ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2012, том 48, № 2, с. 199–205

UDC 577.150.6

ISOLATION AND CHARACTERIZATION OF FEATHER DEGRADING ENZYMES FROM *Bacillus megaterium* SN1 ISOLATED FROM GHAZIPUR POULTRY WASTE SITE

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Received Januray 25, 2011

The SN1 strain of *Bacillus megaterium*, isolated from soil of Ghazipur poultry waste site (India) produced extracellular caseinolytic and keratinolytic enzymes in basal media at 30°C, 160 rpm in the presence of 10% feather. Feathers were completely degraded after 72 h of incubation. The caesinolytic enzyme was separated from the basal media following ammonium sulphate precipitation and ion exchange chromatography. We report 29.3-fold purification of protease after Q Sepharose chromatography. The molecular weight of this enzyme was estimated to be 30 kDa as shown by SDS-PAGE and zymography studies. Protease activity increased by 2-fold in presence of 10 mM Mn^{2+} whereas Ba^{2+} and Hg^{2+} inhibited it. Ratio of milk clotting activity to caseinolytic was found to be 520.8 activity for the 30–60% ammonium sulphate fraction in presence of Mn^{2+} ion suggesting potential application in dairy industry. Keratinase was purified to 655.64 fold with specific activity of 544.7 U/mg protein and 12.4% recovery. We adopted the strategy of isolating the keratinolytic and caesinolytic producing microorganism by its selective growing in enriched media and found that feather protein can be metabolized for production of animal feed protein concentrates.

Milk-clotting enzymes, isolated from microbial sources *Endothia parasitica, Bacillus cereus*, *Mucor pusillus lindt* and *Mucor miehei* are used and reported in production of cheese, cottage cheese, sour cream and Emmentaler cheese. The major application of proteases in the dairy industry is in the manufacture of cheese. The milk-coagulating enzymes fall into three main categories, - animal rennets, microbial milk coagulants, and genetically engineered chymosin. In food industry, rennet prepared from the abomasum (fourth stomach from unweaned calves) is used in the production of cheese. Its supply has become less available and expensive. The shortage of calf's rennet has also highly increased due to religious restriction and ethnic regulations against the use of animal secretion in food.

Most commercial proteases (mainly neutral and alkaline) are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5.0 to 8.0) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than the animal proteinases and hence they are valuable for use in the food industry. A world shortage of calf rennet due to the increased demand for cheese production has intensified the search for alternative microbial milk coagulants too. The keratinases (EC 3.4.99.11) belong to the group of hydrolases that are important for hydrolyzing feather, hair, wool, collagen and casein. They are large serine or metalloproteases capable of degrading the structure forming keratinous proteins. Keratin chain is very tightly packed in the α-helix (α-Keratin) and β-sheets (β-keratin) into super-coiled polypeptide chain [1] and produces mechanical stability resistant to common proteolytic enzymes such as pepsin, trypsin and papain. Keratinolytic enzymes are known to have important use in biotechnological processes involving keratin-containing waste from poultry and leather industries, through the development of non-polluting processes [2, 3]. After hydrolysis, the feather can be converted to feed stuffs, fertilizers, glues and films [4].

The aim of the study is to isolate and to characterize extracellular proteases and keratinases by *Bacillus megaterium* SN1 that can degrade the poultry waste feather and clot milk thus having potential application in bioremediation of feather waste and dairy industry.

MATERIALS AND METHODS

Selection of protease-producing strains on the skim milk agar. Soil isolates showing maximum protease activity were plated on the skim milk agar (10% skim milk powder, 0.1% peptone, 0.5% NaCl and 2% agar). Plates were incubated at 37°C for 24 h and the colonies that showed clear zone were selected and subcultured in the LB broth. The bacterial isolate was further incubated in cultivation media checked for protease activity.

Morphological studies of isolated bacterial strains. Bacterial strain of *Bacillus megaterium* was identified, maintained and kept as glycerol stock. Bacterial iden-

tification was conducted by morphological, cultural and biochemical tests. Results were compared with Bergey's Manual [5] and Genus Bacillus: Agriculture Handbook [6]. The strain was also identified by chromogenic method on the bacillus differential agar M1651 from Himedia (India), recommended for rapid identification of *Bacillus* species from a mixed culture [7].

Production of enzyme in cultivation media. Seed culture of *B. megaterium* were prepared in 500 ml Erlenmeyer conical flask containing 100 ml of feather meal medium that composed of (g/l): NH_4Cl – 0.5; NaCl – 0.5; K_2HPO_4 – 0.3; KH_2PO_4 – 0.4; $MgCl_2 \cdot 6H_2O$ – 0.1; yeast extract – 0.1 and 10% washed feather, pH 7.5. Cultivation was performed at 30°C at 160 rpm for 72 h and the fresh overnight culture was inoculated in cultivation media. Pigeon feathers (10%), hair (10%) or nail (10%) were also used instead of chicken feathers (10%) to compare the growth of *B. megaterium* as well as enzyme production after 7 days. Biomass of bacteria was monitored by taking absorbance at 600 nm on spectrophotometer.

Purification of enzyme. Feather meal media with pigeon feather as substrate was selected for keratinase and protease production, the broth was harvested in 72 h of the growth for the enzyme assay. Isolated *B. megaterium* SN1 was allowed to grow in 500 ml conical flask containing 100 ml of the culture medium at 30°C at 160 rpm for 72 h and fresh culture was inoculated in cultivation media. Cells were harvested by centrifugation (10.000 g, 4°C, 10 min). The 30–60% ammonium sulfate precipitate was obtained from the cell free crude culture broth. The resulting precipitate was collected by centrifugation (10.000 g, 4°C, 30 min) and dissolved in a minimal volume of 10 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer overnight. Then dialysate was loaded on 10 ml Q Sepharose. The 2–4 mM NaCl eluate was collected and protein, protease and keratinase activity were detected in it. All the fractions with high enzyme activity were separately pooled, dialyzed, concentrated by lyophilization and used for further studies.

Determination of keratinase activity. The keratinase activity was assayed by the modified method of Cheng et al. [8] by using keratin as a substrate. The reaction mixture contained 200 μl of enzyme preparation and 800 μl of 20 μg/ml keratin in 10 mM Tris-HCl buffer, pH 8.0. The reaction mixture was incubated at 45°C for 20 min and the reaction was terminated by adding 1 ml of 10% chilled trichloroacetic acid. The mixture was centrifuged at 10.000 g for 5 min and the absorbance of the supernatant fluid was determined at 440 nm. All assays were done in triplicate. One unit (U) of enzyme activity was the amount of enzyme that caused a change of 0.01 of absorbance unit at 440 nm in 20 min at 45°C.

Determination of protease activity. Protease activity was assayed in the various fractions by a modified method of Tsuchida et al. [9] by using casein as substrate. 100 μl of the enzyme solution was added to 900 μl of substrate solution (2 mg/ml casein in 10 mM Tris–HCl buffer, pH 8.0).The mixture was incubated at 50°C for 20 min. Reaction was stopped by the addition of an equal volume of 10% chilled trichloroacetic acid and then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 10.000 g for 10 min at 4°C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na_2CO_3 solution. The color developed after adding 0.5 ml of 3 fold diluted Folin–Ciocalteau reagent was measured at 660 nm. All assays were done in triplicate. One protease unit was defined as the amount of enzyme that releases 1 μmol of tyrosine per ml per minute. The specific activity was expressed in the units of enzyme activity per mg of protein.

Determination of milk-clotting activity. It was determined according to the method of Arima [10], which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g of the skim milk powder in 40 min at 35°C. In brief, 0.5 ml of tested materials was added to a test-tube containing 5 ml of the reconstituted skim milk solution (10 g of dry skim milk in 100 ml of 10 mM $CaCl_2$ and 10 mM $MnSO_4$) preincubated at 35°C for 5 min. The mixture was mixed well and the clotting time T (s) (the time period starting from the addition of test material to the first appearance of clots of milk solution) was recorded and the clotting activity was calculated using the following formula:

$SU = 2400 \times 5 \times D/T \times 0.5$; where T – clotting time (s) and D – dilution of the test material.

Protein concentration. Protein concentration of all the crude and dialyzed fractions of 0–30% and 30–60% ammonium sulphate was determined by the method of Bradford with bovine serum albumin as a standard [11].

Polyacrylamide gel electrophoresis and zymography. SDS-PAGE was performed on a slab gel containing 10% (w/v) polyacrylamide by silver staining according to the method of Switzer et al. [12]. Casein zymography was performed in polyacrylamide slab gels containing SDS and casein (0.12% w/v) as co-polymerized substrate, as described by Choi et al. [13]. After electrophoresis, the gel was incubated for 30 min at room temperature on a gel rocker in 50 mM Tris-HCl (pH 7.4), which contained 2.5% Triton X-100 to remove SDS. Then it was incubated in a zymogram reaction buffer (30 mM Tris-HCl with 200 mM NaCl and 10 mM $CaCl_2$, pH 7.4) at 37°C for 12 h on rocker. The gel was stained with 0.5% Coomassie brilliant blue for 30 min. The activity band was observed as a clear colorless area depleted of casein in the gel against the

blue background when destained in 10% methanol and 5% acetic acid for a limited period of time.

Effect of pH, temperature and various metal ions on enzyme activity. Effect of pH on the purified enzyme activity was measured at various pH ranges (3.0–12.0). The pH was adjusted using the following buffers – 50 mM acetate (pH 2.0–4.0), 50 mM phosphate (pH 5.0–7.0), 50 mM Tris-HCl (pH 8.0) and 50 mM glycine-NaOH (pH 9.0–12.0).

The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 20, 30, 40, 50, 60, 70 and 80°C.

The effects of 10 mM metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+}) on enzyme activity were investigated by adding them to the reaction mixture.

RESULTS AND DISCUSSION

Isolation and identification of protease-producing bacterial strains. Screening of microorganisms that produced protease and keratinase was done on cultures isolated from soil of Ghazipur poultry waste site. Organic waste such as feathers and other poultry waste are essentially composed of proteins. Biodegradation of such samples is generally caused due to the microbial population present at this site. Protease and keratinase producing strains were selected on skim milk agar as described in Methods. Among the cultures tested, the laboratory isolate SN1 showed zone of clearance on these media. The purity of the isolated bacteria was ascertained through repeated streaking (data not shown). Using morphological and biochemical characteristics based on Bergey's Manual the bacterial isolate SN1 was identified as *B. megaterium* SN1.

Protease and keratinase production and effects of different nutrient sources. The growth of isolated colony was detected and protease as well as keratinase activity was measured for 7 days after regular intervals (Figs. 1 and 2). Various substrates like chicken feather, pigeon feather, hair and nail were evaluated for the production of enzymes (Fig. 3). Isolated strain of *B. megaterium* SN1 grown in four nutrient sources produced protease and keratinase. The maximum yield of protease and keratinase was seen in basal media supplemented with pigeon feather. Also complete degradation of the pigeon feather and chicken feather was detected (Figs. 3 and 4).

Purification of protease and keratinase. The extracellular protease and keratinase produced by *B. megaterium* SN1 were purified by 30–60% ammonium sulphate precipitation of culture broth followed by strong anion exchange chromatography on Q Sepharose. The bound protease was eluted with 0.2 and 0.4 M NaCl in 10 mM Tris–HCl buffer pH, 8.0. The fractions showing the presence of protease or caseinolytic activity was pooled (Fig. 5). We report 29.3-fold purification of protease with activity 296.0 U/mg of protein. The re-

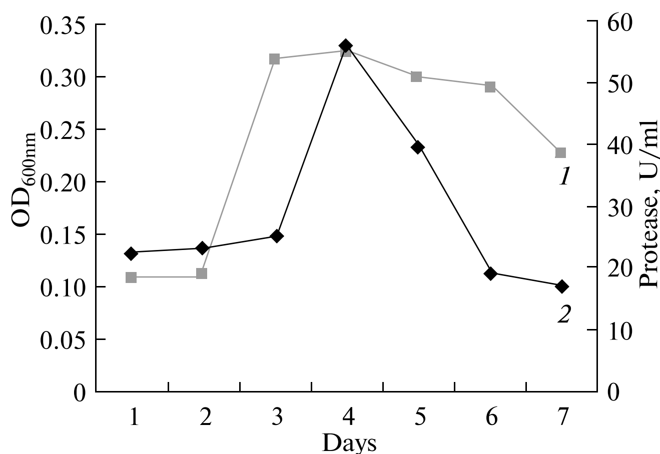

Fig. 1. Growth curve (*1*) and determination of protease activity (*2*) of the SN1 isolate of *B. megaterium*.

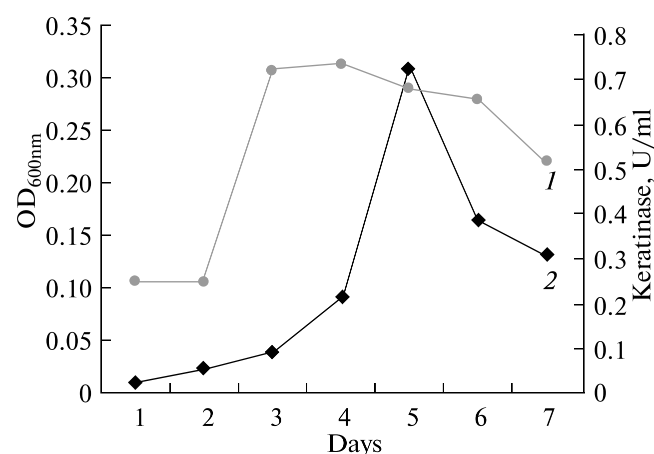

Fig. 2. Growth curve (*1*) and determination of keratinase activity (*2*) of the SN1 isolate of *B. megaterium*.

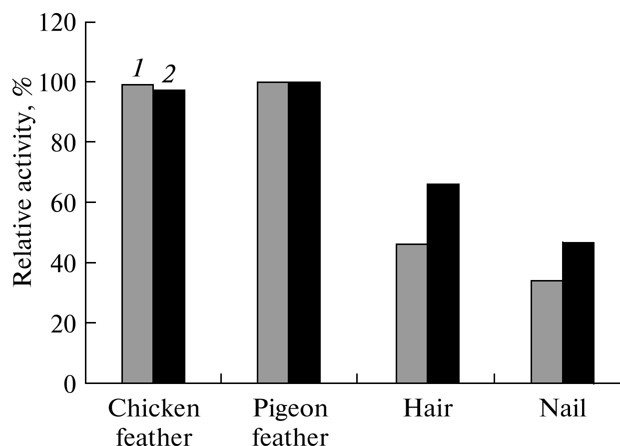

Fig. 3. The relative activity of proteases (*1*) and keratinases (*2*) of *B. megaterium* SN1 in the presence of different proteinaceous substrates.

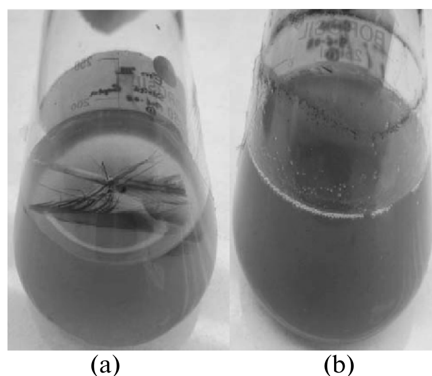

Fig. 4. Degradation of pigeon feathers by the *B. megaterium* SN1 isolated from soil of Ghazipur poultry dumping site (India) in submerged cultivation at 30°C. a – feathers were incubated in the growth medium without the bacterial strain for 72 h of incubation. b – degraded feather after 72 h of incubation with the isolated bacterial strain.

sults of purification of protease from *B. megaterium* SN1 are summarized in Table 1. Keratinase was 655.6-fold purified with specific activity of 544.7 U/mg of protein and 12.4% recovery (Table 2). This keratinolytic-active fraction was further separated on SDS-PAGE for molecular weight determination and zymography studies.

Many authors have suggested various strategies in purification of keratinases. Correa et al. [14] reported that the amazonian bacterium *Bacillus* sp. P7 produced extracellular keratinase that was partially purified by 60% ammonium sulphate precipitation, gel filtration Sephadex G-200, and ion-exchange chromatography on SP Sepharose, DEAE Sepharose FF, resulting in a purification factor of 29.8-fold and a yield of 27%. Zhang et al. [15] studied a new alkaline keratinase extracted from *Bacillus* sp. 50-3 by ammonium sulfate precipitation and DEAE Sephadex-A50 column and 17.7-fold purification with a yield of 46.5%.

The results in Table 3 summarize that the isolated bacterial culture and their ammonium sulphate fractions showed the presence of caesinolytic activity and milk clotting activity. The effect of calcium and magnesium metal ions on the production of milk-clotting enzymes was also studied. Milk clotting activity was detected in crude and ammonium sulphate fraction of *B. megaterium* SN1. The caesinolytic activity was 0.192 at OD_{660} and ratio of milk clotting activity to caseinolytic activity of the 30–60% ammonium sulphate fraction was found to be 520.8 with 100 SU/ml in the presence of Mn^{2+} suggesting potential application in dairy industry. The thermostability and wide pH range shown for the caesinolytic activity are promising for that.

Microorganisms like *Bacillus subtilis, Bacillus licheniformis and Enterococcus faecalis*, produce milk-clotting enzyme which may be potential rennet substitute [16–18]. 685.7 SU/ml of milk-clotting activity (MCA) is reported from *B. subtilis* (natto) enzyme and according to the authors it was found comparable with those of Pfizer microbial rennin and Mucor rennin [19]. Solid-state fermentation resulted in 1.080 and 952.3 U/gds (per g of dried substrate) of milk-clotting protease using soybean meal and rice bran [20]. The protease from *B. licheniformis* had the ability to produce milk curds and exhibited typical milk-clotting kinetics [21]. *B. subtilis* B1, in the presence of optimized medium showed an increase from 782 SU/ml to 1129.05 ± 74.55 SU/ml when wheat bran was used [22]. However, there are no reports on MCE-producing bacteria using feather as substrate.

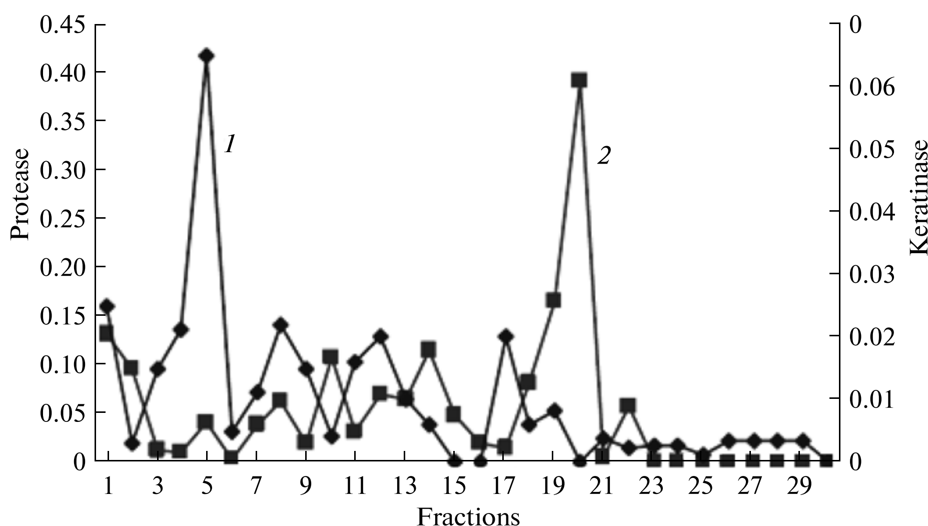

Fig. 5. Activity profile of protease (*1*) and keratinase (*2*) isolated from *B. megaterium* SN1 by Q sepharose ion exchange chromatography.

Table 1. Purification steps of protease from *B. megaterium* SN1

Purification step	Specific activity, U/mg	Purification, fold	Recovery, %
Crude enzyme	10.11	1.0	100
0–30% $(NH_4)_2SO_4$ ppt, dialyzed	3.57	0.35	12.83
30–60% $(NH_4)_2SO_4$ ppt, dialyzed	6.06	0.60	41.46
Q Sepharose	295.98	29.28	1.02

Table 2. Purification steps of keratinase from *B. megaterium* SN1

Purification step	Specific activity, U/mg	Purification, fold	Recovery, %
Crude enzyme	0.84	1	100
0–30% $(NH_4)_2SO_4$ ppt, dialyzed	0.58	0.69	25.09
30–60% $(NH_4)_2SO_4$ ppt, dialyzed	0.49	0.59	40.78
Q Sepharose	544.69	655.64	12.74

SDS-PAGE and zymogram analysis. The Q sepharose fraction was analysed on SDS PAGE (10%) and showed the presence of single band indicating a homogeneous preparation. The enzyme has a molecular weight of 30 kDa. Zymogram activity staining also revealed one clear zone of proteolytic activity against the blue background for purified sample at corresponding positions in SDS-PAGE (Fig. 6, lane 5).

pH optimum, temperature optimum and effect of metal ions. Activity of the enzyme was determined at different pH ranging from 2.0–11.0. The maximum pH recorded was 3.0 for protease and keratinase activity (Fig. 7a). Maximum activity in the acidic range suggests a positive biotechnological potential in the food and detergent industry thus the feather protein can be metabolized and utilized as animal feed protein [23–25]. Additionally, the relative enzyme activity was higher than 40% even at neutral and some alkaline conditions, indicating the potential versatility of such enzyme preparations for diverse applications. Several authors have reported that microbial keratinases typically have optimum pH in more alkaline range [25–29].

The optimum temperature recorded was at 60°C for protease and 70°C for keratinase (Fig. 7b). The protease and keratinase activity was found to be stable in the temperature range from 40°C to 80°C and 50°C to 70°C respectively.

Mn^{2+}, Co^{2+} (10 mM) strongly activated protease activity of *B. megaterium* SN1 by 2.1-fold, 1.3-fold respectively, whereas Mn^{2+}, Co^{2+} and Mg^{2+} strongly activated keratinase activity by 1.2-, 1.1- and 1.1-fold respectively (Fig. 7c). Maybe they act as salt or ion bridges that stabilize the enzyme in its active conformation and might protect the enzyme against thermal denaturation [28, 30]. While Hg^{2+} and Ba^{2+} strongly inhibited protease activity, and Hg^{2+} and Fe^{2+} strongly inhibited keratinase activity. Hg^{2+} is recognized as an oxidant agent of thiol groups, and the enzyme inhibition by this ion could suggest the presence of important –SH groups (such as free cysteine) at or near the active site [26, 31]. However, Hg^{2+} might also react with tryptophan residues and carboxyl groups in amino acids of the enzyme [32].

Table 3. Milk clotting and caseinolytic activities, ratio of milk clotting units to caseinolytic activity of *B. megaterium* SN1

Fractions of purified culture broth of *B. megaterium* SN1	Milk clotting activity, SU/ml*		Caseinolytic activity, OD_{660}	Ratio, Units/OD_{660}	
	$CaCl_2$	$MnSO_4$		$CaCl_2$	$MnSO_4$
Crude enzyme	1.43	2.5	0.250	5.72	10.00
0–30% $(NH_4)_2SO_4$ ppt, dialyzed	10.00	12.5	0.115	86.96	108.69
30–60% $(NH_4)_2SO_4$ ppt, dialyzed	50.00	100	0.192	260.4	520.84

*0.5 ml of tested materials was added to a test-tube containing 5 ml of reconstituted skim milk solution (10 g of dry skim milk/100 ml, 10 mM $CaCl_2$ and 10 mM $MnSO_4$) preincubated at 35°C for 5 min[10].

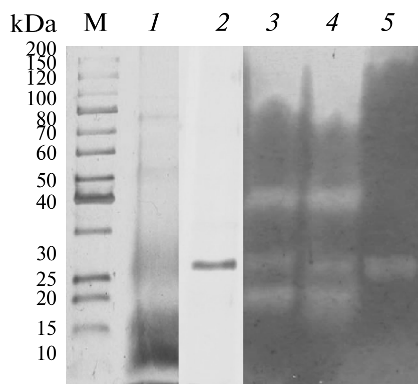

Fig. 6. Silver staining of protease of on 10% SDS PAGE. M – molecular weight markers; *1* – crude enzyme; *2* – 30–60% ammonium suphate fraction; zymogram lanes: *3* – crude enzyme; *1* and *4* – 30–60% ammonium sulphate fraction; *5* – 25 Q sepharose purified fraction.

B. megaterium SN1 was shown to extensively degrade both pigeon and chicken feathers during submerged cultivations, whereas human hair and nail was not degraded. We developed a two step methodology to purify protease by ammonium sulphate precipitation followed by Q Sepharose ion exchange chromatography from this bacterium. The purified enzyme was thermostable at 60°C, pH 3.0 and had a molecular weight of 30 kDa as shown by casein zymography. Ratio of milk clotting activity to caseinolytic activity of the 30–60% ammonium sulphate fraction was found to be 520.84 with 100 SU/ml in presence of Mn^{2+} ion (Table 3). We report isolation of acidic casenolytic protease that showed milk clotting activity, thus it can have high potential in industrial applications as dairy industry in cheese making. The crude extracellular fraction showing the presence of keratinase and protease activities could also degrade feathers completely within 72 h. Keratinase activity was detected at 25 Q Sepharose step of purification showing 655.6-fold purification with specific activity of 544.7 U/mg of protein.

Thus, it could be concluded that both caesinolytic protease and keratinase are produced extracellularly by *B. megaterium* SN1 in feather meal media. They possess moderate acidic stability and are thermostable, which might be desirable features for the efficient control of enzyme reactivity in the processes involving protein hydrolysis. These activities were separated by strong anionic exchanger Q Sepharose.

Food industry needs for acidic proteases active at high temperatures. Keratinase is a useful enzyme for promoting the hydrolysis of feather keratin and improving the digestibility of feather meal and protease is useful in milk industry. The protein hydrolysates resulting from the microbial conversion of feather keratin can be utilized as an ingredient in animal feed or as an organic fertilizer and the milk clotting function of protease can be utilized by the dairy industry in cheese making.

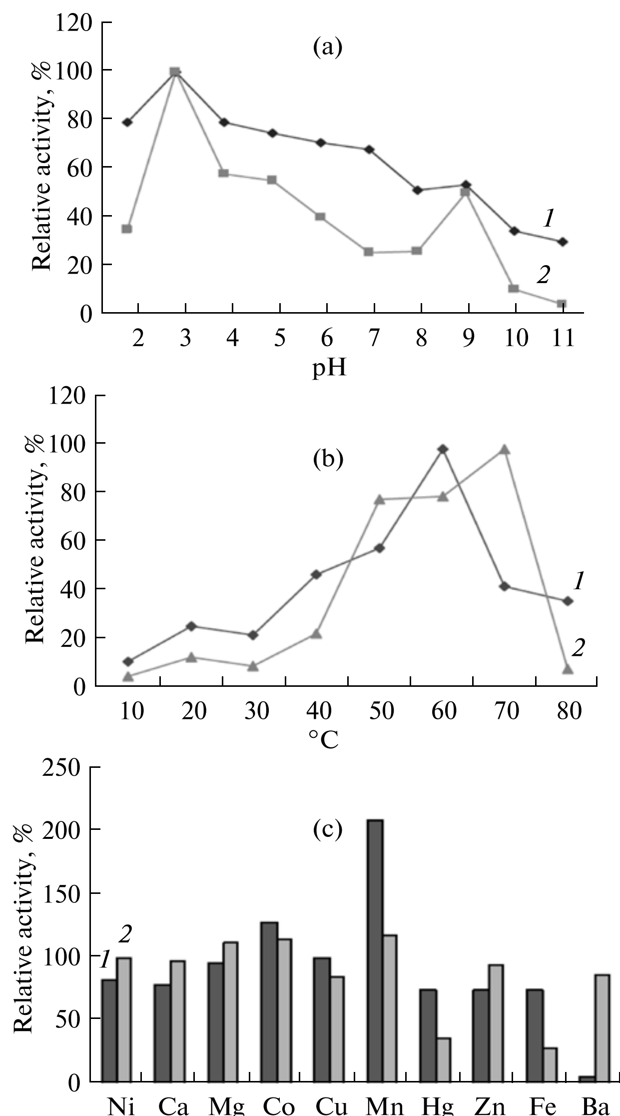

Fig. 7. Effect of pH (a), temperature (b) and of metal ions (c) on caseinolytic (*1*) and keratinolytic (*2*) activities from *B. megaterium* SN1. The maximum of enzyme activity obtained at pH 3.0, temperature 60°C and 70°C for protease and keratinase respectively and without metal ions was considered as 100%.

ACKNOWLEDGMENTS

We are thankful to Jaypee Institute of Information Technology University, Noida (India) for providing the infrastructure facilities for this study.

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