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An efficient cloth cleaning properties of a crude keratinase combined with detergent: towards industrial viewpoint

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ABSTRACT

Production of alkaline keratinolytic protease related to various industrial applications, especially for enzyme detergents combination, was done from a selected bacterial strain *Paenibacillus woosongensis* TKB2. Enzyme yield was maximized by optimizing the culture conditions of a low-cost culture medium under submerged condition with a lab scale fermentor of 5 L capacity. The highest keratinolytic activity was obtained 76.05 U/mL by maintaining fermentation conditions of keratinase production at 30 °C, 40% dissolved oxygen level at 500 rotations per minute (rpm) and an initial pH of media of 8.5 supplemented with 0.76% chicken feathers. In fact, the crude enzyme showed four proteolytic zones in casein zymography which revealed that the crude keratinase contained four hydrolytic enzymes. The crude keratinase was active in broad range of pH (7–10.5) with keratin as a substrate. However, it exhibited highest activities at pH 9. The crude keratinase which was strongly inactivated by Phenylmethylsulfonyl fluoride (PMSF), aprotinin and leupeptin, indicated that it was a serine type protease. The crude enzyme showed remarkable stability with EDTA and was compatible with commercially available detergents. The enzyme bead prepared by 1.5% CMC improved significant storage stability with commercial detergent powder making it suitable for commercial exploitation. The alkaline keratinase of *P. woosongensis* TKB2 showed a potential application in the laundry industry by removing the blood stains from the surgical cloths and gave a remarkable result towards removing the composite stain of blood, egg yolk and chocolate stain in a short period without changing texture, strength (27.18 g/tex) of cloths and cloths fibers. The effluents generates after cloth washing with this detergent formulation does not effect on aquatic ecosystem. Thus, this aqua-friendly detergent formulation can be used to avoid pollution problems associated with effluent generates after cloths washing.

1. Introduction

Keratinolytic proteases occupy a pivotal status with respect to their commercial applications, which are hydrolytic in nature that cleaves peptide bonds in keratin proteins. They are classified into various groups such as serine proteases, cysteine-thiol proteases, aspartic proteases and metallo-proteases (Beg et al., 2003; Nascimento and Martins, 2004). Among proteases, keratinase has been used as an alternative of chemicals to accelerate the efficiency and cost-effectiveness of a wide range of industrial systems and processes (Gupta et al., 2002). Further, highly-alkaline keratinase, which alone accounts for about 40% of the total worldwide enzyme sales (Rao et al., 1998), proved particularly suitable for industrial uses. This is mainly due to its high stability and activity under harsh conditions as well as its significant ability to hydrolyze various proteinaceous substrates. Keratinolytic proteases have been purified from different microorganisms mainly bacteria, such as *Streptomyces* (Jaouadi et al., 2010), Bacillus (Pillai and Archana, 2008) and *Paenibacillus* (Paul et al., 2013a). Among these enzymes, several serine keratinolytic proteases have so far been isolated, purified, and characterized from various species, such as *Bacillus licheniformis* (Beg et al., 2003), *Bacillus pumilus* CBS (Jaouadi et al., 2008), *Bacillus subtilis* (Pillai and Archana, 2008) and *Paenibacillus woosongensis* TKB2 (Paul et al., 2013c). Nevertheless, little data is currently available on characterization of keratinase from *Paenibacillus* sp. These keratinolytic enzymes have been widely used in the detergent industry since their introduction in 1914 as detergent additive. Their use in detergent formulation at present makes up a high percentage (89%) of their total sales.

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Finally, the alkaline keratinase production by *Paenibacillus* strain in submerged fermentation (SmF) was enhanced by production carried out in a process-controlled bioreactor. Because of the low purification yields and high cost for enzyme purification, detergent industries do not prefer the purified enzyme for using detergent formulation.

On the other hand the detergent industries prefer crude keratinase enzyme with high yield and low cost for production. In this context, the main goal of this study was to produce detergent stable crude alkaline serine keratinase in large scale bioreactors and characterized some biochemical properties of the crude enzyme concerning its application in the detergent industry. This study also intended to develop a new method for enzyme bead preparation by CMC to improve the storage stability of enzyme with detergent powder in commercial viewpoint.

2. Materials and methods

2.1. Isolation and cultivation of the organism

Paenibacillus woosongensis TKB2 (GenBank JQ248575), isolated from poultry processing plant, Khirpai, Paschim Midnapore, West Bengal, India is a novel stain which was used in this study. The bacterium produced keratinase enzyme in submerged fermentation (Paul et al., 2013a) and solid state fermentation (Paul et al., 2013b). The bacterial culture was maintained over skim milk agar slants (pH 8.5) and stored at 4 °C.

2.2. Keratinase production in lab scale 5-L stirred-tank bioreactor

The optimized conditions of pH (8.5) and temperature (30 °C) of *P. woosongensis* TKB2, one of gram-positive bacteria, have been previously reported (Paul et al., 2013a). Therefore, the object of this work was to optimize the keratinase production in a 5-L stirred-tank bioreactor, which included three factors (parameters): oxygen inoculation amount, saturation level (pO_2), and feather quantity. The medium consisting of 75 g/L whole feathers, 50 g/L NaCl, 0.5 g/L K_2HPO_4, 0.25 g/L $MgSO_4$, 0.20 g/L CaCO3 and 0.15 g/L $FeSO_4$ with final pH adjusted to 8.5 was inoculated with different volume (100 mL, 120 ml and 140 ml) of mid logarithmic phase (12 h, 14 h and 16 h) bacterial culture.

A total working volume of 3 L was used in a 5-L EYLA-MBF (Japan) Jar fermentor (laboratory-scale) with a Rushton turbine. The initial pH of the substrate was adjusted according to the one variable at a time approach (OVAT) in a shake flask experiment (Paul et al., 2013a) and automatically controlled throughout the fermentation process by adding 2 M NaOH or 2 M HCl into the fermentor. The pH probe was calibrated before measurement of the sterilization of the media, and the pO_2 probe and acid, base, and antifoam pumps were calibrated before the inoculation. The pO_2 probe was calibrated by saturated sodium sulfite. The dissolved oxygen (pO_2) was maintained by agitation of the impeller, which was cascaded to the stirrer only. Temperature, agitation, foaming, pO_2, and pH were maintained automatically by microprocessor control of the bioreactor. The fermentation process for each experiment ran for 3 days and a 30 mL sample was collected from the reactor vessel every day. The sample was centrifuged at 10,000 rpm for 10 min prior to analysis. Each fraction of collected samples was analyzed for keratinase activities and protein concentration.

2.3. Determination of keratinase activity, protein estimation and zymogram analysis

The enzyme assay was performed with keratin substrate (Paul et al., 2013a). One unit (U) of keratinolytic activity was defined as an increase of corrected A_{280} for 0.01 under the assay conditions. Protein estimation was done according to Lowry et al. (1951) method.

Polyacrylamide gel (PAGE) 10% containing 1% casein (HiMedia, India) was prepared for zymogram analysis of crude enzyme. The gel was stained with Coomassie brilliant blue R-250 (Mark, India).

2.4. Determination of optimum pH and stability

Activity of crude enzyme preparation was measured at pH 6–11 at 50 °C using keratin powder (HiMedia, India) as a substrate. The pH stability of the crude enzyme was determined by pre-incubating the enzyme preparation in buffer solutions with different pH values for different periods of time (1, 2, 3 days) at room temperature. Aliquots were withdrawn and residual activity (relative activity, %) was determined at pH 9 and 50 °C. The following buffer systems were used at 50 mM: phosphate buffer for pH 6–8, Tris-HCl for pH 8–9, Glycine-NaOH for pH 9–11.

2.5. Enzyme stability with inhibitors

The effects of different protease inhibitors like PMSF (Sigma–Aldrich, USA), Arrotinin (Sigma–Aldrich, USA), leupeptin (Sigma–Aldrich, USA), pepstain A (Sigma–Aldrich, USA), ethylenediaminetetraacetic acid (EDTA), sodium dodesyl sulphate (SDS), β-marcaptoethanol on keratinase activity (relative activity, %) was investigated by pre-incubating crude enzyme for 30 min at room temperature with each chemical. Enzyme assays were carried out under standard assay conditions.

2.6. Enzyme stability with commercial detergents

The study evaluated the compatibility of the enzyme with commercial solid detergents. The commercial detergents such as Tide™, Nirma™, Rim™, Anmol™, Safed™, Gadi™ and Sunlight™ were diluted in tap water to give a final concentration of 7 mg/mL. The endogenous enzymes in the detergents were inactivated by heating the diluted detergents for 30 min at 100 °C prior to the addition of the keratinase enzyme. The mixture of enzyme and detergent was then incubated for 30 min and 60 min at 50 °C and the residual enzyme activity was determined under optimal assay conditions.

2.7. Study of in-vitro hemolytic effect

In-vitro hemolytic study was done according to Vishalakshi et al. (2009) with necessary modifications. In brief, blood agar medium was prepared with human blood and 1.5% agar. Then pleating well was prepared on to the blood agar followed by poring of 50 μL of crude enzyme in the well. The plate was incubated at 40 °C for 12 h.

2.8. Wash technique to remove blood stain from medicinal aprons

With the aim to ascertain the capability of crude alkaline keratinase for use as a bio-detergent additive, wash performance was evaluated by determining the removal of blood stains from surgical cloths. Aprons stained with human blood were collected from Chatterjee Nursing Home, Midnapore, West Bengal, India. For the scaling up, an apron was cut into two pieces, as shown in Fig. S1. Piece-I was subjected to enzymatic distaining with detergent supplementation, while piece-II to the only detergent process.

2.8.1. Testing for the removal of composite stain of .blood, egg yolk and chocolates

For the efficiency testing of formulated bioactive detergents the composite blood, egg yolk and chocolate stain releasing capacity