Electronic Journal of Biotechnology 18 (2015) 236–243

HOSTED BY

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

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

Abdelnasser S.S. Ibrahim [a,b,*], Ali A. Al-Salamah [a], Yahya B. Elbadawi [a], Mohamed A. El-Tayeb [a], Shebl Salah Shebl Ibrahim [c]

[a] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[b] Department of Chemistry of Natural and Microbial Products, National Research Center, El-Buhouth St., Dokki, Cairo 12311, Egypt
[c] College of Science, Center of Excellency for Biotechnology, King Saud University, Riyadh 11451, Saudi Arabia

ARTICLE INFO

*Article history:*
Received 12 January 2015
Accepted 10 March 2015
Available online 11 April 2015

*Keywords:*
Alkaline protease
Alkalophiles
*Bacillus* sp.
Fermentation
Optimization

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

* Corresponding author.
*E-mail address:* ashebl@ksu.edu.sa (A.S.S. Ibrahim).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.04.001

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.