Original Article

ALKALINE PROTEASE FROM A NEW HALOTOLERANT ALKALIPHILIC *Bacillus agaradhaerens* STRAIN AK-R ISOLATED FROM EGYPTIAN SODA LAKES

PROTEASE ALCALINA DE UMA NOVA ESTIRPE AK-R HALOTOLERANTE E ALCALÓFILA DE Bacillus agaradhaerens *ISOLADA A PARTIR DOS LAGOS ALCALINOS EGÍPCIOS*

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ABSTRACT: Alkaline proteases are hydrolytic enzymes that cleave peptide bonds in proteins and peptides in alkaline conditions, which occupy a pivotal importance with respect to their industrial applications. This study aimed to isolate new alkaline protease producing alkaliphilic bacteria from Egyptian soda lakes and optimize the fermentation process to enhance the enzyme production. The extensive screening process of the samples collected from Egyptian soda lakes resulted in isolation of a potent alkaline protease producing alkaliphilic strain AK-R. The isolate was identified as *Bacillus agaradhaerens* strain AK-R based on 16S rRNA gene analysis (99%). Wheat bran and gelatin supported maximum alkaline protease production as carbon and nitrogen sources, respectively. Strain AK-R is halo-tolerant thermo-tolerant alkaliphilic bacterium in nature, as it can grow over a wide range of NaCl concentrations (up to 25%) and up to 55 °C, with maximal growth and enzyme production at 2.5-5%, and pH 11 at 35 °C. Among the tested cations, only Mg^{2+} and Ca^{2+} ions significantly enhanced the enzyme production by about 1.2, and 1.3 fold compared to control, respectively. Alkaline protease secretion was coherent with the growth pattern, reaching maximal yield after about 32 h (mid stationary phase). In conclusion a new halo-tolerant thermo-tolerant alkaliphilic alkaline protease producing *Bacillus agaradhaerens* strain AK-R was isolated from Egyptian soda lakes. Optimization of the nutritional and cultivation conditions resulted in increase of enzyme yield by 20 fold. Strain AK-R and its extracellular alkaline protease with salt, pH and temperature, tolerance signify their potential application in laundry and pharmaceuticals industries.

KEYWORDS: *Bacillus agaradhaerens*. Soda lakes. Alkaline protease. Enzyme production. 16S rDNA. Fermentation

INTRODUCTION

Proteases are hydrolytic enzymes that cleave peptide bonds in proteins and peptides that occupy a pivotal importance with respect to their commercial and industrial applications (JOSHI et al., 2013). Within the enzyme market, proteases alone contribute approximately 60% of the total sales in the world; and microbial proteases constitute approximately 40% of the total worldwide production of enzymes (JAIN et al., 2012; JOSHI et al., 2013). Alkaline proteases, characterized by high activity and stability in high alkaline range, have various industrial applications including leather, pharmaceuticals, foods, diagnostic reagents, soy processing, peptide synthesis, re-cycling of X-ray film for silver extraction, and bioconversion of chitinous materials (SHAH et al., 2010; JELLOULI et al., 2011; JOSHI et al., 2012). However, the main application of alkaline proteases is in detergent industry, accounting for approximately 30% of the total world enzyme production, which is due to the alkaline pH of laundry detergents ingredients (HADDAR et al., 2009a). Alkaline proteases are used in detergents formulations, with other hydrolytic enzymes, as cleaning additives to facilitate the breakdown and release of proteins (JAIN et al., 2012). The increased commercial demand for highly active alkaline proteases with high specificity and stability continues to stimulate the search for new enzymes (VIJAYARAGHAVAN; VINCENT, 2012). As the origin of the alkaline protease is the key factor in determining its activity and properties, scientists are searching nature for better performing alkaline proteases. When searching nature for novel alkaline protease, samples from unexplored environments should be used; and one of such environments is extreme environment in which extremphilic microorganisms are enriched, i.e. halophilies, alkalophiles, thermophiles and others (HORIKOSHI et al., 2011).

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Alkaliphilic bacteria are group of microorganisms that grow better in alkaline environments, making them candidate strains for exploration of novel alkaline proteases for various biotechnological potential (JAYAKUMAR et al., 2012; GOHEL; SINGH, 2015). Soda lakes are one of the main natural habitats of alkaliphilic bacteria, representing the major types of naturally occurring highly alkaline environments with pH >11 (HORIKOSHI, 1999). One of those environmental niches, which have not been studied in details, is hyper saline soda lakes located in Wadi El-Natrun valley (Egypt) (HORIKOSHI, 1999). However, the low productivity of enzymes and metabolites from extremophiles represent one of the major bottlenecks in their industrial applications, thus it is very important to optimize production medium and cultivation conditions in order to obtain high and commercial yields of alkaline protease (PATEL et al., 2005; PATEL et al., 2006). The present study focused on isolation of new alkaline producing alkaliphilic bacteria from Wadi El-Natrun soda lakes and optimization of the fermentation process in order to enhance the enzyme production.

MATERIAL AND METHODS

Collection of Soil and Water Samples

Water and sediment samples were collected from different hyper saline soda lakes located Wadi El-Natrun valley. This valley is an elongated depression approximately 90 km northwest of Cairo (Egypt) that extends in a northwest by southeast direction between latitudes 30°15' north and longitude 30°30' east. The bottom of Wadi El-Natrun valley is 23 m and 38 m below sea level and water level of Rosetta branch of the Nile, respectively, with average length and width of 60 km and 10 km, respectively (TAHER, 1999). The collected samples were kept at 4 °C, and transferred within few days to the laboratory at King Saud University (Riyadh, Saudi Arabia).

Isolation of alkaline protease producing alkalipilic bacteria

Alkaline protease producing alkalipilic bacteria were isolated from the collected samples using Horikoshi-I alkaline medium with some modification (HORIKOSHI, 1999). The modified Horikoshi-I alkaline medium (pH 10.5) contained: glucose (10 g/L), yeast extract (5 g/L), peptone (5 g/L), K_2HPO_4 (1 g/L), $Mg_2SO_4.7H_2O$ (0.2 g/L), NaCl (50 g/L), Na_2CO_3 (10 g/L), agar (15 g/L), in addition to 10% (w/v) skim milk as an indicator of alkaline protease production (SUNDARARAJAN et

al., 2011). Sediment samples were suspended and serially diluted in glycine-NaOH buffer (50 mM, pH 10). Aliquots (0.2-0.5 mL) of different dilutions were spread on the alkaline agar medium and incubated for several days at 30 °C, 40 °C, 50 °C, and 60 °C. 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 protease activity (SUNDARARAJAN et al., 2011; VIJAYARAGHAVAN; VINCENT, 2012). The alkaline protease producing alkaliphilic isolates were sub cultured several times in fresh agar plates until single homogeneous colonies were obtained; and glycerol stocks of each strain were prepared and stored at -80 °C.

Bacterial identification

The alkaline protease producing bacterium was identified through sequencing and analysis of 16S rRNA gene as previously reported (LANE, 1991). Total bacterial DNA was extracted from overnight culture using DNeasy Blood& Tissue Kits (Qiagen) according to the manufacturer's instructions. Universal 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 (LANE, 1991). The PCR reaction contained (50 μL): 18 μl nuclease-free water, 25 μL GoTaq® Green Master Mix (2×) (Promega), 1 μL 16F27 (10 μM), 1 μL 16R1525, and 5 μL DNA template (200 ng). The PCR reaction was run for 35 cycles under the following thermal profile in a DNA thermal cycler: 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 product was run in 1.5% agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen,) according to the manufacturer's instructions. The purified 16S-rDNA amplicon was sequenced using an automated sequencer (Macrogen, Korea). The obtained sequence was aligned with the reference 16S-rDNA sequences available in National Centre for Biotechnology information (NCBI) homepage using the BLAST algorithm.

Production of alkaline protease

The alkaline medium used for bacterial isolation, without skim milk and agar, was used for alkaline protease production by the selected isolates. A loopful of the isolates culture from agar plates

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was inoculated into 50 mL-glass tube containing 5 ml of the liquid production medium, and incubated overnight at 40 °C and 150 rpm. This culture was then inoculated into 250 mL capacity Erlenmeyer flask containing 50 mL of the same medium and incubated at 40 °C for about 24 h. After the incubation period the culture was centrifuged at 10000 rpm for 15 min at 4 °C to remove the cells and any insoluble materials. The cell-free supernatant was used to measure the alkaline protease activity as described below.

Assay of alkaline protease

Proteolytic activity was assayed by a modified method of Kunitz (KUNITZ, 1947). A 0.5-mL of suitably diluted culture supernatant was mixed with 0.5 mL of 50 mM glycine–NaOH buffer (pH 10) containing 1% (w/v) casein and 10 mM $CaCl_2$; and incubated at 50 °C for 20 min. Then, the reaction was terminated by addition of 0.5 mL of trichloroacetic acid (20%, w/v), and the mixture was allowed to stand at room temperature for 15 min before centrifugation at 10000 g for 15 min to remove the precipitate. The acid-soluble materials were estimated using Lowry method (LOWRY et al., 1951). 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.

Optimization of alkaline protease production
Effect of carbon source

The influence of different carbon sources on the bacterial growth and alkaline protease production by the selected isolate were investigated by replacement of glucose in the production medium with other carbon sources (1%, w/v) including: galactose, fructose, xylose, glucose, lactose, sucrose, starch, and wheat bran. Carbon sources were autoclaved separately and added to the medium on an equal carbon basis. In addition, the effects of different concentrations of the best carbon source, supporting maximal enzyme production, in the range of 0 to 2.5% were also investigated. The growth and enzyme activity were determined after 24 h of incubation at 40 °C, in shaking incubator (150 rpm).

Effect of nitrogen source

Effect of different nitrogen sources on the bacterial growth and enzyme production by the alkaline protease producing isolate was investigated

by substituting peptone and yeast extract in the production medium (Horikoshi-1) with different sources of organic and inorganic nitrogen sources at concentration of 0.5% (w/v). Organic nitrogen sources included peptone, yeast extract, trypton, alkali soluble casein, insoluble casein, skim milk, gelatin, and beef extract; while inorganic nitrogen sources included ammonium nitrate, ammonium sulphate, sodium nitrate, and urea. Furthermore, the effects of different concentrations (0-2%) of nitrogen source that support maximum enzyme yield were investigated. Bacterial growth and enzyme activity were measured after 24 h incubation at 40 °C in orbital shaking incubator (150 rpm).

Effect of salinity and cations

In addition to carbon and nitrogen source testing, the effects of NaCl concentration ranged from 0 to 25%, and various metal ions, including Mg^{2+}, Mn^{+2}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, and Ba^{2+} at concentrations of 1 mM, 5 mM, and 10 mM, on the bacterial growth and alkaline protease production was investigated, keeping the other parameters constant. The growth and enzyme activity were measured after 24 h incubation at 40 °C in orbital shaking incubator. All experiments and enzyme assays were carried out in triplicate and the mean values were reported.

Effect of temperature, pH, and aeration

Influence of incubation temperature on the cells growth and enzyme production by the selected strain was investigated by varying the growth temperature in the range of 30 to 55 °C, keeping the other parameters constant. Similarly, in order to investigate the influence of initial pH of the production medium on growth and alkaline protease production, the isolate was grown in medium with different initial pH values ranged from 5.0 to 12.0 at the optimum growth temperature. The bacterial growth and enzyme activity were measured as described above after 24 h incubation period. Furthermore, influence of the aeration level during fermentation on growth and protease production was investigated by incubating the culture in shaking incubators with different rpm values ranged from zero (static) to 250 rpm.

Bacterial growth versus alkaline proteases production

A loopful of the isolate culture from agar plate was inoculated into 50 mL-glass tube containing 5 mL of the liquid medium, and incubated overnight at 200 rpm and 35 °C. This culture was then inoculated into 500 mL capacity

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Erlenmeyer flask containing 250 mL of the same medium and incubated at 35 °C for 48 h. Samples (2 mL) were withdrawn at 2 h interval up to 48 h for measurement of cells growth and alkaline protease activity. The samples were centrifuged at 10000 rpm at 4 °C; and the pellets obtained were washed twice using Tris buffer (50 mM, pH 8) and resuspended in 1 mL of the same buffer. Absorbance was measured at 600 nm against 50 mM Tris Buffer (pH 7) as blank, was reported as growth of the organism. Alkaline protease activity was measured in the cell-free supernatant as described above. Triplicate of each time period was taken to calculate growth and enzyme activity and the mean values were reported.

Statistical analysis

All the experiments and assays were performed in triplicate; and the standard deviation for each experiment was calculated using SPSS 14.0 and are indicated in the figures as error bars (JAYAKUMAR et al., 2012).

RESULTS AND DISCUSSION

Isolation of the microorganism

Isolation of alkaline protease producing alkaliphilic bacteria was carried out using rich alkaline agar medium containing skim milk. Formation of clear zone around the colonies was considered as indication of alkaline protease production. After the incubation period, several morphologically distinct colonies showed zone of hydrolysis indicating production of extracellular alkaline protease and subsequent casein hydrolysis (**Figure 1**). Individual positive isolates were purified through repeated streaking on fresh agar plates. It has been established that there is not necessarily good correlation between zones of clearing around colonies on milk-agar plates and levels of proteinase activity (COOLBEAR et al., 1991). Therefore, all the positive isolates were cultivated in the alkaline production medium and the proteolytic activity was measured. The results indicated that strain AK-R showed high alkaline protease activity (36.3 U/mL) and was selected for further investigation.
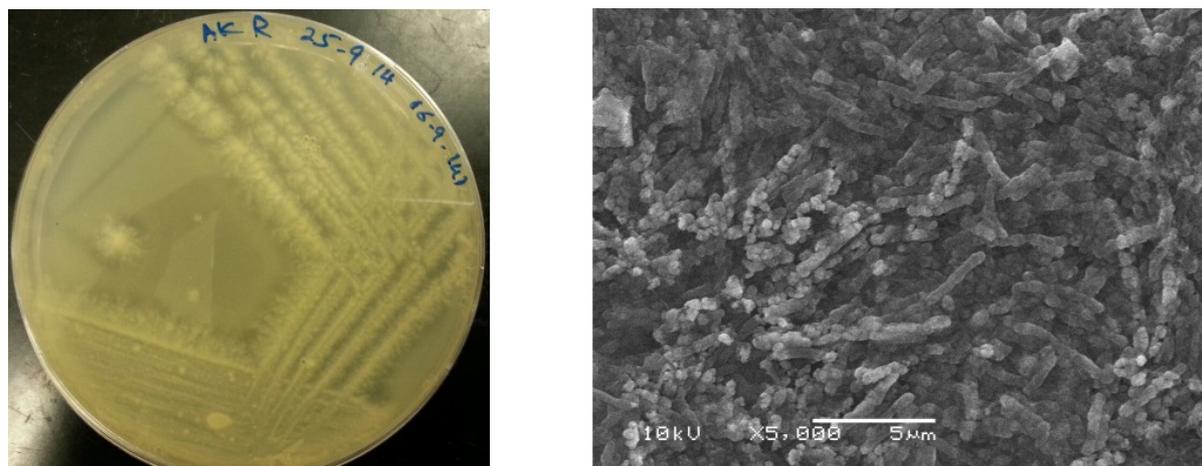

Figure 1. A; Isolation of alkaline protease alkaliphilic bacteria using modified Horikoshi-I agar plate containing 10% (w/v) skim milk. The clear zone indicated casein hydrolysis due to alkaline protease production. **B**: Scanning Electron Microscope (SEM) image of strain AK-R.

The alkaline protease producing strain AK-R, grown in the alkaline agar medium showed white rhizoid colonies with filamentous margin which characterized by its adhesion to agar (Figure 1). Culture grown in alkaline liquid medium for 24 h showed motile short rod-shaped cells with length of about 2.5 μm and 0.3 μm in diameter. Cells existed as single, paired or short chain. Strain AK-R was able to grow in the presence of NaCl up to 25 % (w/v), showing growth at 30 ℃ to 55 ℃ but no growth was observed at 60 ℃ after 48 h incubation period. It could grow at pH value from 8 to 12, with

poor growth detected at pH 7 after 48 h incubation. In order to determine the phylogenetic position of strain AK-R, 16S rDNA sequence analysis was performed. A total of 1462 nucleotides of strain AK-R 16S rRNA gene were determined. It showed highest similarity with *Bacillus agaradhaerens* DSM 8721 (99%) and was designed as *Bacillus agaradhaerens* strain AK-R. Figure 2 shows the phylogenetic tree of strain AK-R and its closest bacteria based on 16S rRNA gene sequences. The sequence of strain AK-R 16S rRNA gene was deposited in GenBank with accession number

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KP316022. *Bacillus agaradhearens* was firstly described as a new alkaliphilic *Bacillus* species by Nielsen et al (NIELSEN et al., 1995). A variety of polysaccharides-degrading enzymes including cellulase, xylanase, xyloglucanase, mannanase, and pectate lyase from *B. agaradhaerens* have been studied for other applications (SCHULEIN, 1999; XU, 2000). In addition, new isolate identified as *Bacillus agaradhaerens* strain nandiniphanse5 has proved to produce alkaline protease but not yet investigated (PHANSE et al., 2013).
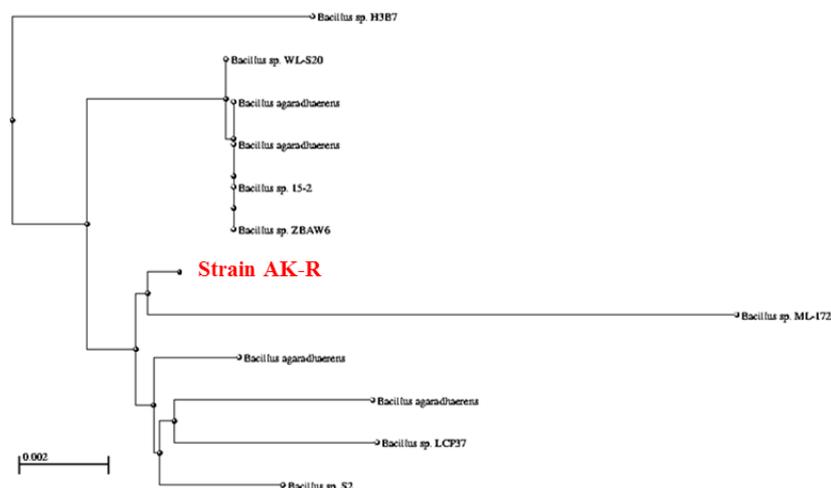
Figure 2. Neighbor-joining phylogenetic tree of the isolated strain AK-R and its closest bacteria based on 16S rRNA gene sequences.

Production optimization of alkaline proteases

It is clear from the literature that production of extracellular protease is highly influenced by media components including carbon and nitrogen source, presence of simple sugars, and salts. In addition to the fermentation conditions including culture aeration level, media pH, growth temperature, and incubation time (GOUDA, 2006; MEENA et al., 2013). Therefore it is essential to optimize the fermentation process in order to obtain high and commercial yields of alkaline protease by the new isolate of *Bacillus agaradhaerens* strain AK-R.

Effect of carbon source

Influence of different carbon sources, including mono-, di- and polysaccharides, in addition to wheat bran on strain AK-R growth and alkaline protease production was investigated. Despite all tested carbon sources supported the cells growth; various carbon sources were found to have different impact on the production of protease by strain AK-R (Figure 3). The results revealed that wheat bran was the best carbon source that enhanced the alkaline protease production by about 3.8 fold compared to the control as carbon source. This was followed by fructose, starch, xylose, and lactose, respectively. However, glucose, galactose, maltose, and sucrose caused severe reduction of protease production by strain AK-R, with production yield of 26.6%, 24.4%, 15.5%, and 13.3% in comparison to maximal yield obtained using wheat bran, respectively. The production of alkaline protease was further monitored at various concentration of wheat bran, as the best carbon source.

As shown in Figure 4 both cells growth and alkaline protease production were increased by increasing the wheat bran concentration, reaching maximal bacterial growth and enzyme production at 1.5%. This result is in accordance with that reported for alkaliphilic *Bacillus* sp. MIG (GOUDA, 2006), *Pseudomonas aeruginosa* (MEENA et al., 2013), and *Bacillus cereus* strain CA15 (MEENA et al., 2013), where wheat bran supported maximum alkaline protease production. The use of wheat bran in the production medium is very important, because it is one of the cheap and readily available carbon sources. The estimated cost of wheat bran was found to be 0.002 $ for one liter production medium (GOUDA, 2006). On the other hand, other carbon sources were reported for maximal protease production based on the source organism (JOSHI et al., 2008; SHIVANAND; JAYARAMAN, 2009; JAYAKUMAR et al., 2012). Less production of alkaline protease by *B. agaradhaerens* strain AK-R in the presence of simple sugar like glucose, galactose, sucrose, and maltose is mostly due to catabolite repression of protein biosynthesis (KANEKAR et al., 2002; DENG et al., 2010).
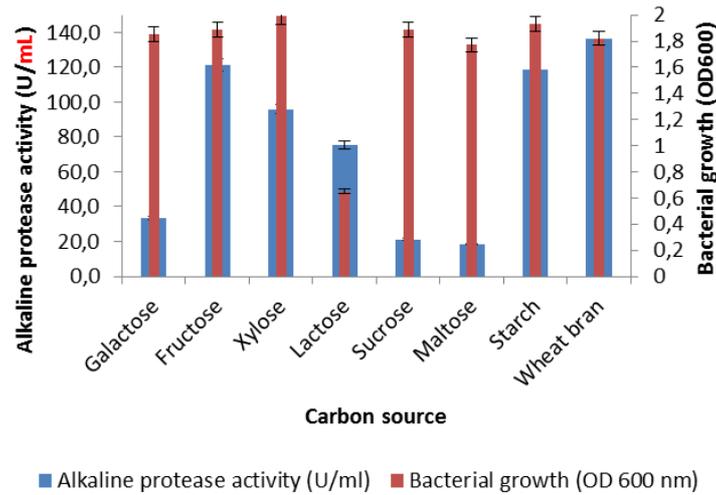
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Figure 3. Influence of various carbon sources on strain AK-R growth and alkaline proteases production by strain AK-R. Bacterial growth and alkaline protease activity were determined after incubation for 24 h at 40 °C under shaking conditions (150 rpm). Standard deviations (n=3) are reported as error bars.
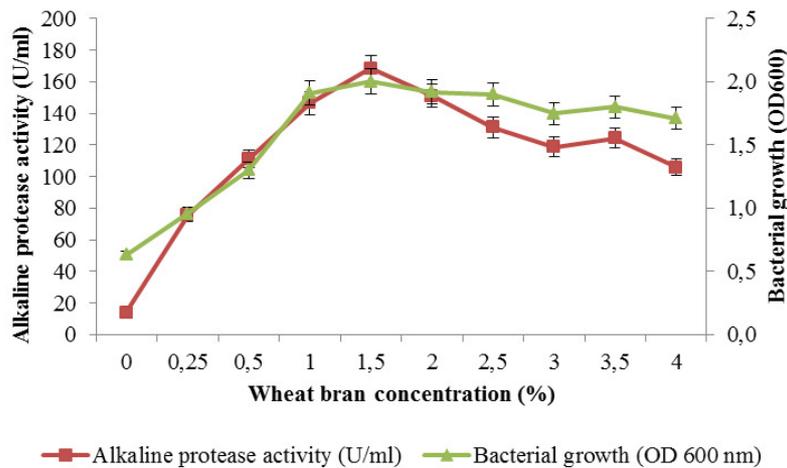

Figure 4. Effect of wheat bran concentration on growth and alkaline proteases production by strain AK-R. Cells growth and alkaline protease activity were determined after incubation for 24 h at 40 °C under shaking conditions (150 rpm). Error bars represent the standard deviations (n=3).

Effect of nitrogen source

The effect of different nitrogen sources on *B. agaradhaerens* strain AK-R growth and alkaline protease production was evaluated using wheat bran as a carbon source. The results shown in Figure 5 indicated that several organic nitrogen sources supported both bacterial growth and alkaline protease production, showing maximum enzyme production in medium containing gelatin, followed by skim milk, and alkali soluble casein, that the enzyme yield increased by about 1.6, 1.3, and 1.2 compared to control, respectively. Regarding inorganic nitrogen source, while urea showed less growth and protease production than the control, ammonium sulphate and ammonium nitrate were unfavorable for either cells growth or alkaline protease production with enzyme yield of about 9.0% and 10.9% of the maximum production, respectively.
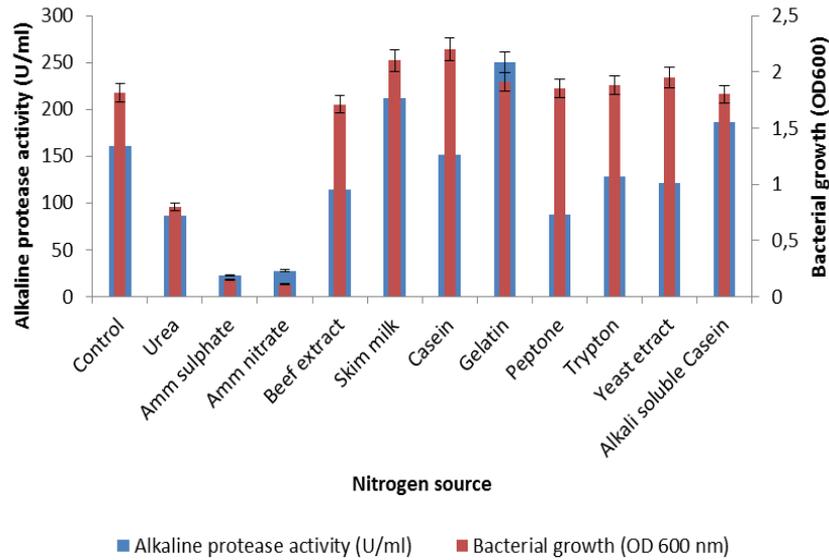
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Figure 5. Influence of nitrogen sources on bacterial growth and alkaline protease production by strain AK-R, using wheat bran as a carbon source. Error bars represent the standard deviations (n=3).

The production of alkaline protease by strain AK-R was further monitored at various concentration of gelatin. Both bacterial growth and enzyme production was increased by increasing the gelatin concentration, showing maximum enzyme yield at concentrations of 1% (Figure 6). Further increase of gelatin led to slight decrease of the enzyme production, with no effect on the bacterial growth. This result was in agreement with that reported for other *Bacillus* sp where alkaline protease production was maximal using gelatin and significantly reduced using inorganic nitrogen sources (SHAH et al., 2010; RAJA et al., 2012).

However, other organic nitrogen sources were found to support protease production in other microorganisms including yeast extract (DENG et al., 2010); beef extract (KUMAR et al., 2014), casamino acids (JAIN et al., 2012), skim milk (GOUDA, 2006), peptone (OSKOUIE et al., 2008), and others (SRIVIDYA; MALA, 2011). Slight decrease of alkaline protease production by strain AK-R at high gelatin concentration may due to repression role of excessive amino acid and ammonium ions in alkaline protease production (JOO et al., 2003; CHU, 2007).
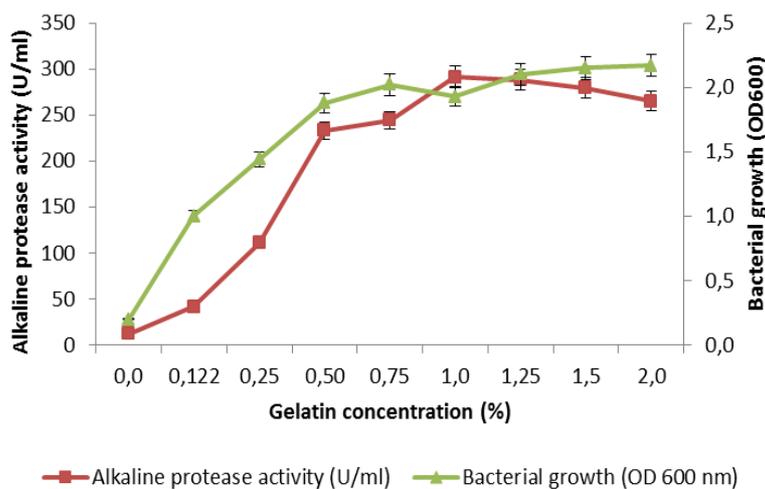

Figure 6. Influence of gelatin concentration on bacterial growth and production of alkaline proteases by strain AK-R. Bacterial growth and alkaline protease activity were determined after incubation for 24 h at 40 °C under shaking conditions (150 rpm). Standard deviation (n=3) are indicated as error bars.

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Effect of salinity

In order to investigate the effect of salinity on the cells growth and alkaline protease production by *B. agaradhaerens* strain AK-R, the production medium was supplemented with various concentration of NaCl. The results revealed that strain AK-R can grow over a wide range of NaCl concentrations from 0 to 25%, showing maximal growth and enzyme production at NaCl concentration of 2.5-5.0%. While at 7.5% NaCl there was slight decrease in the protease production to about 79 % of the maximal yield, drastic decrease in enzyme production occurred at 10% NaCl to about 20% of the maximal protease production (Figure 7). The ability of strain AK-R to grow over

a wide range of NaCl concentrations (up to 25%), with maximum cells growth at 2.5-5% NaCl, indicated the halo-tolerance nature of this organism (JAIN et al., 2012). Studies on the effect of salinity on 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 decrease of growth rate causing reduced enzyme production (TANG, X. M. et al, 2004). Strain AK-R and its extracellular alkaline protease with salt tolerance signify their potential application in laundry industry (HADDAR et al., 2009b).
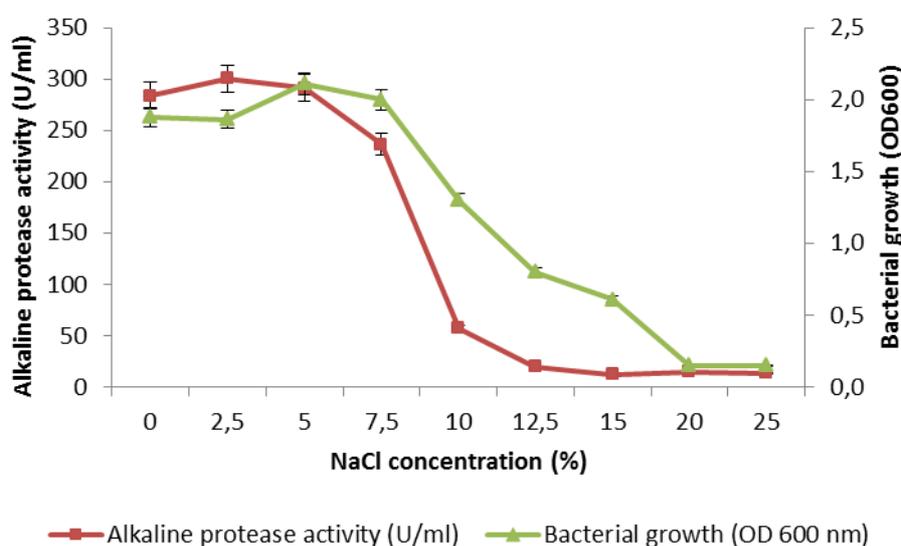

Figure 7. Influence of sodium chloride concentration on growth and production of alkaline proteases by strain AK-R. Bacterial growth and alkaline protease activity were determined after incubation for 24 h at 40 °C under shaking conditions (150 rpm). Standard deviation (n=3) are indicated as error bars.

Effect of various metals

In order to investigate the influence of various metals on strain AK-R growth and alkaline protease yield, the production medium was supplemented with different concentrations of metals salts. Among the tested cations, only Mg^{2+} and Ca^{2+} ions significantly enhanced the enzyme production by about 1.2, and 1.3 fold compared to the control, respectively (Figure 8). On the other hand, while Ba^{2+} caused significant decrease in the protease production, Fe^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and ions led to drastic inhibition of the enzyme production to less than 6% of the yield compared to the control. This result is in agreement with that reported for

production of alkaline protease production by other *Bacillus* sp (NADEEM et al., 2007; UYAR et al., 2011) and alkaliphilic Haloalkaliphilic Bacterium S-20-9 (JOSHI et al., 2008), where Ca^{2+}, Mg^{2+} ions significantly enhanced the enzyme production. These cations probably protect the enzyme against thermal denaturation and therefore maintain the active conformation of the enzyme at high temperature (CHU, 2007). In addition; the inhibitory effect of other ions on alkaline protease is mostly attributed to metal catalyzed oxidation of amino acid residues essential to the enzyme activity (TANG et al., 2004).
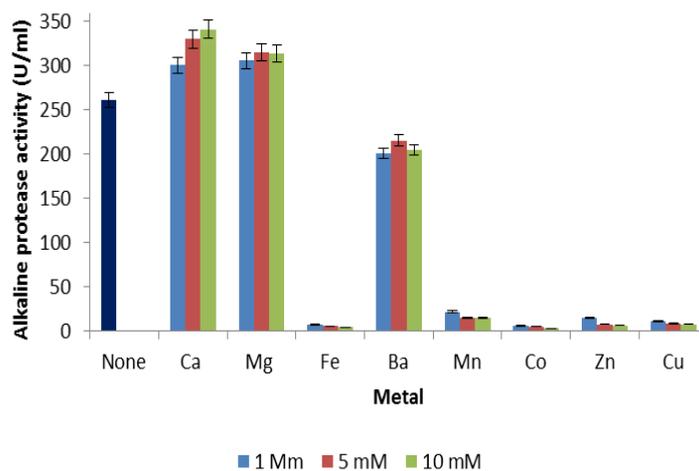
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Figure 8. Influence of various cations on alkaline protease production by *Bacillus agaradhaerens* AK-R. The medium was supplemented with various metals at concentrations of 1 mM, 5 mM and 10 mM. Standard deviation (n=3) are indicated as error bars.

Effect of incubation temperature, media pH, and aeration level

The incubation temperature is an important factor affecting the bacterial growth and enzyme production (CHU, 2007; SRIVIDYA; MALA, 2011). The cells growth and alkaline protease production by strain AK-R were studied at various growth temperatures ranged from 30 °C to 55°C.

Maximum bacterial growth and enzyme production were seen at 35 °C (Figure 9). At higher temperature enzyme production decreased to 65.2% and 36.7% of the maximum yield at growth temperatures of 40 °C and 45 °C, respectively. *B. agaradhaerens* strain AK-R can grow up to 55 °C with no growth seen at 60 °C, indicating that this organism is thermo-tolerant bacterium (SRIVIDYA; MALA, 2011).
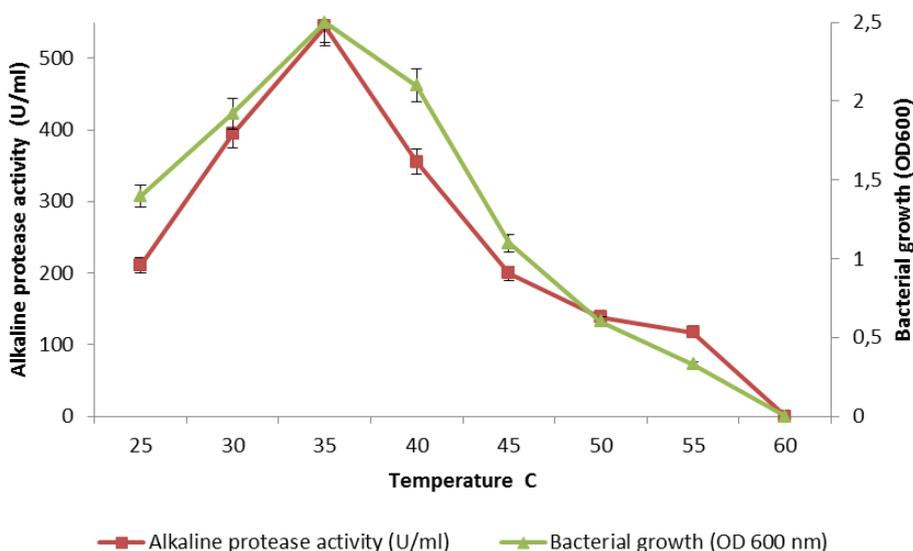

Figure 9. Influence of incubation temperature on bacterial growth and alkaline protease production by strain AK-R. Cells growth and alkaline protease activity were determined after incubation for 24 h at 40 °C under shaking conditions (150 rpm). Error bars represent the standard deviations (n=3).

The effects of initial medium pH values on the cells growth and protease production were investigated and the results are shown in Figure 10. *B. agaradhaerens* strain AK-R 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. However, enzyme production was decreased to about 49.5% of the maximum yield at pH 12. In addition, no growth was seen at pH 5 or 6. Requirement of alkaline pH for optimum growth and protease production obviously indicated the alkaliphilic nature of this

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bacterium and its enzyme. Alkaliphilic microorganisms are characterized by optimum

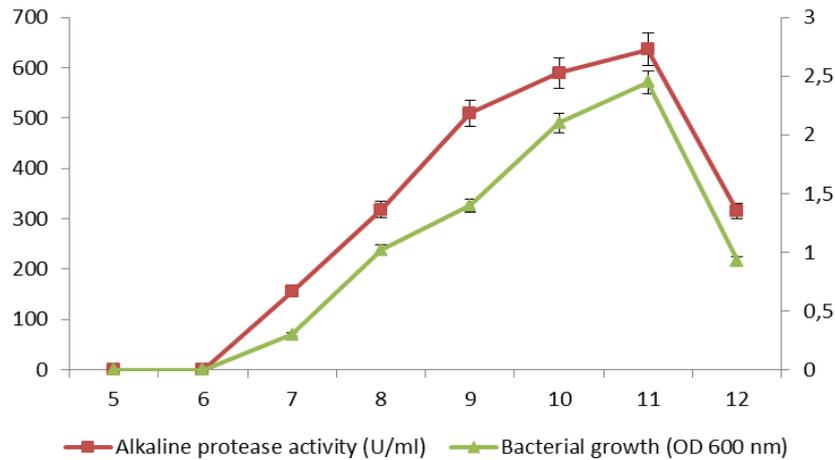growth pH ranged from 9 to 11 (HORIKOSHI et al., 2011).

Figure 10. Influence of the initial pH of the culture medium on bacterial growth and alkaline protease production by strain AK-R. Error bars represent the standard deviations (n=3).

Finally, the effect of culture aeration level on bacterial growth and protease production by strain AK-R was studied by incubating the cultures at various shaking speeds ranged from 0 to 250 rpm. The growth was significantly affected under static conditions and due to poor growth; protease production was reduced to about 16.7% of the maximal enzyme yield (Figure 11). Both bacterial growth and enzyme production increased with increasing aeration level up to 200 rpm. Sever reduction of cells growth and alkaline protease

production under static condition indicated the aerobic nature of strain AK-R and the importance of high aeration level for alkaline protease production by this isolate. This result was relatively similar to that that reported for Halophilic Bacterium MBIC3303 (JOSHI et al., 2008) and *Bacillus mojavensis* (BEG et al., 2002; JOSHI et al., 2008), where the bacterial growth was completely reduced under static condition and increased significantly by increasing of the aeration level.
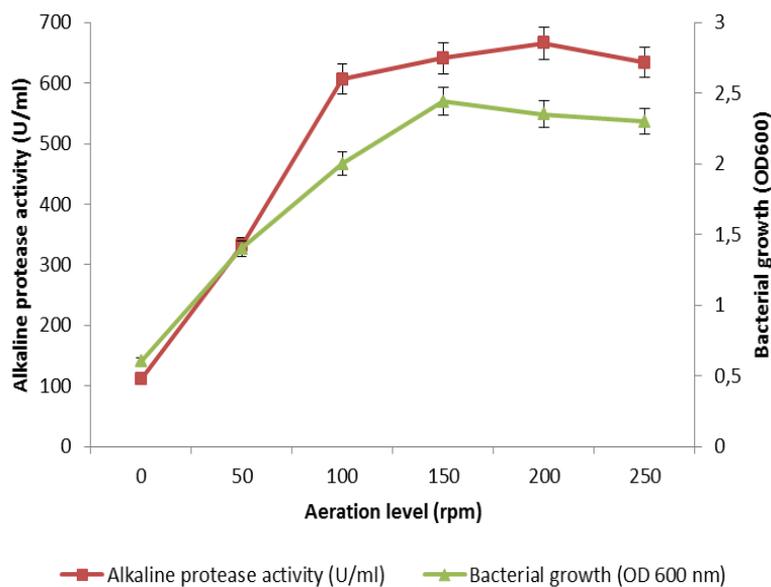

Figure 11. Influence of aeration level (shaking rpm) on cell growth and alkaline protease production by strain AK-R. Cells were propagated under the optimized medium with pH 11 and incubated in shaking incubators with various rpm at 35 °C for 24 h. Standard deviations (n=3) are seen as error bars.

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Time course of bacterial growth versus and alkaline proteases production

B. agaradhaerens strain AK-R was grown under the optimized medium and culture conditions for 48 h; and both cells growth and alkaline protease production were measured 2 h intervals. As shown in Figure 12 strain AK-R entered into the exponential phase after about 8 h and stationary phase started after about 28 h. Production of extracellular alkaline protease was coherent with the cells growth, started shortly after the beginning of the exponential phase and reached maximum yield in the mid stationary phase with the highest activity detected after about 32 h (686.7 U/mL). The enzyme production thereafter remained nearly constant at maximal level along with the stationary phase up to 42 h. Production of the alkaline protease during the stationary phase indicated the significant role of extracellular proteases in metabolism and survival of this organism (JOSHI et al., 2008). This secretion pattern of alkaline protease is quite similar to that of haloalkaliphilic *Bacillus* sp. Po2 and alkalophilic *Bacillus* sp. B001 where maximal protease production was detected at the mid stationary phase (PATEL et al., 2005; RAJA; PRABHAHAR, 2012). However, protease section by *Bacillus pumilus* MCAS8 was found to be at late stationary phase (48 h) (JAYAKUMAR et al., 2012).
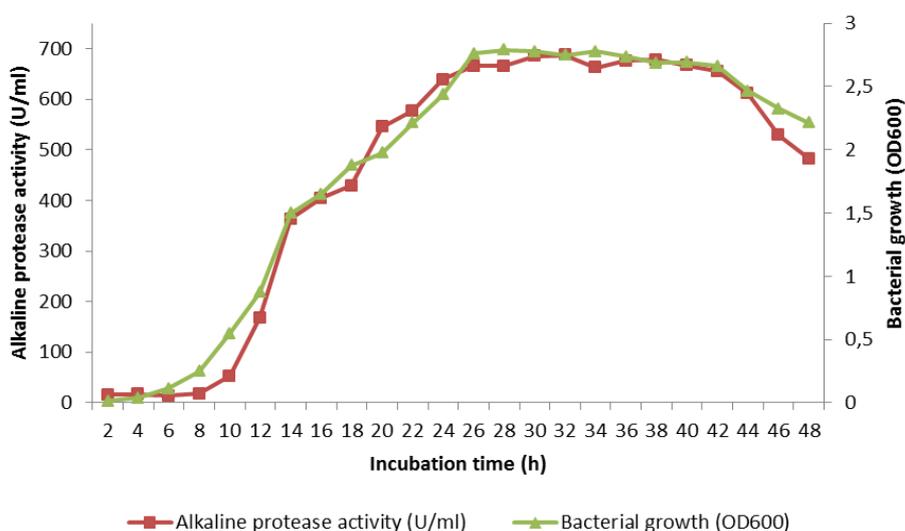

Figure 12. Time course of bacterial growth versus and alkaline proteases production by *Bacillus agaradhaerens* AK-R. Cells were grown in the optimized alkaline production medium and conditions, at pH 11 for 48 h at 35 °C and 200 rpm. Samples were withdrawn at 2 h interval for the determination of cell growth. Standard deviations (n=3) were in range of 1.2 to 3.5%

CONCLUSIONS

When searching nature for novel alkaline protease, samples from unexplored environments should be used; and one of such environments is hyper saline soda lakes in Wadi EL-Natrun valley.

The features of Wadi EL-Natrun valley created an ecosystem that considers as rich sources for isolation of alkaliphilic, haloalkalipilic and thermoalkaliphilic microorganisms.

A new potent alkaline protease producing alkaliphilic strain AK-R was isolated from the collected samples and identified as *Bacillus agaradhaerens* strain AK-R.

Extensive optimization of the nutritional and cultivation conditions of growth and alkaline protease production resulted in increase of enzyme yield by 20 fold, indicating significance of optimization of the fermentation parameters to obtain commercial yield of the enzyme.

B. agaradhaerens strain AK-R is halo-tolerant thermo-tolerant alkaliphilic bacterium with high alkaline protease production yield. Hence, this new isolate is a promising candidate for alkaline protease production for potential industrial applications. To best of our knowledge this is the first report about optimization of protease production from *Bacillus agaradhaerens*. Purification and characterization of alkaline protease from *B. agaradhaerens* strain AK-R are in progress to be published elsewhere.

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RESUMO: Proteases alcalinas são enzimas hidrolíticas que quebram ligações peptídicas em proteínas e peptídeos em condições alcalinas, o que ocupa uma importância fundamental em relação às suas aplicações industriais. Este estudo teve como objetivo isolar novas proteases alcalinas e produzir bactérias alcalófilas a partir dos lagos salgados alcalinos egípcios e otimizar o processo de fermentação para aumentar a produção de enzimas. O extensivo processo de triagem das amostras coletadas dos lagos salgados alcalinos egípcios resultou no isolamento de uma protease alcalina potente produzindo uma estirpe alcalófila AK-R. O isolado foi identificado como sendo a estirpe AK-R de Bacillus agaradhaerens baseado na análise de genes 16S rRNA (99%). O farelo de trigo e a gelatina suportaram a produção máxima de protease alcalina como fontes de carbono e nitrogênio, respectivamente. A estirpe AK-R é uma bactéria alcalófila halotolerante e termotolerante, pois pode crescer dentro de uma vasta gama de concentrações de NaCl (até 25%) e até 55ºC, com crescimento e produção de enzimas máximos a 2.5-5% e pH 11 a 35ºC. Dentre os cátions testados, somente os íons Mg^{2+} e Ca^{2+} aumentaram significativamente a produção de enzimas em cerca de 1.2 e 1.3 em comparação ao controle, respectivamente. A secreção de protease alcalina foi coerente com o padrão de crescimento, atingindo o rendimento máximo após 32h (fase estacionária média). Pode-se concluir que uma nova estirpe AK-R de Bacillus agaradhaerens halotolerante, termotolerante e alcalófila produtora de protease alcalina foi isolada a partir dos lagos salgados alcalinos egípcios. A otimização das condições de nutrição e cultivo resultou num aumento da produção de enzima em 20 vezes. A estirpe AK-R e a sua protease alcalina extracelular com tolerância ao sal, pH e temperatura tornam significantes as suas potenciais aplicações nas indústrias farmacêutica e de lavanderia.

PALAVRAS-CHAVE: Bacillus agaradhaerens. Lagos salgados alcalinos. Protease alcalina. Produção de enzimas. 16S rDNA. Fermentação.

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