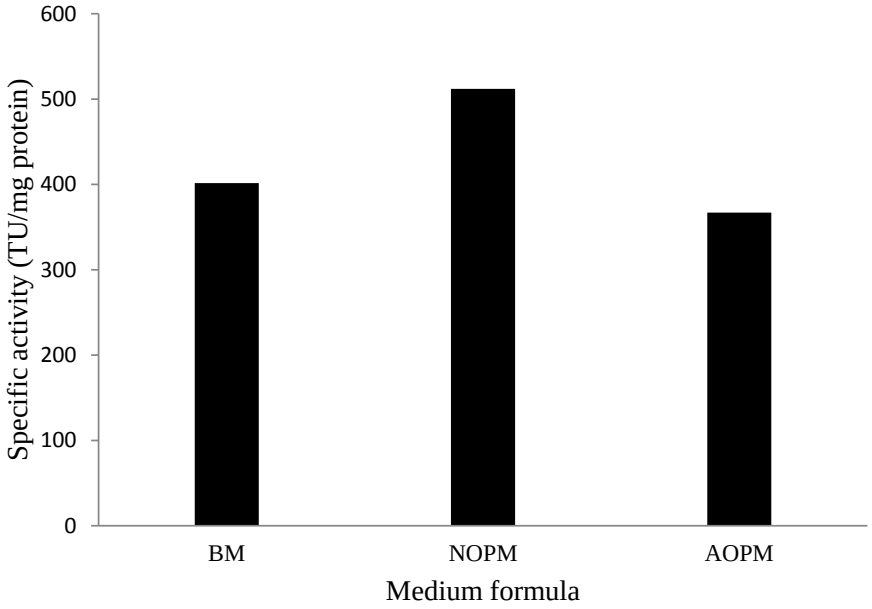

Figure 6. Specific activity of alkaline proteases produced by isolate NA7 grown on basal, near-optimized and anti-optimized medium. BM, the basal medium; NOPM, near-optimized medium and AOPM, the anti-optimized medium.

of enzymes that retain their activities under extreme temperatures, extreme pH and aggressive chemicals. In this work, the isolate NA7 has been chosen as a promising alkaline protease producer among thirteen haloalkaliphilic phenotypes isolated from wild plant root samples collected from Wadi Natrun desert in Egypt. In addition to being Gram positive, rod-shaped and spore former, the isolate was further characterized based on partial 16S rDNA sequencing as a *Bacillus* species. The sequence showed 99% identity when compared to the homologous sequence of two *B. haloalkaliphilus* genotypes with the accession numbers, DQ157468.1 and AJ238041.1. To the best of our knowledge no single report that investigates the expression of alkaline protease by a *B. haloalkaliphilus* strain has been published.

It is well known that protease production by *Bacillus* cells is directly related to biomass production and is associated with the stationary phase of cell growth (Bascarán et al., 1990; Ferrero et al., 1996; Kumar and Takagi, 1999; Puri et al., 2002; Gupta et al., 2002). The study of NA7 growth kinetics with reference to protease production demonstrated that, prior to the end of the exponential phase, the culture showed marked protease production efficiency with relatively stable maximum activity records (0.610-0.625 TU) for more than 60 h during the stationary phase. When compared to other similar halotolerant *Bacillus* shake flask condition (Prasad et al., 2014), the expression phase of the proteolytic activity is very much longer.

Multiple starvation-induced cell states in the genus *Bacillus* such as competence, development of endospores and expression of various extracellular digestive enzymes are possible. Nevertheless, cells can be environmentally programmed to express certain valuable postexponential secondary metabolites rather than others (El-Helow et al., 2000). The main purpose of the second part of this work was to find out a culture condition that shifts the metabolic activity of NA7 cells towards more expression of alkaline protease. To fulfil this objective, it is recommended to express the experimental response results in the form of enzyme specific activity units (e.g. TU/mg protein) rather than activity units. The studied factors included temperature and initial pH, each examined separately as one variable at a time, followed by medium components investigated through a multi-factorial experiment to reflect their effects without neglecting their interactions.

The pH of the culture strongly affects many enzymatic processes, transport of compounds across the cell membrane and stability of the ionic groups on the active sites of enzymes (Ellaiah et al., 2002). It is also known that almost all currently used detergent compatible enzymes are alkaline and thermostable in nature with a high optimum pH because the pH of laundry detergents is generally in the range of 9.0-12.0 (Saracoglu et al., 2013). The results revealed that NA7 cells are able to grow in a pH range of pH 8 to 12 with a maximum specific alkaline protease activity of 354.9 TU/mg protein at pH 10. Raising the incubation temperature to 40°C increased the specific proteolytic activity up to 357 TU/mg protein. Comparable pH and temperature results obtained by phenotypically related *Bacillus* strains have been previously reported (Gessesse and Gashe, 1997;

Denizci et al., 2004; Vidyasagar et al., 2006).

Nutritional regulation studies on the expression of alkaline protease in NA7 were carried out by applying a 2-level Plackett-Burman multi-factorial experiment (Plackett and Burman, 1946). Such statistical experimental designs are powerful tools for finding out the key factors rapidly from a multivariable system. They also minimize the error in determining the effect of multiple parameters and have been successfully used in biotechnological applications (Niladevi et al., 2009). The results presented here showed that most of the examined nutritional variables appeared to cause upregulatory effects on protease expression at their high levels, especially, NaCl followed by $MgSO_4$. Similar to the results of another halotolerant *Bacillus* strain (Shivanand and Jayaraman, 2009), maximum protease specific activity record in this experiment was achieved in the presence of 20% (w/v) NaCl concentration. This result suggests that the alkaline protease expression by NA7 is mainly dependent on high salt concentrations and that the enzyme is a halo-tolerant protease as it retained its activity at this relatively high NaCl concentration.

Alkaline protease expression by *Bacillus* cells is greatly dependent on the quality and quantity of both carbon and nitrogen sources in the medium (Moon and Parulekar, 1991; Chu et al., 1992). The present results demonstrated that an increased supply of yeast extract as well as peptone stimulated the expression of alkaline protease by the *Bacillus* strain NA7. These positive effects of the two protein-rich complexes on growth and protease synthesis by *Bacillus* cells match previous findings (Kaur et al., 2001; Patel et al., 2005; Anbu, 2013). Though the presence of casein enhances protease production in some *Bacillus* strains (Belmessikh et al., 2013), it is likely that casein is not among the best protease inducers for the experimental *Bacillus* strain. Similarly, slight negative effects of casein on enzyme production by other related bacterial strains have been formerly reported (Nilegaonkar et al., 2007; Daroit et al., 2011).

A marked negative effect on the production of the enzyme has been observed in the presence of a relatively high level of casamino acids, the hydrolysis product of casein. This result suggests that catabolite repression plays a role in the regulation of alkaline protease expression in NA7. Other reports have indicated similar repressive role of excessive casamino acids on alkaline protease production by other *Bacillus* strains (Gusek et al., 1988; Ferrero et al., 1996; Gupta et al., 2002; Chauhan and Gupta, 2004). Catabolite repression by simple sugars is also well known in the regulation of protease production in many microorganisms (Sato and Sudo, 1999; Gupta et al., 2002; Sandhya et al., 2005). However, enhanced protease yields have been reported by some researchers on supplementation of glucose and lactose (Gessesse and Gashe, 1997; Mabrouk et al., 1999; Nisha and Divakaran, 2014). Within the glucose concentration range examined in this investigation, the high level (15 g/L) showed a minor positive effect on alkaline protease expression by NA7.

Based on the prediction studies of this work and a verification experiment, it can be concluded that a medium formula containing the examined high levels of NaCl, $MgSO_4$, yeast extract, peptone and glucose together with the low levels of casamino acid and casein reallocates the metabolic regulatory mechanisms in NA7 cells towards more production of extracellular alkaline protease. The protease activity achieved under this growth condition is comparable to those recorded by other *Bacillus* genotypes (Saxena and Singh, 2010; Gaur et al., 2014; Mhamdi et al., 2014).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. S. Sabry and Dr. H. Ghozlan (Faculty of Science, Alexandria University) for providing information and practical support for isolation of extremophilic bacterial phenotypes.

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