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Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes

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ABSTRACT

Background: Alkaline proteases are among the most important classes of industrial hydrolytic enzymes. The industrial demand for alkaline proteases with favorable properties continues to enhance the search for new enzymes. The present study focused on isolation of new alkaline producing alkaliphilic bacteria from hyper saline soda lakes and optimization of the enzyme production.
Results: A new potent alkaline protease producing halotolerant alkaliphilic isolate NPST-AK15 was isolated from hyper saline soda lakes, which affiliated to *Bacillus* sp. based on 16S rRNA gene analysis. Organic nitrogen supported enzyme production showing maximum yield using yeast extract, and as a carbon source, fructose gave maximum protease production. NPST-AK15 can grow over a broad range of NaCl concentrations (0–20%), showing maximal growth and enzyme production at 0–5%, indicated the halotolerant nature of this bacterium. Ba and Ca enhanced enzyme production by 1.6 and 1.3 fold respectively. The optimum temperature and pH for both enzyme production and cell growth were at 40°C and pH 11, respectively. Alkaline protease secretion was coherent with the growth pattern, started at beginning of the exponential phase and reached maximal in mid stationary phase (36 h).
Conclusions: A new halotolerant alkaliphilic alkaline protease producing *Bacillus* sp. NPST-AK15 was isolated from soda lakes. Optimization of various fermentation parameters resulted in an increase of enzyme yield by 22.8 fold, indicating the significance of optimization of the fermentation parameters to obtain commercial yield of the enzyme. NPST-AK15 and its extracellular alkaline protease with salt tolerance signify their potential applicability in the laundry industry and other applications.

1. Introduction

Proteases (EC 3.4.21) are a large group of hydrolytic enzymes that catalyze the hydrolysis of the proteins by cleavage of the peptide bonds between the amino acid residues in other proteins [1]. Proteases constitute one of the most important groups of industrial enzymes, accounting for more than 65% of total industrial enzyme market [2,3]. Moreover, microbial proteases constitute approximately 40% of the total worldwide production of enzymes [4,5]. Alkaline proteases, with high activity and stability in high alkaline range, are interesting for several bioengineering and biotechnological applications. However, their main application is in the detergent industry, accounting for approximately 30% of the total world enzyme production [6] because the pH of laundry detergents is in the range of 9.0–12.0. Alkaline proteases are used in detergent formulations, with other hydrolytic enzymes, as cleaning additives to facilitate the breakdown and release of proteins [7,8]. In addition, alkaline proteases have various other industrial applications including leather, pharmaceuticals, protein processing, foods, diagnostic reagents, soy processing, peptide synthesis industries, and extraction of silver from used X-ray film [9,10]. Therefore, the industrial demand for highly active alkaline proteases with high specificity and stability of pH, temperature, and organic solvents continues to enhance the search for new enzymes [11].

Extremophiles are microorganisms that have evolved to live in a variety of unusual habitats, the so-called extreme environments. They fall into a number of different classes including halophiles, alkalophiles, thermophiles, psychrophiles, and others [12]. The groups of bacteria that can grow under alkaline conditions in the presence of NaCl are referred to as halotolerant alkaliphiles and haloalkaliphiles. The dual extremity of these extremophiles, high pH, and salt concentration make them attractive strains for exploration of novel

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alkaline proteases for biotechnological potential [13,14]. One of the major natural habitats of alkaliphilic bacteria are hyper saline soda lakes which represent the major types of naturally occurring highly alkaline environments (pH > 11.5), in addition to high NaCl concentration [15]. Despite the worldwide prevalence of the soda lakes, few of such lakes have been explored from the microbiological point of view as a result of their inaccessibility. One of those environmental niches, which have not been studied in details, are Wadi El-Natrun Valley hyper saline soda lakes, located in northern Egypt. The features of Wadi EL-Natrun Valley created an ecosystem considered as rich sources for isolation of alkaliphilic, haloalkaliphilic, and thermo-alkaliphilic microorganisms [15,16,17].

In order to obtain commercially viable yields, it is essential to optimize fermentation media for the growth and protease production. Considering the above mentioned facts, the present study focused on isolation of new alkaline producing alkaliphilic bacteria from Wadi El-Natrun hyper saline soda lakes, and optimization of the enzyme production by investigation of the effect of various physio-environmental parameters.

2. Materials and methods

2.1. Collection of soil and water samples

Sediment and water samples were collected from hyper saline soda lakes in Wadi El-Natrun Valley that are located in northern Egypt (Fig. 1). The Valley contains alkaline inland saline lakes in an elongated depression approximately 90 km northwest of Cairo (capital of Egypt). The average length of the valley is nearly 60 km, and its average width is approximately 10 km. Wadi El-Natrun Valley extends in a northwest by southeast direction between latitude 30°15′ North and longitude 30°30′ East. The bottom of the Valley is 23 m and 38 m below sea level, and water level of Rosetta branch of the Nile, respectively [18]. Sediment and water samples were collected from various hyper saline soda lakes in sterile containers, kept at 4°C, and transferred within few d to the laboratory at King Saud University (Riyadh, Saudi Arabia).

2.2. Isolation of alkaline protease producing alkaliphilic bacteria

Isolation of alkaline protease producing alkaliphilic bacteria was carried out using Horikoshi-I alkaline medium with some modification [15]. The alkaline agar medium (pH 10.5) contained glucose (10 g/L), yeast extract (5 g/L), peptone (5 g/L), K_2HPO_4 (1 g/L), $Mg_2SO_4 \times 7H_2O$ (0.2 g/L), NaCl (50 g/L), Na_2CO_3 (10 g/L), and agar (15 g/L), in addition to 10% (w/v) skim milk, as an indicator of alkaline protease production [2]. The skim milk, glucose, and Na_2CO_3 were autoclaved separately before the addition to the medium. Sediment and water samples were suspended and serially diluted in a 10% (w/v) NaCl solution prepared in 50 mM glycine–NaOH buffer, pH 10. Aliquots (200 μL) of various dilutions were spread on the alkaline agar medium and incubated at different temperatures for several d. The formation of clearing zone around the colonies resulted from the production of alkaline protease, and subsequent casein hydrolysis was considered as an initial indication of enzyme activity [2,11]. Positive strains were streaked several times on fresh plates until single uniform colonies were obtained, and glycerol stocks of each strain were prepared and stored at -80°C till further analysis.

2.3. Bacterial identification

The selected strain was identified using 16S rRNA gene sequence analysis as per the standard protocols [19]. The bacterial isolate was grown overnight in 5 mL alkaline broth medium. Total DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen, USA) according to the manufacturer's instructions. Eubacterial-specific forward primer: 16F27 (5′-AGA GTT TGA TCC TGG CTC AG-3′), and reverse primer: 16R1525 (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used to amplify 16S rDNA gene [20,21]. PCR amplification was performed in a final reaction volume of 50 μL. The reaction mixture contained 2 × 25 μL GoTaq® Green Master Mix (Promega, USA), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) 5 μL DNA template (200 ng) and 18 μL nuclease-free water. The PCR reaction run for 35 cycles in a DNA thermal cycler, under the following thermal profile: Initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, primers annealing at 52°C for 1 min, and extension at 72°C for 1.5 min. The final cycle included extension for 10 min at 72°C to ensure full extension of the products. PCR products were ran on agarose gel electrophoresis, and then purified using a QIAquick gel extraction kit (Qiagen, USA). The purified 16S-rDNA was sequenced using an automated sequencer (Macrogen, Korea), and the obtained sequence was aligned with reference 16S-rDNA sequences available in NCBI homepage (National Center for Biotechnology Information) using the BLAST algorithm.

2.4. Inoculum preparation and alkaline protease production

Colonies of the positive strains, showing clearing zone around their margins, were transferred to 50 mL glass tube containing 5 mL of the liquid production medium, with the same composition as the solid medium, except for the presence of agar and skim milk, and incubated overnight at 40°C in shaking incubator (150 rpm). This culture was used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of the same medium and cultivated under the same conditions for approximately 24 h. Cells and insoluble materials were removed by centrifugation at 6000 × g for 15 min at 4°C, and cell-free supernatant

Fig. 1. a) Map of Egypt showing the location of Wadi El-Natrun Valley (red circle). b) A photo of a hypersaline soda lake in the Wadi Natrun area (photo taken by authors). c) Isolation of alkaline protease alkaliphilic bacteria using modified Horikoshi-I agar plate containing skim milk. The clear zone indicated casein hydrolysis due to alkaline protease production.

was filtered through a 0.45 μm pore-size membrane filter, and the alkaline protease activity was measured.

2.5. Assay of alkaline protease

Alkaline protease activity was measured using previously reported method with some modifications [22]. 0.5 mL of 50 mM glycine–NaOH (pH 10.0) containing 1% (w/v) casein and 10 mM $CaCl_2$ was pre-incubated for 5 min at 50°C. Then, 0.5-mL aliquot of suitably diluted culture supernatant was mixed with the substrate solution, and incubated for 20 min at 50°C. After that, the reaction was terminated by the addition of 0.5 mL of 20% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand at room temperature for 15 min and centrifuged at 6000 × g for 15 min to remove the precipitate. The acid-soluble materials were estimated using Lowry method [23]. Control was prepared by adding TCA before adding the enzyme. A standard curve was generated using solutions of 0–100 μg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions. All enzyme assay experiments were carried out in triplicate, and the mean values were recorded.

2.6. Optimization of the production medium

2.6.1. Effect of carbon source

To investigate the influence of different carbon sources on the bacterial growth and alkaline protease production by the selected strain, glucose in the production medium was substituted with other carbon sources, including 1% (w/v) xylose, glucose, galactose, fructose, maltose, lactose, sucrose, starch, and wheat bran as a sole source of carbon. The various carbon sources were autoclaved separately and added to the medium on an equal carbon basis. Furthermore, the effects of different concentrations (0–4%) of the best carbon source were also investigated. The growth and enzyme activity were monitored after 24 h incubation at 40°C, under shaking conditions (150 rpm). All experiments and enzyme assays were performed in triplicate, and the mean values were reported.

2.6.2. Effect of nitrogen source

The effect of different nitrogen sources on the bacterial growth and alkaline protease production by the selected isolate was investigated by substituting the peptone and yeast extract in the production medium (Horikoshi-1), with different sources of organic and inorganic nitrogen sources (0.5%, w/v). Organic nitrogen sources included peptone, yeast extract, tryptone, alkali soluble casein, insoluble casein, skim milk, gelatin, and beef extract; while inorganic nitrogen sources included ammonium nitrate, ammonium sulfate, sodium nitrate, and urea. Furthermore, the effects of different concentrations (0–2%) of nitrogen source that support maximum enzyme yield were also investigated. The growth and enzyme activity were measured after 24 h incubation at 40°C, under shaking conditions of 150 rpm.

In addition to carbon and nitrogen source testing, the effects of NaCl concentration (0–25%), and various metal ions, including Mg^{+2}, Mn^{+2}, Zn^{+2}, Ca^{+2}, Cu^{+2}, Co^{+2}, Fe^{+2}, and Ba^{+2} (1 mM, 5 mM, and 10 mM), on the bacterial growth and alkaline protease production were investigated.

2.6.3. Effect of temperature, pH, and aeration

The influence of temperature on the bacterial growth and alkaline protease production by the selected strain was investigated by varying the growth temperature (30–60°C), keeping the other parameters constant. Similarly, in order to investigate the influence of initial pH of the production medium on growth and protease production, the isolate was grown in medium with different pH (5.0–12.0) at optimum growth temperature. The growth and enzyme activity were measured as described above. Moreover, the effect of the aeration level during fermentation on growth and protease production was studied by incubating the culture in shaking incubators with different rpm values which ranged from zero (static) to 250 rpm.

2.7. Growth kinetics and proteases production

Colonies of the selected isolate were transferred to 250 mL Erlenmeyer flasks containing 50 mL of the optimized production medium and incubated at 40°C under orbital shaking (200 rpm). These cultures were used to inoculate 1 L flasks containing 250 mL of the same medium and cultivated under the same conditions. Samples (1 mL) were withdrawn at 2 h intervals up to 48 h for the measurement of growth and alkaline protease activity. The samples were centrifuged at 6000 × g at 4°C, and the pellets obtained were washed twice using Tris buffer (pH 8), and resuspended in 1 mL of the same buffer. Absorbance was measured at 600 nm against Tris Buffer (pH 7) as blank, and was reported as growth of the bacterium. The cell-free supernatant was filtered through a 0.45-μm pore-size membrane filter, and the alkaline protease activity was measured as described above. The triplicate of each period was taken to calculate the growth and enzyme activity and the mean values were reported.

2.8. Statistical analysis

All the experiments, enzyme assay, and cells growth measurement were carried out in triplicate, and the standard deviation for each test was calculated using SPSS 14.0 [14]. The standard deviations ($n = 3$) are indicated as error bars.

3. Results and discussion

3.1. Isolation and identification of the microorganism

The sediment and water samples were spread over the modified Horikoshi-I agar medium containing skim milk as an indicator of protease production. After the incubation period, morphologically distinct colonies were observed on the agar plates. Some of the colonies were showing zone of hydrolysis indicating the production of extracellular alkaline protease (Fig. 1). Individual colonies were purified through repeated streaking on fresh agar plates. The extensive screening process resulted in isolation of 15 alkaline protease producing alkaliphilic bacterial strains that showed clear zone around their colonies. All the positive isolates were cultivated in alkaline production liquid medium, and the proteolytic activity was measured. The strain NPST-AK15 showing the highest alkaline protease activity (55.1 U/mL) was selected for further investigation.

Strain NPST-AK15 is a Gram-positive, spore former, catalase-positive, oxidase-negative bacterium, which forms yellow colonies on the agar surface. Cells were motile, short rods, with lengths of about 1.5 μm and 0.5 μm in diameter, and the cells existed as single, paired or short chain (Fig. 2). NPST-AK15 can utilize several carbon sources including glycogen, mannose, glucose, xylose, citric acid, and starch. The growth of NPST-AK15 occurred with 0–20% (w/v) NaCl (optimally at 0–5%), at pH 7.0–12.0 (optimally at pH 11) and can grow at temperature between 25°C and 55°C (optimally at 40°C). Considering the physiological and biochemical tests performed, and according to Bergey's Manual of Determinative Bacteriology, the strain was identified as *Bacillus* sp. [14]. The organism was further identified by 16S rRNA gene sequencing having a length of 1449 bp nucleotides. Phylogenetic analysis of its 16S rRNA gene sequence revealed that NPST-AK15 is affiliated with the genus *Bacillus* and closely related to several alkaliphilic *Bacillus* sp. with 99% similarity (Fig. 3). This sequence was deposited in GenBank with accession number KP295749, and the isolate was tentatively named as *Bacillus* sp. strain NPST-AK15.

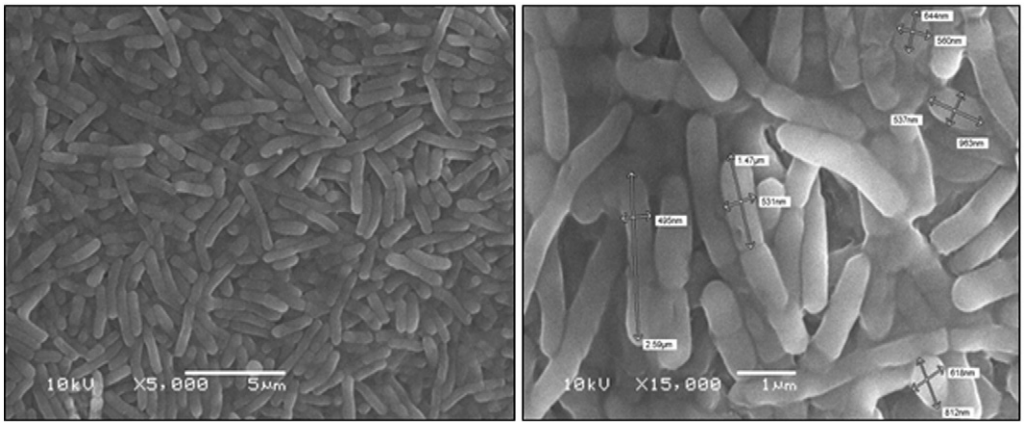

Fig. 2. Scanning electron microscope (SEM) images of strain NPST-AK15.

3.2. Optimization of alkaline protease production

The small productivity of enzymes and metabolites from extremophiles represents one of the major bottlenecks in their industrial applications [17]. It has been established that the production of alkaline protease is highly affected by media composition, *viz.* variation in C/N ratio, and the presence of metabolizable sugars, ions, and salts, in addition to other fermentation parameters including aeration level, pH, temperature, and incubation time [24,25,26]. Hence, it is essential to optimize production medium and cultivation conditions for the growth and enzyme production, in order to obtain high and commercial yields of alkaline protease.

3.2.1. Effect of carbon source

The influence of different carbon sources, including mono-, di- and polysaccharides, on the cells growth and production of alkaline protease was investigated. The results indicated that different carbon sources have a different impact on the production of extracellular protease from *Bacillus* sp. NPST-AK15. All tested carbon sources supported the growth of strain NPST-AK15. However, among the various carbon sources, fructose was found to support maximum protease production (Fig. 4a). Protease production in the presence of glucose or maltose led to a drastic decrease in the enzyme production up to 16.3% and 18.1% of the maximum yield. In addition, the enzyme yield decreased to 25.5, 27.3, 44.9, and 48.9% of the maximum production in case of wheat bran, galactose, xylose, and lactose, respectively. The production of alkaline protease was further monitored at various concentrations of fructose, as the best carbon source. As shown in Fig. 4b, both growth and alkaline protease production were increased by increasing the fructose concentration, with maximal bacterial growth and enzyme production observed at 2%. Further increases in fructose concentration led to the decrease of the enzyme production. These results are in accordance with some previous studies which showed that some pure carbon sources, mainly glucose and maltose, inhibit protease production, owing to catabolite repression of protein biosynthesis [25,27,28]. The production of protease from moderately halophilic *Pseudoalteromonas* sp. strain CP76 [29], halophilic

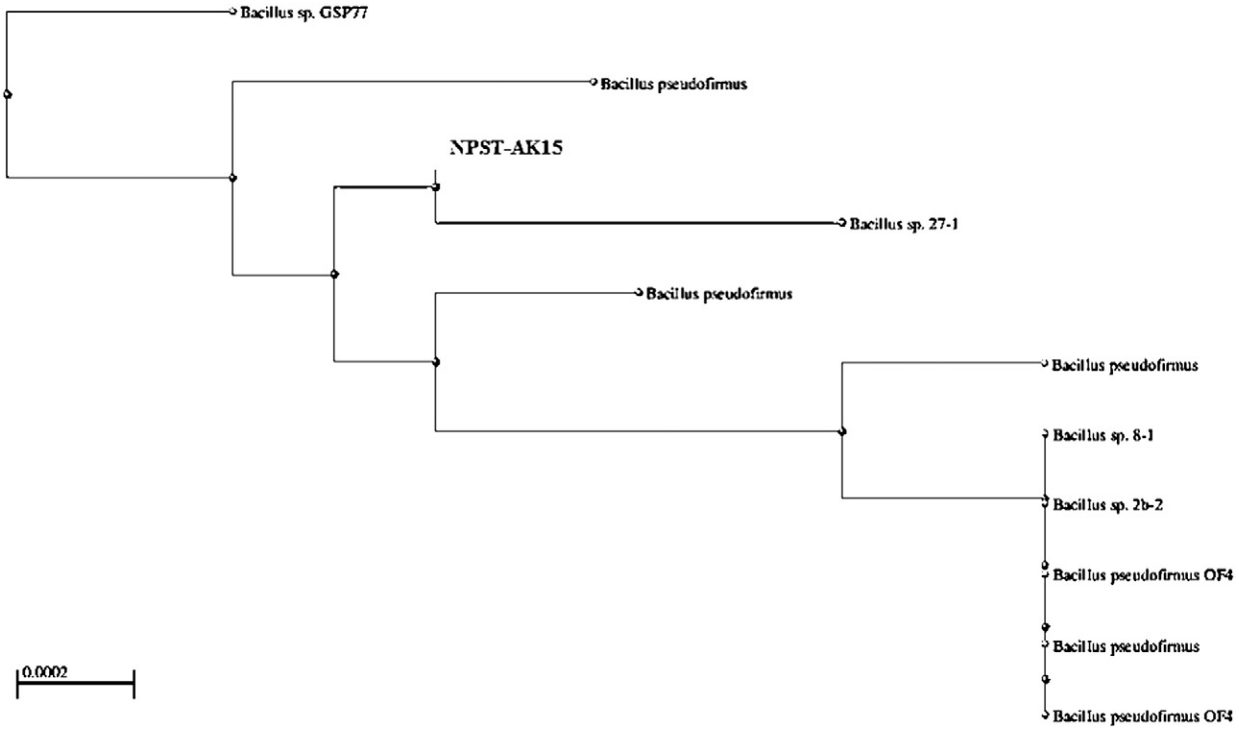

Fig. 3. Neighbor-joining phylogenetic tree of the isolated strain NPST-AK15 and its closest *Bacillus* strains based on 16S rRNA gene sequences.

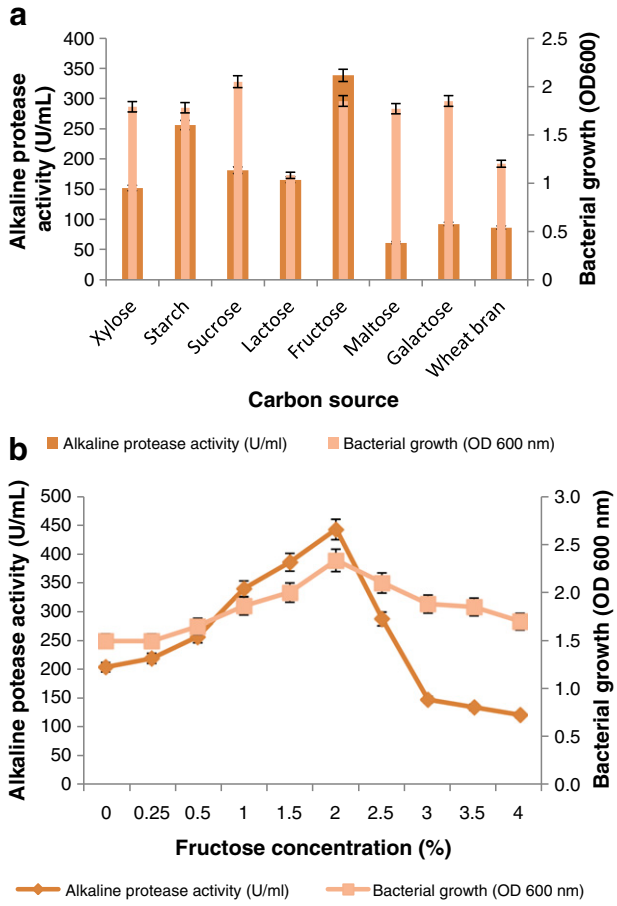

Fig. 4. (a) Effect of carbon sources (0.5%, w/v) on growth and alkaline proteases production by strain NPST-AK15. Samples were taken after incubation of 24 h at 40°C under shaking conditions (150 rpm), for the determination of bacterial growth and alkaline protease activity. Standard deviations (n = 3) are reported as error bars. (b) Effect of fructose concentration on bacterial growth and alkaline proteases production by strain NPST-AK15. Error bars represent the standard deviations (n = 3).

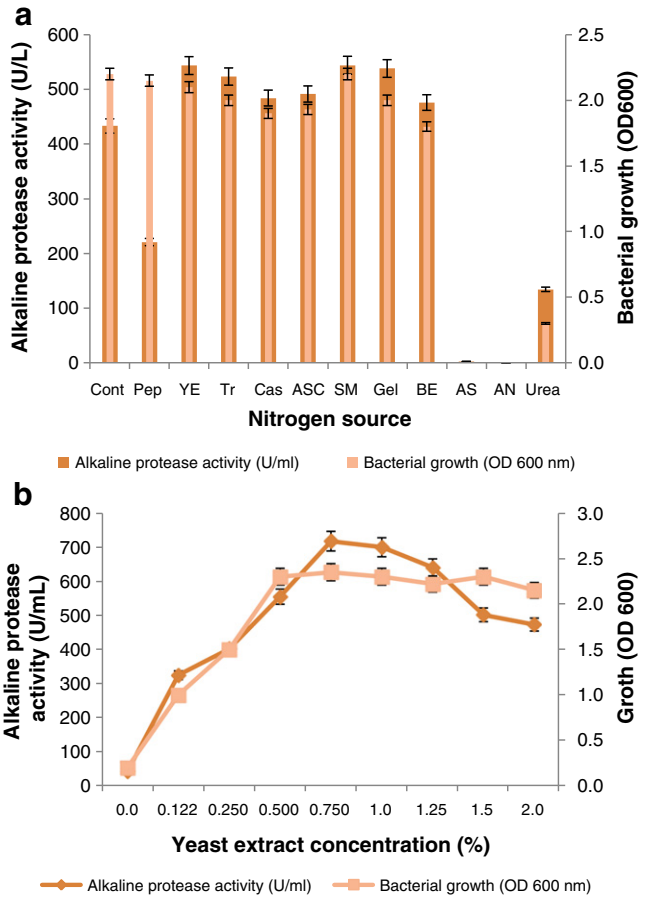

Fig. 5. (a) Effects of various nitrogen sources on cell growth and alkaline protease production by strain NPST-AK15, using fructose as a carbon source. Samples were taken after incubation of 24 h at 40°C under shaking conditions (150 rpm). Cont: Control; Pep: Peptone; YE: Yeast extract; Tr: Tryptone; Cas: Casein; ASC: Alkali soluble casein; SM: Skim milk; Gel: Gelatin; BE: Beef extract; AS: Ammonium sulfate; AN: Ammonium nitrate. Standard (n = 3) deviations are shown as error bars. (b) Effect of yeast extract concentration on bacterial growth and production of alkaline proteases by strain NPST-AK15. Standard deviations (n = 3) are indicated as error bars.

Bacillus S-20-9 [17] was significantly inhibited in the presence of maltose, glucose, and lactose. In addition, repressed growth and enzyme production at higher concentration of the substrates might be due the catabolic repression, or substrate inhibition, a traditional property of batch fermentation processes [30]. On the other hand, different carbon sources were reported for maximal protease production based on the organism of origin [14,17,31].

3.2.2. Effect of nitrogen source

The influence of various nitrogen sources on the cells growth and alkaline protease production was evaluated using the production medium containing fructose as the sole carbon source. The results indicated that among the nitrogen sources used, several organic sources supported both growth and enzyme production by strain NPST-Ak15, with maximum yield in medium containing yeast extract, followed by skim milk, gelatin, casein, and other organic nitrogen sources, respectively (Fig. 5a). On the other hand, inorganic nitrogen sources proved to be unfavorable, where enzyme production yield drastically reduced to 0.0, 0.5, and 31% of the maximum output level, in case of ammonium nitrate, ammonium sulfate, or urea, respectively. This result was in agreement with that reported for marine *Bacillus* sp. MIG [32], alkaliphilic *Bacillus pumilus* MCAS8 [14], alkaliphilic *Bacillus licheniformis* KBDL4 [25], and *Bacillus cluasii* [33] where alkaline protease production was maximal using yeast extract, and significantly reduced using inorganic nitrogen sources. In earlier reports, it was found that other organic nitrogen sources supported protease production in other microorganisms including skim milk [34], peptone [35], casamino acids [7], beef extract [10], and others, depending on the source organism [7,36,37]. The production of alkaline protease by strain NPST-Ak15 was further monitored at various concentrations of yeast extract. As shown in Fig. 5b, both growth and alkaline protease production were increased by increasing the yeast extract concentration, showing maximum enzyme yield at concentrations of 2%. However, further increase of yeast extract led to a significant decrease in the enzyme production, with no effect on the bacterial growth. There are some reports on the repressive role of organic nitrogen sources and excessive amino acid and ammonium ions in alkaline protease production [38,39].

3.2.3. Effect of salinity

The results presented in Fig. 6 show the influence of NaCl concentration on the cell growth and alkaline protease production by strain NPST-AK-15, which revealed that strain NPST-AK15 can grow over a wide range of NaCl concentrations from 0 to 20%. However, maximal growth and enzyme production were seen in medium which contained 0–5% of NaCl. Increasing the salt concentration to 7.5% caused a relative decrease in both bacterial growth and protease production to 78 and 85.9% of the maximal enzyme yield and cell growth, respectively. However, higher NaCl concentration led to a drastic reduction in both growth and protease production. The ability of strain NPST-AK15 to grow over a wide range of NaCl concentrations

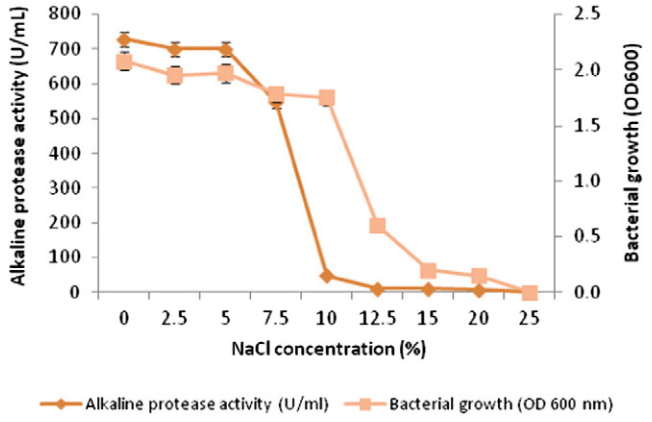

Fig. 6. Effect of sodium chloride concentration on growth and production of alkaline proteases by strain NPST-AK15. Samples were taken after incubation of 24 h at 40°C under shaking conditions (150 rpm), for the determination of cell growth and protease activity. All experiments were carried out in triplicate and the mean values were recorded and the standard deviations (n = 3) are indicated as error bars.

(0–20%), with a maximum cell growth of 0–5%, indicated the halotolerant nature of strain NPST-AK15 [7]. Previous studies on the effect of salinity on the growth of halotolerant bacteria have shown a change in the polar lipid composition of the cell membranes, and an increased salt concentration creates change in the lipid resulting in a decrease of growth rate causing reduced enzyme production [30]. Strain NPST-AK15 and its extracellular alkaline protease with salt tolerance signify their potential applicability in the laundry industry in a better way than the other reported proteases from *Bacillus* sp. [6,7,40]. Halophiles produce a large variety of stable and unique biomolecules that may be useful for practical applications. Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNAases, lipases, amylases, gelatinases and proteases) capable of functioning under conditions that lead to precipitation or denaturation of most proteins [12]. The high salt tolerance is also a characteristic feature of halophiles enzymes, which have a number of future applications in biotechnological process that depends on high salinity or osmotic pressures. In addition, halophilic proteins compete effectively with salts for hydration, a property that may result in resistance to other low-water-activity environments, such as in the presence of organic solvents. Novel halophilic biomolecules may also be used for specialized applications, *e.g.* bacteriorhodopsin for biocomputing, gas vesicles for bioengineering floating particles, pigments for food coloring, and compatible solutes as stress protectants [15,16].

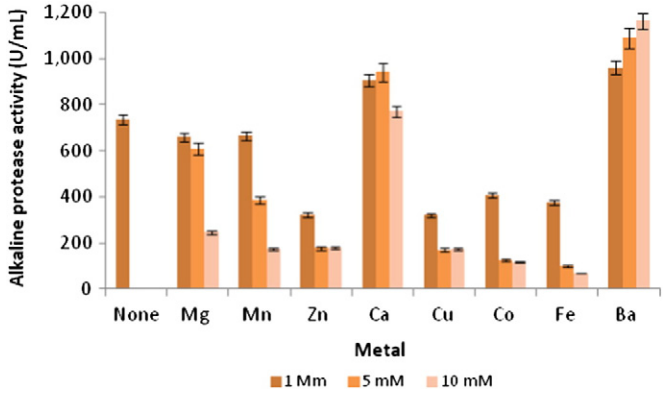

Fig. 7. Effects of various metal ions on alkaline protease production by strain NPST-AK15. The medium was supplemented with various metals at concentrations of 1 mM, 5 mM and 10 mM. Standard deviations (n = 3) are indicated as error bars.

3.2.4. Effect of metals ions

The influence of various metal ions on cell growth and alkaline protease production was evaluated, and the results are demonstrated in Fig. 7. Among the cations tested Ba and Ca ions significantly enhanced the protease production by about 1.6 fold and 1.3 fold compared to the control, respectively. However, most of the other tested ions caused a significant reduction of the enzyme yield particularly at high concentration. Calcium ion has been reported to

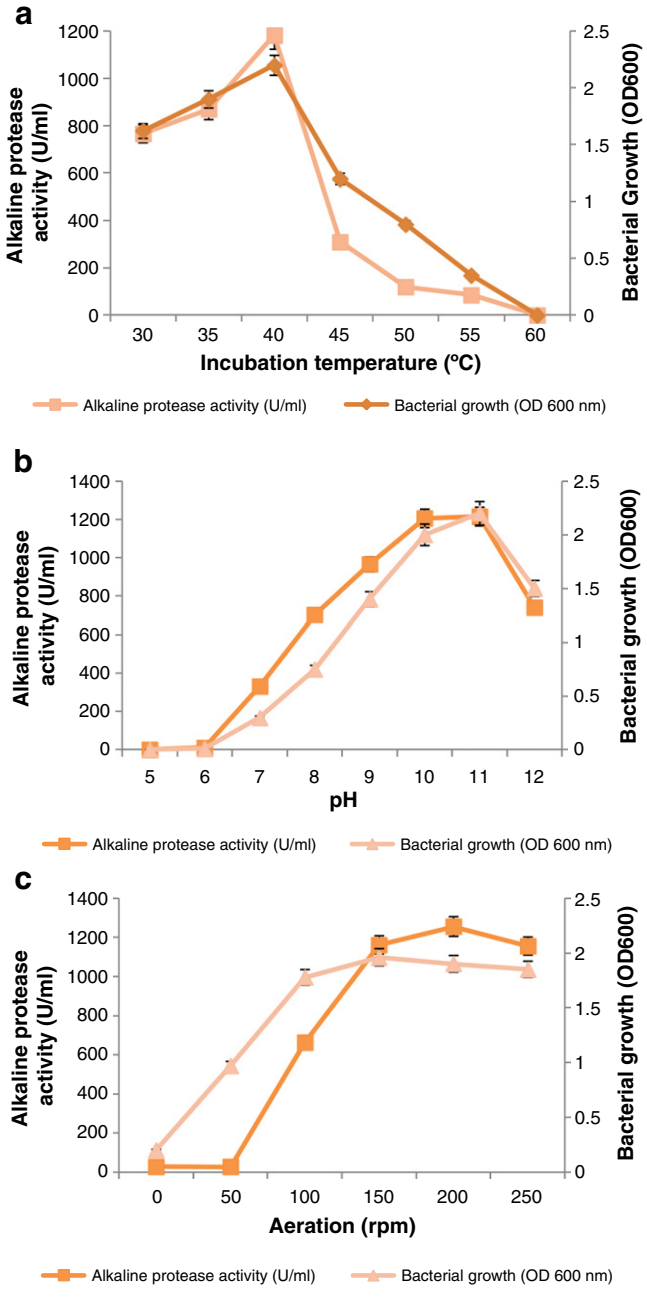

Fig. 8. (a) Influence of growth temperature on cell growth and alkaline protease production by strain NPST-AK15. Cells were propagated under the optimized medium with pH 10 and incubated at various growth temperatures for 24 h in orbital shaker (150 rpm). Error bars represent the standard deviations (n = 3). (b) Influence of pH on bacterial growth and alkaline protease production by strain NPST-AK15. Cells were propagated under the optimum temperature (40°C) in media with different initial pH and incubated for 24 h in orbital shaker (150 rpm). Error bars represent the standard deviations (n = 3). (c) Influence of aeration level (shaking rpm) on cell growth and alkaline protease production by strain NPST-AK15. Cells were propagated under the optimized medium with pH 10 and incubated in shaking incubators with various rpm at 40°C for 24 h. Standard deviations (n = 3) are seen as error bars.

enhance protease production in several organisms [41,42]. In addition, Ba ion enhanced protease production by *Bacillus* sp. [43]. It has been reported that these metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature [17]. On the other hand, supplementation of the medium with Zn, Cu, Fe, and Co caused severe inhibition of protease production by NPST-AK15, particularly at high cation concentrations.

3.2.5. Effect of incubation temperature, media pH, and aeration level

The effects of growth temperature, media pH and culture aeration are critical parameters affecting the bacterial growth and enzyme production [28,37]. Therefore, cell growth and alkaline protease production by strain NPST-AK15 were studied at various growth temperatures (30–60°C). The optimum temperature for both alkaline protease production and cell growth was found to be at 40°C (Fig. 8a). There was a drastic decrease in the enzyme production at higher growth temperatures that the enzyme yield decreased to 26.2 and 10.1% of the maximum production at 45°C and 50°C, respectively. *Bacillus* sp. NPST-AK15 can grow up to 55°C with no growth at 60°C, indicating that this bacterium is a thermotolerant organism.

With respect to pH, strain NPST-AK15 could grow and produce alkaline protease over a wide pH range from 7 to 12, with maximal growth and enzyme production observed at pH 11 (Fig. 8b). At pH 5–7, the bacterial growth and protease production were significantly reduced, which indicated the importance of growth pH in metabolic reactions which lead to the alkaline protease production in this bacterial strain. In addition the requirement of alkaline pH for optimum growth and protease production, clearly suggested an alkaliphilic nature of the organism and enzyme [15,16]. The optimum pH range between 9 and 10 for growth and protease production is common among alkaliphilic and haloalkaliphilic organisms [15,26,27,28].

Culture aeration is one of the most important parameters affecting microbial growth and enzyme production. The effect of the aeration level on bacterial growth and protease production by strain NPST-AK15 was investigated by incubating the cultures at various shaking speeds ranging from 0 to 250 rpm. As shown in Fig. 8c, the growth was drastically affected under static conditions and due to poor growth; protease production was reduced to less than 3% of the maximal enzyme yield. Both bacterial growth and enzyme production increased with an increasing aeration of up to 200 rpm, indicating the aerobic nature of NPST-AK15 and the importance of high-level aeration for alkaline protease production by this isolate. This finding was in agreement with that reported for Halophilic Bacterium MBIC3303, that its growth was completely reduced under static condition [17].

3.3. Growth kinetics and proteases production of strain NPST-AK15

The production of alkaline proteases by strain NPST-AK15 was investigated during 48 h of cultivation under the optimized medium compositions and conditions. As shown in Fig. 9 after about 4 h of lag phase, the growth was exponential up to 26 h followed by a stationary phase. Alkaline protease secretion was coherent with the growth pattern, and increased with the increasing growth, which started at the beginning of the exponential phase (8–10 h), and was maximal in the mid stationary phase with the highest activity recorded at 36 h (1263.3 U/mL). After that, the enzyme production remained nearly constant at the maximal level along with the stationary phase of up to 40 h. This secretion pattern of alkaline protease is quite similar to haloalkaliphilic *Bacillus* sp. Po2 [24] and alkaliphilic *Bacillus* sp. B001 [25], where maximal protease production was detected at the stationary phase. However, protease section by *B. pumilus* MCAS8 was found to be at late stationary phase (48 h) [14]. These findings on the production of the enzyme during stationary phase clearly suggest the prominent role of extracellular proteases in ecological sustenance, metabolism and survival of this organism [17,24].

4. Conclusion

A new potent alkaline protease producing halotolerant alkaliphilic isolate NPST-AK15 was isolated from hyper saline soda lakes of Wadi El-Natrun Valley, located in northern Egypt. The organism was affiliated to *Bacillus* sp. based on biochemical tests and 16S rRNA gene analysis. The characterization of NPST-AK15 indicated that this bacterium is halotolerant, thermotolerant and alkaliphilic in nature. Optimization of various fermentation parameters resulted in an increase of enzyme production yield by about 22.8 fold, indicating the significance of optimization of the fermentation process to obtain commercial yield of the enzyme. Alkaline protease secretion was coherent with the growth pattern, showing maximum enzyme production in mid stationary phase. NPST-AK15 and its extracellular alkaline protease with salt, pH, and temperature tolerance signify their potential applicability in the laundry industry and other applications of alkaline protease. Complete purification and characterization of alkaline protease from NPST-AK15 are in progress to be published.

Conflicts of Interest Statement

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

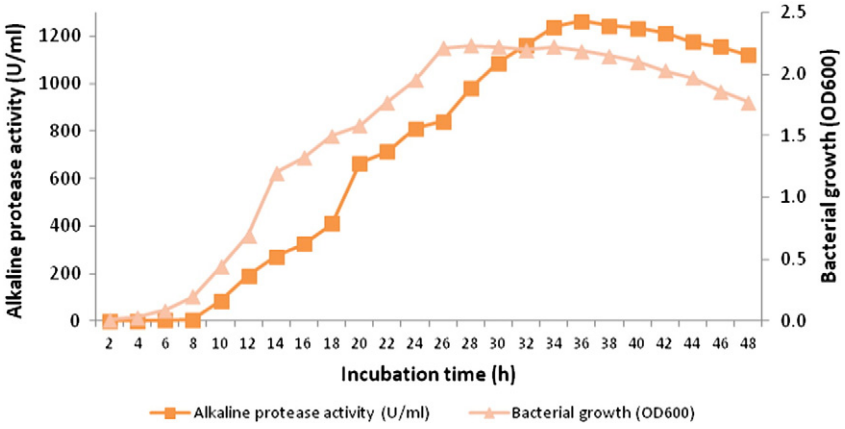

Fig. 9. Growth kinetics and alkaline protease production from strain NPST-AK15. Cells were grown in the optimized alkaline production medium and conditions, at pH 11 for 48 h at 40°C and 150 rpm. Samples were withdrawn at 2 h interval for the determination of cell growth. Standard deviations (n = 3) were in a range of 1 to 3%.

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