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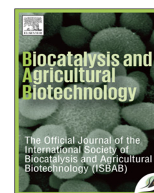

Optimization for production of liquid nitrogen fertilizer from the degradation of chicken feather by iron-oxide (Fe_3O_4) magnetic nanoparticles coupled β-keratinase

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ABSTRACT

There is an escalating demand for balancing crop yields with a reduction in the environmental impacts caused by synthetic fertilizers, and focusing on sustainable agricultural practice using organic fertilizers high in nitrogen and increased soil water retention capability. The current study demonstrates a significantly improved chicken-feather hydrolyzate prepared by *Bacillus licheniformis* AS-S24-I β-keratinase coupled iron-oxide magnetic nanoparticles. Biochemical and proteomic characterization suggests alkarnase as a serine protease in nature with broad pH stability as well as incubation temperature. The Scanning Electron Microscope (SEM) study reveals that iron-oxide (Fe_3O_4) magnetic nanoparticles coupled β-keratinase can degrade 80–93% of chicken-feather keratin post 48 h of incubation. Chicken-feather hydrolyzate demonstrated the release of six volatile compounds post treated with iron-oxide (Fe_3O_4) magnetic nanoparticles coupled β-keratinase via Gas Chromatography–Mass Spectrophotometry (GC–MS) and matrix-assisted laser desorption/ionization-time-of-flight MS (MALDI–TOF–MS) analyzes, respectively. The release of low volatile compounds post degradation recommended that it could be a sustainable method of eco-friendly organic fertilization. The results of the physiochemical analysis suggested improved nitrogen and amino acid levels indicating that chicken-feather hydrolyzate was a good organic fertilizer source. Filtered sterilized chicken-feather hydrolyzate revealed a significant increase in the length and growth of Bengal gram seed germination, as well a rise in the soil microbial population. Thus, the present study offers significant biotechnological applications of chicken-feather hydrolyzate, which could serve as a cheap source of liquid organic fertilizer.
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1. Introduction

Over the past six decades, chemical fertilizer application has played a crucial role globally in increasing crop yield and maintaining adequate food supplies. Crop production and landscape management had created a large-scale market for chemical fertilizer consumption. However, these land-use practices subsequently encountered environmental challenges, due to the low efficiency of fertilizer use and subsequent nutrient release into the surface or ground water, and emission of gases into the atmosphere (Yang et al., 2013). Therefore, over the last decade, great efforts have been taken to replace the chemical fertilizers with environmental friendly bio-fertilizers or slow nitrogen release organic fertilizers (Bose et al., 2014).

During the recent years, keratinous wastes have been gaining great popularity for their use as organic fertilizers due to the high protein content in the form of "keratin", a structurally insoluble protein (Choi and Nelson, 1996; Rai and Mukherjee, 2010; Gupta et al., 2013). Keratin constitutes a major component of the epidermis and its appendages viz. hair, feathers, nail, horns, hoofs, scales and wools (Gupta et al., 2013) making them more rigid and hard to degrade using the conventional approach (Gupta et al., 2013). Valuing its high protein content and amino acid worth, we developed a modified method for the efficient degradation of feather-keratin using a special class of proteolytic enzyme called "Keratinase" capable of degrading highly insoluble keratin substrates into nutritional value-added products (Rai et al., 2009).

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To enhance the enzyme performance under nonconducive conditions, "enzyme immobilization" has been demonstrated as an efficient technique in improving the stability as well as the activity of the immobilized enzyme (Wang et al., 2003; Farag and Hassan, 2004; Mateo et al., 2007). Although a large number of nano-scaled carriers have been applied prior in the enzyme immobilization process (Kim et al., 2006), however, from an industrial perspective there are several disadvantages in this process owing to the difficulty experienced in recovering and handling the nanoparticles at the industrial level. Alternatively, the use of iron-oxide magnetic nanoparticles (MNPs) might overcome such problems because utilizing magnets enables the simple recovery of the catalyst post binding with MNP (Tong et al., 2001; Konwarh et al., 2009). Besides the use of iron-oxide MNP as a support to immobilize the enzymes there are several other advantages including those of higher specific surface area resulting in the binding of a large quantity of enzyme (Konwarh et al., 2009), lower mass transfer resistance and less fouling (Huang et al., 2003) as well as its strong magnetism and low toxicity (Liang et al., 2007).

Our earlier study had shown an improvement in the catalytic efficiency, heat-tolerance and storage stability of the *Bacillus subtilis* strain RM-01β-keratinase post immobilization onto the iron-oxide MNP (Konwarh et al., 2009). In this study, we report the improved hydrolysis and recycling of native chicken feather by the β-keratinase iron-oxide MNP bio-conjugate. The results suggest that the application of chicken-feather hydrolyzate, as an organic fertilizer to the soil enhances seed germination and plant growth (Gousterova et al., 2005; Kim et al., 2005; Vasileva-Tonkova et al., 2009; Paul et al., 2013).

2. Materials and methods

2.1. Isolation and identification of alkaline β-keratinase producing bacterium

Following our procedure described earlier (Mukherjee et al., 2008) the initial screening was conducted for the alkaline β-keratinase producing bacteria from the various soil samples of Assam. One alkaline β-keratinase producing bacterium (AS-S24-I) isolated from a soil sample from the Sonitpur district of Assam, was selected for further study. The bacteria were sub-cultured on nutrient-agar plates before being utilized as the inoculum for β-keratinase production under submerged fermentation using M9 medium with the following composition (in g/l): 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.5 g of NaCl, 0.014 g of $CaCl_2$, 0.245 g of $MgSO_4 \cdot 7H_2O$, and 10 mg of thiamine hydrochloride (Mukherjee and Das, 2005).

The bacterium was identified using the polyphasic approach viz. by biochemical and phenotypical characterization (Cappuccino and Sherman, 1983), analysis of the bacterial cellular fatty acid methyl esters (Pandey et al., 2002), and 16S rRNA gene sequencing (Rai et al., 2009). The genomic DNA of the bacteria was isolated as described earlier by Ausubel (2002). The rDNA was amplified by PCR using the universal primers that were designed to amplify approximately 1.5 kbp segment of the 16s rDNA. The forward primer was the 5′-GAGTTTGATCCTGGCTCAG-3′ and the reverse primer was 5′-CGGCTACCTTGTTACGACTT-3′. PCR was conducted as described by Rahman et al. (2007) in the PCR system thermal cycler (GeneAmp* PCR system 9700, Applied Biosystems). The purified PCR product (PCR purification kit, Qiagen, Germany) was used directly for the automated DNA sequencing employing 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The sequence thus deduced was subjected to the BLAST search tool from the National Center of Biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov) to retrieve the homologous 16s rRNA gene sequences in the GenBank.

2.2. Phylogenetic analysis

The multiple sequence alignment program MEGA4 was used for aligning the partial 16S rDNA sequence with reference sequences showing the sequence homology from the NCBI database. Phylogenetic trees were constructed utilizing the distance matrix-based cluster algorithms viz. the unweighted pair group method with averages (UPGMA), neighbor-joining, maximum-likelihood, and maximum-parsimony analyzes (Kluge and Farris, 1969; Saitou and Nei, 1987). All positions containing gaps or missing data were eliminated from the dataset (complete deletion option). The trees were rooted using *E. coli* KesE6 (Accession No. EU884314) as the out-group. The stabilities of the trees obtained by cluster analysis were assessed through the BOOTSTRAP program in sets of 1000 resamplings (MEGA 4).

2.3. Keratinolytic assay

Unless otherwise stated, the cell-free culture supernatant isolated at 4 °C was used for the assay of the alkaline β-keratinase activity, as explained earlier (Mukherjee et al., 2008), using 1.0% (w/v) keratin as the substrate at pH 10.0 (100 mM Glycine–NaOH), and incubated at 45 °C for 30 min. The unit (U) of alkaline β-keratinase activity is defined as the μg of tyrosine liberated min^{-1} ml^{-1} of enzyme (Rai et al., 2009). To assay for substrate specificity of the enzyme, the procedure mentioned above was followed except that keratin was replaced by any of the following protein substrates viz., casein, gelatin, hemoglobin, bovine serum albumin, globulin, fibrinogen, human hair and collagen. The protein content was determined following the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the protein standard. The specific activity of enzyme was represented as units of enzyme activity per mg of protein.

2.4. Substrate preparation

Broiler chicken feathers were collected from the local vendors located at Napam, near the Tezpur University Campus. The chicken feathers were washed in boiling water for about 5 min to remove any bloodstains and/or any other adherent materials. The feathers were then oven dried at 50 °C until they regained their original fluffiness. These were then cut into about 5–8 mm size pieces with a pair of scissors. Before using as them as substrate, the feathers were sterilized by heating under pressure at 121 °C for 15 min.

2.5. Optimization procedure and experimental design

2.5.1. Screening the factors affecting β-keratinase production

Our preliminary screening study revealed that from among the sources tested for carbon (glucose, fructose, galactose, maltose, sucrose, lactose, carboxymethyl cellulose, and starch) and nitrogen (yeast extract, beef extract, casein, tryptone, ammonium sulfate, potassium nitrate, ammonium nitrate, and sodium nitrate), glucose and yeast-extract showed best results in terms of keratinase production (data not shown). Next, the Plackett–Burman factorial design was employed to identify the most significant factors affecting the keratinase production by the strain under study. Six factors, viz., the glucose level, yeast extract level, incubation time, agitation rate (rpm), pH of the production medium and incubation temperature were used to determine the key parameters significantly affecting the β-keratinase production. Based on the Plackett–Burman factorial design, each factor was examined at two levels: −1 for the low level and +1 for high level, and a center point was run to evaluate the linear and curvature effects of

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