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Advances in Bioresearch

## ORIGINAL ARTICLE

# Production of keratinase by *Bacillus* sp. ND6D under Solid State Fermentation and its application

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**ABSTRACT**
*A newly isolated Bacillus sp. ND6D potential was evaluated to produce keratinase using white chicken feathers as sole energy source under solid state fermentation. Optimization of the fermenting medium was carried out using the one-variable-at-a-time approach and the fermenting parameters optimized includes: moistening agent-50 mM potassium phosphate buffer, pH 7.6 ± 0.2, temperature 50 °C, ions, incubation time 96 h, 10% (v/v) active culture. The maximum enzyme production by Bacillus sp. ND6 under optimized conditions was taken place in Erlenmeyer flasks of capacity 2000 ml with 26.278 ± 0.331 U/g of Protease activity, 76.207 ± 0.960 U/g of keratinase activity, protein 57.844 ± 0.729 mg/g, 26.132 ± 0.329% feather degradation. Most of the tested supplemented organic sources, surfactants and ions in fermenting media showed negative or neutral effect on enzyme production and other parameters. But among all the presence of copper sulfate and zinc sulfate drastically reduced the enzyme production and feather degradation process. While the presence of 20 mM $MgSO_4$ and 300 mM NaCl enhanced both the enzyme production and feather degradation. The keratinase obtained from the fermenting medium showed blood stain removal activity from cotton cloth at broad temperature range (5 to 80 °C). The crude enzyme also showed antimicrobial activity against Escherichia coli, Pseudomonas fluorescence and Staphylococcus aureus. While inspite of inhibition, the keratinous hydrolysate enhanced the biomass of the fungi Alternaria alternata, Aspergillus niger, A. regenerata, Curvularia and Penicillium notatum in the antifungal activity assay.*
***Keywords:*** *keratin, chicken feathers, protease, degradation, antibacterial, antifungal*

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## INTRODUCTION

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries, especially microbial proteases which represent one of the largest groups of industrial enzymes. Keratinase is an example of such enzyme which is highly commercially exploited in last thirty decades particularly due to their potential in numerous industrial applications such as in the animal feeds, fertilizers, detergents, leather, glues, films, and pharmaceutical industries. Keratinases have gained major impetus with the discovery of prion decontamination by keratinase KerA of *Bacillus licheniformis* PWD-1 by Shih's group in 2003 [14]. Since then, the use of keratinases has also been advocated in skin and nail medications [13].

Keratinases (E.C 3.4.21/24/99.11) are a special class of proteolytic enzymes that display the capability of degrading of both soluble and insoluble keratinous substrates and display a great diversity in their biochemical characterisitics [21]. Keratinases are robust enzymes with a wide temperature and pH activity range. Exceptionally, microbial keratinases are predominantly of the metallo, serine or serine-metallo type with the exception of keratinase from yeast which belongs to aspartic protease [27]. For its production from laboratory to industrial level, several other important factors plays a key role viz. keratinolytic microorganisms presence, substrate availability in the form of agro-waste, substrate abundance and its demand in different fields from medical to food industry etc. Keratinolytic degraders

can be found in diverse groups of microorganisms: from fungi, actinomycetes to bacteria. But predominantly bacteria were favoured more for keratinase production due to ease in- handling, cultivation, less pathogenicity, time etc. And the isolated bacterial strains known to degrade keratin or produce the keratinase are primarily composed of *Bacillus*; it includes *B. subtilis* and *B. licheniformis*. Keratin-"substrate for keratinase production", is one of the most abundant biopolymers in the world. Keratinous material is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin, and papain due to the composition and molecular conformation of the amino acids found in keratin, which leads to their accumulation in nature thereby constituting nuisance to the environment [28]. And its abundance increased with the growth of vertebrate animal-based industries, predominantly, 'poultry industries'. For example, the global poultry meat processing industry alone produces $40\times10^6$ ton of waste feathers annually [32], which represent 4 to 7 % of the total weight of mature chickens [1]. The utilization of keratin-based organic waste for enzyme production not only economically favourable for industries but also helps in waste management.

In general, submerged fermentation has been reported for keratinase production by bacteria. There are very few reports which deals with the production of keratinase by different organisms using a keratinous substrate in solid state fermentation (SSF) system irrespective of its several advantages viz. low moisture requirement, less labour intense, ease in handling, cost effective etc. It may be due to requirement of high moisture for bacterial metabolism as compared to fungi, so employment of SSF for metabolite production using bacteria is generally restricted.

Considering several aspects, the present study was conducted to optimize the keratinase production by *Bacillus* sp. ND6D through SSF, using sequential execution of one variable at a time (OVAT) and the application of crude enzyme was also investigated.

## MATERIAL AND METHODS

**Bacterial culture:** A keratinolytic bacterium, *Bacillus sp.* ND6D isolated from bird droppings in our research laboratory was used for keratinase production. The 16S rRNA gene sequence data of *Bacillus sp.* ND6D was submitted to GenBank, NCBI, NIH (SUB5426186 gbMK757681).

**Substrate:** Washed, dried and thread like-grinded native white chicken feathers which were collected from local poultry farm, Kurukshetra, Haryana, India.

**Fermentation medium and culture conditions**

Chicken Feather Medium (CFM) containing 1% w/v **native** white chicken feathers in 0.5 M Potassium-phosphate buffer supplemented with 20 mM $MgSO_4$ and 300 mM NaCl, pH 7.0 inoculated with 18 h old culture of *Bacillus* sp. ND6D was incubated at 37**°C** for keratinase production under SSF**.** Fermented medium was filtered through muslin cloth to remove large debris followed by centrifugation at 10,000 rpm for 10 min at 4 °C to remove all debris and supernatant (crude enzyme) was collected and used for estimation of protein, protease activity and keratinase activity. All debris was collected separately and treated to find the degradation of feathers.

**Optimization of fermentation parameters:** Various conditions for keratinase production by *Bacillus* sp. ND6D were studied by varying one factor at a time, while keeping others as constant. The fermentation parameters studied include incubation temperature (15-70 °C), incubation period (24-168 h), moisture content (0-25 ml/g), pH (4.2-11.0), supplementary sources at varying concentration (carbon, nitrogen, surfactants, and inorganic ions), inoculum age (2-36 h), inoculum size (5-35% v/v) and surface area by changing Erlenmeyer flask size from 50 to 2000 ml . Flasks containing CFM inoculated with active culture were incubated considering all optimized conditions in previous experiment. After incubation, experimental parameters were investigated. All the experiments were conducted twice in triplicates.

**Protein determination**: Protein content was determined as described by Lowry et al. [15] using Bovine serum albumin (BSA) as standard.

**Protease assay**: Sigma's non-specific protease assay method described by Cupp-Enyard [6] was used for the determination of protease activity.

**Keratinase assay:** Keratinase activity was assayed by replacing casein with soluble keratin in sigma's non-specific protease assay method described by Cupp-Enyard (2008). And soluble keratin was prepared from white chicken feathers with some modifications in the method of Wawrzkiewicz et al [36] and the complete method is described in our earlier publication [8].

Concentration of tyrosine produced during the protease and keratinase assay was determined for estimating the enzyme production with the help of standard graph of tyrosine. A unit of enzyme activity (U) is defined as amount of enzyme required to liberate 1.0 µmol of tyrosine per minute under the assay conditions. Enzyme yield was expressed as the activity of enzyme per gram dry substrate (U/g). To

distinguish enzymatic activity unit of protease and keratinase assay initial P and K is added before U i.e. PU- for protease activity unit and KU for used for keratinase activity unit.

**Degradation of feathers:** methodology used is given in Dabas and Garg (2019) and calculated on the basis of following equation: Degradation (%) = $\frac{C-R}{C} \times 100$; C-feather residues left in control, R-feather residues left in inoculated flask.

**Application of Keratinase**

**Stain removal activity**: White test fabric (cotton) cloth piece (approximately 5×5 cm) stained with animal blood was allowed to dry in open air for 12 hr. The stained cloth was put in 250 ml Erlenmeyer flask and treated with 10 ml crude enzyme for 30 min at different temperature (5 to 80 °C). Phosphate buffer was used as control under the same condition. Protein stain removal was checked qualitatively by visualization

***In vitro* antimicrobial activity**

**Antibacterial activity via well diffusion method:** Antibacterial activity of the crude enzyme was tested against *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas fluorescence* and *Staphylococcus aureus*, by the agar well diffusion method. All the test cultures were adjusted to + 0.5 McFarland standards which are visually comparable to the bacterial suspension of 1.5 ×$10^8$ CFU $ml^{-1}$. The microbial suspensions was swabbed over the nutrient agar surface using a sterile cotton swab to ensure uniform growth. After 20 min, 3 wells of 8 mm diameter were cut with the help of cork-borer and filled with 1 ml of negative control (distilled water), 1 ml positive control (ampicillin 30 µg/ml) and 1 ml test compound (enzyme). Then plates were incubated at 37 °C and 50 °C for 48 under continuous observation at an interval of 12 h for the development of inhibition zone.

**Antifungal activity:** The antifungal activity of the crude enzyme was investigated by using poisoned food technique. The growth of molds viz. *Alternaria alternata, Aspergillus niger*, *A. regenerata, Curvularia* and *Penicillium notatum* was carried out on potato dextrose agar (PDA) at 25 °C for 5 days and availed as inocula. Then 5 crude enzyme was transferred in a sterilized Petri plates and then 20 ml of molten sterilized PDA was added to each Petri plate and granted to solidify at room temperature. The solidified poisoned agar plates were inoculated at the centre with fungal plugs, obtained from actively growing colony and incubated at 25 °C for 5 days. Fluconazole was taken as a positive control. The diameter of fungal colonies was measured and antifungal index was calculated by using formula:

$Antifungal\ activity = [1 - \frac{Da}{Db}]$ ; where Da -diameter of growth zone in test plate; Db-diameter of growth zone in control plate.

**Statistical analysis**

The mean and standard deviation from mean were calculated for each treatment. The data were expressed as means ± standard deviations. Differences among various tested variables were determined using Analysis of Variance (ANOVA) with a software MS-Excel 2013. Significance was considered where calculated p-value > 0.05.

The current research work was conducted in the Research Laboratory, Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana-136119, India during the academic period from March 2017 to March 2022 as a part of Ph. D research work.

## RESULTS AND DISCUSSION

**Keratinase Production and Optimization**

In the course of investigation of Keratinase Production by *Bacillus* sp. ND6D under SSF, four different parameters viz. protease activity, keratinase activity, soluble protein content and degradation of substrate i.e. residual feathers were recorded. All the experimental measured parameters indicates keratinase enzyme production as well as its action in terms of feather degradation and enhancement in enzyme production due to optimization of cultural conditions. Effect of various physical and cultural conditions were discussed below.

**Temperature** For keratinase production the optimum temperature is 50 °C as per measured experimental parameters: 12.982 ± 0.016 PU/g; 36.922 ± 0.316 KU/g; 15.649 ± 0.401 mg/g and degradation of feathers 15.235 ± 0.753%. From 37 to 50 °C, enzyme production increased significantly as noticed in terms of enhancement in protease activity (44.94%) & keratinase activity (43.15%) and protein content remained almost constant as shown in Fig. 2a. With further increase in temperature, enzyme production starts declining with only 4% at 55°C and 8% reduction at 60°C, however at temperatures of 65 °C and 70 °C there was significant reduction of 50% and 58% respectively. It was also observed in our results that high temperature supported %degradation of feathers as with rising

temperature from 15°C to 60°C % degradation was also increased from 5.463 ± 0.345 to 15.844 ± 0.298 and on further increasing the temperature to 70°C it became constant

Independent researchers across the globe have suggested that the optimum temperature varied widely for microbial keratinase production. Similar to *Bacillus* sp. ND6D, Rai et al. (2009) reported 50 °C as the optimum temperature for keratinase production by *B. subtilis* RM-01. Even some organisms *Aspergillus flavus* S125 [17] and *Fervidobacterium islandicum* AW-1 [19] showed higher optimum temperature of 55 °C and 70 °C for keratinase production respectively.

**Incubation period:** The optimum time duration was found to be 96 h (15.226 ± 0.258 PU/mg; 45.206 ± 0.766 KU/g; protein: 32.941 ± 0.558 mg/g; degradation of feathers: 21.638 ± 0.367% as with extending incubation time to 120 h, keratinase production was enhanced only by ~2% and declined after further incubation (Fig. 2b). Whereas it was also observed that feather degradation and protein content kept on increasing with the duration of incubation time. But our purpose was to enhance enzyme production with efficient utilization of substrate & energy, so for further optimization work 96 h was chosen for incubation. In case, the objective was to degrade only feathers, then the incubation time could be extentded up-to 7 days in SSF conditions.

Not only in SSF, but under submerged fermentation (SmF) conditions also the optimum keratinase production may take up-to 96 h depending upon the organism and fermentation conditions. For example, *Bacillus licheniformis* isolate S3 showed optimum keratinase production in 96 h at 40 °C with 80% degradation of feathers at 150 rpm [29]. *Bacillus* sp. IIB-B9 showed optimum keratinase production after an incubation period of 96 h at 50 $^{o}$C, 160 rpm [2].

**Moisture content:** As shown in Fig. I (c), the optimum level of moistening agent (MA) was 10 ml/g required for maximum keratinase (45.146 ± 0.370 KU/g) production under SSF conditions. By further raise in moisture content, enzyme production was declined whereas degradation of feathers and protein content were increased. Due to requirement of high moisture level per gram of substrate to moisten the feathers or other keratinous substrates, this process is also sometimes categorized as semi- solid-state fermentation. Similar to these findings other researchers also reported the need of high moisture level during SSF for production of keratinase using keratin source as substrate. For example, 15 ml/g MA was required for *B. weihenstephanensis* PKD5 for keratinase production under SSF using white chicken feathers as substrate [25]. Whereas *Bacillus* sp. MD24 required 500% water content for optimum keratinase production under SSF using chicken feathers as main substrate [20]. *B. subtilis* (MTCC9102) required 100% (v/w) moisture level using horn meal as substrate[12] for keratinase production.

**pH:** As shown in Fig. 2d, the optimum pH for keratinase production was found to be 7.6. The enzyme production and protein content were maximum between pH 7.6 to 8.6. By shifting the pH of the chicken feather medium from acidic (4.2) to alkaline (9.6), % feather degradation were increased from 13.468 to 23.153, on further increasing the pH to 10 and 11 there was respectively 2% and 8% reduction in %feather degradation.

In similar studies it was suggested that alkaline conditions favour keratinolysis and also with degradation of feathers pH of the medium changes towards alkaline (Muhsin & Hadi, 2002; Jain et al., 2012), which may adversely affect released amino acids or polypeptide structure. At higher pH of 10.0 and 11.0 Keratinase and protease reduced by more than 25% and 45% respectively which might have affected the degradation of feathers at these pH. Kumar et al., [11]; Sahoo et al., 2015 and Ahmed et al. (2019), in their studies with SSF of keratinous substrates have reported optimum pH as 7.0, 7.79 and 7.0 respectively for keratinase production using *B. subtilis* (MTCC9102), *B. weihenstephanensis* PKD5 and *Bacillus* sp. IIB-B5.

**Carbon sources:** As shown in Fig. 2e, the addition of all tested sugars viz. cellobiose, fructose, galactose, glucose, maltose, mannitol, mannose, soluble starch, sorbitol, sucrose, trehalose and xylose cause decline in enzyme production. As per data obtained from experiments, supplementation of sugars in CFM causes more harm in terms of enzyme production in comparison to degradation of feathers and protein content as 18 to 41% decline in enzyme production and 5 to 32% in %feather degradation is observed. Whereas protein content increased only by 4. 35% and 5.96% on addition of maltose and sucrose respectively; comparable to control on addition of the sugars cellobiose, glucose, sorbitol and declined by 7-25% on the addition of xylose, mannitol, trehalose, mannose, galactose, soluble starch and fructose. Content of protein is either least affected or increased on addition of other carbon sources which are easily utilized by the organism for its metabolism and growth and thus might have resulted in synthesis of protein in due course. In accordance with these findings, keratinase production was reduced due to supplementation of sugars by *Bacillus pumilus* GHD in SSF [4]. Similar to this, glucose and galactose inhibited the keratinase production by *B. wehenstephanesis* PKD5 strain (Sahoo et al., 2012).

Like bacteria, in fungus also keratinase production is decreased on supplementation of carbon sources. For example, Saber et al. (2010) reported that the supplementation of carbon sources in feather meal lead

to a decline in both keratinase production and feather solubilization by *Alternaria tenusissima* and *Aspergillus nidulans*. Although in the case of *Myrothecium verrucaria* slight enhancement of keratinase production was noted on supplementation of carbon sources [7]. Keratinase production by *Streptomyces sp.* MS-2 is also inhibited due to the supplementation of starch and glucose [16].

**Nitrogen sources:** From the bar graph given in Fig. 2f, it was observed that additional nitrogen sources in CFM did not enhance the enzyme production. Although in comparison to carbon sources, presence of nitrogen sources in CFM causes very less decline in enzyme production by *Bacillus* sp. ND6D in SSF. Addition of tryptose, urea and yeast extract declined 4 to 6% protease production and presence of any other nitrogen source affected the protease enzyme production only by ±3% in comparison to control. Though, more reduction i.e. 5 to 15% in keratinase production was observed on supplementation of nitrogen sources in CFM production medium. In accordance to these results, el-Naghy et al. [9], Son et al. [30] and da-Gioppo et al., [7] also reported a partial or complete catabolite repressive effect on keratinase production by *Chrysosporium georgiae, Bacillus pumilis* and *Myrothecium verrucaria* respectively due to additive nitrogen sources.

In terms of degradation of feathers, any additional nitrogen source enhanced it, for example on addition of BSA, casein, gelatin, SMP more than 9% increase was noticed irrespective of keratinase production. It might be due to release of complex of protease enzymes in the presence of other nitrogen sources in CFM or due to some unexplained reasons. In nature also, inspite of single enzyme, complex of enzymes degrades substrates more efficiently. Veselal & Friedrich [33] also reported that the degradation of different keratinous wastes was enhanced on addition of nitrogen sources by *Paecilomyces marquandii*.

**Surfactants effect:** The effect of surfactants viz. EDTA, SDS, Triton X-100, Tween 20, Tween 80 on addition in CFM was shown in Fig. I (g). On analyzing the results presented in Fig. I (g) it was indicated that on supplementation of surfactants in CFM keratinase production was reduced between 10 to 20%, except SDS, on addition of which there was 5% reduction only. On addition of 5% (v/v) Triton X-100, Tween 20 & Tween 80 in CFM although keratinase production was reduced more than 10% but the protein content and % feather degradation were increased by ~6.64 to ~15.66% and ~14.56 to ~20.78% respectively in comparison to control. Performance of EDTA was very poor among all the tested surfactants w.r.t. all the experimental parameters. Yusuf et al. [36] reported that addition of surfactants viz. EDTA, SDS, Triton X-100 and Tween 20 reduced keratinase production by *Bacillus* sp. Khayat. EDTA and SDS affected it less significantly but Triton X-100 and Tween 20 drastically affect it, inspite of not a significant change in growth. According to Suntornsuk & Suntornsuk (2003), there was no effect on feather degradation due to addition of Tween 80 and Triton X-100 in the production media of *Bacillus* sp. FK 46.

**Inorganic ions effect (Fig. I-h):** Copper sulfate and zinc sulfate usually inhibit bacterial growth and thus enzyme production, this has also been observed in the present study. Among various inorganic ions, supplementation of 50 mM copper sulfate in CFM lead to a maximum 41-45% decrease in enzyme production, protein content and % feather degradation with respect to control by *Bacillus* sp. ND6D under SSF. It was followed by zinc sulfate, due to which ~36% enzyme production, ~12.58% protein content & ~30% feather degradation (%) decreased in comparison to control. However by addition of Molybdenum trioxide, keratinase production was reduced only by 15% whereas 35% reduction in protease production and 25% in % feather degradation and minor increase (2%) in protein content were observed. Supplementation of other than these three salts caused a 1 to 9% decline in keratinase and 3 to 28% in protease production. Though protein content was either slightly reduced or increased by 1 to 9% as compared to control. Feather degradation was enhanced when ammonium, calcium and potassium salts were used additionally. As $MgSO_4$ and NaCl were found to enhance enzyme production by *Bacillus* sp. ND6D under submerged fermentation (SmF) (data not published). Accordingly these salts in their optimized concentration were used as ingredient of moistening agent in CFM. However to study the effect of inorganic ions, CFM was supplemented with these salts additionally. But the values of all analyzed parameters were equivalent to control; it might be due to the fact that their addition and content in the moistening agent was already optimized and there was no need of further addition of these salts. As not much research data is available for comparison with effect of ions on keratinase production under SSF so the results of the present study has been discussed in the light of related data available for SmF. Similar to our observations Awad et al. [4] also reported the positive effect of $Na^+$ and $Mg^{2+}$ ions on keratinase production under SmF by *Bacillus pumillus* GHD, with some negative effects of $MnCl_2$ and drastic effect of $CuSO_4$. Different effect of Zinc ions on keratinase production has been reported by the various workers as Wang et al (2017) reported negative effect of Zinc and ammonium sulfate on keratinase production by *Thermoactinomyces* sp. YT06 and Awad et al.[4] reported positive effect of Zinc.

Yusuf et al. [37] has reported the negative effect of $Cu^{2+}$, $CaCl_2$ and $Fe_2SO_4$ on keratinase production by *Bacillus* sp. khayat under SmF, in the present study also copper ions showed 42% reduction in keratinase production however $CaCl_2$ and $Fe_2SO_4$ has shown only ~5% reduction. $Na_2SO_3$, $Na_2S_2O_3$, $(NH_4)_2S_2O_8$ and $H_2O_2$ are harsh chemicals and are generally used as bleaching agents. These can adversely affect the metabolism of the microorganisms. The pattern of their effect was almost similar as seen in SmF on keratinase production by isolate *Bacillus* sp. ND6D. Although their effect on *Bacillus* sp. ND6D under SSF did not seem to affect its metabolism much. As in comparion to control irrespective of 5 to 23% decline in enzyme production, protein content equivalent to control, while % feather degradation increased 3 to15%. These results indicate that presence of bleaching agents might have supported keratinolysis.

**Inoculum age** of isolate *Bacillus* sp. ND6D did not seem to affect the enzyme production in SSF (Fig. 2j) which might be due to long hours of incubation, culture attains the same metabolic state irrespective of initial state. These results are contrary to those reported by Cai et al., [5], Abdel-Fattah et al., [1] on the significance of inoculum age.

**Inoculum content:** Increasing inoculum (Fig. I-k) content of *Bacillus* sp. ND6D in CFM, did not affect the keratinase production and other analyzed experimental factors. Increasing the inoculum level from 5 to 10% (w/v), enzyme production was increased by more than 8%, on further increasing the inoculums content it was almost constant and slightly declined at still higher content of more than 25%. So 10% (v/v) inoculum content was sufficient for the optimum enzyme production. These results are in accordance with results reported by Rajkumar et al., [23] & Vijayaraghavan et al., [34]. Kumar et al. [12] reported even higher inoculum content 50% (v/v) as optimum for keratinase production under SSF using horn meal as substrate.

**Surface area:** With increase in surface area, increase in enzyme production, protein content and% degradation of feathers was observed as shown in Fig. I-l. By increasing the capacity of flask from 100 ml to 2000 ml, enzyme production as well as %degradation of feathers were increased respectively from 11.676 ± 0.033 PU/g to 26.278 ± 0.331 PU/g, 32.693 ± 0.083 KU/g to 76.207 ± 0.960 KU/g, 22.682 ± 0.058 mg/g to 57.884 ± 0.729 mg/g and from 14.923 ± 0.038% to 26.136 ± 0.329% (Fig. I-L). However best results were observed when flask capacity was increased from 100 to 250 ml with more than 155% increase in enzyme production and 180% protein content, whereas 123.846% increase in feather degradation%. But, this scenario was not observed in further increase in Erlenmeyer flask capacity, especially in case of shift from 1000 to 2000 ml capacity flask. In that case just 5 to 10% increment in enzyme production was noticed, while degradation of feathers almost constant in both flasks. The maximum enzyme production by *Bacillus* sp. ND6 was take place in Erlenmeyer flasks of capacity 2000 ml with 26.278 ± 0.331 PU/g, 76.207 ± 0.960 KU/g, protein 57.844 ± 0.729 mg/g, 26.132 ± 0.329% feather degradation. For selection of optimum flask capacity for fermentation need to evaluate other parameters also viz. energy, surface area, productivity, economy etc.

**Stain removal activity**

The crude keratinolytic enzyme obtained from *Bacillus* sp. ND6D was able to remove blood stain (Fig. 3) from the white cotton cloth piece at all the tested temperature. Although mild scrubbing required to remove the stain completely.

**Antimicrobial activity**

The crude enzyme i.e. keratinous hydrolysate obtained from fermenting CFM by *Bacillus* sp. ND6D was shown antibacterial activity against both Gram-positive and Gram-negative bacteria. Antibacterial activity of the enzyme varied from species to species as maximum inhibition of bacterial growth were shown as clear zone around well (Fig. 4) in case of *Escherichia coli* (150 ± 0.4 mm) followed by *Pseudomonas fluorescence* (110 ± 0.5 mm), *Staphylococcus aureus* (80 ± 0.6 mm), *Bacillus subtilis* (10 ± 0.5 mm) and *B.cereus* (0 mm).

**Antifungal activity**: The crude keratinolytic enzyme of *Bacillus* sp. ND6D was unable to inhibit the growth of all tested fungi viz. *Alternaria alternata, Aspergillus niger*, *A. regenerata, Curvularia* and *Penicillium notatum*. Irrespective of growth inhibition, in the presence of crude enzyme fungal biomass of all tested fungi increased more than 20 % in comparison to control.

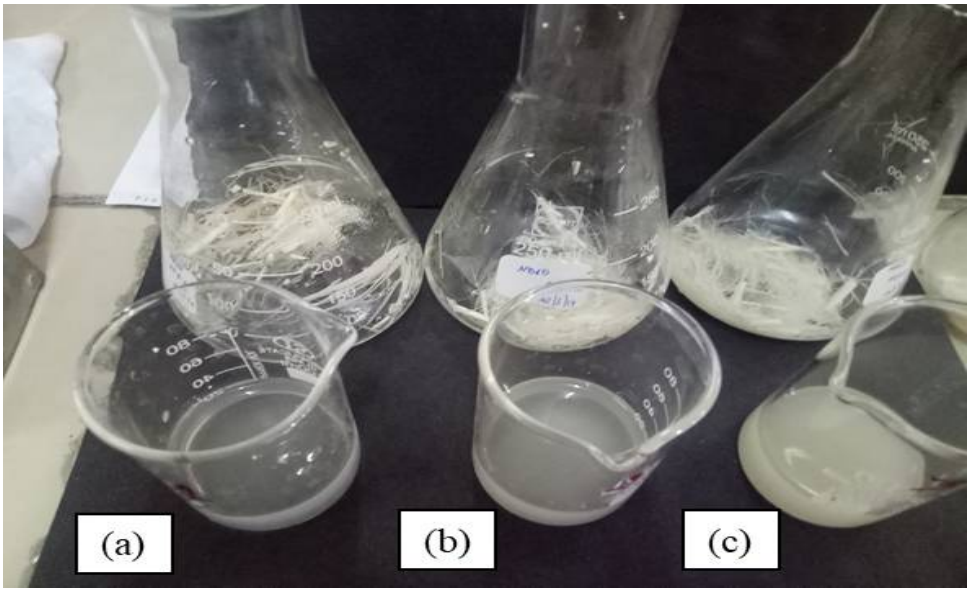


Fig.1 In beaker the supernatant was collected after muslin cloth filtration from the Erlenmeyer flasks containing fermented chicken feather medium by *Bacillus* sp. ND6D at different temperature (a) 15 °C (b) 25 °C and (c) 35 °C.

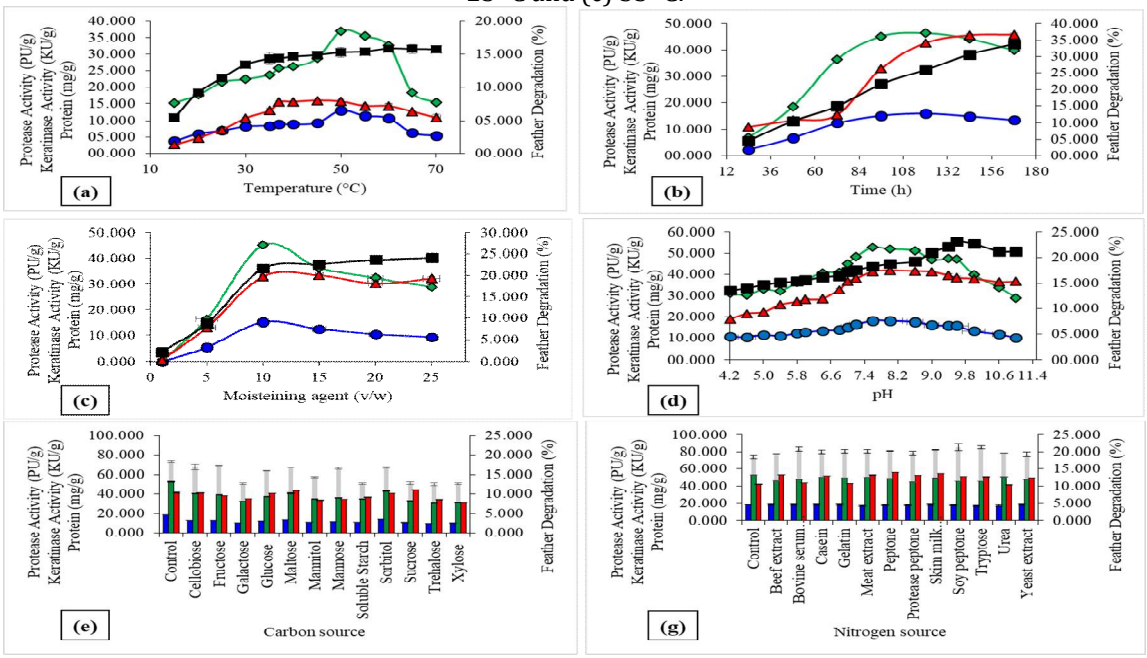


Fig. 2 (continue..)

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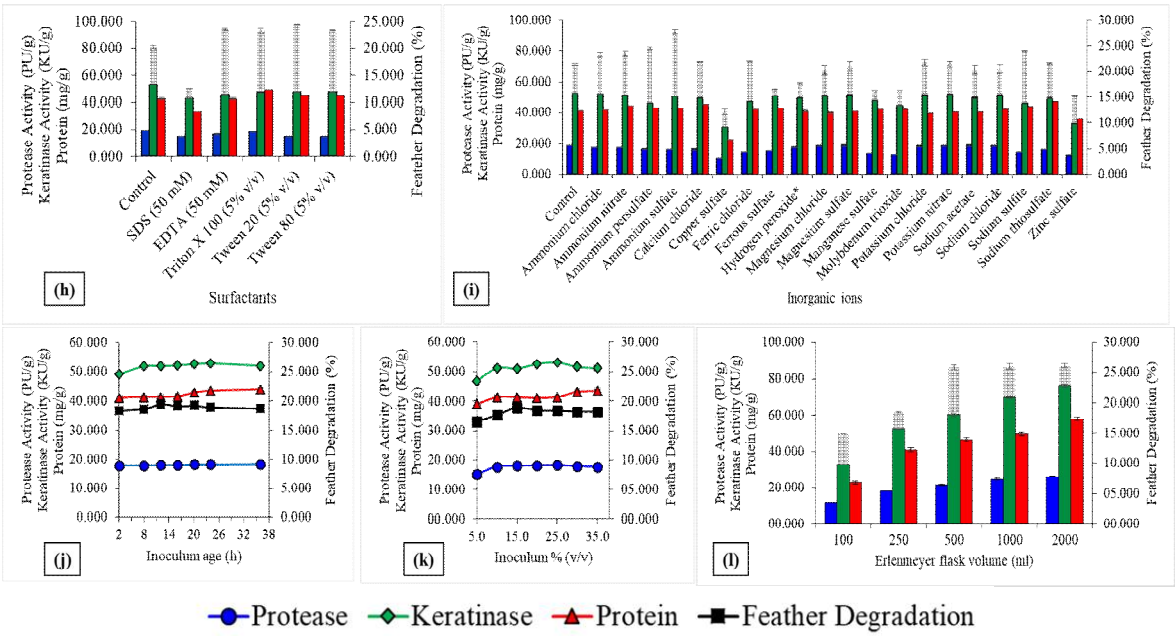


**Fig. 2** Effect of **(a)** temperature **(b)** incubation time **(c)** moisture content **(d)** pH, supplementary sources (**(e)** 0.5% w/v carbon sources **(f)** 0.5% w/v nitrogen sources **(g)** surfactants (**h)** 50 mM inorganic ions) in CFM **(J)** inoculum age **(k)** inoculum content and **(l)** surface area on keratinase production and its associated factors under SSF.

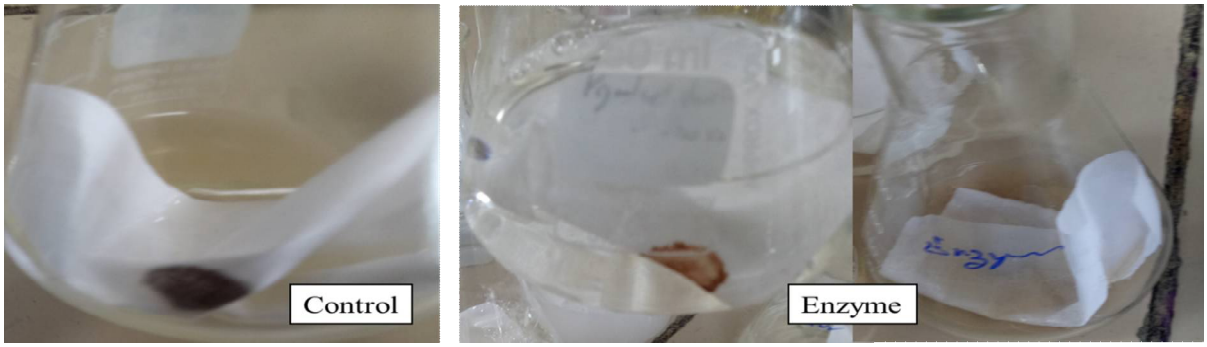


Fig. 3 Visual evaluation of crude keratinase of Bacillus sp. ND6D for removal of blood stain from the white cotton cloth.

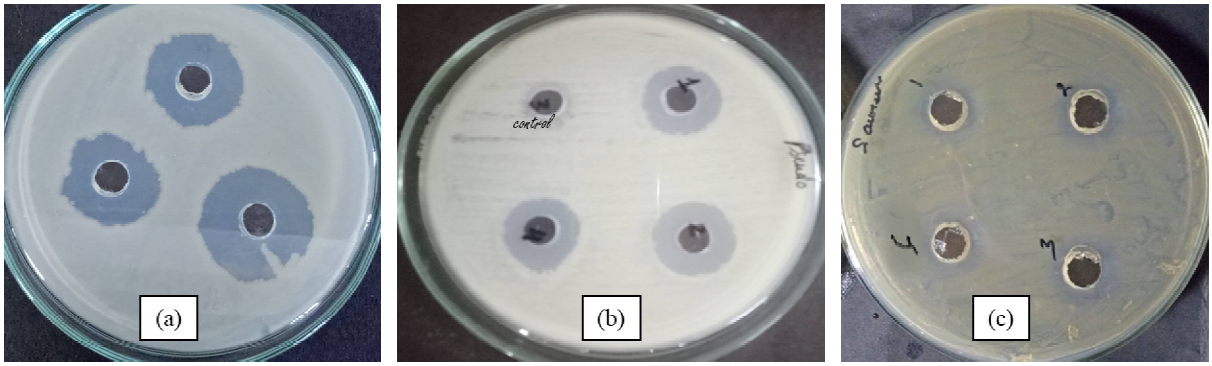


Fig. 4 Antibacterial activity of crude keratinolytic enzyme of *Bacillus* sp. ND6D against (a) *Escherichia coli* (b) *Pseudomonas fluorescence* and (c) *Staphylococcus aureus*.

## CONCLUSION

The *Bacillus* sp. ND6D able to utilize a highly recalcitrant keratin i.e. white chicken feathers as substrate for keratinase production as sole source of energy under solid state fermentation. The enzyme was able to remove blood stain at broad range of temperature, in future its efficiency can be exploited as detergent additive. The efficiency of enzyme to remove stain at broad range of temperature indicates its possibilities. The crude keratinolytic enzyme showed antibacterial activity against both Gram positive

and Gram negative bacteria but not antifungal activity. But the enhancement of fungi biomass on supplementing of keratinous hydrolysate indicates that in future keratinous hydrolysate can be tested as supplementing material in the cultivation media of fungi.

**Conflict of Interest:** There is no conflict of interest.

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