ALKALINE PROTEASE PRODUCTION BY BACILLUS SP. HAP-1, A HALOPHILIC BACTERIUM ISOLATED FROM A SOLAR EVAPORATION POND

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ABSTRACT

A halophilic alkaline protease producing bacterium was isolated by screening bacteria obtained from the salt samples collected from solar evaporated salt ponds of Vasai Road East, Mumbai, India, using skim milk agar. By the 16s r DNA sequencing and BLAST analysis, the organism was identified as Bacillus sp. HAP-1. Bacillus sp. HAP-1 was found to be growing at salt concentrations of 10-20%. The present study was conducted to isolate a halophilic alkaline protease producing bacteria which can be used in industries as their proteins can withstand high salt concentrations.

KEYWORDS: Alkaline protease, Bacillus sp. HAP-1, Halophiles.

INTRODUCTION

Halophiles are microorganisms that live and grow in high saline environments. The saline content in halophilic environment is usually 10 times the salt content of normal ocean water. For the halophiles to survive their cytoplasm must be isotonic with its environment. Usually halophiles adopt two strategies: compatible solute strategy and salt in strategy for surviving in saline environments (Kunte et al 2001). Depending upon the NaCl requirement for halophiles, they are classified as slightly halophilic (2-5%), moderately halophilic (5-20%) and extremely halophilic (20-30% NaCl) (DasSarma and Arora 2001). Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNAses, lipases, amylases, gelatinases and proteases) capable of functioning under conditions that lead to precipitation or denaturation of most proteins. All exoenzymes excreted by halophiles have to be active in the presence of the high salinities found in their
medium, even when the organisms that produce them maintain lower intracellular ionic concentration (Oren, 2002).

Proteases are proteolytic enzymes, functioning as molecular knives cutting long amino acid sequences into fragments, the process that is essential for the synthesis of all proteins, controlling their size, composition, shape, turnover and ultimate destruction (Seife, 1997). Microbial proteases are of three types; acidic, neutral and alkaline based on the optimum pH of their action. Among all proteases, alkaline proteases are robust in nature and are primarily used as detergent additives (Prakasham et al. 2006). Alkaline proteases occur widely in plants, animals and microorganisms. The increased interest in microbial protease was due to the inability of plant and animal protease to meet the current world demand (Kumar et al., 2008). They are used in all types of laundry detergents and in automatic dishwashing detergents, which function to degrade proteinaceous stains. They are also used for cleaning of membrane used in protein ultrafiltration (El Enshasy et al. 2008). Microbes serve as a preferred source of alkaline protease enzymes because of their rapid growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications (Anwar and Saleemuddin 1998; Gupta et al., 2002). As alkaline proteases have been found to be present in halophiles and it can be reasonably assumed that they possessed interesting properties which might be useful. However very few works have been reported on alkaline protease production from halophiles.

The present work was aimed at isolating a suitable halophilic bacterium for alkaline protease production in large scale.

MATERIALS AND METHODS

1. Isolation and culturing of halophiles

The salt samples were collected from the solar evaporated salt ponds of Vasai Road East, Mumbai, India. Halophilic bacteria were isolated by enrichment culture techniques. 5gm samples were inoculated into 100ml DSC-97 broth medium (Casamino acids- 7.5g, Yeast extract-10g, trisodium citrate-3g, KCl-2g, MgSO₄·7H₂O -20g, FeCl₂·4H₂O-0.036g, NaCl-200g, distilled water-1000 ml, pH-7.4) and incubated for 7days at 38˚C. After 7 days the subcultures were made on DSC-97 agar plates.
2. **Screening for Alkaline protease activity**

The purified cultures were screened for the production of alkaline protease by streaking them on alkaline skim milk agar plate (skim milk 1%, NaCl 10%, Agar 2% and pH 9). Colonies with surrounding zone of clearance were collected.

3. **Identification of bacteria**

   a. **Identification based on phenotypic properties**
   
   The bacterium was identified tentatively studying the cultural, morphological, biochemical and physiological properties.

   b. **Identification by 16S rDNA sequencing**
   
   Genomic DNA was isolated from the bacteria using Cell Lysis method and the 16S rDNA was amplified by Mycycler™ (Bio-Rad, USA), using the universal primers 27F (5'-AgA gTT TgA TCM Tgg CTC-3') and 1525R(5'-AAg gAg gTg WTC CAR CC-3') respectively.

   The 16SrDNA sequence data was subjected to BLAST analysis. For the phylogenetic analysis, the related sequences were retrieved from NCBI. The selected sequences were first aligned with ClustalW program using Bioedit software. The aligned data was used for further phylogenetic analysis using MEGA5.

4. **Protease production by solid substrate fermentation**

   10 gm of substrate was taken in a 500ml Erlenmeyer flask and was moistened with 20ml of moistening solution (K₂H₂PO₄-0.1%, NaCl-10%, MgSO₄-0.01% and NH₄NO₃-0.5%, pH 9). The content of the flask was mixed thoroughly and autoclaved at 121°C, 15 lbs pressure for 20 mins. Then the flask was cooled to room temperature and inoculated with 2 ml of 24 hrs grown bacterial culture with turbidity corresponding to MacFarland standard 0.5 (approximately 1.5×10⁸ CFU/ ml) and incubated at 38°C for 3 days in a humidified incubator.

5. **Protease extraction from bacterial bran**

   For the protease extraction from bacterial bran, a known quantity of bacterial bran was mixed with 50 ml distilled water and the flask was placed on the shaker for 1 hour at 38°C. The slurry was then squeezed through a muslin cloth. The culture filtrate was then centrifuged at 10,000 rpm for 20 mins. The supernatant was used as the crude alkaline protease preparation.

6. **Assay for proteolytic activity**

   The alkaline protease activity was assayed by the method of Meyers and Ahearn (1977) with some modifications. The protocol followed is given below.
The test contained enzyme-0.5ml, glycine-NaOH buffer (0.2M, pH 9)-0.5ml and casein solution (1% in 0.2M glycine-NaOH buffer) - 1ml, while in control instead of buffer TCA (5%)-0.5ml was added. Reaction mixture was incubated for 20 mins at 45°C. The reaction was terminated by adding 4ml of 5% TCA to both test and control preparations. The tubes were incubated for one hour at room temperature. Filtered through Whatman no.1 filter paper and the filtrate was collected and assayed for tyrosine concentration.

For colour development for the assay of tyrosine in the filtrate, 5ml of 0.4 M sodium carbonate and 0.5ml of Folin phenol reagent (1N) were added to 1ml of filtrate. Vortexed immediately and incubated for 20 mins at room temperature. OD was taken at 660 nm. Concentration of tyrosine in the filtrate was read from the standard curve for tyrosine already prepared.

One unit of enzyme activity was defined as the amount of enzyme liberating one microgram of tyrosine per minute per ml under the defined conditions. The dry weight of the bacterial bran was determined by drying it at 80°C to a constant weight. The protein was expressed as U/g DBB (dry bacterial bran).

I. RESULTS AND DISCUSSION

Of the bacterial strains which were protease positive, the isolate HAP-1 obtained from salt sample showed the largest zone of clearance. Colonies of HAP-1 on DSC-97 agar plates were flat, irregular, rough cream colored with lobate edges. In broth, growth was indicated by the formation of sediments and appeared slimy. The results of Gram staining showed that the HAP-1 was Gram positive rods with spores. The biochemical characters of the isolate is shown in table 1.

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Plate 1: Bacillus HAP1 strain in alkaline skim milk agar plate
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Table 1 Biochemical characteristics of the isolate HAP-1

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<td>MR test</td>
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<td>VP test</td>
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<td>Citrate utilization test</td>
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<td>Catalase test</td>
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<td>Urease test</td>
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<td>Nitrate reduction test</td>
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<td>Hydrogen sulphide production test</td>
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<td>Oxidase test</td>
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<td>Carbohydrate fermentation test</td>
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<tr>
<td>Mannitol</td>
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<td>Glucose</td>
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Table 2 Alkaline protease production by *Bacillus* sp. HAP-1 using different substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Alkaline protease production (U/g DBB)</th>
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<tbody>
<tr>
<td>Wheat bran</td>
<td>51.79</td>
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<tr>
<td>Rice bran</td>
<td>30</td>
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<tr>
<td>Coconut oil cake</td>
<td>45.66</td>
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<tr>
<td>Groundnut oil cake</td>
<td>24.49</td>
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Fig: 1 Phylogenetic analysis of 16s rDNA sequence of the isolate HAP-1.

The optimum salt concentration for the growth of bacterium was found to be 20%, which indicated that it was a moderate halophile.

The result of BLAST analysis showed the isolate HAP-1 to have maximum identity of 99% to both *Bacillus sonorensis* and *Bacillus licheniformis* (Fig: 1). So the isolate HAP-1 can be
identified as a member of genus *Bacillus* and the bacterium was designated as *Bacillus* sp. HAP-1.

Alkaline protease production by *Bacillus* sp. HAP-1 using different substrates such as wheat bran, rice bran, coconut oil cake and ground nut oil cake were studied. Results are shown in Table 2.

Higher production was obtained with wheat bran (51.79U/g DBB) and lowest production with ground nut oil cake. SSF are extensively influenced by the nature of solid substrate. The solid substrates play a dual role, in supplying nutrients to the microbial culture growing and anchorage for the growing cells (Uyar and Baysal 2004, Prakasahm et al., 2006, Akcan and Uyar 2011). The bran proved as the best substrate for the alkaline protease production in this study is a good source of carbohydrates, protein, vitamin B and mineral ions 1gm of wheat bran contains 0.64gm carbohydrate, 0.15gm protein, 6.1mg magnesium. Its fat content is less. Thus it will be suitable for growth of bacteria.

**II. CONCLUSION**

The present study reports alkaline protease production from a novel bacterial strain *Bacillus* sp. HAP-1, which could not be included under any of the *Bacillus* species reported earlier. This alkaline protease production by *Bacillus* sp. HAP-1 is economically feasible since the solid substrate used was wheat bran, which is a cheap, agricultural byproduct. Extra supplementation of carbon and nitrogen were not needed. Since the bacterium is halophilic, the presence of higher salt concentration in the medium may prevent contamination during fermentation. Since the bacterium used in this study is a novel one and moderate halophile, further studies on the scaling-up of fermentation process and purification and characterization of the enzyme will be of use.

**REFERENCES**


