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Microbial biodegradation of proteinaceous tannery solid waste and production of a novel value added product – Metalloprotease

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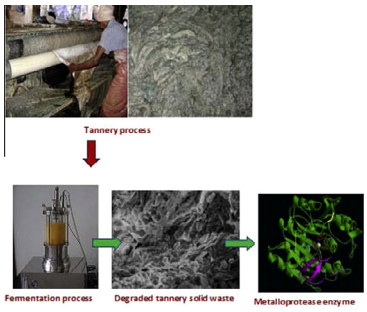
HIGHLIGHTS

- Utilization of proteinaceous waste for production of novel extracellular acid metalloprotease.
- Reduction of pollutant load through the microbial degradation of tannery solid waste.
- Hydrolysis of animal fleshing through protease activity in the anaerobic fermentation process.
- *Clostridium limosum*, a potential candidate for simultaneous degradation of tannery waste and production of metalloprotease.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, animal fleshing (ANFL) was utilized as a substrate for the production of extracellular protease by *Clostridium limosum* through central composite rotatable design (CCRD) and response surface methodology (RSM). Optimum protease production of 433 U/ml was achieved and the purified enzyme was identified as acidic metalloprotease, a monomeric protein. The molecular weight of the enzyme was 71 kDa, whose activity was enhanced by bivalent metals such as Zn^{2+} and Mg^{2+}. Scanning electron microscopy (SEM) examination also revealed the hydrolysis/microbial degradation of ANFL through protease activity in the anaerobic fermentation process. Simultaneous hydrolysis of ANFL and production of an enzyme with the potential for different industrial applications provide an attractive methodology for the disposal of tannery solid waste.

1. Introduction

One of the most polluting industries in the world is the leather industry, which gives rise to toxic solid and liquid wastes which are then abandoned due to their non utilization or under utilization, resulting in environmental pollution (Chowdhury et al., 2013) Indian tanneries produce 305 million kg of solid waste per annum, which create disposal problems as well as environmental pollution (Chandra et al., 2011; Ahamed and Kashif, 2014). In this regard, looking for alternatives can minimize the amount of waste generated and maximize the return by producing value-added products. The structural constituent of proteins and other complex constituents of tannery solid waste, and the chemical contaminants derived from the tanning process, are highly resistant to microbial degradation (Ravindran et al., 2012). This

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waste when dumped on soil makes it unfit for agricultural activities and pollutes the groundwater system. The main solid waste produced during pre-tanning operations is animal fleshing (ANFL) and it contains high protein (50–60%) and collagen content (3–5%) (Ravindran et al., 2008, 2012).

The recovery of content from solid waste that can be commercially utilized has not been studied sufficiently. Recently, enzyme and fermentation-based techniques have been explored and found to be viable for utilizing tannery ANFL waste (Kumar et al., 2010; Ravindran et al., 2012). Microorganism assisted enzymatic degradation is considered a cleaner process as it breaks down complex compounds into smaller intermediates without any impact on the environment (Okoroma et al., 2012; Fang et al., 2013). Reports on utilization of ANFL are scanty and there are only limited reports on the use of ANFL for the protease production from *Selenomonas ruminantium* (Ravindran et al., 2012), *Synergistes jonesii* (Kumar et al., 2008a), *Pseudomonas aeruginosa* (Kumar et al., 2008b) and *Pediococcus acidolactici* (Bhaskar et al., 2007).

Jana et al.(2014) reported that the green biotechnology encourages the application of enzymes for the production of fuels, chemicals, secondary metabolites and others in industrial level, preferably from renewable sources. Proteases are enzymes with a broad range of commercial applications including in the food, detergent and leather industries, as well as in the biotechnology, medical and basic research fields. Acid proteases are extensively used in the pharmaceutical, leather and textile industries (Spelzini et al., 2005). The current study focuses on the production of acid protease using ANFL as a substrate by means of the newly isolated bacteria *Clostridium limosum*. There have been only a few reports on the production of acidic aspartic protease using ANFL as a substrate (Kumar et al., 2008a). To the best of our knowledge, the present study is the first regarding the production of acid metalloprotease from *C. limosum*.

In industrial-scale fermentation processes, the cost of the nutrient medium and enhancement of enzyme production are relevant factors (Thirunavukarasu et al., 2015). The statistical design of experiments can assist in the selection of optimal conditions and processes and so yield more reliable information than conventional approaches (Edwinoliver et al., 2009). The microbial degradation of ANFL is of interest for two reasons: the conversion of waste into value-added products, and the neutralization of a pollutant making it safe for disposal. Therefore, this study investigated the role of *C. limosum* in the production of extracellular acid protease in minimal media (optimized through central composite rotatable design (CCRD)), a tool of response surface methodology (RSM) using ANFL as a substrate, and characterized the biochemical properties of the purified protease enzyme.

2. Materials and Methods

2.1. Raw material and bacterial isolation for degradation

The ANFL utilized in this study was collected from the division of tannery at Council of Scientific and Industrial Research – Central Leather and Research Institute (CSIR – CLRI), India. The isolated bacterial strain belonged to the genera *Clostridium* under Clostridiaceae family. The working stock culture was maintained in an anaerobic egg broth medium (Hi media) at 37 °C. Due to the predominance of protein in the tannery waste (ANFL) being studied, we focused on extracellular protease enzyme activity.

2.2. Protease production optimization by response surface methodology (RSM)

The composition of the production media was optimized using a five-level four-factorial CCRD tool for the four critical factors. The factors were selected by means of the classical method (Ravindran and Sekaran, 2009). The four factors selected are ammonium chloride (A), dipotassium hydrogen orthophosphate (B), potassium dihydrogen orthophosphate (C) and sodium chloride (D) with an α value of ±1.414. Factorial design of the equations are given below:

$$Y_c = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 \text{ (intercept and main effects)} + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 \text{ (quadratic effects)} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \text{ (interactions)}$$

These equations explain the connection between the response Y_c and the coded variables $X_1 - X_4$, where Y_c is the protease activity (U/ml) and X_1, X_2, X_3 and X_4 represent the variation factors A, B, C and D, respectively. Here β_0 represents the regression coefficient and β_1, β_2, β_3, etc. represent the coefficient estimated by the interactive effects of X_1, X_2, X_3 and X_4 factors on the protease activity along with ANFL as substrate. The combinations of variation were designated in three blocks each containing 10 runs with a total of 30 runs. Each run contained 100 ml of minimal medium consisting of different factor combinations along with a constant composition of trace elements (200 µl) and 1 g of ANFL. All runs were incubated at an optimized temperature of 37 °C for 72 h (Ravindran and Sekaran, 2009). The data was analyzed using the Design Expert software package (version 7.0.3, Stat-Ease Inc., Minneapolis, MN).

2.3. Experimental design for ANFL degradation

The anaerobic batch fermentation experiments consisted of 1 g ANFL (size of 0.25 cm) in 100 ml minimal medium, composed of (g/l) sodium chloride, 0.5; ammonium chloride, 0.75; dipotassium hydrogen orthophosphate, 0.5; and potassium dihydrogen phosphate, 0.75 optimized from RSM. A trace element solution of 2 ml/L minimal medium was added. The trace element solution was composed of (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. The media was dispensed into serum bottles, sealed with butyl-rubber stoppers, then clamped with aluminium caps. The desired gas (N_2) was flushed into the media three times alternating with a vacuum. The media was autoclaved at 121 °C at 15 psi for 15 min. Fermentation was carried out by seeding the medium with inoculum of *C. limosum* (5%, v/v) and incubating without prior agitation at 37 °C. Experiments were carried out in duplicate and repeated three times.

2.4. Effect of harvest time, pH and temperature on protease production

The anaerobic batch fermentation process lasted 120 h. The protease activity was determined by harvesting a sample every 24 h. The effect of pH (3–8) and temperature (20–60 °C) on protease production was monitored.

2.5. Determination of protein and protease activity

The protein concentrations of the samples were determined by Bradford method (Bradford, 1976). Enzyme preparations were routinely assayed using the Germaine et al. method (Germaine et al., 1978) with modification, using 1% BSA as substrate in a citrate buffer (100 mM, pH 5.0) used for a assay at 37 °C for 10 min. The absorbance was measured at 280 nm and calculated to microgram tyrosine with a standard curve.