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Wealth from waste: Optimized alkaline protease production from agro-industrial residues by *Bacillus alcalophilus* LW8 and its biotechnological applications

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Abstract

An efficient alkaline protease producer was isolated from the water of the hyperalkaline-saline Lonar soda lake and identified as *Bacillus alcalophilus* LW8 by culture-dependent techniques by its morphological, microscopic, biochemical, physiological and molecular characteristics. The 16S rRNA gene sequence was submitted to the GenBank nucleotide repository under accession number KC689353. Alkaline protease production was optimized by adopting a one-variable-at-a-time approach in a submerged fermentation system in modified fermentation medium (MFM). The optimized components of MFM were (w/w) casein (1%), sugarcane molasses (1%), NaCl (1%), ammonium sulphate (0.5%), KH_2PO_4 (0.05%), K_2HPO_4 (0.05%) and Na_2CO_3 (1%). Optimized culture conditions were used for alkaline protease production. The final yield of partially purified alkaline protease after dialysis was 53.35%. The molecular mass of the dialysed alkaline protease was 27 kDa. On a Lineweaver–Burk plot, the calculated K_m and V_{max} values were 24 mg/mL and 1000 U/mg, respectively. The enzyme was remarkably stable in the pH range 7.0–12.0, with optimum activity at pH 10.0. LW8 alkaline protease was completely inhibited by phenylmethylsulphonyl fluoride at 10 mmol/L, indicating that the enzyme belongs to the serine protease class. The metal ions Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+} and Mn^{2+} increased the catalytic activity of partially purified alkaline protease. The protease effectively decomposed the gelatinous coating on an X-ray film, hydrolysed blood clot, a blood-stain from a piece of cotton fabric and hairs from a piece of goat skin.

Keywords: Alkaliphiles; Alkaline protease; Lonar soda lake; Optimization; Blood clot; MEGA 6.06

1. Introduction

Lonar crater (19°58' N, 76°31' E) is a simple, bowl-shaped, near-circular, remarkably well-preserved depression in the otherwise featureless Deccan Plateau in Buldhana district, Maharashtra State, India [1]. It was first brought to international notice in 1823 by a British officer CJE Alexander [2]. Lonar soda lake is a habitat conducive for alkaliphiles and alkali-stable extracellular enzyme producers.

Isolation of microorganisms to be used in industrial applications from sources such as alkaline habitats is much preferred, as these strains produce enzymes that are stable under high alkaline conditions and can resist chemical denaturants present in detergents. There is a growing need to develop a cost-effective, environment-friendly method for synthesizing such enzymes, to replace traditional carbon and nitrogen sources in various media. Use of agro-industrial residues as carbon and nitrogen sources has become popular in the synthesis of eco-enzymes. The aim of this study was to optimize

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alkaline protease production by *Bacillus alcalophilus* LW8 and to investigate its biotechnological applications.

2. Material and methods

2.1. Isolation and screening of alkaline protease producers

Water samples were collected from Lonar Lake, and their pH was measured. A composite water sample was inoculated into basal medium containing (g/L) glucose 2.0, casein 0.5, peptone 0.5, yeast extract 0.5, Na_2CO_3 10.0 and salt solution 50 mL [salt solution: (g/L) KH_2PO_4 5.0, $MgSO_4{\cdot}7H_2O$ 5.0 and $FeSO_4$ 0.1] enriched at 30 °C and 100 rpm for 24 h, then incubated in an orbital shaking incubator [3]. The enriched broth was diluted and spread on Horikoshi and Akiba agar plates [4], which were incubated at 30 °C for 48 h. Isolated colonies were cultivated on Horikoshi and Akiba agar slants. Cultures were incubated on alkaline casein agar [5], and amido black solution was flooded onto the plates [6] to screen for alkaline protease production. Cells (1%, v/v) from each isolate were grown overnight and inoculated into modified fermentation medium (MFM) comprising (g/L) casein 10.0, K_2HPO_4 0.5, KH_2PO_4 0.5 and Na_2CO_3 10.0 and incubated at 30 °C and 200 rpm for 72 h. The cultures were then centrifuged at 10 000 rpm for 10 min at 4 °C [7]. Cell-free supernatants were subjected to the qualitative casein cup assay [3,6,7], and a modified Anson's quantitative assay for alkaline protease activity [8] was carried out. One proteolytic unit was defined as the amount of the enzyme that releases 1 μmol/min of tyrosine under the assay conditions [8,9]. Total protein content was determined with bovine serum albumin as the standard [10]. The isolate that yielded the highest alkaline protease activity, LW8, was selected for further studies.

2.2. Preliminary and molecular identification

The LW8 isolate was characterized by determining its morphological, microscopic and sugar use characteristics, enzyme profile, antibiotic sensitivity and physiological attributes [4,9,11–15]. Further, it was subjected to 16S rRNA molecular analysis. DNA was extracted by InstaGene Matrix (Bio-Rad, USA) treatment from cell pellets [16], and the 16S rRNA gene was amplified in a thermocycler (Applied Biosystems, USA) with pair of primers (forward (27f) AGAGTTTGATCMTGGCTCAG and reverse (1492r) TACGGYTACCTTGTTACGACTT) [7,17,18]. The amplified 16S rDNA PCR product was gel-purified with a QIAquick Gel Extraction kit (Qiagen, USA) [19] and sequenced in an ABI Prism™ 377 automated DNA sequencer (Applied Biosystems, USA) [18,20]. The deduced sequence was compared with GenBank data by the BlastN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [21,22]. Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 6.06 software [23]. A phylogenetic tree was constructed by the neighbour joining method [24]. Bootstrap analysis 1000 replication was applied [25]. The evolutionary distances were computed by the maximum composite likelihood method [26].

2.3. Optimization of physicochemical parameters for maximum alkaline protease production

Factors that affect protease production, such as inducers, carbon and nitrogen sources, pH, temperature and salt concentration, were optimized by a search technique, i.e. a one-variable-at-a-time approach in a submerged fermentation system in MFM. The range of optimum parameters achieved by one step was fixed subsequently in the next step [3,7]. The effect of time and agitation on protease production by the LW8 isolate was determined. A culture of LW8 grown overnight, with $A_{600} = 0.9154$ was inoculated into MFM and incubated with shaking (50–200 rpm) and under static conditions at 30 °C for 96 h. Samples were withdrawn aseptically every 24 h, and the catalytic activity of alkaline protease was determined under standard assay conditions [3,7,8]. The parameters tested for maximum protease production were pH (8.0–12.0), temperature (20–60 °C), salt concentration (0–7% NaCl) and inoculum size (1–5%) [3,7]. The inducers tested were casein, skimmed milk, gelatin, peptone, tryptone, casein hydrolysate, yeast extract, beef extract, meat extract, flour made of seeds (corn, pumpkin, soybean, green gram, chick pea), dried powdered cow dung [27,28], feathers and defatted seed meals (soybean, mustard, corn, groundnut, cotton, sesame, sunflower and safflower), amended at a concentration of 1% (w/w) in MFM. The best inducer was evaluated for optimum concentration between 0.5% and 3.0% [3,7,29]. The effects on alkaline protease production by the LW8 isolate of synthetic carbon sources (arabinose, glucose, glycerol, lactose, mannitol, ribose, sorbitol, sucrose and xylose), low-cost agricultural residues (dried powder of wheat bran, rice bran and sugarcane bagasse), industrial residue (sugarcane molasses), inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium chloride) were investigated at a concentration of 1.0%. The optimum carbon and nitrogen source concentration for enhanced enzyme production was also determined