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Purification of extracellular acid protease and analysis of fermentation metabolites by *Synergistes* sp. utilizing proteinaceous solid waste from tanneries

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Abstract

The untanned proteinaceous tannery solid waste, animal fleshing (ANFL), was used as a substrate for acid protease production by *Synergistes* sp. The strain was isolated from an anaerobic digester used for the treatment of tannery solid waste and was selected for its enhanced protease production at activity 350–420 U/ml. The optimum pH was in the acidic range of 5.5–6.5 and optimum temperature was in mesophilic range of 25–35 °C. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the zymogram analyses of the purified protein indicated an estimated molecular mass of 60 kDa. This protease could be classified as aspartic protease based on its inhibition by aspartate type protease inhibitor pepstatin and on non-inhibition by 1,10-phenanthroline, EDTA, EGTA and phenylmethylsulfonyl fluoride. The degradation of ANFL was confirmed by Gas Chromatography–Mass Spectroscopy (GC–MS), Proton Nuclear Magnetic Resonance Spectroscopy (H^1 NMR) and Scanning Electron Microscopy (SEM) analyses. In this study we found that the activity of acid protease depended on factors such as calcium concentration, pH and temperature. Based on these lines of evidence, we postulate that this protease is a highly catalytic novel protease of its type.

Keywords: Acid protease; *Synergistes* sp.; Animal fleshing (ANFL); Tannery solid waste; Surfactant resistant enzyme

1. Introduction

The microorganisms represent an exceptional source of protease owing to their extensive biochemical diversity and susceptibility to genetic manipulation. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (Ward, 1985). The proteolytic enzymes have many physiological functions, ranging from the generalized protein digestion to the more specific regulated processes (Kalisz, 1988; Kaminishi et al., 1994). In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell. In contrast to the multitude of the roles contemplated for proteases, knowledge on the mechanisms and the metabolic pathways in which the proteases play an integral role in the fermentation of proteinaceous substrates is very limited (Mala et al., 1998). There have been several studies of the different aspects of proteolytic enzymes and they have been used in various industrial fields. In the case of anaerobic digestion, the unique hydrolytic enzyme production by the bacterial isolate will enhance the hydrolysis of varied organic substrates. Thus, there has been a constant search for anaerobic bacterial strain with the higher proteolytic activity, which enhances the hydrolysis of proteinaceous tannery solid waste.

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In India, approximately 1,50,000 ton offals in the form of raw hide trimmings, limed animal fleshings, green animal fleshings, hide splits and chrome shavings are available, which are not utilized or under utilized thus creating a solid waste disposal problem in tanneries (Rao et al., 1994). The present work focuses on the utilization of animal fleshing (ANFL), as a substrate for the microbial production of acid protease. The ability of microorganisms to grow and produce appreciable level of enzyme, using ANFL as substrate could offer tremendous potential for the development of biotechnological methods for the rapid hydrolysis of ANFL.

Beside the numerable properties of protease, it was considered essential to investigate further on the potential of the protease, particularly the stability towards surfactants, the temperature and the requirements of divalent cation (Ca^{2+}) for its activity. The utility of enzymes depends on their stability in the presence of denaturing agents (e.g., detergents). Generally, proteins are denatured in 0.1% surfactant (Lee et al., 1999; Joo and Chang, 2006; Han and Damodaran, 1997; Venugopal and Saramma, 2006).

As the acid proteases have been extensively used in pharmaceutical industry (Spelzini et al., 2005), the production of acid protease appears to be a very important one. However, reports on the utilization of ANFL for the production of acid protease is very little or perhaps nil. Moreover, the mechanism of action of the proteases has been recent subject of interest to researchers. Understanding the mechanism of proteases activity towards myoproteins and its characterization can provide a new insight in biotechnology. Therefore, considering the applications of acid protease and the need for disposal of solid wastes generated in tannery, an investigation has been carried out on optimization of fermentation conditions for the extracellular acid protease production from ANFL and studying biochemical properties of protease.

2. Methods

2.1. Substrate preparation

The limed animal fleshing predominant solid waste generated during the process of leather manufacture was collected from the tannery division of Central Leather Research Institute, Chennai and was treated with ammonia solution (25% v/v) for 3–4 h to remove the adhered calcium salts on ANFL. The delimed fleshing was suspended in water and the pH was adjusted to 7.0 ± 0.2. The ANFL particles of 0.25 cm, 0.5 cm, 0.75 cm and 1.0 cm in dimensions were obtained by manual scissoring. The sized ANFL particles were stored at 4 °C until the start of the experiments.

2.2. Strain and culture conditions

The strain was isolated anaerobically from an anaerobic digester used for the treatment of tannery solid wastes and maintained in anaerobic egg broth (High media) medium at 37 °C. The batch fermentation experiments were conducted for 1 g ANFL in 100 ml minimal medium of composition (g/l) NaCl – 0.9; NH_4Cl – 0.01; K_2HPO_4 – 0.75; and KH_2PO_4 – 0.5. The trace element solution of 1 ml, containing composition (g/l) $MgSO_4$ – 0.49; $FeSO_4$ – 0.055; $CoCl_2$ – 0.028; $MnCl_2$ – 0.019; $CaCl_2$ – 0.147; and $NH_4Mo_7O_{24}$ – 0.123 was added. The media were autoclaved at 120 °C at 15 psi for 15 min and incubated, without agitation, at 37 °C. All experiments were carried out in duplicates and repeated thrice.

2.3. 16 S rDNA gene sequencing and phylogenetic analysis

The DNA was isolated according to the procedure of Marmur, 1961 and the small subunit rRNA gene was amplified, using the two primers 16S1 (5-GAGTTTGATCCTGGCTCA-3) and 16S2 (5-CGGCTACCTTGTTACGACTT-3). The purified DNA product, of approximately 1.5 kb, was sequenced, using five forward and one reverse primer as described earlier (Reddy et al., 2000). The deduced sequence was subjected to BLAST search tool, for the closest match in the database. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16 S rDNA database to obtain the closely related sequences, and the phylogenetic tree was constructed based on evolutionary distances that were calculated, according to the distance matrix method using the Phylip package (Felsenstein and Phylip, 1993).

2.4. Analytical procedures, CHNS analysis and high-performance liquid chromatography

The ANFL sample was characterized for TOC (total organic carbon), TKN (total Kjeldhal nitrogen), ash and moisture content in accordance with standard methods for analysis of water and wastewater (Lenore et al., 1989). The percentages of carbon, hydrogen, nitrogen and sulphur were determined, using Elementar Analysen systeme GmbH vario EL, German CHN analyzer. The amino acid composition of ANFL was determined, using C18 column in Agilent model 1100 HPLC analyzer (Ramakrishnan et al., 1996).

2.5. Measurement of enzyme activity

Protease activity was determined as described previously (Shimogaki et al., 1991) with modification, using casein as the substrate. Enzyme solution (0.5 ml) was added to 3.0 ml of substrate solution (0.6% casein in 20 mM sodium phosphate buffer, pH 6.0) and the mixture was incubated at 37 °C for 20 min. The reaction was terminated by the addition of 3.2 ml of TCA mixture (containing 0.11 M trichloro acetic acid), and the mixture was kept at room temperature for 30 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was measured at 280 nm. One unit of protease activity is defined as the