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Optimization for Keratinase Enzyme Production Using *Bacillus thuringiensis* TS2

[1]T. Sivakumar, [1]T. Shankar, [1]P. Vijayabaskar and [2]V. Ramasubramanian

[1]Department of Microbiology,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu, India
[2]Department of Plant Biology and Plant Biotechnology,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu, India

Abstract: Keratinase producing bacterial strains were isolated from the chicken feather dumping site. Keratinase producing bacterial strains were identified by physical, biochemical characteristics, fatty acid methyl ester (FAME) and 16S rDNA sequences. Medium optimization of the selected keratinase producing bacterial strain (*Bacillus thuringiensis* TS2) was checked with various parameters such as incubation time (52.3±1.2 U/mg), substrate concentration (41.3±1.2 U/mg), inoculums concentration (43.54±0.89 U/mg), carbon sources (76.20±0.34U/mg), nitrogen sources (85.60±3.12U/mg), pH (90.78±0.97U/mg), temperature (92.78±0.59U/mg) and chemical inhibitors (112.17±0.02U/mg) that were tested with specific keratinase activity.

Key words: Keratin · Keratinase · Optimization · *Bacillus thuringiensis* TS2 · Feather Waste

INTRODUCTION

Microbial proteases represent one of the largest groups of industrial enzymes and they have extensive applications in a range of industrial and household products including detergents, food, leather, silk and pharmaceuticals industries. Microbial keratinase is an enzyme capable of degrading the insoluble structural protein found in feathers, hair and wool known as keratin. This protein is resistant to degradation by proteolytic enzymes such as trypsin, pepsin, papain due to the composition and molecular conformation of the amino acids found in keratin [1, 2]. Consequently, huge amount of keratinase wastes accumulate in nature and impose a great concern for the environment [3, 4]. Bacillus strains are known to produce and secrete large quantities of extracellular enzymes and constitute a major group of industrial enzyme producers due to the robust nature of the organism as well as its enzymes. Several Bacillus isolates produce extracellular proteases which act on feathers, e.g., *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus* and *B. pseudofirmus* [5].

Cultivation conditions are essential in successful production of an enzyme and optimization of parameters such pH, temperature and media composition is important in developing the optimum fermentation conditions. Keratinase production is affected by various nitrogen sources and its concentration, such as raw feather, feather meal, powdered chicken nails, bovine hair or wool [6]. Besides these, several other physical factors such as aeration, inoculum's density, pH, temperature and incubation time also affect the amount of keratinase production [7].

Bacillus subtilis (MTCC9102) isolate is shown to produce significant amount of keratinase under optimized conditions in solid state fermentation using horn meal as a substrate. Optimized value for moisture, inoculums and aeration are found to be 100% (v/w), 50% (v/w) and 150% (w/w) respectively and the optimum nitrogen source is peptone and carbon source is dextrose. Maximum keratinolytic activity is observed at 48h after incubation and the optimum time (24h) of inoculums is significant [7].

The proteolytic activity is slightly stimulated by $CaCl_2$ and $MgCl_2$ and strongly inhibited by $CuSO_4$ and $ZnSO_4$ whereas as $COCl_2$ and $MnSO_4$ cause a moderate inhibition. From these inhibition assays, it could be inferred that *Bacillus* sp.45 secretes diverse proteolytic enzymes during growth on feather meal optimization [4]. At the industrial level, biochemical and process engineers

Corresponding Author: T. Sivakumar, Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi-626124, India. Mob: +919443310246.

use several strategies to obtain high yields of protease in a fermentor. Controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability have successfully increased keratinase production by 10 fold [8]. In a recent study, the overall keratinase yield from *B. licheniformis* PWD-1 was improved up to 10 times those found during regular batch culture using multistage continuous and fed-batch operations [9]. The present study was mainly focused on the optimization of cultural conditions tested by *Bacillus thuringiensis* TS2 to maximize the production of the keratinase enzyme.

MATERIALS AND METHODS

Isolation and Identification of Microorganism: The soil sample was collected from the feather dumping site at Sivakasi, Tamilnadu, India. The selected isolate shows clear zone around the colony in skim milk agar plates. Then biochemical, carbohydrate test, fatty acid methyl ester (FAME) analysis and 16S rDNA sequences were performed (References??). The organism was submitted at NCBI (National Center for Biotechnology Information) with accession number *Bacillus thuringiensis* TS2 (FJ377887).

Keratinase Assay: To begin the process, 5mg of azokeratin was added to a 1.5ml centrifuge tube along with 0.8ml of 50mM potassium phosphate buffer (pH 7.5) at 37°C for 1h with constant agitation (900rpm). This mixture was agitated until the azokeratin was completely suspended. A 0.2ml aliquot of supernatant of crude enzyme (from culture supernatent) was added to the azokeratin, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2ml of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity.

The absorbance of the filtrate was measured at 450nm with a UV-160 spectrophotometer. A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity is defined as a 0.01 unit increase in the absorbance at 450nm as compared to the control after 15min of reaction [10].

Effect of Various Inoculum`s Size on Keratinase Production: Feather minimal medium was prepared and inoculated with inoculums of various sizes *Bacillus thuringiensis* TS2 such 1, 2, 3, 4 and 5% and kept for 24 h at 37°C with shaking (120rpm). After incubation, the culture from feather minimal medium was centrifuged at 5000 rpm for 15 minutes and supernatant was taken and enzyme activity was estimated by enzymatic hydrolysis of azokeratin, using potassium phosphate buffer (pH 7.5) at 50°C for 15 min.

Effect of Various Incubation Times on Keratinase Production: Feather minimal medium was prepared and incubated at various time intervals (24, 48, 72 and 96 hrs). 0.5% of inoculums from 24h nutrient broth of the culture *Bacillus thuringiensis* TS2 was inoculated in 100 ml of basal feather medium and kept in shaker with 120 rpm at 37°C for various time intervals.

Effect of Various Substrate Concentrations on Keratinase Production: Feather minimal medium was prepared and inoculated with various substrate concentrations (2, 4, 6, 8, 10, 12 and 14mg). 0.5% inoculums from 24h nutrient broth of the culture *Bacillus thuringiensis* TS2 was inoculated in seven different 100 ml of basal feather medium kept in shaker with 120 rpm at 37°C for 24h.

Effect of Various Carbon Sources on Keratinase Production: Feather minimal medium was prepared with different carbon sources of 1% strength (glucose, fructose, sucrose, lactose, galactose, starch, glycerol, maltose, mannitol and dextrose). 0.5% inoculums from 24h nutrient broth culture of *Bacillus thuringiensis* TS2 was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37°C for 24 h.

Effect of Various Nitrogen Sources on Keratinase Production: Feather minimal medium was prepared with different nitrogen sources of 1% strength (ammonium molybdate, ammonium chloride, sodium nitrate, urea, yeast extract, tryptone, peptone, soybean meal, ammonium nitrate and casein). 0.5% inoculums from 24 h culture of the organism *Bacillus thuringiensis* TS2 was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37°C for 24 h.

Effect of pH on Keratinase Production: Feather minimal medium was prepared in different pH (4, 5, 6, 7, 8, 9, 10, 11 and 12). 0.5% inoculums from 24 h nutrient broth of the culture *Bacillus thuringiensis* TS2 was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37°C for 24 h.

Effect of Temperature on Keratinase Production: Feather minimal medium was prepared and 0.5% inoculums from 24 h nutrient broth of the culture *Bacillus thuringiensis* TS2 was inoculated in 100 ml of basal feather medium subjected to various temperature (30, 40, 50, 60, 70 and 80°C) and kept in shaker with 120rpm for 24 h.

Effect of Various Chemical Inhibitors on Keratinase Production: Feather minimal medium was prepared and incubated with various chemical inhibitors like (PMSF, DMSO, EDTA, β-mercaptoethanol, SDS, Mg^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Ca^{2+}, H_2O_2 and Triton X-100). 0.5% inoculums from 24 h nutrient broth of the culture *Bacillus thuringiensis* TS2 was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37°C for 24h.

RESULTS

Effect of Inoculum's Size on Keratinase Production: Maximum keratinase production was recorded at 96hrs in 4% of inoculum for specific activity (43.54±0.89 U/mg) and total activity (178.04±0.89 U/ml) (Table 1). Minimum keratinase production was recorded at 12 hrs in 1% of inoculum (Specific activity 2.11±0.51 U/mg and total activity 12.6±1.8 U/ml) (Table 1).

Effect of Incubation Time on Keratinase Production: Maximum keratinase production was recorded at 96 hrs (specific activity 52.3±1.2 U/mg and total activity 208.5±4.91 U/ml). Minimum keratinase production was recorded at 24 hrs (specific activity 12.0±0.11 U/mg and total activity 50.6±1.8 U/ml) (Fig. 1).

Effect of Substrate Concentration on Keratinase Production: Maximum keratinase production was recorded at 10 mg (specific activity 41.3±1.2 U/mg and total activity 160.5±4.91 U/ml). Minimum keratinase production was recorded at 2 mg (specific activity 2.0±0.11 U/mg and total activity 95.6±1.8 U/ml) (Fig. 2).

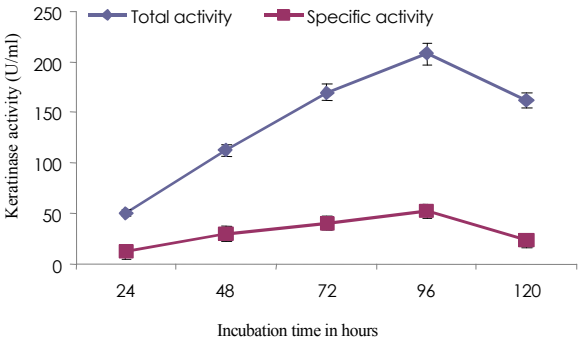

Fig. 1: Effect of incubation time on keratinase production

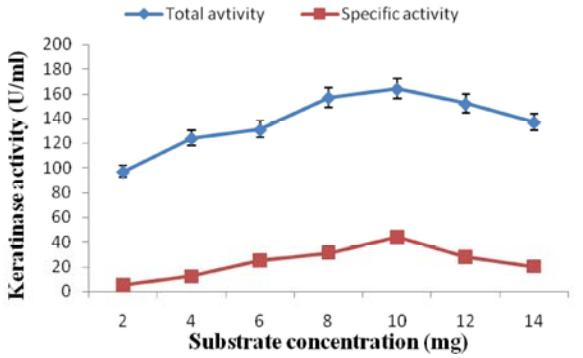

Fig. 2: Effect of substrate concentration (Azokeratin) on keratinase production

Effect of Different Carbon Sources on Keratinase Production: The maximum keratinase production was recorded in mannitol (specific activity 76.26±0.34 U/mg and total activity 130.80±2.6 U/ml) supplemented medium. The minimum keratinase production was recorded in lactose (specific activity 10.73±1.04 U/mg and total activity 41.29±0.51 U/ml) added medium (Fig. 3).

Effect of Nitrogen Sources on Keratinase Production: Maximum amount of enzyme production was found in peptone (specific activity 85.60±3.12 U/mg and total activity 197.60±3.15 U/ml) supplemented medium and minimum amount of keratinase production was observed in ammonium nitrate (specific activity 17.20±0.05 U/mg and total activity 42.80±0.43 U/ml) supplemented medium (Fig. 4).

Table 1: Effect of inoculum's size on keratinase production (specific activity)

		Keratinase activity (U/ml)				
Strain	Inoculum's size %	12h	24h	48h	72h	96 h
TS2	1	2.11±0.51	7.78±0.87	8.44±0.48	9.88±0.49	11.55±0.47
	2	4.10±0.54	9.25±0.94	10.78±0.51	11.11±0.48	25.69±0.89
	3	7.49±0.64	9.45±0.84	17±0.54	20.22±0.87	19.53±0.45
	4	7.45±0.58	11.25±0.99	28±0.55	21.54 ±0.56	43.54±0.9
	5	3.14±0.24	8.44±1.20	21.89±0.84	19.45±0.94	25.47 ±0.12

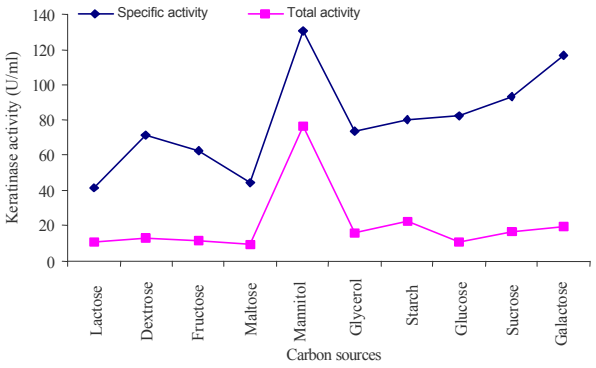

Fig. 3: Effect of different carbon sources on keratinase production

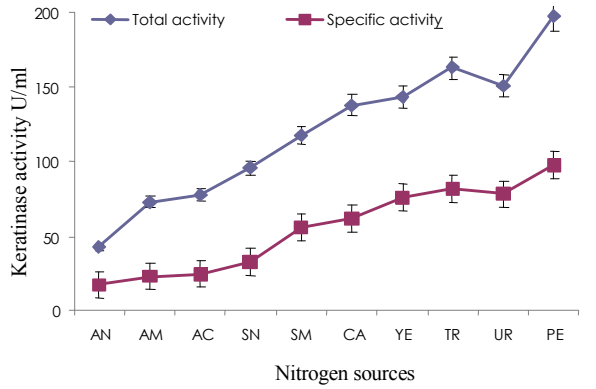

Fig. 4: Effect of nitrogen sources on keratinase production.
AN - Ammonium nitrate; AM - Ammonium molybdate; AC - Ammonium chloride; SN - Sodium nitrate; SM - Soybean meal; CA - Casein; YE - Yeast extract; TR - Tryptone; UR - Urea; PE - Peptone.

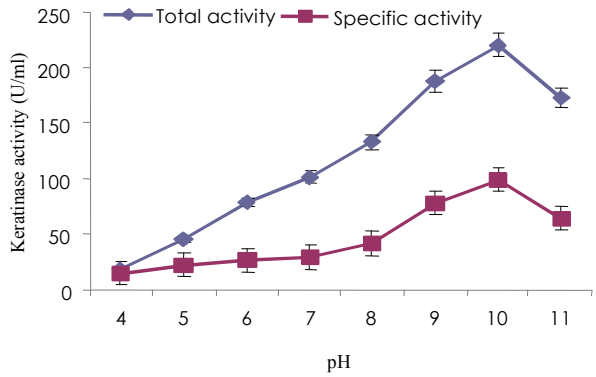

Fig. 5: Effect of different pH on keratinase production

Effect of Different Ph on Keratinase Production: Maximum keratinase production was recorded at pH 10.0 (specific activity 90.78±0.97 U/mg and total activity 220.34±1.47 U/ml). Minimum keratinase production was recorded at pH 4.0 (specific activity 15.14±1.97 U/mg and total activity 18.35±2.12 U/ml) (Fig. 5).

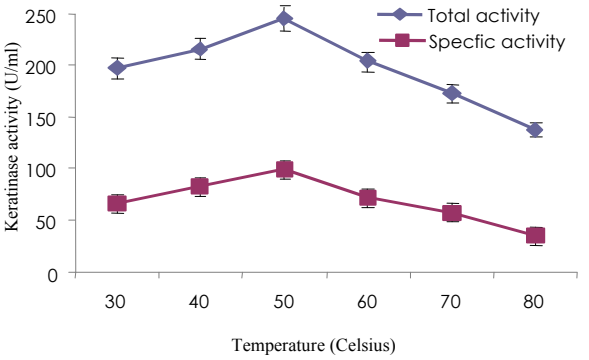

Fig. 6: Effect of different temperatures on keratinase production

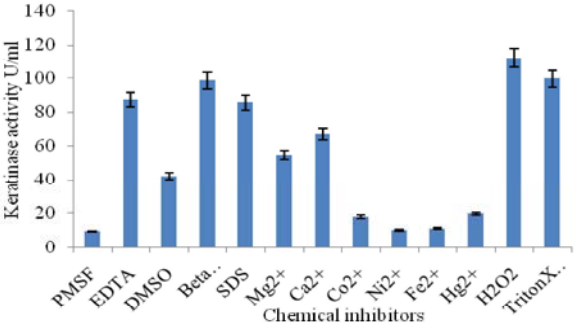

Fig. 7: Effect of various chemical inhibitors on keratinase production

Effect of Different Temperatures on Keratinase Production: Maximum keratinase production was recorded at 50°C (specific activity 92.78±0.59 U/mg and total activity 245.79±0.54 U/ml). Minimum keratinase production was recorded at 80°C (specific activity 34.32±2.78 U/mg and total activity 137.35±2.32 U/ml) (Fig. 6).

Effect of Various Chemical Inhibitors on Keratinase Production: Maximum keratinase production was recorded in H_2O_2 supplemented medium (specific activity 112.17±0.02 U/mg). Minimum keratinase production was recorded in PMSF supplemented medium (specific activity 9.1±0.54 U/mg) (Fig. 7).

DISCUSSION

Effect of Carbon and Nitrogen Sources on Keratinase Production: The proteolytic activity of crude keratinase is assessed by incubating keratinase with different carbon and nitrogen sources. Extra carbon (starch) and nitrogen

(yeast extract) have positive effects on keratinase production while other carbon and nitrogen sources have negative effects. Starch stimulates keratinase production strongly and is selected as an extra carbon source in further optimization (Ref.??). Any effects of extra carbon and nitrogen sources as product formation are normally optimized further. In the case of keratinase, addition of extra sources has been discussed widely (Ref??).

Ramnani and Gupta [11] reported that in optimization of medium for keratinase production by *Bacillus subtilis* RGI, glucose and peptone were found to have positive effects. Usually glucose has negative effects on microbial proteinase (keratinase included) production. For example, the keratinase produced by strain *Aspergillus fumigatus* [12]. *Thermoactinomycetus candidus* and *Stenotrophomonas* sp. D-[13] are partially inhibited by glucose. As for other carbon sources, Antarctic actinomycetes strains of *Streptomyces flavis* 2BG and *Microbiospora aerata* IMBAS-IIA produced much more keratinase in wool substrate upon the addition of starch [14]. The effects of nitrogen sources on keratinase production also vary. Supplementation of yeast extract resulted in maximal keratinase production by *Stenotrophomonas* sp. D-I [13].

The low cost substrates were screened for the maximum production of keratinase. Some cost effective substrate such as soybean meal have been successfully used [15]. The strain grew well and produced appreciable level of alkaline keratinase using feather as sole source of nitrogen. In most cases, keratin serves as the inducer, however, soy meal is also known to induce enzyme production [16, 17].

Tatineni *et al*. [18] observed that, in presence of sucrose, starch or glucose (1%), the protease activity was almost constant. However it decreased significantly in the absence of carbon sources. The activity obtained with 1% starch was similar to that with 2% starch in SS medium. The protease activity was enhanced 4 fold in presence of starch. Effect of different inorganic nitrogen sources shows highest protease activity with soybean meal. Soybean meal acts as inducer for protease production from *Conidiobolusoronatus* sp. [19]. An Enhancement of 7.4 fold increase in protease activity was achieved by supplementation of 0.3% $CaCO_3$. This indicated that the calcium ion was necessary for enzyme production [20]. Thus, the optimization studies resulted in the following findings: the most suitable nutrient medium starch (1%), soybean meal (1%) and $CaCO_3$ (0.3%) of initial pH 9.0, temperature 30°C, 1% inoculum and period of incubation at 6h (References??).

Different bacteria have different preferences for either organic or inorganic nitrogen for growth and enzyme production although complex nitrogen sources are usually used for alkaline protease production [21, 22]. *B. subtilis* MTCC9102 strain has shown a better performance for organic nitrogen sources compared to inorganic nitrogen for keratinase production, but surprisingly, *B. subtilis* MTCC9102 required ammonium sulfate as second preference which yields high keratinase. From this observation, organic nitrogen sources like peptone and yeast extract were found to suppress the protease production an alkalophilic strain of *Arthrobaeter ramosus* MCMB 351 [23].

Chen *et al*. [24] described complete inhibition of the extracellular protease production from *Geobacillus caldoproteolyticus* strain SF03 in presence of glucose, a versatile source of carbon. However, *Bacillus subtilis* MTCC 9102 shows that keratinase synthesis is enhanced when dextrose and other carbohydrates are supplied as co-carbon sources to the fermentation medium.

Bacillus licheniformis ER15 produce maximum keratinase after 48h of incubation at 37°C and 200 rpm in feather peptone medium (FM1) which is much higher in comparison to *B. licheniformis* MZK-3 and RG1 [25]. Starch releases carbon slowly favouring enzyme production and glucose exhibited catabolite repression and inhibited enzyme production [26].

The optimum keratinolytic activity produced by *Bacillus pumilus* AI is achieved with peptone as nitrogen sources followed by the yeast extract. The present study showed that *Bacillus thuringiensis* TS2 keratinase production was enhanced when mannitol was supplied as co-carbon and peptone supplied as co-nitrogen sources. The choice of carbon and nitrogen sources has a major influence as the maximum yield of enzymes.

Johnvesly *et al*. [27] observed maximum activity keratinase in the presence of 2% yeast extract on the first day with a decrease thereafter, Maximum keratin hydrolyzing activity was achieved at higher yeast extract concentrations in a shorter period than in the presence of lower yeast extract concentrations. Because higher concentration of yeast extract provides higher concentrations of amino acids, proteins and vitamins that are essential for improved cell growth and synthesis of enzymes such as proteases [28]. This would add an economic advantage to the enzyme production and its use in hydrolysis of abundant feather waste.

Optimization of pH: Hossain *et al.* [25] and Najafa *et al*. [29] reported that the bacterium could grow over a wide pH range (7–12) while keratinase production is limited to pH 7–10 with maximum production of 244 U/ml at pH 7.

In the present study *Bacillus thuringiensis* TS2 showed maximum keratinase activity at pH 10.0 Similarly, keratinolytic proteases produced by other *Bacillus* sp. isolated from the Amazon basin were reported to be optimally active at pH 9.0 [30, 31]. Most of the studies on *Bacillus* sp reported the production of alkaline proteases, with particular emphasis on their utilization as detergent additives and in the leather industries [32, 30].

Different organisms show maximum enzyme production at different pH levels. For example, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus* produce maximum enzyme at pH levels of 7.0, 5.9 and 5.6 respectively. According to Kunert [33] dermatophytes and non dermatophytes metabolize free or combined cystein as a source of sulphur and nitrogen. The products of cystine metabolism by fungi are inorganic sulphur and other intermediate products and also indicated that the excessive sulphur is excreted back to the medium in the oxidized form as sulphate and sulphite. At neutral to alkaline pH sulphite reacts with cystine, cleaving it to cystein and S-sulphocystein.

Khardenavis *et al*. [34] reported that the enzyme showed very little activity at pH 4.0–6.0 after which an increase in activity was observed up to pH 10.0. The activity at pH 10.0 was 1.8 fold higher than at pH 7.0. Activity decreased slightly at pH 11.0, though it was still 1.6 fold higher than at pH 7.0. Thus, pH 10.0 was found to be optimal for high enzyme activity and the enzyme was found to be active between the neutral to alkaline ranges of pH.

The enzyme activity is studied for *Bacillus thuringiensis* TS2 over a broad range of temperature (30-80°C) and it is found to be optimal at 50°C. Further increase in the temperature to 80°C reduces the relative activity. The high thermo stability allows performance of the industrial process at high temperature and minimizes the risk of microbial contamination. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperature [35]. Some mesophilic bacteria exhibit the optimal enzymes production and activity from 20-30°C [36].

Lin *et al*. [35] demonstrated that the three keratinolytic protease-producing isolates were thermophiles *B. subtilis* and *B. pumilus* were able to grow at 55°C but the optimal temperature for enzyme production was 40°C. Meanwhile, *B. cereus* was able to grow at 40°C with the optimal temperature for enzyme production at 30°C. In the feather medium, the best temperature range for the production of keratinolytic protease by feather degrading bacilli was observed in *B. licheniformis* and *B. brevis* between 40° and 45°C. *Staphylococcus* sp. was able to grow at 50°C, but its optimal temperature for enzyme production was 37–40°C. The temperature for maximum enzyme production is slightly lower than that for growth, which is consistent with the present results. Williams and Shih [37] also reported that maximum growth of *B. licheniformis* PWD-1 was observed at 50°C. In the modified culture method, both starter and main cultures were incubated at 40°C for *B. subtilis* and *B. pumilus*, but at 30°C for *B. cereus*.

Laxman *et al*. [38] observed that the enzyme showed broad temperature specificity with a maximum activity at 60°C. The relative activity of enzyme was found to be higher in the temperature range of 50–70°C than at the normal assay temperature (40°C), while the enzyme retained nearly 50% of its activity at 80°C.

Effect of Various Chemical Inhibitors on Keratinase Production: In the present work, *Bacillus thuringiensis* TS2 was totally inhibited by PMSF and partially inhibited by $CaCl_2$ and it was stimulated by H_2O_2, Triton X-100 and β-mercaptoethanol. Several reports have shown that the serine proteases are slightly affected by metalloprotease inhibitors [39, 40]. Heavy metal ions such as Cu^{2+} [41-43], Mg^{2+} [42] and Zn^{2+} [43] have inhibitory effects on keratinolytic activity. Contrarily, Ca^{2+}, Mg^{2+} and Mn^{2+} stimulate some bacterial keratinase [41].

From the economic point of view, keratinase production by *Bacillus thuringiensis* TS2 can be carried out in the optimized parameters like incubation time, substrate concentration, inoculums concentration, carbon sources, nitrogen sources, pH, temperature and chemical inhibitors which were tested with specific activity.

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