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Optimization of Cultural Condition for Keratinase Production Using *Bacillus cereus* TS1

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Abstract: Method: Three different soils were collected from various feather dumping sites at Sivakasi area for this study, the samples were screened for efficient protease producing microorganism. Among the eleven strains isolated from the sample, one strains (TS1) produced zone of inhibition on skim milk agar (20 mm), respectively. Azokeratin assay was performed with 24 h culture supernatant and keratin assay experiment was conducted for isolate (TS1 showed higher keratinase activity. Maximum keratinase specific activity was observed in TS1 (53.2±1.5 IU mg^{-1}) which was selected for further studies. Bacterial strain was identified by physical, biochemical characteristics and Fatty Acid Methyl Ester (FAME) and 16S rDNA analysis. **Results:** The maximum keratinase production was recorded in 4 mg of substrate concentration and the inoculums size of 4% showed higher enzyme activity (51.01±0.64 IU mg^{-1}). After medium optimization process, It was revealed that, the after 72 h of incubation period maximum keratinase production was recorded in starch (specific activity 84.47±0.74 IU mg^{-1}) supplemented medium for *Bacillus cereus* (TS1). *Bacillus cereus* TS1 showed maximum amount of enzyme production in yeast extract (specific activity 241.42±0.01 IU mg^{-1}) supplemented medium Regarding the favourable pH for maximum keratinase production for *Bacillus cereus* (TS1) at pH 9.0. Interestingly, the favorable temperature for maximum production of keratinase are found to be 50°C. **Conclusion:** The proteolytic activity was slightly stimulated by $CaCl_2$ and $MgCl_2$ and strongly inhibited by $CuSO_4$ and $ZnSO_4$, whereas $CoCl_2$ and $MnSO_4$ caused a moderate inhibition for *Bacillus cereus* TS1.

Key words: Keratin, keratinase, optimization, *Bacillus cereus*, feather waste

INTRODUCTION

Poultry feather accumulates as a waste after processing the chicken for human consumption; thus the waste carries potent polluting implications, especially with promising global poultry production.Feathers are poultry products by rich in protein (mainly α and β keratin keratin) generated in very large amounts as a waste product from poultry processing industry. This type of protein is not easily brokendown by applying enzymes such as trypsin, pepsin etc. Proteolytic enzyme is easily hydrolyze the insoluble keratins more powerfully than other proteases are called keratinases. They are classified into into various groups based on whether they are acidic, neutral, or alkaline conditions and catalytic mechanism and further classified into serine proteases, aspartic proteases, cysteine proteases and metalloproteases by the action of enzyme to specific attachment site.. Most proteases are active in a neutral and alkaline pH and its different characteristics and different origins. Generally they are belonging to the extracellular enzymes (Rao *et al*., 1998).

The Keratinokytic enzymes capable of degrading keratinous proteins which give rise to the simple polypeptides and amino acids which have some nutritional values. Onifade *et al.* (1998) and Farag and Hassan (2004). Specific proteases extracted intracellularly or extracellularly by keratin degrading microorganisms are named keratinases or keratinolytic enzymes. These enzymes have the capacity to act on compact substrates better than other comparable proteolytic enzymes; this distinguishes keratinase from other proteases and peptidases.

Medium 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.The enzyme is greatly influenced by the various nitrogen sources, carbon sources and its concentration, inoculums concentration, substrate concentration and metal ions (Riffel and Brandelli, 2006). Besides these, several combination of other physiogical factors by response surface methodology using central composite design had

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significantly effect the affect the amount of keratinase production (Sangali and Brandelli, 2000).

This study was mainly focused on the medium optimization of the selected bacterial strain (*B. cereus* TS1) with various parameters such as incubation time, substrate concentration, inoculums concentration, carbon sources, inorganic nitrogen sources, organic nitrogen, pH, temperature and chemical inhibitors were tested for the significant production of keratinase enzyme.

MATERIALS AND METHODS

Optimization of physiological factors for keratinolytic activity: The factors like pH, temperature, carbon, nitrogen and metal ions concentration which were expected to affect the production of keratinase by the selected strain were optimized by selecting one parameter at a time.

Effect of various inoculums sizes on keratinase production: Feather minimal medium was prepared and inoculated with inoculums of various sizes such 1, 2, 3, 4 and 5% and kept for 24 h from nutrient broth of *B. cereus* TS1 culture at 37°C with 120 rpm.

Effect of various incubation times on keratinase production: Feather minimal medium was prepared and incubated at various time intervals (24, 48, 72 and 96 h). A 0.5% inoculum from 24 h nutrient broth of the culture (*B. cereus* TS1) was inoculated in 100 ml of basal feather medium and kept in shaker at 37°C for various time intervals with 120 rpm.

Effect of various substrate concentrations on keratinase production: Feather minimal medium was prepared and incubated at various substrate concentrations (2, 4, 6, 8, 10, 12 and 14 mg). A 0.5% inoculum from 24 h nutrient broth of the culture (*B. cereus* TS1) was inoculated for 24 h in seven different 100 mL of basal feather medium and kept in shaker at 37°C with 120 rpm.

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). The 0.5% inoculum from 24 h nutrient broth culture of *B. cereus* TS1 was inoculated in two different 100 mL of basal feather medium and kept in shaker at 37°C for 24 h with 120 rpm.

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). A 0.5% inoculum from 24 h culture of the organism *B. cereus* TS1 was inoculated in 100 mL of basal feather medium and kept in shaker at 37°C for 24 h with 120 rpm.

Effect of pH on keratinase production by the isolates: Feather minimal medium was prepared at different pH (4, 5, 6, 7, 8, 9, 10, 11 and 12). A 0.5% inoculum from 24 h nutrient broth of the culture *B. cereus* TS1 was inoculated in 100 mL of basal feather medium and kept in shaker at 37°C for 24 h with 120 rpm.

Effect of temperature on keratinase production by the isolates: Feather minimal medium was prepared and 0.5% inoculum from 24 h nutrient broth of the culture *B. cereus* TS1 were inoculated in two different 100 mL of basal feather medium subjected to various temperature (30, 40, 50, 60, 70 and 80°C) and kept in shaker for 24 h with 120 rpm.

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). A 0.5% inoculum from 24 h nutrient broth of both the culture *B. cereus* TS1 was inoculated in 100 mL of basal feather medium and kept in shaker at 37°C for 24 h with 120 rpm.

Assay condition: After incubation, the culture from feather minimal medium was centrifuged at 5000 rpm for 15 min 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.

RESULTS

Effect of inoculum size on keratinase production by *B. cereus* (TS1): Keratinase production by *B. cereus* (TS1) was assayed after various inoculum sizes of 1, 2, 3, 4 and 5% (Table 1). Maximum keratinase production was observed by *Bacillus cereus* (TS1) at 72 h in 4% of inoculum for specific activity (51.05± 0.64 IU mg^{-1}) and total activity (231.12±0.15 IU mL^{-1}). Minimum keratinase production for *B. cereus* (TS1) was recorded at 12 h (specific activity 4.01±0.54 IU mg^{-1}) and total activity 24.61±2.73 IU mL^{-1}) (Table 1).

Effect of incubation time on keratinase production by *B. cereus* (TS1): Keratinase production by *B. cereus* (TS1) isolated from feather waste dumping soil was

Table 1: Effect of inoculum size on keratinase production (specific activity) by *Bacillus cereus* (TS1)

Strain	Inoculum size (%)	Keratinase activity (IU mL^{-1}) 12 h	24 h	48 h	72 h	96 h
TS1	1	4.01±0.54	5.12±0.45	7.57±0.21	9.14±0.54	2.36±0.25
	2	3.14±0.84	5.17±0.46	8.11±0.32	10.12±0.55	2.65±0.58
	3	5.89±0.45	7.45±0.51	10.45±0.24	16.58±0.59	3.11±0.20
	4	9.11±0.48	13.24±0.52	34.21±0.37	51.01±0.64	1.25±1.20
	5	4.95±0.62	12.12±0.54	29.13±0.41	37.22±0.55	1.89±1.50

Valuies are mean of triplicate values

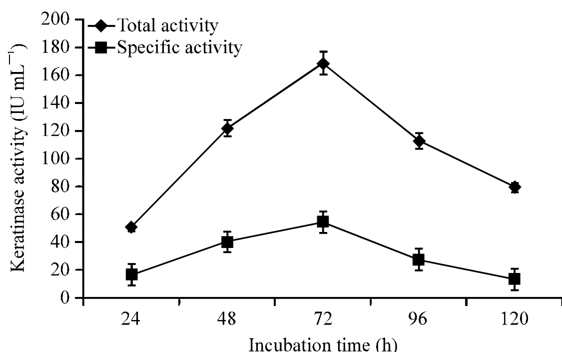

Fig. 1: Effect of incubation time on keratinase production by *B. cereus* (TS1)

adjusted under varying cultural conditions. The keratinase production was assayed after various incubation time intervals like 24, 48, 72, 96 and 120 h at 37°C (Fig. 1). Maximum keratinase production for *B. cereus* (TS1) was recorded at 72 h (specific activity 54.10±1.34 IU mg^{-1}) and total activity 167.4±4.1 IU mL^{-1}). Minimum keratinase production for *B. cereus* (TS1) was recorded at 120 h (specific activity 13.2±0.51 IU mg^{-1} and total activity 50.3±2.73 IU mL^{-1}.

Effect of substrate concentration on keratinase production by *B. cereus* (TS1): The keratinase production was assayed at various substrate concentrations such as 2, 4, 6, 8, 10, 12 and 14mg at 37°C. Maximum keratinase production for *B. cereus* (TS1) was recorded at 10 mg (specific activity 46.12±1.34 IU mg^{-1} and total activity 175.34±4.1 IU mL^{-1}). Minimum keratinase production for *B. cereus* (TS1) was recorded at 2mg (specific activity 7±0.51 IU mg^{-1} and total activity 100.4±2.73 IU mL^{-1} (Fig. 2).

Effect of different carbon sources on keratinase production by *B. cereus* (TS1): The effect of various carbon sources such as lactose, dextrose, fructose, maltose, mannitol, glycerol, starch, glucose, sucrose and galactose checked for keratinase production by the candidate species *B. cereus* (TS1) after 24h of incubation period at 37°C is given in Fig. 3. Here, the maximum

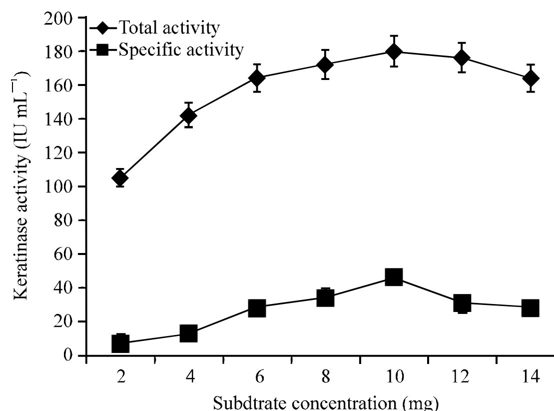

Fig. 2: Effect of substrate concentration on keratinase (azokeratin) production by *B. cereus* (TS1)

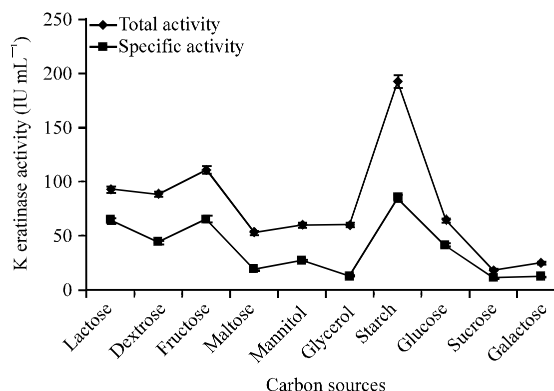

Fig. 3: Effect of different carbon sources on keratinase production by *B. cereus* (TS1)

keratinase production was recorded in starch (Specific activity 84.47±0.74 IU mg^{-1} and total activity 192.60±4.87 IU mL^{-1}). The minimum keratinase production was recorded in sucrose (Specific activity 5.59±0.14 IU mg^{-1} and total activity 18.4±3.61 IU mL^{-1} supplemented medium.

Effect of nitrogen sources on keratinase production by *B. cereus* (TS1): The effect of different nitrogen sources such as ammonium nitrate, ammonium molybdate,

ammonium chloride, sodium nitrate, soybean meal, casein, yeast extract, tryptone, urea and peptone checked for keratinase production after 24 h of incubation period at 37°C showed maximum amount of enzyme production in yeast extract (specific activity 241.42±0.01 IU mg^{-1} and total activity 293.90±2.5 IU mL^{-1}) supplemented medium and minimum amount of keratinase production in ammonium nitrate (specific activity 9.26±0.67 IU mg^{-1} and total activity 43.40±0.56 IU mL^{-1}) supplemented medium (Fig. 4).

Effect of different pH on keratinase production by *B. cereus* (TS1): The keratinase production was assayed after 24 h of incubation at 37°C under various pH like 4, 5, 6, 7, 8, 9, 10 and 11 (Fig. 5). Maximum keratinase production for *B. cereus* (TS1) was recorded at pH 9.0 (specific activity 95.60±0.5 IU mg^{-1} and total activity 197.20±3.78 IU mL^{-1}). Minimum ker tin se production for *B. cereus* (TS1) w s recorded t pH 4.0 (specific ctivity 19.71±1.87 IU mg^{-1} nd tot l ctivity 102.78±0.21 IU mL^{-1}).

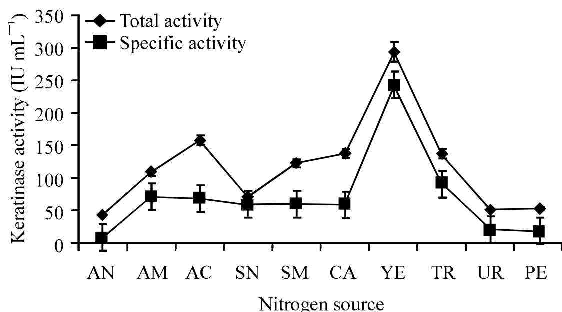

Fig. 4: Effect of various nitrogen sources on keratinase production by *B. cereus* (TS1), AN: Ammonium nitrate, AM: Ammonium molybdate, AC: Ammonium chloride, SN: Sodium nitrate, SM: Soybean meal, CA: Casein, YE: Yeast extract, TR: Tryptone, UR-Urea, PE: Peptone

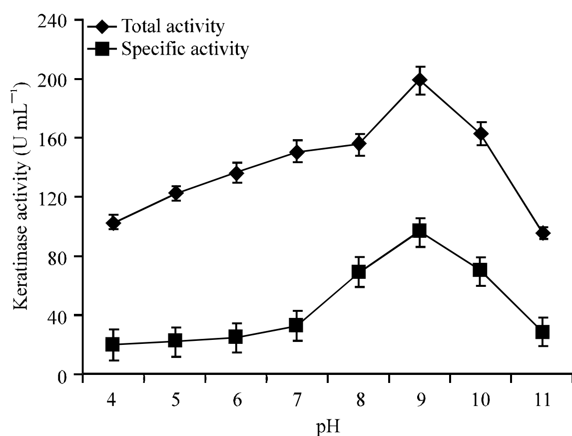

Fig. 5: Effect of different pH on keratinase production by *B. cereus* (TS1)

Effect of different temperature on keratinase production by *B. cereus* (TS1): The keratinase production was assayed under various temperatures like 30, 40, 50, 60, 70 and 80°C at 37°C for 24h incubation (Fig. 6). Maximum keratinase production for *B. cereus* (TS1) was recorded at 50°C (Specific activity 99.50±0.72 IU mg^{-1} and total activity 273.24±0.43 IU mL^{-1}). Minimum keratinase production for *B. cereus* (TS1) was recorded at 80°C (Specific activity 31.12±1.83 IU mg^{-1} and total activity 89.78±2.32 IU mL^{-1}).

Effect of various chemical inhibitors on keratinase production by *B. cereus* (TS1): The keratinase production was assayed after 24 h at 37°C under 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 (Fig. 7). Maximum keratinase production for

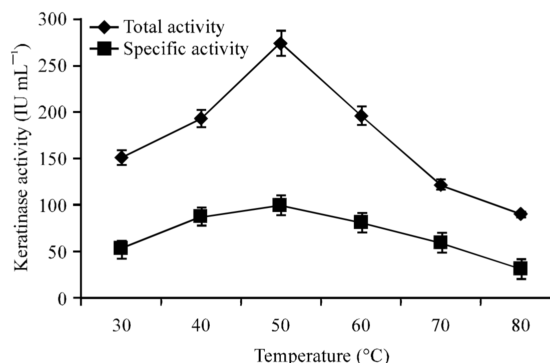

Fig. 6: Effect of different temperature on keratinase production by *B. cereus* (TS1)

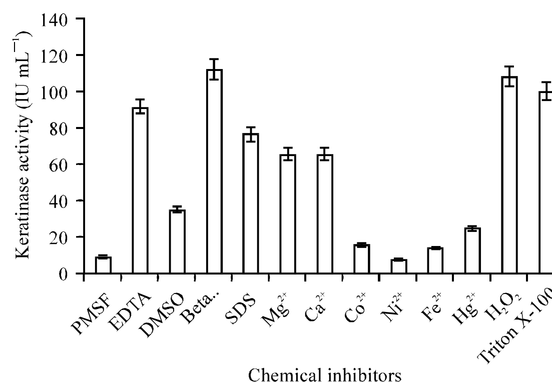

Fig. 7: Effect of various chemical inhibitors on keratinase production by *B. cereus* (TS1)

B. cereus (TS1) was recorded in β-mercaptoethanol with supplemented medium (specific activity 112.0±5.5 IU mg^{-1}). Minimum keratinase production for *B. cereus* (TS1) was recorded in Ni^{2+} supplemented medium (Specific activity 7.41±3.72 IU mg¯3).

DISCUSSION

Effect of carbon and nitrogen sources in keratinase production: The proteolytic activity of crude keratinase was assessed by incubating keratinase with different carbon and nitrogen sources. Extra carbon (starch) and nitrogen (yeast extract) had positive effects on keratinase production while other carbon and nitrogen sources had negative effects. Starch stimulated keratinase production most and was selected as an extra carbon sources in further optimization. Any effects of extra carbon and nitrogen sources as product formation are normally optimized further. In the case of keratinase, addition of extra sources had been discussed widely.

Ramnani and Gupta (2004) reported in optimization of medium for keratinase production by *B. 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* (Santos *et al*., 1996) *Thermoactinomycetus candidus* (Ignatova *et al*., 1999) and *Stenotrophomonas* sp. D-1 (Yamamura *et al*., 2002) is 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 (Gousterova *et al*., 2005). The effects of nitrogen sources on keratinase production also vary. Supplementation of yeast extract resulted in maximal keratinase production by *Stenotrophomonas* sp. D-I (Yamamura *et al*., 2002).

The low cost substrates were screened for the maximum production of keratinase. Some cost effective substrate such as soybean meal have been successfully used (Vidyasagar *et al*., 2007). 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 (Gradisar *et al*., 2000; Manczinger *et al*., 2003).

Tatineni *et al*. (2008) 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 enhanced 4 fold in presence of starch. Effect of different inorganic nitrogen sources shows highest protease activity with soybean meal. Soybean meal acted as inducer for protease production from *Conidiobolusoronatus* sp. (Thanikaivelan *et al*., 2004). The protease activity was increased when production medium was supplemented with potassium, magnesium, iron and calcium ions. An Enhancement of 7.4 fold activity was achieved by supplementation of 0.3% $CaCO_3$. This indicated that the calcium ion was necessary for enzyme production (Boopathi and Abidha, 2008). 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 6 h.

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 (Panday *et al*., 2000; Prakasham *et al*,. 2006) *B. subtilis* MTCC9102 strain has show 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 yield 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 (Nilegaonkar *et al*., 2007).

Chen *et al*. (2005) described complete inhibition of the extracellular protease production from *Geobacillus caldoproteolyticus* strain SF03 in presence of glucose, a versatile source of carbon. However, *B. 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 produced maximum keratinase after 48 h 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 (Ramnani and Gupta, 2004; Hossain *et al*., 2007). When starch, releases carbon slowly favouring in enzyme production and glucose exhibited catabolite repression and inhibited enzyme production (Cheng *et al*., 1995; Wang and Shih, 1999; Hossain *et al*., 2007).

The optimum keratinolytic activity produced by *Bacillus pumilus* AI was achieved with peptone as nitrogen sources followed by the yeast extracts. Present study shows that in *Bacillus cereus* TS1 keratinase production is enhanced when starch is supplied as co-carbon and yeast extract as co-nitrogen source to fermentation medium. The choice of carbon and nitrogen sources has a major influence as the maximum yield of enzymes.

Johnvesly *et al*. (2002) observed maximum activity 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 provided higher concentrations of amino acids, proteins, vitamins that were essential for improved cell growth and synthesis of enzymes such as proteases. However, in present study, α 3-foldhigher enzyme activity was obtained with 5-fold lower yeast extract concentration within a shorter time period than that reported by Mehta *et al.* (2006). This would add an economic advantage to the enzyme production and its use in hydrolysis of abundant feather waste.

Optimization of pH: Najafa *et al.* (2006) and Hossain *et al.* (2007) reported that the bacterium could grow over a wide pH range (7-12) while keratinase production was limited to pH 7-10 with maximum production of 244 IU mL^{-1} at pH 7.

In the present study *B. cereus* TS1 show maximum keratinase activity at pH 9.0 and shows proteolytic activity which is in agreement with other reports. Similarly, keratinolytic proteases produced by other *Bacillus* sp. isolated from the Amazon basin were reported to be optimally active at pH 9.0 (Giongo *et al.*, 2007; Correa *et al.*, 2010). Most of the studies on *Bacillus* sp. report the production of alkaline proteases, with particular emphasis on their utilization as detergent additives and in the leather industries (Joo *et al.*, 2002; Giongo *et al.*, 2007).

Different organisms show maximum enzyme production at different pH levels. For example, *B. cereus*, *Bacillus subtilis* and *Bacillus pumilus* produce maximum enzyme at pH levels of 7.0, 5.9 and 5.6, respectively. According to Kunert (1992) dermatophytes and non dermatophytes metabolize free or combined cystein as a source of sulphur and nitrogen. The products of cystine metabolism by fungi were inorganic sulphur and other intermediate product and also indicated that the excessive sulphur is excreted back to the medium in the oxidized form as sulphate and sulphite. A neutral to alkaline pH sulphite reacts with cystine, cleaving it to cystein and S-sulphocystein.

Khardenavis *et al.* (2009) 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.

Optimization of temperature: The enzyme activity was studied for *B. cereus* TS1 over a broad range of temperature (30-80°C) and it was found to be optimal at 50°C. Further increase in the temperature to 80°C reduced 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 (Lin *et al.*, 1999). Some mesophilic bacteria exhibit the optimal enzymes production and activity from 20-30°C (Allpress *et al.*, 2002).

Lin *et al.* (1992) 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 was slightly lower than that for growth, which was consistent with the present results. Williams and Shih (1989) 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.* (2004) 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.

Chemical inhibitors: The keratinase from *B. cereus* TS1 was totally inhibited by Ni_2 and partially inhibited by $CaCl_2$ and slightly inhibited by EDTA and PMSF. It could be possible that some metal ions are required for the keratinase activity 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 (Bockle *et al.*, 1995; Bressollier *et al.*, 1999). Heavy metal ions such as Cu^{2+} (Nam *et al.*, 2002; Riffel *et al.*, 2003a; Thys *et al.*, 2004), Mg^{2+} (Riffel *et al.*, 2003b) and Zn^{2+} (Thys *et al.*, 2004) have inhibitory effects on keratinolytic activity. Contrarily, Ca^{2+}, Mg^{2+} and Mn^{2+} stimulate some bacterial keratinase (Nam *et al.*, 2002; Riffel and Brandelli, 2002).

CONCLUSION

The isolated microorganisms *Bacillus cereus* that produced keratinolytic enzymes with novel properties. After medium optimization process, revealed that, theafter 72 h of incubation period maximum keratinase production was recorded in starch (specific activity 84.47±0.74 IU mg^{-1} and total activity 192.60±4.87 IU mL^{+}) supplemented medium for *Bacillus cereus* (TS1). Regarding the favourable pH for maximum keratinase production for *B. cereus* (TS1) it was pH 9.0. Interestingly, the favorable temperature for maximum production of keratinase in both strains was found to be 50°C. The proteolytic enzymes produced by *B. cereus* which have prominent neutral metalloprotease characters.

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