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Microbiology

Study of Keratinolytic Activity of Thermophilic and Alkaliphilic Actinomycetes: *Saccharomonospora Viridis* SJ-21

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Absract

Keratins are insoluble proteins from feathers, wool, silk, collagens, elastin, horn, hair and nail. They are not easily degraded by common proteolytic enzymes like trypsin, pepsin and papain.The resistant property of these compounds are due to their disulphide bonds, hydrogen bonds, salt linkages and cross linkages and hydrophobic interactions. Actinomycetes are known to digest keratins by synthesizing specific class of extracellular enzymes called alkaline thermo stable proteases which degrade keratin into small peptides that can be utilized by cell. Alkaline protease producing thermophilic actinomycete strain was screened from hot water spring of Tulsishyam Gujarat and was identified as *Saccharomonospora viridis* SJ-21 on the basis of colony characters, biochemical activity, spore nature, growth patterns and pigmentation and 16 S r RNA sequencing.The partially purified protease of *Saccharomonospora viridis* SJ-21 and the isolate itself were employed to check feather degradation. The feathers were degraded successfully within 72h at 45ºC. The degraded samples were analyzed for release of various amino acids by HPLC- Fluorescence with post column Derivatization. The aminoacids released were tyrosine, phenylalanine, leucine, valine, cysteine, arginine, methionine, etc. *S. viridis* SJ-21 is found having a significant keratinolytic activity and may serve dual purpose for degradation of poultry waste and production of amino acid rich feed supplement. The protein rich, concentrated feather meal can also be used for organic farming as semi-slow release, nitrogen fertilizer.

Highlights

- Screening of alkaline protease producing thermophilic actinomycetes .
- Identification of the potent protease producing thermophilic actinomycete.
- Alkaline Protease production and characterization
- Checking feather degrading capacity of isolate and protease produced by the isolate.
- Amino acid profiling of degraded feather sample by HPLC.

Keratin occurs in nature mainly in the form of hair, horn, nails, feathers and cornified tissue. Keratin by virtue of its insolubility and resistance to proteolytic enzymes is not attacked by most of living organisms. Nevertheless, keratin does not accumulate in the nature and therefore biological agencies may be presumed to accomplish its removal (Laemmli, U. K., 1970). Along with bacteria and fungi, some insects, including cloth mouth larvae, carpet beetles and chewing lice are known to digest keratin (Riffel, A, 2007). They hydrolyze the keratin by synthesizing specific class of extracellular enzymes called alkaline thermostable proteases which degrade keratin into small peptides that can be utilized by cells. Several feather-degrading bacteria have been isolated from soil, poultry wastes, hair debris and animal skin and most of the isolates were confined to genera *Streptomyces* and *Bacillus* (Dalev, P. (1990).

The keratinolytic microorganisms and technologies developed for feather degradation are not only to remove the waste feathers efficiently from the nature but also for making the by-products of the process as valuable protein supplement. The protein rich, concentrated feather meal can also be used for organic farming as semi-slow release, nitrogen fertilizer (Kaul, S. and Sumbali, G. 1999), Friedrich, A.B., and Antranikian, G. 1996). In this study we emphasized on keratinolytic activity of thermophilic actinomycetes *Saccharomonospora viridis* SJ-21 and its protease.

Materials and Methods

1. Isolation and Identification of *S. viridis* SJ-21

Samples collected from hot water spring, soil and Areetha extract were serially diluted using sterile distilled water to spread evenly on skim milk agar medium of pH 8.5 and incubated at 55ºC for 3-4 days to allow the organisms to grow. The well isolated colonies showing zone of casein hydrolysis were marked and colony characters and morphological characters were noted at an interval of 24 h. Diameter of zone of clearance due to hydrolysis of casein was recorded which provided a measure of their proteolytic activity. On the basis of zone of casein hydrolysis, potent isolates were selected for further study.

The isolate SJ-21 showing zone of casein hydrolysis on milk agar plates was preserved on Nutrient casein agar slants and sent to Gujarat State Biotechnology Mission (GSBTM, Gandhinagar) for 16S rRNA sequencing and the BLAST match was used to confirm identification of the isolate. The sequences obtained from GSBTM, Gandhinagar were analyzed at NCBI server (www.ncbi.nim.nih.gov) using BLAST tool and have been submitted to Gene Bank. The phylogenetic tree of the isolate was constructed with MEGA version 4.0 using the neighbor joining method (Tamura, K. *et al.,* 2007).

2. Micro-organisms and inoculum preparation

Fresh growth of *S. viridis* SJ-21 was obtained on milk agar medium (pH 8.5) and transferred in to 25 ml broth containing glycerol 0.5 ml, peptone 1 gm, yeast extract 0.5 ml, NaCl 0.5 gm in 100 ml distilled water and pH 8.5 and incubated at 50ºC on a rotary shaker at 100 rpm for 48 h. This actively growing culture was used as inoculum for the protease production (O.D. 0.75 at 660 nm).

3. Enzyme production.

The *Saccharomonospora viridis* SJ-21 JX 262282*,* was inoculated in the optimized medium, having composition: casein, 250mg%; peptone, 200mg%; K2HPO$_4$20mg%, $KH_2PO_4$20mg%, $MgSO_4$,10mg%, $CaCl_2$ 10mg% , and Glycerol 0.5ml in 100 ml of Distilled water,(pH 9.5.) (28); under shaking conditions (100 rpm), for 96 h at 55°C. The culture broth was centrifuged at 10,000 × g for 20 min at 4ºC and the culture supernatant was used as a source of protease. Activity of enzyme was measured in terms of unit (µg/ml/min). One unit of enzyme is defined as the quantity of enzyme required to release 1µg of tyrosine per minute, under the standard assay conditions (Hagihara, B. (1958).

Protein content was measured by Lowry's method with bovine serum albumin (BSA) as a standard protein.

4. Ammonium sulphate Precipitation and Dialysis of enzyme:

250 ml of the cell free filtrate was subjected for precipitation by different concentrations of (20- 100%) solid ammonium sulphate (Dixon, M. and Webb, E. G., 1964). The precipitated protein was obtained by centrifugation at 10000 rpm for 15 minutes at 4ºC. Both enzyme activity and protein content were determined for each separate fraction (Khalil B.Q. and Gupt, R. (2003). The concentrated enzyme was collected in glycine NaOH buffer and dialyzed overnight against the same buffer till the ammonium sulphate was completely removed.

The caseinolytic activity of this partially purified enzyme preparation was determined by Hagihara method (Hagihara, B. 1958).

5. Characterization of the protease by determination of molecular weight and type of enzyme

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was preformed according to method of Laemmli (Laemmli, 1970) as modified by Studier (1973). This method was used to determine the molecular weight of the partially purified protease. Molecular weight of purified alkaline protease was measured using a series of protein with standard molecular weight markers (Sigma, USA)

The purified enzyme was preincubated with different inhibitors at 5 mM concentrations for 10 and 60 min before the addition of substrate at room temperature (Adinarayana, K.*et al.,* 2003). The inhibitors used were: phenyl methyl sulfonyl fluoride (PMSF) serine inhibitor, dithiothreitol (DTT) a reducer of disulfide bonds, perachloromercuric benzoate (PCMB) thiol group containing amino acid inhibitor and ethylene diamine tetraacetic acid (EDTA) chelator of divalent metal ions. The caseinolytic activity was determined at 70°C using glycine NaOH buffer (pH 9.5).

The relative activity was calculated with respect to the control without treatment with inhibitors.

Table 1. Profile of free amino acids released by *S. viridis* from feathers at 45°C. (Analyzed at NDDB, Anand (3.6 g feathers in 50 ml sample)) (HPLC- fluorescence with post column derivetization.)

Aminoacids	µg/ml of sample	µg/g of feather
Aspartic acid	Less than 0.5	Less than 7.0
Threonine	Less than 0.5	Less than 7.0
Serine	1.40	19.6
Glutamic acid	5.45	76.30
Glycine	4.02	56.28
Alanine	1.15	16.1
cysteine	7.56	105.84
Valine	13.45	188.3
Methionine	6.37	89.18
Isoleucine	5.60	78.4
Leucine	13.35	186.9
Tyrosine	21.65	303.1
Phenyl alanine	19.74	276.36
Lysine	5.74	80.36
Histidine	2.13	29.82
Arginine	6.67	93.38

6. Degradation of feathers by S. viridis SJ-21.

Chicken feathers (whole feathers) were collected from chicken shop. Feathers were first extensively washed in tap water and finally with double distilled water. Feathers were then steam sterilized and stored at 5°C until used. 3 ml fresh culture suspension of SJ-21 of optical density 0.75 at 680 nm was inoculated in basic salt solution along with feathers (feathers were the sole source of carbon and nitrogen) (Adriano Brandelli 2008). Total volume of 50 ml in tube with two feathers each weighing 1.8 g. Control was maintained with same contents without inoculating the organism. The set was incubated at 45°C for 96 h and observed for keratinolytic activity at the interval of 24 h.

7. Degradation of feathers by partially purified protease

10 ml of partially purified protease of *S. viridis* SJ-21 was inoculated in basic salt solution along with feathers as described above. Control was maintained with same contents without inoculating the enzyme. Total volume of 50 ml in tube with two feathers each weighing 1.8 g. The set was incubated at 45°C for 96 h and observed for keratinolytic activity at the interval of 24 h and results were noted visually and photographs were taken as well.

8. Amino acid analysis from degraded feather samples

The samples of above mentioned feather degradation experiment were sent to National Dairy Development Board, Anand, Gujarat for amino acid analysis. The amino acids released due to feather degradation by *S. viridis* SJ-21 were analyzed by HPLC- Fluorescence with post column Derivatization.

Results and Discussion

Isolation of alkaline protease producing actinomycetes from various sources was carried out using alkaline skim milk agar medium. Growth of the organism is shown in Figure 1. On the basis of colony characters, biochemical activity, spore nature, growth patterns and pigmentation, organism was identified as *Saccharonomospora spp.* (Lacey, J., 1997). And for confirmation, we relied on 16S r RNA Sequencing. Phylogenetic analysis based on 16 S r-RNA sequence of SJ-21 showed that the sequence exhibited a high level of homology with *Saccharomonospora* (Figure 2). The sequence was submitted to Gene Bank under the NCBI accession number **JX 262282.** Based on all these experimental data, it was confirmed that isolate represented a novel species of *Streptomyces* and designated as *Saccharomonospora viridis* SJ-21 JX 262282.

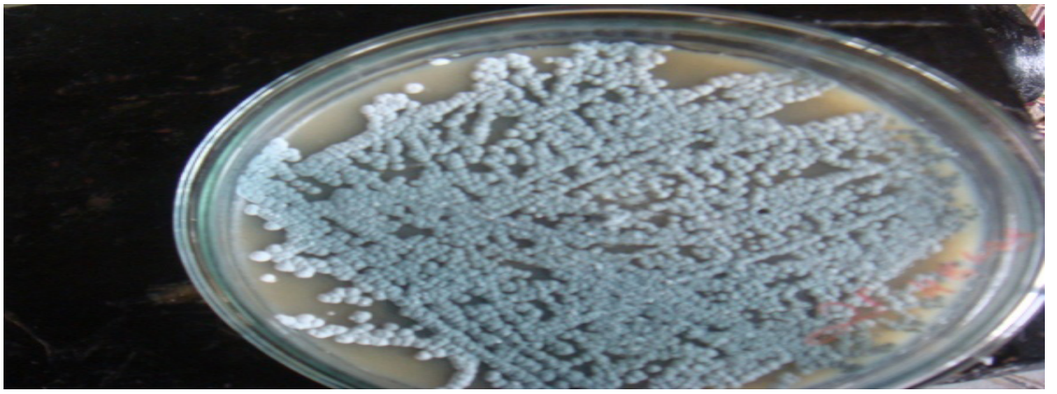

Figure 1. Growth of *Saccharomonospora viridis* on skim milk agar medium

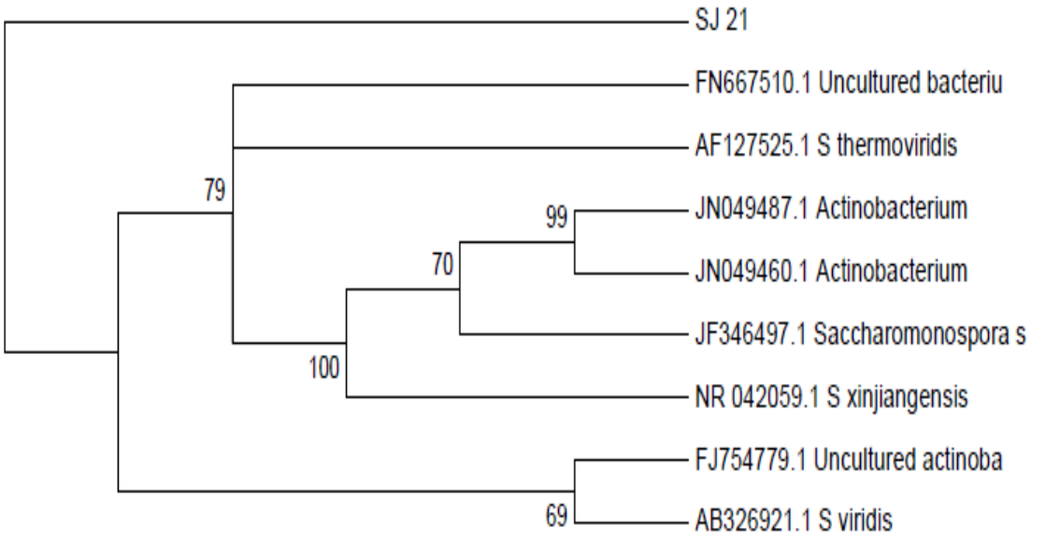

Figure 2: Phylogram of strain SJ-21(*Saccharomonospora viridis*)

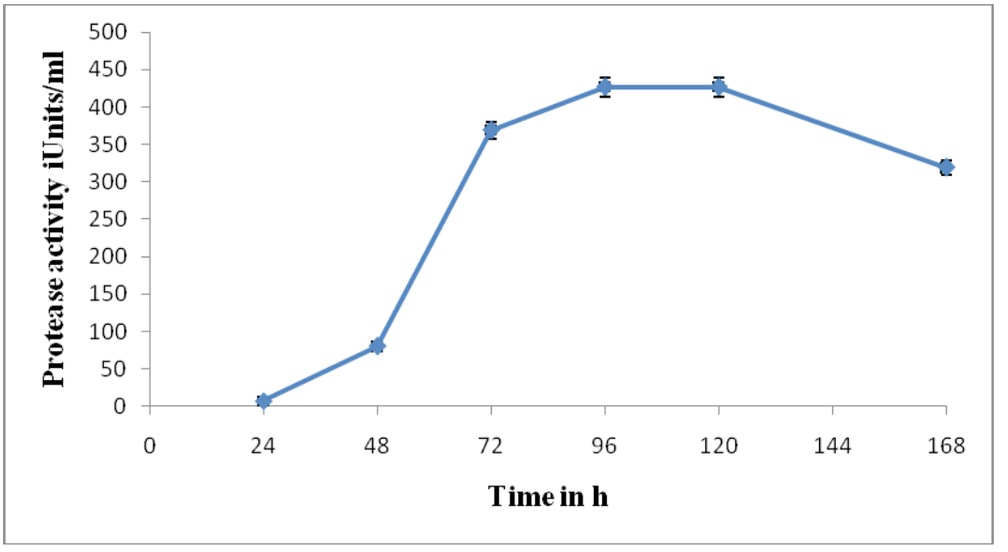

Figure 3. Protease productions by *S. viridis*.

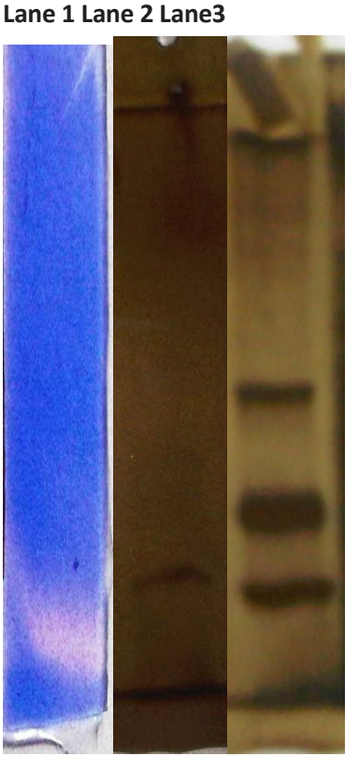

Figure 4. SDS PAGE for molecular weight determination and activity staining (Zymogram) (Lane-1 is activity staining of purified protease. lane-2, pure protein fraction Lane-3 Markers)

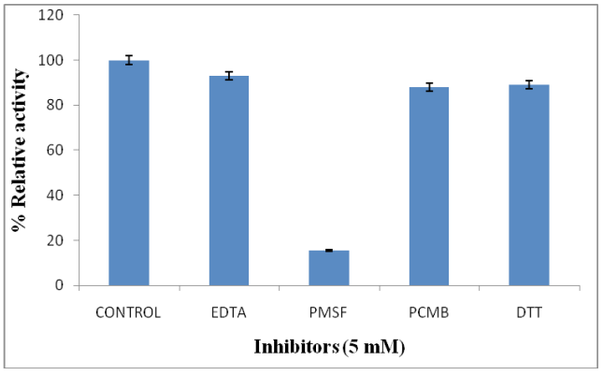

Figure 5: Effect of Inhibitors on protease activity

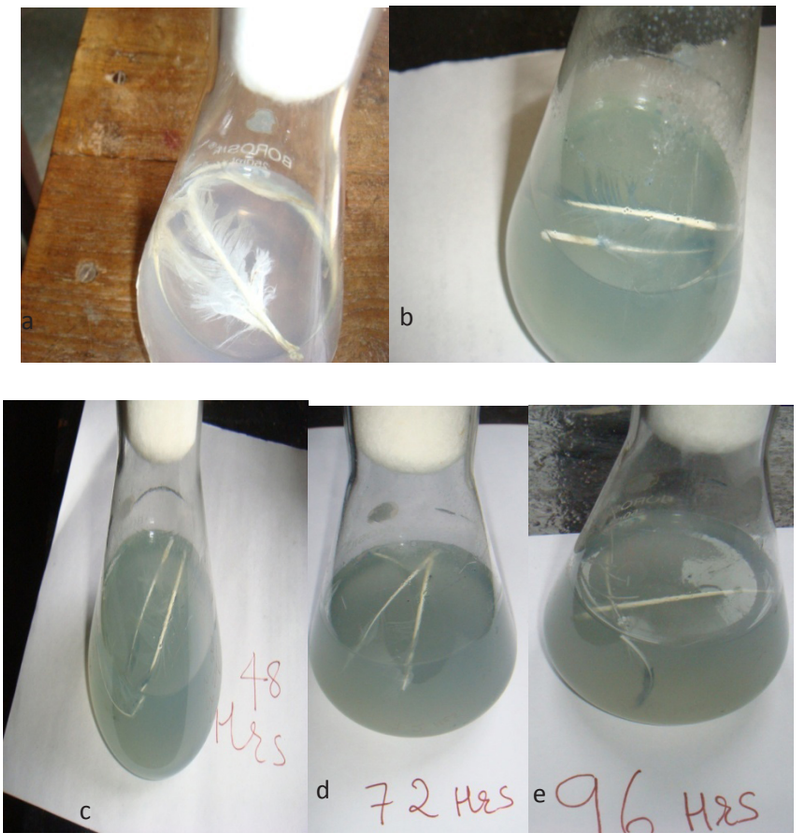

Figure 6: Feather degradation by *S. viridis* at different time intervals
a: Control , b: after24h , c : after 48h, d : after 48h, e : after 48h, at 45°C

Crude enzyme sample collected from production medium after 96 h of incubation was analyzed for its enzyme activity and it was found that the crude sample was having 427 units/ml of enzyme. (Figure-3) (Shilpa Jani, *et al.,* 2012)

The molecular weight of purified protease of *S. viridis* SJ-21 was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the purified protease was 18.4 KDa (Figure 4 lane-2). Our result exactly matches with the low molecular weight (18.4 KDa) of protease identified from *Streptomyces griseus* (Kumar *et al.,* 2006), and that of serine protease from S*treptomyces albidoflavus* (18 KDa) (Bressollier *et al.,* 1999).

Generally, the molecular masses of alkaline protease from microorganisms range between 15 and 36 KDa (Gupta *et al.,* 2002b).

The protease was strongly inhibited by 5 mM PMSF indicating the enzyme as a serine protease. Negligible inhibition was observed in presence of 5 mM EDTA or 5 mM PCMB and 5 mM DTT, indicating that it is neither a metalloprotease nor a cysteine protease. The majority of the proteases from thermophiles are of the serine type (de Vos *et al.,* 2001). Reports indicate that most of the alkaline proteases from thermophilic actinomycetes are of serine type.

Degradation of feathers: keratinolytic protease activity of *S.viridis* SJ-21.

The *S.viridis* SJ-21 showed excellent keratinolytic activity as it started degrading feathers within 24 h. The feathers were degraded completely in 72 h. As no other nutrition was provided it could be confirmed that organisms were

Figure: 7 Degradation of feather by *S. viridis*

utilizing feathers as sole source of carbon and nitrogen with keratinolytic enzyme. The blue colour developed in the flask confirmed the growth of SJ-21 due to production of blue green pigment. (Figure-6)

This kind of results were also reported for *Bacillus* sp. (Patil, C.*et al.,* 2010), for *Bacillus licheniformis* MZK-3 (Mohammad *et al.,*, 2007), for *Bacillus licheniformis* (Yasuhiko, T. 2004)), for *Nocardiopsis* sp. TOA-1 (Mitsuiki, S., *et al.,* 2002), for *Streptomyces* sp. Strain 16 (Xie, F. *et al.,* 2010), for *Streptomyces albidoflavus* (Bressollier, P.*et al.,* 1999), *Streptomyces pactum* (Böckle, B. and Müller, R. 1997, Böckle, B.*et al.,* 1995), for *Streptomyces gulbagensis* (Syed, D.G., *et al.,* 2009), for *Streptomyces thermoviolaceus* (Chitte, R. *et al*.,1999).

It was observed that feathers were completely degraded not only by organism but also by partially purified protease of *S. viridis* SJ-21 (Figure 8).

The degraded sample was sent to NDDB Anand, Gujarat for amino acid analysis by HPLC- fluorescence with post column derivetization. It was observed that *S.viridis* SJ-21 released most of all amino acids from the feathers. The maximum aminoacid released were tyrosine (21.65 µg/ml), Followed by phenylalanine (19.74 µg/ml), leucine (13.35 µg/ml), valine (13.45 µg/ml), cysteine (7.56 µg/ml), arginine (6.67 µg/ml), methionine (6.37 µg/ml), etc (Table 1). Results indicated that, organism was efficient for degrading feathers with significant amount of amino acid release at 45°C (to make the process economic, we incubated the tubes at 45°C).

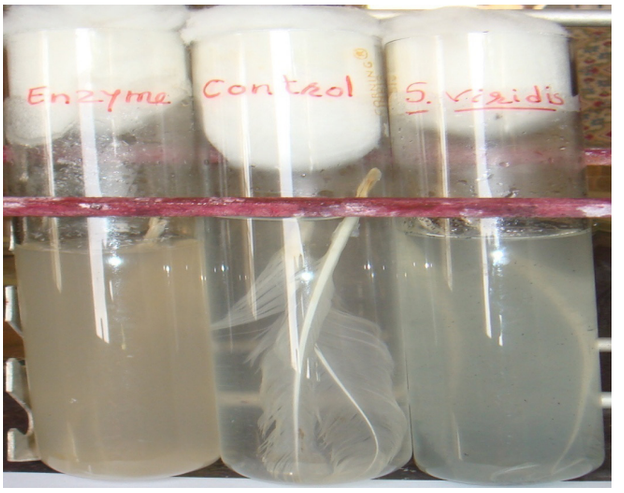

Figure 8. Photograph showing feather degradation

The result obtained confirms the use of the keratinolytic activity of the organism in feed industry (Böckle, B. and Müller, R., 1997). Thus the use of *S. viridis* SJ-21 as keratinolytic agent may serve dual purpose for degradation of poultry waste and production of aminoacid rich feed supplement. Reports of keratinase activity of the actinomycetes are as follows: *Streptomyces pactum* DSM 40530, serine protease having molecular mass 30 KDa, active at pH 7 to 10, was reported by Böckle *et al.,*. (Böckle,B., *et al.,* 1995). *Streptomyces albidoflavus* K1-02, serine protease having molecular mass 18 KDa, active at pH 6 to 9.5, reported by Bressolier *et al.,* (1999). Chitte *et al.,* (1999) reported an actinomycete strain *Streptomyces thermoviolaceus* that can grow and degrade

keratin at temperatures higher than 50°C. All these reports match with our result as our protease was also found having similar characteristics molecular mass 18.4 KDa and a serine protease. The activity of keratin degrading protease is normally associated with serine proteinase (Lin, X., *et al.,* 1992, Bressolier *et al.*, 1999), but not all serine type proteases are able to degrade keratin (Eggen H., *et al.,* 1990). The keratinolytic strain *Streptomyces pactum* DSM 40530 produces a combination of serine proteinases and metalloproteinases (Böckle, B., *et al.*, 1995). Feather degradation by *Streptomyces* sp. 594 has also been reported (De Azeredo, L., *et al.,* 2006).

Conclusion

The use of keratinolytic enzymes is becoming attractive in biotechnological applications. The enzymes are used for removing hair and feather in the poultry industry, upgrading of feather meal, converting feather into feed protein, and clearing obstructions in the sewage system (Gradisar, H., *et al.,* 2000, Pissuwan, D, and Suntornsuk, W. 2001, Riessen S, and Antranikian G. 2001). Keratinase can also be produced by some thermophilic microbes, such as *Fervidobacterium penavorans* (Friedrich, A.B., and Antranikian, G. 1996), *Streptomyces* sp. (Böckle, B., *et al.,* 1995, Letournaeu, F., *et al.*, 1998, Bressolier *et al.*,., 1999), and *Bacillus* sp. (Lin, X., *et al.,* 1992, Takagi, H., *et al.,* 1992, Cheng, S., *et al.,* 1995).

The requirements of amino acids in animals are well defined in various sets of recommendations such as those of NRC (National Research Council), USA, etc. Requirements vary depending on the species and age of animals. Amino acids should be supplied either in the form of protein or crystalline amino acids in feed to meet requirements. The industrial application of amino acids for feed has an almost 40-year history. In the late 1950s and 1960s, DL-Methionine, produced by chemical synthesis, began finding its way into poultry feed. In addition to the amino acids DL-methionine, lysine, threonine, tryptophan, the next limiting amino acids, isoleucine, valine and arginine, will be introduced in the near future (Yasuhiko, T., 2004). The drawbacks of the high-temperature treatment impel the use of microbial keratinases that serve as attractive alternatives to hydrolyze feather into nutritionally rich animal feed (Onifade, A., *et al.,*, 1998). The application of keratinases/keratinolytic microbes for improvement of feather as poultry feed has been extensively reviewed by Onifade *et al.,* (1998). Feather meal is relatively inexpensive and is shown to be superior to soybean meal in terms of total cysteine, valine and threonine content, and the hydrolyzed meal can replace soybean meal at 7% dietary level. The crude enzyme can also serve as a neutraceutical product, leading to significant improvement in broiler performance. In addition, nutritional enhancement can be achieved by hydrolysis of feather meal/raw feather using keratinolytic microorganisms. The use of keratinolytic microorganisms may represent an alternative for the development of nitrogen sources for fertilizer utilization. The enzymatic capability of the feather-degrading bacteria to accelerate the composting of dead chickens or feather waste could be an economical and environmentally safe method of recycling these organic materials into high-nitrogen fertilizers (Hagihara, B., 1958).

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