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Cloning, over expression and functional attributes of serine proteases from *Oceanobacillus iheyensis* O.M.A$_1$8 and *Haloalkaliphilic bacterium* O.M.E$_1$2

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ABSTRACT

Cloning, over-expression, characterization and structural and functional analysis of two alkaline proteases from the newly isolated haloalkaliphilic bacteria: *Oceanobacillus iheyensis* O.M.A$_1$8 and *Haloalkaliphilic bacterium* O.M.E$_1$2 were carried out. The cloned protease genes were over-expressed in *Escherichia coli* within 6 h of the IPTG induction. The protease genes were sequenced and the sequence submitted to the GenBank with the accession numbers, HM219179 and HM219182. The recombinant proteases were active in the range of pH 8–11 and temperature 30–50 °C. The amino acid sequences of the alkaline proteases displayed hydrophobic character and stable configurations. The amino acids Asp 141, His 171 and Ser 324 formed the catalytic triad, while Ile, Leu and Ser were other amino acid moieties present in the active site. The characteristics of the recombinant proteases were compared and found to be similar to their native counterparts. On the basis of the *in-silico* analysis and inhibitor studies, the enzymes were confirmed as serine proteases. The study hold significance as only limited enzymes from the haloalkaliphilic bacteria have been cloned, sequenced and analyzed for the structure and function analysis.

1. Introduction

Proteases are the largest selling enzymes, accounting for about 60% of the global enzyme market [1–4]. Alkaline proteases are produced by a wide range of organisms; bacteria, moulds, yeasts and mammalian tissues [5]. The microbial alkaline proteases are commercially the most viable enzymes and are derived from the various strains of *Bacillus* sp. [6–8]. Most of the studies on the haloalkaliphilic bacteria have focused on the diversity and molecular phylogeny and only limited reports are available on their enzymatic and other biotechnological potential. Genes from some extremophiles have been cloned and over-expressed in different hosts to obtain large quantities of the recombinant enzymes [9,10]. It is necessary and interesting to compare the folding and functioning of the recombinant enzyme to its counterpart produced in native bacteria. Therefore, during the recent years, gene cloning from extremophiles into mesophilic host has gathered considerable attention. However, with particular reference to alkaline proteases from halophiles and haloalkaliphiles, only few enzymes have been purified and characterized [9]. The saline habitats along the Gujarat Coast in Western India exhibits significant diversity of natural microbial flora. However, it is rarely explored and investigated for molecular biological properties and enzymatic potential [2–4,10–14].

Developments in molecular approaches to improve the cloning and expression of genes, from halophilic and other extremophilic organisms, for enhanced solubilization of the expressed proteins would add to the prospect of enzyme-driven catalysis [13–19]. In this report, we describe the cloning and expression of two alkaline protease genes from haloalkaliphilic bacteria into *Escherichia coli* as host, followed by the characterization of the recombinant enzymes to establish the structure and function relationship.

2. Materials and methods

2.1. Bacterial strains and plasmids for cloning and expression study

The bacterial strains used for cloning and expression were *E. coli* TOP$_{10}$ and BL21 (DE$_3$) (Invitrogen, USA), while pET21a+ (Invitrogen, USA) plasmid was used as vector.

2.2. Sample collection

The haloalkaliphilic bacteria were isolated from salt enriched soil near a salt pan located in Okha-Madhi (Latitude 22.20 N, Longitude 70.05 E), the coastal region

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Table 1
One-step purification of the recombinant enzyme by Nickel chromatography, where samples were eluted by increasing concentrations of immidazole (0–200 mM).

	Enzyme Preparations	Total activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
A. O.M.$A_1$8 Alkaline proteases	Recombinant fraction	726	1.45	500.69	–	100
	Purified enzyme	640	0.20	3200.00	6.40	88.15
B. O.M.$E_1$2 Alkaline proteases	Recombinant fraction	640	0.690	927.54	–	100
	Purified enzyme	586	0.192	3052.00	3.29	91.5

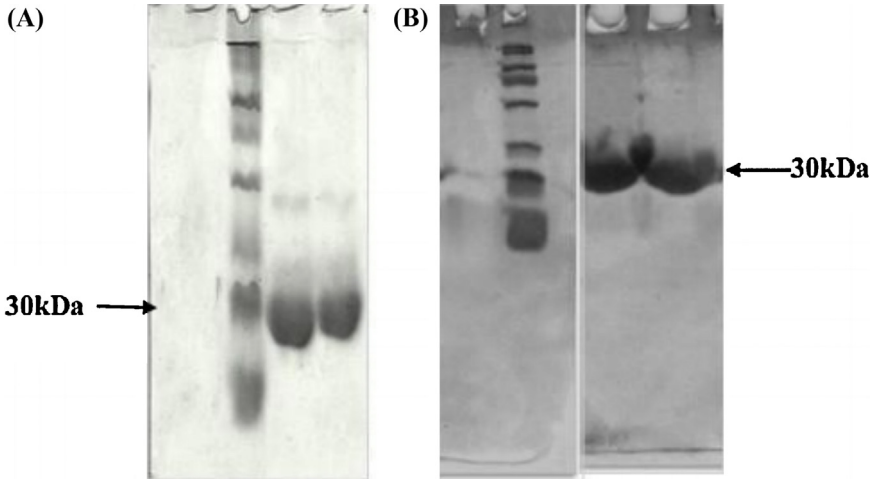

Fig. 1. SDS–PAGE of recombinant enzymes:
Panel A: Over-expression of alkaline proteases at 27 °C and 1 mM IPTG induction. L1, Pre-induction; L2, marker; L3, induction at 6 h for O.M.$A_1$8 alkaline protease; L4, induction at 6 hrs for O.M.$E_1$2 protease.
Panel B: One-step purification of the serine proteases using nickel matrix. L1, Pre-induction; L2, marker; L3, Purified O.M.$A_1$8 protease; L4, Purified O.M.$E_1$2 protease.

of Gujarat, India [20]. Based on the 16S rRNA gene homology, the organisms were related to their nearest homologs and the sequences deposited in NCBI as *Oceanobacillus iheyensis* O.M.$A_1$8 (Gene bank accession no. EU680961) and *Haloalkaliphilic bacterium* O.M.$E_1$2 (Gene bank accession no. EU680960) [20].

2.3. Cloning of the protease genes

Genomic DNA was isolated by the enzymatic method and used as template. A set of the forward (5′-ggatccgccgcc gag gacgac-3′) and reverse (5′-gga tccgccgcc gag gac gac-3′) primers were designed on the basis of the conserved sequences of the haloalkaliphilic *Bacillus* species [21–23]. PCR was performed in a gradient thermocycler (Eppendorf) with the conditions: [95 °C × 5 min] × 1, [95 °C × 1 min/50 °C × 45 s/and 72 °C × 1 min] × 30, [72 °C × 1 min] × 1, using 100 ng of DNA as the template, 25 pmol of each forward and reverse oligonucleotides primer, 25 μl of 2X Red Mix Plus (Merk, Life Sciences).

Plasmid pET21a+ (200 ng) and insert DNA (50 ng) were digested in 30 μl reaction mixtures with BamHI and Sal I under the conditions specified by the manufacturer (Merck Life Science). The digested samples (10–15 μl) were resolved on 1.2% agarose gel along with Broad range DNA marker (Merck Life Science) to analyze the restriction patterns. The digested products were ligated at 4 °C for 12 h. The ligated recombinant plasmids were transformed into *E. coli* strain Top_{10} (Novagen) [21]. Further, the bacterial clones were selected on LB agar medium containing 0.5 mM IPTG (isopropyl-D-thiogalactopyranoside) and 50 μg/ml ampicillin as a marker. Plasmid pET21a+ was extracted from Top_{10} for further confirmation of the positive clones. The protease genes were sequenced from both ends using T_7 promoter and terminator sequence by chromosome walking method (Merck Life Sciences) and confirmed to be in-frame to the vector. The recombinant plasmid was re-transformed into over-expression host, BL_{21} (DE_3), using standard the calcium transformation procedures [21].

2.4. Screening for positive clones

The positive clones were grown on gelatin agar plates to analyze protease expression. Plate diffusion assay was performed by dispensing soluble fraction from induced recombinant cells into wells bored in gelatin agar plate (2% Agar + 1% gelatin in 0.1 M Glycine–NaOH pH 10.0 buffer). Thereafter, the plates were incubated overnight at 37 °C and observed for the development of the zone of clearance of protein hydrolysis around the wells. The alkaline protease activity and total protein content from the positive clones were measured by Anson-Hagihara's and Bradford methods, respectively [22].

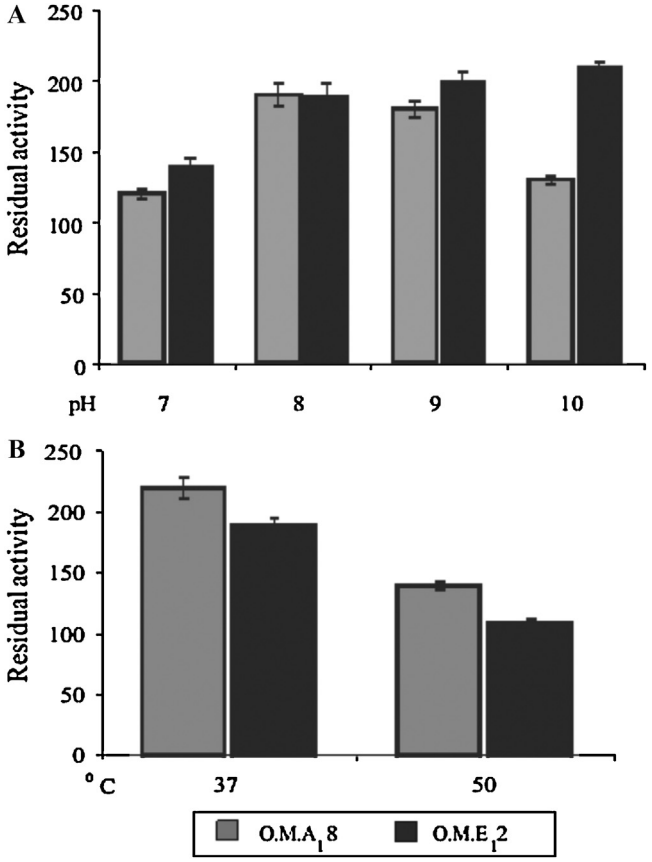

Fig. 2. Effect of temperatures (37–50 °C) and pH (7–10) on the secretion of the recombinant alkaline proteases of O.M.$A_1$8 (A) and O.M.$E_1$2 (B).