

IMPROVEMENT OF *BACILLUS LICHENIFORMIS* MZK05 BY MUTATION FOR INCREASED PRODUCTION OF KERATINASE

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Abstract

Bacillus licheniformis MZK05 was subjected to mutation by ultraviolet radiation for enhanced production of keratinase. Of 750 isolates from irradiated plates, 200 colonies that showed zone of casein hydrolysis on Skim Milk Agar were cultured in liquid Feather Meal Medium containing digested feather as carbon and nitrogen source in shake culture at 37°C. The mutant *B. licheniformis* MZK05M9 (*BIM9*) exhibited highest enzyme activity of 170 ± 5.63 U/ml as compared to 74 ± 5.29 U/ml by the wild MZK05. Both the strains were examined for the presence of gene encoded for keratinase (*kerA* gene) by PCR using primer which showed the product sizes 1156 bp and 520 bp, respectively for MZK05 and *BIM9*. The keratinase from both strains exhibited a thermal stability of about 97% for 2 hrs at 40°C whereas the keratinase of the mutant strain showed less stability (55%) at 50°C. The *BIM9* while cultivated in batch culture in 7 litre bioreactor for production of the keratinase in the Feather Meal Medium, the productivity was found to be double (17,608 U/L/hr) than that of in the shake flask culture (8,525 U/L/hr). This improved strain thus will be very useful for large scale production of keratinase enabling its technical applications in industry.

Introduction

Keratinase that has the capability of degrading insoluble keratin is very important for the hydrolysis of keratinous substrates and other applications⁽¹⁾. Thus, keratinolytic enzymes may have important uses in the dehairing of hide and skin in leather processing, biotechnological conversion of keratin-containing wastes from poultry and leather industries through the development of non-polluting processes. Insoluble feather keratins can be converted, after keratinolytic hydrolysis, to feedstuffs, fertilizers, and films^(2,3).

The main source of microbial keratinolytic enzymes are fungi, actinomycetes and bacteria and have been frequently isolated from soils where keratinous materials are deposited^(4,5). *Bacillus subtilis* and *B. licheniformis* are attractive industrial organisms for

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keratinase production particularly for their high growth rate leading to short fermentation period, extracellular secretion of the enzyme and generally regarded as safe (GRAS) organism.

In view of the above fact, some *Bacillus* species were isolated from effluents of poultry farm and tannery and identified by biochemical and 16S rRNA gene sequence analysis⁽⁶⁾ revealing *B. licheniformis* MZK05 (MZK05) as keratinase producer on feather as inducer⁽⁶⁻⁸⁾. It was found that the quality of the MZK05 keratinase was very suitable in the technical applications⁽⁷⁻¹⁰⁾. However, the enzyme productivity in shake flask culture was not enough for meeting the commercial standard. Hence, the present study was undertaken to improve the wild strain for enhanced production of the keratinase by random mutation technique brought forward by ultraviolet irradiation.

Materials and Methods

Bacillus licheniformis MZK05 was obtained from “Enzyme and Fermentation Biotechnology” laboratory of the Department of Microbiology, University of Dhaka. This strain was isolated previously from feather-decomposed soil of poultry farm and identified by both biochemical and 16S rRNA typing in the International Center for Biotechnology, Osaka University, Japan⁽⁶⁾.

One ml of appropriately diluted bacterial suspension was centrifuged at 5000 rpm for 10 min and pellet was resuspended in phosphate buffer (1 ml, 0.1M, pH 7.4). Two hundred microlitre of this suspension was plated on nutrient agar medium and treated with UV irradiation for 90 min with 15 min time interval under UV lamp (lamp distance: 10 cm from the plates) having a wavelength of 254 nm with 220 V at 50 Hz. Then plates were incubated overnight at 37°C. Individual colonies were selected at random based on morphological differential characteristics and plated on Skim Milk Agar medium. Colonies showing large clear zones of casein hydrolysis were selected and enzyme fermentation was carried out in Feather Meal Broth (FMB) medium.

For keratinase production *B. licheniformis* MZK05 was cultivated in FMB medium comprising of following constituents (%): NH₄Cl 0.05, NaCl 0.05, KH₂PO₄ 0.03, K₂HPO₄ 0.04, MgSO₄ 0.01, feather meal 1.0 and yeast extract 0.01 in a 250 ml Erlenmeyer flask and incubated at 37°C and 150 rpm in an orbital shaker for about 48 hrs. After separation of solid liquid by centrifugation, the supernatant was used for assaying keratinase titre and protein estimation.

Keratinase activity was determined with azocasein (Sigma Co. St. Louis. Mo.) as a substrate by a modified procedure described by Kreger and Lockwood⁽¹¹⁾. In brief, 400 µl crude enzyme solution was incubated with 400 µl of 1% azocasein solution in 0.05 M Tris-HCl buffer at pH 8.5 for 1 hr at 37°C in a water bath. The reaction was terminated by addition of 135 µl of 35% trichloroacetic acid (TCA) and keeping the mixture at 4°C for 10 min. The reaction mixture was then centrifuged at 13,000 rpm for 10 min. Then 0.75 ml

supernatant was mixed with 0.75 ml of 1.0 M NaOH and the absorbance was read at 440 nm against the control. The control was treated in the same way, except TCA was added to the crude enzyme before mixing with azo-casein solution. One unit of keratinolytic activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 under the assay conditions. The soluble protein concentration in the culture supernatant was estimated according to the Bradford method⁽¹²⁾.

A single colony was placed on the PCR tube containing 10X PCR buffer (with 15 mM MgCl₂) 3 µl, 10 µM forward *kerA*(F) primer (5'ACGGATCCATGATGAGGAAAAA GAGT-3') 3 µl, 10 µM reverse *kerA*(R) primer (5'ATCTCGAGTTATTGAGCGGCA GCTTC 3') 3 µl, 2.5 mM dNTP mix 2.5 µl, 5 U/µl commercial Taq polymerase (Promega, USA) 0.3 µl and PCR grade water to make final volume 30 µl⁽¹³⁾. The PCR reactions were performed in a thermal cycler (MJ Mini™ BIO RAD, USA) with initial denaturation of template DNA at 94°C for 4 min, followed by 30 cycles of the following steps: Denaturation at 94°C for 1 min, annealing at 56°C for 1.5 min. and extension at 72°C for 1 min. A single final extension was done at 72°C for 10 min. PCR amplification was checked by horizontal electrophoresis on 1.0% agarose gel slab in Tris-borate EDTA (TBE) buffer. 1 kb ladder (Fermentas, USA) was used to ensure amplification of the desired gene with exact product size. The gel was then stained in staining solution (0.5 µg/ml EtBr in TBE) for 15 - 30 min and destained in distilled water for 15 min. The EtBr stained DNA bands were observed on a gel doc system (AlphaImager Mini) and photographs were captured. The molecular weight was determined by Alphaview SA software V 3.4.0.0.

Bioreactor facilities in the Pilot Plant Lab. at the Center for Advanced Research in Sciences of University of Dhaka were used. The vessel volume of the stirred tank bioreactor (model: BIO FLO 110 Fermentor/ Bioreactor; New Brunswick Scientific) was 7.0 liter and the working volume was 3.0 liter. The bioreactor was equipped with instrumentation in order to measure and control the agitation, pH, temperature, foam, dissolved oxygen and gas flow. The fermentation was carried out continuously for 36 hrs.

Results and Discussion

In the present study UV irradiation was applied to develop a high keratinase producing mutant. Upon UV exposure, 750 colonies were taken into concern for determining activity on Skim Milk Agar medium. Based on zone ratio of casein hydrolysis, the number of colonies to be screened was narrowed down to 200. Among them, *Bacillus licheniformis* MZK05M9 (*BIM9*), *B. licheniformis* MZK05M31 (*BIM31*), *B. licheniformis* MZK05M3 (*BIM3*) and the wild strain *B. licheniformis* MZK05 showing the higher zone ratio (diameter of the clear zone divided by diameter of the colony) of 3.33, 2.67, 1.09 and 2.0, respectively were selected for their enzyme titre in shake culture on

FMB medium at 37°C and 150 rpm (Fig. 1). *BIM9* and *BIM31* showed enzyme activity of 170 ± 5.63 and 122.35 ± 4.8 U/ml, respectively while the wild strain showed 74 ± 5.29 U/ml in FMB (Fig. 2). Based on these results, it was revealed that the *BIM9* was the most potential mutant, giving approximately 2.3-fold higher keratinase activity over the wild.



Fig. 1. Zone of casein hydrolysis by *B. licheniformis* mutants on Skim Milk Agar medium.

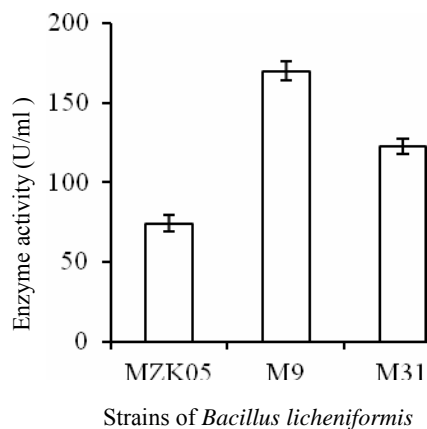


Fig. 2. Titre of keratinase in FMB by wild and mutant strains.

The *kerA* genes were isolated through PCR using same forward and reverse primers. In case of wild-type strain, *B. licheniformis* MZK05, the PCR product possessing 1156 bp was found, whereas in case of the mutant strain *BIM9*, approximately 520 bp long PCR

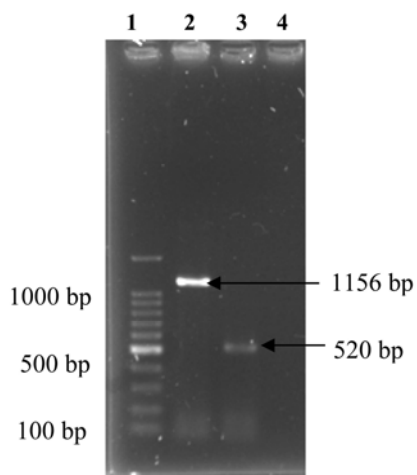


Fig. 3. PCR products from *Bacillus licheniformis* MZK05 and *BIM9*.

Lane 1: marker, lane 2: MZK05, lane 3: *BIM9* and lane 4: Negative control.

product was found (Fig. 3). The amplified *kerA* gene product size of the mutant strain was quite different in size than that of the wild-type strain suggesting gross changes with

the genome which needs further critical studies including sequencing of both the products to ascertain the type of mutation occurred.

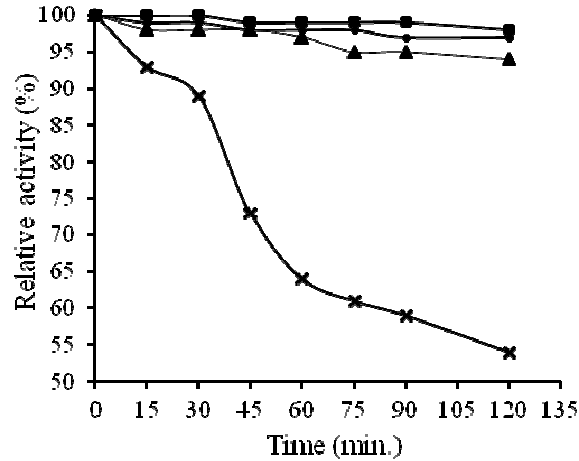


Fig. 4. Thermal stability of keratinase produced by MZK05 and *BIM9* at 40 and 50°C. (■) MZK05 keratinase at 40°C, (●) *BIM9* keratinase at 40°C, (▲) MZK05 keratinase at 50°C and (×) *BIM9* keratinase at 50°C.

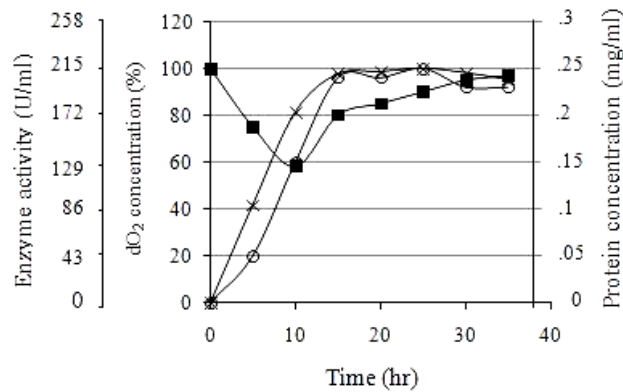


Fig. 5. Time course for keratinase and soluble protein production by *BIM9* in 7 litre bioreactor culture. (■) dissolved O₂ concentration (%), (×) enzyme activity (U/ml) and (o) protein concentration (mg/ml).

At 40°C, the enzymes from both wild and mutant strains showed significant stability for 2 hrs. Enzyme produced by the mutant and wild type retained 97 and 98% enzyme activity, respectively. On the other hand, at 50°C, the enzyme produced by the wild strain was found to be more stable (95 %) than the keratinase from the mutant that gradually decreased to 55% (Fig. 4).

Evaluation of the mutant *BIM9* for keratinase production at bioreactor condition was performed. In the fermentation, medium pH and dissolved O₂ (dO₂) were not kept controlled. Rather, the change of pH and dO₂ was observed. dO₂ started to decrease while the cell growth started to increase. After 9 hrs, dO₂ concentration reached to plateau indicating the stationary phase of cell. From protein concentration and enzyme activity curve, it was also evident that the enzyme production reached its maximum only after 12 hrs (Fig. 5). The enzyme production in bioreactor was growth associated with a higher productivity of 17,608 U/L/hr found as compared to the same in the shake flask culture (8,525 U/L/hr) (Fig. 6). After that, dO₂ concentration started to increase indicating the equilibrium cell growth attained and protein concentration as well as enzyme activity remained stable up to 36 hrs until the fermentation completed.

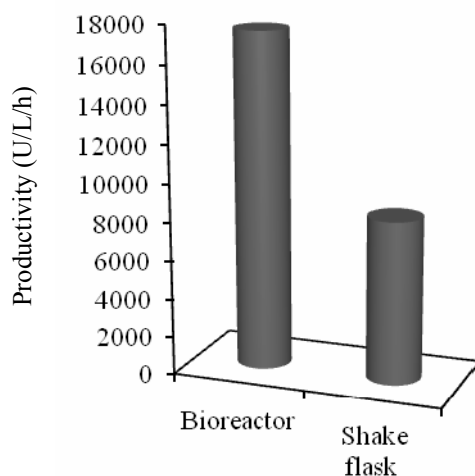


Fig. 6. Keratinase productivities in bioreactor and shake flask fermentation by *BIM9*.

From the bioreactor study, it was also found that the mutant strain was very stable and exhibited a two-fold higher productivity in bioreactor than that of shake culture using FMB medium. Hence, it can be said that the mutant *BIM9* is very much reliable for industrial applications in case of keratinase production.

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