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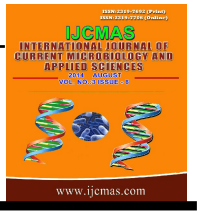


**Original Research Article**

# Screening, isolation and characterization of keratin degrading actinomycetes: *Streptomyces* sp. and *Saccharothrix xinjiangensi* and analyzing their significance for production of keratinolytic protease and feed grade aminoacids

**Shilpa Ashok Jani[1*], Rishit Soni[2], Hetal Patel[2], Brinda Prajapati[2] and Gayatri Patel[2]**

[1]Department of Microbiology, J and J College of Science, Nadiad, Gujarat University, Gujarat, India
[2]Department of Microbiology, School of Sciences, Ahmedabad, Gujarat University, Gujarat, India
**Corresponding author*

**A B S T R A C T**

Keratin degrading protease producing actinomycetes were isolated from soil samples, collected from Dalhousie rocks of Himachal Pradesh and Poultry farm waste of Sheriyaj Gujarat. Initially alkaline protease producing actinomycetes were screened on the basis of zone of casein hydrolysis.The isolates were tested for their relative enzyme activity on solid media. The isolates were characterized on the basis of their morphological characters, biochemical activity, spore nature, growth patterns and pigmentation and 16 S r RNA sequencing. The isolates identified as *Streptomyces* sp. (A1) and *Saccharothrix xinjiangensi* (A2) were employed to check feather degradation. The feathers were degraded successfully within 48h at 37ºC. The degraded samples were analyzed for release of various amino acids by TLC-Thin Layer chromatography. The aminoacids detected were histidine, lysine, tryptophan and methionine. Isolates were found having a significant keratinolytic activity and may serve dual purpose for degradation of poultry waste and production of amino acid rich feed supplement. Isolates were also employed for production of keratinolytic protease production and it was found that they produced keratinolytic protease optimally at 72h and 48h respectively. The pH and temperature optima for production were found to be and 8.5 and 40°C for A1 and 9.5 and 40°C for A2.

**Keywords**

keratinolytic protease, actinomycetes, feather degradation

## Introduction

The capacity of *Streptomycetes* to produce new compounds remains unsurpassed through members of other microorganisms. Actinomycetes genera are becoming increasingly important as a source of novel products (Nord *et al.*, 1998). These microorganisms are particularly abundant in alkaline soils rich in organic matter. Keratin is an insoluble structural protein of skin, and its derivatives like feather, hair, wool and horn, are known for their high stability. Actinomycetes producing keratinases are

having high applications in feed, fertilizer, and leather, pharmaceutical and biomedical applications. Isolated, feather degrading actinomycete strains were investigated for their ability to produce keratinase enzyme. Keratin-containing materials are abundant in, nature but have limited uses in practice since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen for plants. Each organism or strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999) so we also decided to check pH and temperature optima for keratinolytic protease production. 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.

## Materials and Methods

### Source / Samples for screening. Medium and procedures used for isolation, Screening and preservation of protease producing microorganisms

Various samples of soil and poultry waste were collected from different regions of Gujarat and outside Gujarat which included; alkaline rock soils from Dalhousie and poultry waste of Sheriyaj, Junagadh district, Gujarat). Samples were aseptically collected from top soil surface and were serially diluted using sterile distilled water, stirred thoroughly and 100µl aliquots were plated on skim milk agar plates. (Table-1), pH: 8.5 and incubated at 37 °C for 3-4 days to allow the colonies to grow. The well isolated colonies were marked and colony characters and morphological characters were noted at the interval of 24 h, diameter of zone of clearance of casein was also measured which provided a measure of their Proteolytic activity. Each isolate was transferred on milk agar plates two to three times to get confirm isolated pure cultures. Purity of cultures was confirmed by Gram staining (Bergey David *et al,* 1994). Cultures were preserved at 4°C on casein agar slants. Sub culturing was carried out every 30- day's interval.

### Selection of potent isolates and study of their cultural characters

### Measure of Proteolytic Activity on solid media

Fresh culture isolates were put in the middle of skim milk agar plate and incubated at 50°C for 5-6 days and at interval of 24 h., zone of casein hydrolysis and diameter of growth were measured and relative enzyme activity (REA) was calculated (Jain *et al,* 2009) (REA =Diameter of zone of casein hydrolysis/ Diameter of colony in mm.)

Based on REA, organisms were categorized into three groups showing excellent (REA>5), good (REA>2.0 to, 5.0) and poor (REA<2) producer of protease.

### Study of Cultural, Morphology and Biochemical characteristics

The isolates showing zone of casein hydrolysis on milk agar plates, were studied for their colony characteristics, and morphological characteristics along with their spore arrangement by performing Grams Staining (Bergey *et al*, 1994) by visual and microscopic observations respectively. The isolates were also characterized for their biochemical activity. The biochemical tests carried out for the isolates were**:** indole production test,

methyl red test, Voges proskauer test , citrate utilization test, nitrate reduction test, ammonia production test, catalase test, urea utilization test, gelatin hydrolysis test, hydrolysis of starch, $H_2S$ production test, dehydrogenase test. Growth pattern in broth**,** Carbohydrate utilization test:

**Degradation of feathers by actinomycete isolates**

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 A1 and A2 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 37°C for 96 h and observed for keratinolytic activity at the interval of 24 h.

**Measure of keratinolytic protease production with reference to growth of isolates**

To compare the keratinolytic protease activity of enzyme produced by the isolates, isolates were inoculated in production medium consisting of feathers 0.5gm, glycerol 0.5%, $K_2HPO_4$ 20mg, $KH_2PO_4$ 20mg, $MgSO_4$ 10mg, $CaCl_2$ 10mg, casein 200mg, peptone 200mg, $NaNO_3$ 100mg, 100ml distilled water, pH-8.5(Rao and Narasu 2007) and one set is put in medium consisting of feathers 0.5gm, $K_2HPO_4$ 20mg, $KH_2PO_4$ 20mg, $MgSO_4$ 10mg, $CaCl_2$ 10mg, $NaNO_3$ 100mg, 100ml distilled water, pH-8.5( feathers as sole source of carbon and nitrogen) put on an environmental shaker at 100 rpm at 40ºC for 168 hours and checked for enzyme activity at interval of 24 hours. The supernatant was collected after centrifugation at 15000 rpm at 4ºC temperature for 15 minutes and used as crude enzyme source keratinolytic protease activity in the supernatant was determined by using spectrophotometer method, given by Anson - Hagihara (1958) with minor modifications. Simultaneously, optical density was measured at 680 nm at the interval of every 24 h and graph of time vs. Optical density was plotted to get growth curve.

**16 S rRNA Sequencing of Potent Isolates**

Isolates preserved on Nutrient casein agar slants were sent to Gujarat State Biotechnology Mission (GSBTM, Gandhinagar) for 16S rRNA sequencing and the BLAST match was used for confirm identification of the isolates A1 and A2. The phylogenetic trees of both the isolates were constructed with MEGA version 4.0 using the neighbor joining method. (Tamura *et al,* 2007)

**Optimization of certain parameters**

**Feather as an inducer of keratinase production**

Three different types of medium were used. I.e. production medium [Rao and Narasu, 2007] with (0.5 g %) feather meal, only feather meal as sole source of carbon and nitrogen and production medium [Rao and Narasu, 2007] without feather meal. Culture isolates were inoculated in the respective media and then incubated at 40±2$^{o}$C on the environmental shaker at 100rpm. After 48-72 h sampling was done and the activity of keratinolytic protease activity was checked by Anson-Hagihara (1958) method. The growth curve was prepared simultaneously

to check relationship of growth and keratinase production.

**Effect of incubation temperature on keratinase production**

3ml of fresh culture was inoculated in the production medium (salt solution+0.5% feather meal) of optical density of 0.75 at 670nm and was kept at different temperature like room temperature, 40±2$^{o}$C and 50±2$^{o}$C for 96 h. The supernatant was collected after centrifugation at 4000 rpm at room temperature and was used as crude enzyme and activity was measured by Anson-Hagihara (1958) method.

**Effect of initial pH on keratinase production**

3ml of fresh culture of optical density of 0.75 at 670nm was inoculated in the production medium (salt solution+0.5% feather meal) and was kept at different pH like 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 and 12.0 for 96 h. The supernatant was collected after centrifugation at 4000 rpm at room temperature and was used as crude enzyme and activity was measured by Anson-Hagihara (1958) method.

**Effect of shaking and static condition on keratinase production**

3ml of fresh culture was inoculated in the production medium (salt solution+0.5% feather meal) of optical density of 0.75 at 670nm. The flasks for static condition were kept in room temperature and 40±2$^{o}$C and 50±2$^{o}$C in incubator. The flasks for shaking condition were kept on environmental shaker at 60±2$^{o}$C. The supernatant was collected after centrifugation at 4000 rpm at room temperature and was used as crude enzyme and activity was measured by Anson-Hagihara (1958) method.

**Amino acid analysis from degraded feather samples**

The samples of A1 and A2 were employed to check amino acids released due to feather degradation by thin layer chromatography against four known amino acids (histidine, methionine, lysine and tryptophan).A sheet (13×10 cm) of TLC was taken and the above mentioned amino acids along with the samples of A1 and A2 were placed 1.5cm above the TLC sheet. Then the TLC sheet was kept in solvent system of (acetic acid: water) and the system was allowed to run till the 2cm above the TLC sheet. Then the TLC sheet was air dried and 0.5% of Ninhydrin solution was sprayed on it and was kept in hot air oven to dry for 5 minutes till the colour develops. Then the TLC sheet was taken out and its Rf value was calculated.

## Results and Discussion

**Isolation of protease producing microorganisms**

Isolation of alkaline protease producing bacteria from various sources was carried out using alkaline skim milk agar medium. Nineteen potent isolates were collected showing zone of casein hydrolysis surrounding their colonies.

All were alkaliphilic and having diverse morphological characters. Out of nineteen isolates, seven potent actinomycetes A1 to A7 were selected for the further studies. The cultural characteristics of these seven protease producing actinomycetes are represented in Table 2 and morphological and spore nature of the isolates are presented in Table 3 and in photographs of the gram staining Figure 2 and 3 respectively.

**Comparative REA (relative enzyme activity) of isolates**

On the basis of REA, A2 and A5 were found potent protease producers as compared to other isolates. (Figure 2).

On the basis of calculated REA and spot test it was found that A2 and A5 were giving highest zone of casein hydrolysis after 72 h compared to other isolates. Both were giving same REA of 2.6. (Figure: 1,2). (The zone diameter of A2 and A5 were reported as 26mm and their colony size was 10mm).Similar reports were made by Jain *et al.* (2009) for various *Streptomyces* species like, *Str.exfoliates*CFS1068 (REA= 10), *Str. Somaliensis* GS 1242 (REA= 8.8), *Str. Sampsonii*GS 1242 (REA= 9.6) by the similar method. Similar report were made by Jani S.A *et al*. (2012) for various alkalophilic and thermophilic Actinomycetes species like *Thermoactinomyces vulgaris* (REA=2.44) and *Saccharomonospora viridis* (REA=5.5).

**Feather degradation for checking the keratinolytic activity of actinomycetes**

Fresh culture suspension of all seven actinomycete isolates of optical density 0.75 at 670 nm was inoculated in basic salt solution along with feathers (feathers were the sole source of carbon and nitrogen) [Adriano Brandelli 2008]. The set was incubated at 37±2°C for 96 h and observed for keratinolytic activity at the interval of 24 h and the results obtained were presented in Table:3. Results indicate that isolates A1 and A2 were degrading feathers very efficiently within 72h ( Figure:3, Table:3) where as other isolates were very slow. This kind of results were also reported for *Bacillus* sp. [Patil *et al.*, 2010], for *Bacillus licheniformis*MZK-3 [Mohammad, 2007], for *Bacillus licheniformis* [Vigneshwaran, 2010], for *Nocardiopsis*sp. TOA-1 [Mitsuiki *et al.*, 2002], for *Streptomyces* sp. Strain 16 [Xie *et al.*, 2010], for *Streptomyces albidoflavus* [Bressollier *et al.*, 1999], *Streptomyces pactum*[Böckle *et al.*, 1997 and 1995], for *Streptomyces gulbagensis* [Syed *et al.*, 2009], for *Streptomyces thermoviolaceus* [Chitte *et al.*, 1999], and for *Streptomyces thermonitrificans* [Mohamedin, 1999].

The Figure: 4 shows the comparison of the keratinolytic activity of the degraded feather for isolates A1 and A2 against control.

When we checked for keratinolytic activity for all seven isolates by performing feather degradation, even though A5 was having high REA, it was found weak in feather degradation (Table: 1) with compared to A1 and A2. Hence, we selected A1 and A2 for further studies and identification for which we relied upon: cultural characteristics like, colony characters, reverse side pigments and spore nature

**Biochemical Activity of isolates**

Results of biochemical tests carried out for selected isolates A1 and A2 are presented in Table: 5. Biochemical activity revealed that A1 may be *Streptomyces* sp. and A2 may be *Saccharothrix* sp.

**16S rRNA sequencing and phylograms of potent isolates**

A molecular approach was necessary to support unambiguous identification of isolates at species level. Significance of phylogenetic studies based on16S rRNA sequences is increasing in the systematic of bacteria and actinomycetes [Yokota, 1997].

Depiction of the phylogenetic tree derived from 16S rRNA sequences of isolate A1and

A2 is presented in Figure:6 which showed that the sequence exhibited a high level of homology of A1 with *Streptomycesand* of A2 with *Saccharothrix* Based on morphological, biochemical and molecular data, it was confirmed that isolate A1 represented a species of *Streptomyces* and designated as *Streptomyces* sp.*1720570 A1*. Whereas isolate A2 represented a species of *Saccharothrix* and designated as *Saccharothrix xinjiangensis 1720577 A2.*

**Measure of keratinolytic protease production with reference to growth of isolates**

Results indicated that *Steptomyces*sp. (A1) produced keratinolytic protease in logarithmic phase of growth (Figure.7a). Maximum Enzyme production was observed at 72 h indicating that keratinolytic protease is produced maximally in logarithmic phase of growth by isolate *Steptomyces sp.* [Mehta *et al.* (2006)] showed that synthesis of protease from *Streptomyces*sp.(A1)starts in early stationary phase of growth (110 h).

Results indicated that *Saccharothrix xinjiangensis* (A2) produced keratinolytic protease activity in between the end of stationary phase of growth and start of decline phase of growth (Figure 7b). Maximum Enzyme production was observed at 48 h indicating that keratinolytic protease is produced maximally in end stationary phase of growth and start of decline phase of growth by isolate *Saccharothrix xinjiangensis* (A2).

**Optimization of Incubation time**

Study on growth kinetics and enzyme production is described in section 2.7. It was observed that incubation period for optimum keratinolyticprotease production, for *Streptomyces* sp. (A1). It was 72 h and for *Saccharothrix xinjiangensis*(A2) it was 48 h. (Figure 8). Hence, throughout the study at 72 h and 48 h were considered as optimized incubation period for the fermentation.

**Feather as an inducer of keratinase production**

In commercial practice, the optimization of medium composition is done to maintain balance between various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward: (i) evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes (ii) requirement of divalent metal ions in the fermentation medium and (iii) optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production [Kumar and Takagi, 1999].

The cost of enzyme production is a major obstacle in its successful industrial application. In view of the promising applicability of the keratinase protease, it should be produced in high yields in a low-cost medium. With this context, the purpose of the present study was to design the optimum culture medium and the best fermentation conditions for the maximum protease production using A1 and A2.The results(Figure: 9,10) obtained in this showed that Both the isolates produced kertinolytic protease production maximum in the medium in which the feathers were used as sole source of carbon and nitrogen. *Streptomyces* sp. (A1) gave (92.81units/ml) and *Saccharothrix xinjiangensis* (A2) gave

(90.56 units/ml) followed by production medium with feather meal and least keratinase production was found in case of medium without feather meal incorporation. *Streptomyces* sp. (A1) gave (105.75 units/ml) and *Saccharothrix xinjiangensis* (A2) gave (87.18 units/ml) respectively.

These results are very attractive as no extra expensive ingredients are required for the production of enzyme.

**Effect of incubation temperature on keratinolytic protease production**

It is well known that temperature is one of the most critical parameter which has to be controlled in bioprocess [Chi and Zhao, 2003]. The growth and enzyme production are greatly influenced by incubation temperature. The effect of different incubation temperatures on keratinolytic protease production was evaluated and it was found that 40±2˚C was the most favorable temperature for keratinolytic protease production by the isolates. (Figure 11a, b).

Highest amount of enzyme production was observed at 40±2˚C by the isolate *Streptomyces* sp. (A1) (70.31 units/ml)(figure11a). The growth optimum for the enzyme production was 40˚C-50˚C. The results of *Streptomyces* sp. (A1) matches with the strain of *Bacillus* sp. SCB-3 [Lee *et al.*2002] and strain of *Stenotrophomona smaltophilia* [Cao *et al.* 2009] which gave highest enzyme activity at 40˚C and also with the strain of *Streptomyces sp*. S7 [Tatenie*t al.* 2008] which gave enzyme activity at 45˚C which falls in the temperature optima of the *Streptomyces* sp. (A1).

Highest amount of enzyme production was observed at 40±2˚C by the isolate *Saccharothrix xinjiangensis*(A2) (21.37 units/ml) (figure:11b).The results of *Saccharothrix xinjiangensis*(A2) matches with the strain of *Bacillus* sp. SCB-3 [Lee *et al*.2002] and strain of *Stenotrophomonas maltophilia*[Cao *et al*. 2009]which gives highest enzyme activity at 40˚C.

**Effect of initial pH on keratinolytic protease production**

pH of the production medium greatly affects enzyme production [Kumar and Takagi1999]. The results showed that the enzyme production was maximum at pH 8.5 and 9.5

Growth and keratinolytic protease production were highest at pH 8.5-10. For *Streptomyces* sp.(A1). Highest growth of enzyme production was seen at pH 8.5(105.75 units/ml) (Figure 3.15). The result matches with the strain *B. licheniformis*FK14 [Suntornsuk*et al.* 2005] which gives highest enzyme production at pH 8.5 while strain *Streptomyces gulbagensis*DAS 131 gives highest enzyme production at pH 9.0 which comes in the range of pH optima of the *Streptomyces* sp. (A1).

Growth and keratinolytic protease production were highest at pH 8.5-10.5. For *Saccharothrix xinjiangensis*(A2) highest enzyme production was seen at pH 9.5(104.56 units/ml). (Figure 3.16) The result almost matches with the strain of *Thermoanaerobacter sp.* (Kublanov *et al.* 2009a) which gave highest enzyme production at pH 9.3.

**Effect of static and shaking condition on keratinolytic protease production.**

Both the isolates *Streptomyces* sp.(A1) and *Saccharothrix xinjiangensis*(A2) were

giving maximum keratinolytic protease production at shaking condition than the static condition (Figure 3.19). This indicated that both the isolates need continuous aeration for the production of the keratinolytic ptotease .

When keratinase production by both the isolates was compared in static and shaking conditions, it was found that both produced keratinase in good amount**:** *Streptomycetes* sp. (A1)(70.310 Units/ml) in static condition at 72h, in shaking condition (92.810 Units/ml) at 72 h, while in *Saccharothrix xinjiangensis* (A2)(21.37 Units/ml) in static condition at 48h, in shaking condition (90.560 Units/ml) at 48 h from this results it indicates that in both the isolates the results are better in shaking condition as compare to static condition.

**Release of amino acids by *Streptomyces* sp. (A1) and *Saccharothrix xinangensis* (A2) by feather degradation**

Currently, feathers are converted to feather meal by steam pressure cooking, which require high-energy input. Feather meal has been used on a limited basis as an ingredient in animal feed, as it is deficient in methionine, histidine, and tryptophan [Papadopoulos *et al.*, 1986; Wang and Parsons, 1997]. The use of keratinases to upgrade the nutritional value of feather meal has been described [Onifade *et al.*, 1998; Grazziotin *et al.*, 2006].So we decided to check the release of these amino acids by performing TLC of sample of feather degradation.

Different kinds of amino acids were released when Thin Layer Chromatography (TLC) was performed for four known amino acids like histidine, lysine, methionine and tryptophan (Figure 14). Thus four different types of amino acids were released in *Streptomyces* sp. (A1) and three of them were related to the known amino acids like histidine, lysine and methionine according to the Rf value. Whereas, many different types of amino acids were released in *Saccharothrix xinangensis*(A2) and four of them were related to the known amino acids like histidine, lysine, tryptophan and methionine according to the Rf value.

**Figure.1** Spot test of protease producing actinomycetes.(REA)

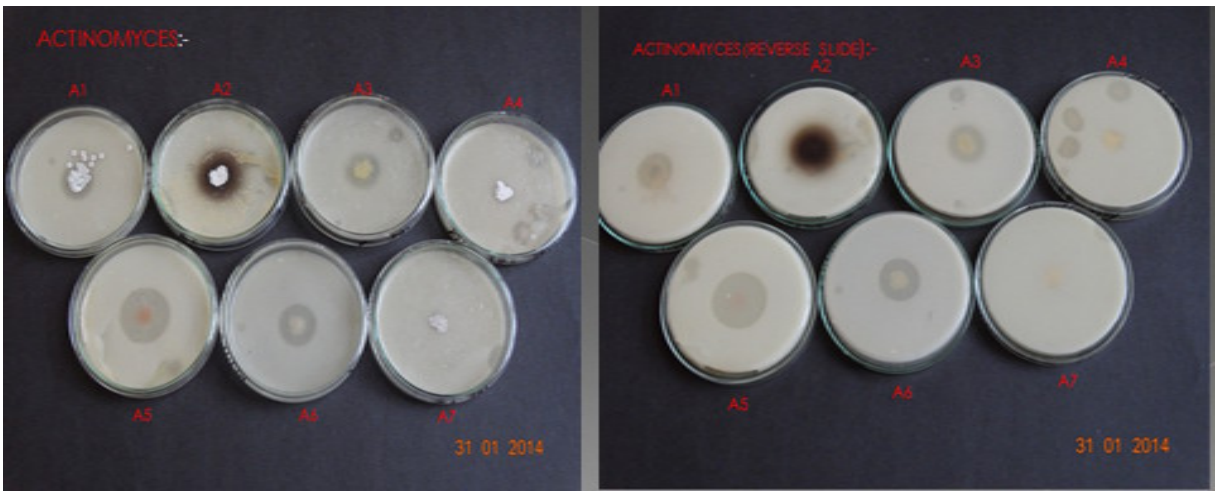

**Figure.2** Relative enzyme activity of protease producer potent isolates

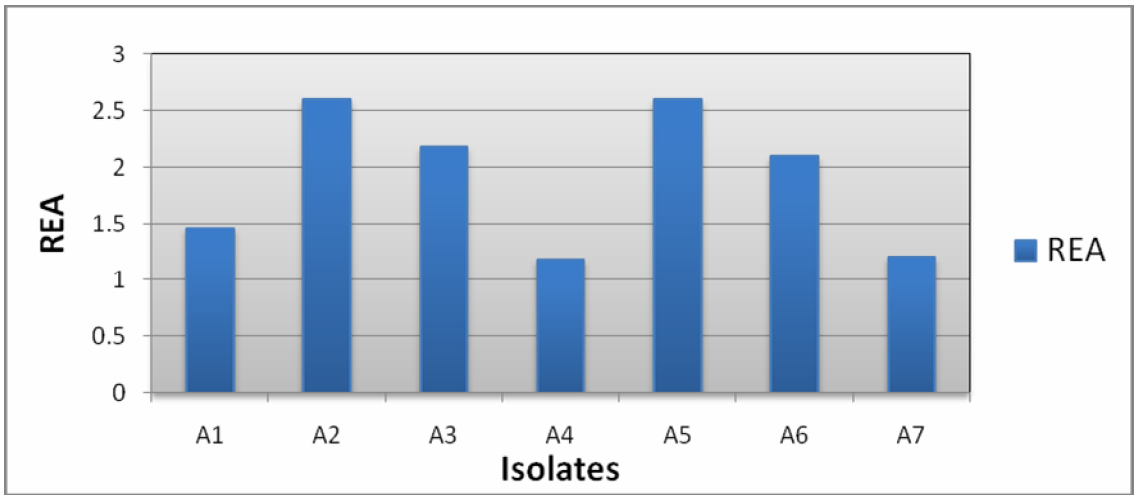


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**Figure.3** Feather degradation for checking the keratinolytic activity of actinomycetes.

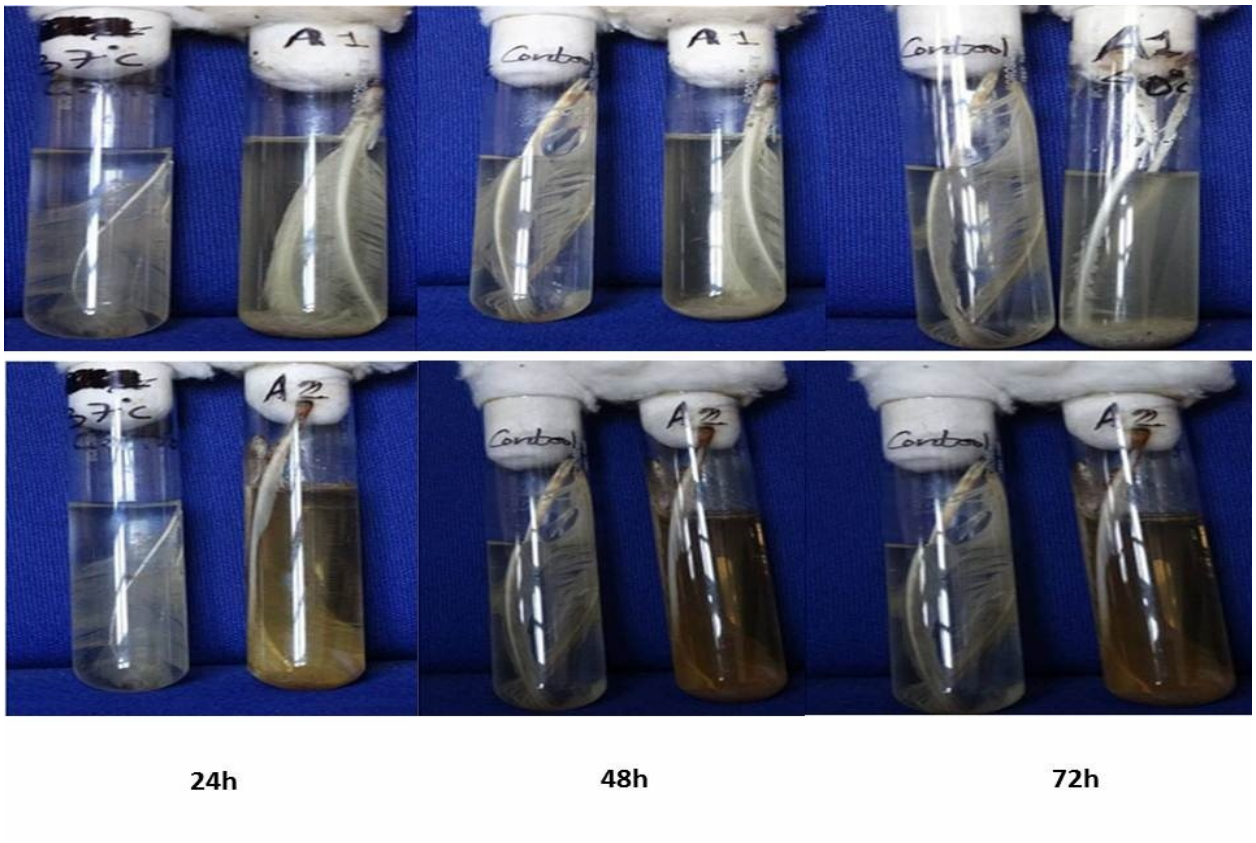
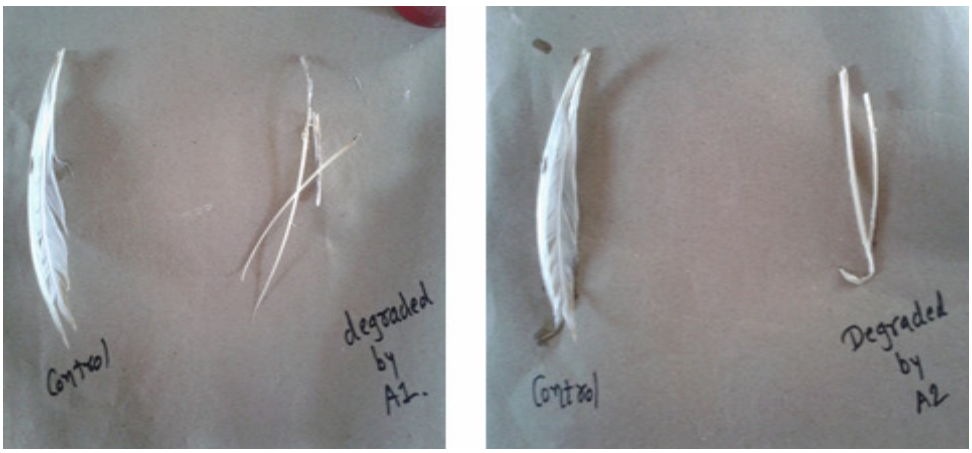


**Figure.4** Degraded feather by isolate A1 and A2 after 72h.




**Identification of potent keratinase producing isolates A1 and A2.**

**Figure.5** Growth characteristics of isolate A1 and A2 on skim milk agar plates.

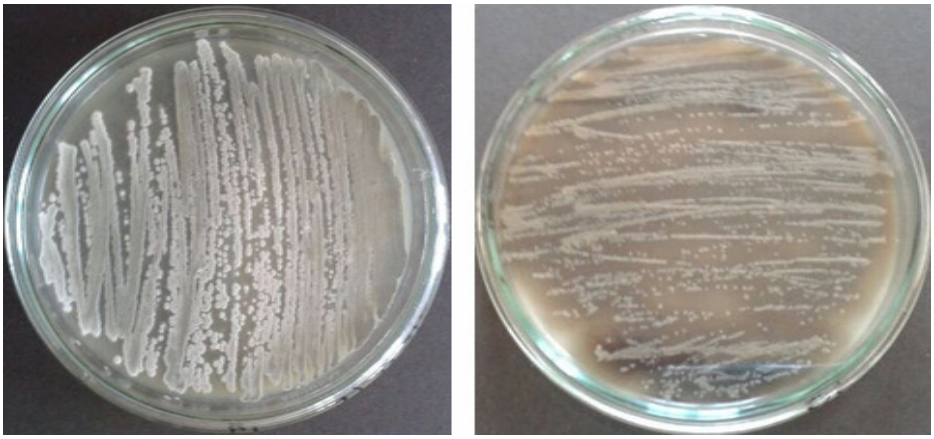
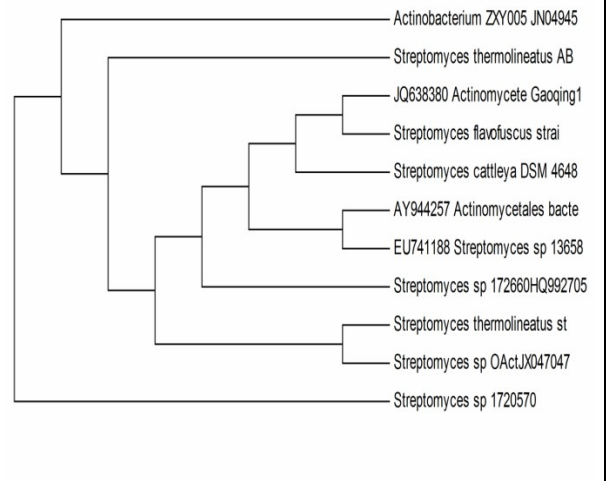
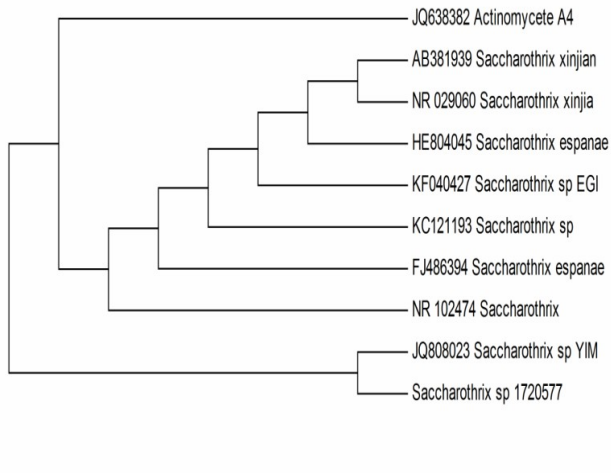


**Figure.6** Phylograms of isolates




Phylogram of strain *Streptomyces* sp. *(*A1)




Phylogram of strain *Saccharothrix xinjiangensis* (A2)

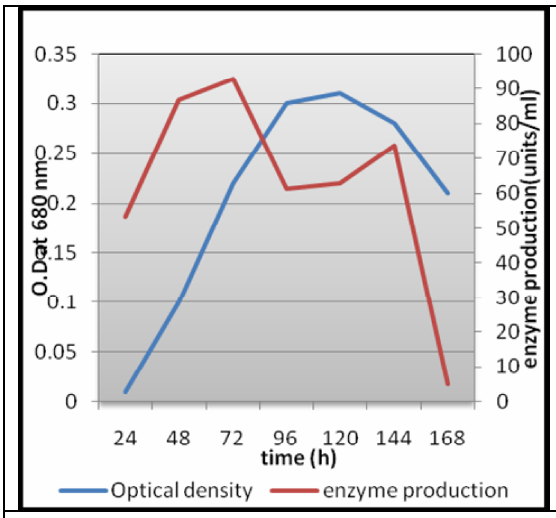



Figure: 7a: Growth and keratinolytic protease production by Streptomycessp. (A1) in production medium

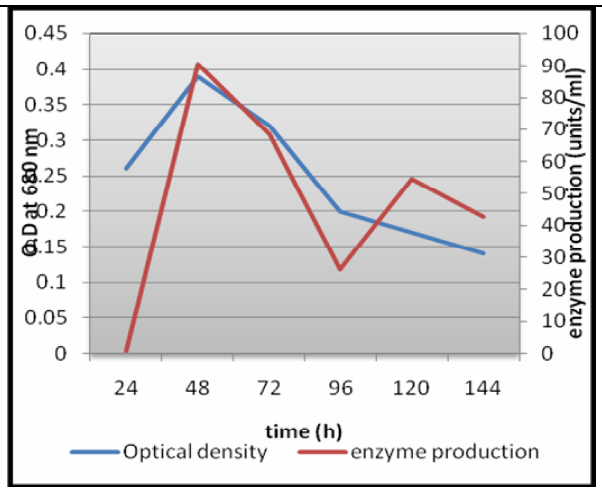



Figure:7b: Growth and keratinolytic protease production by Saccharothrix xinjiangensis(A2) in production medium.

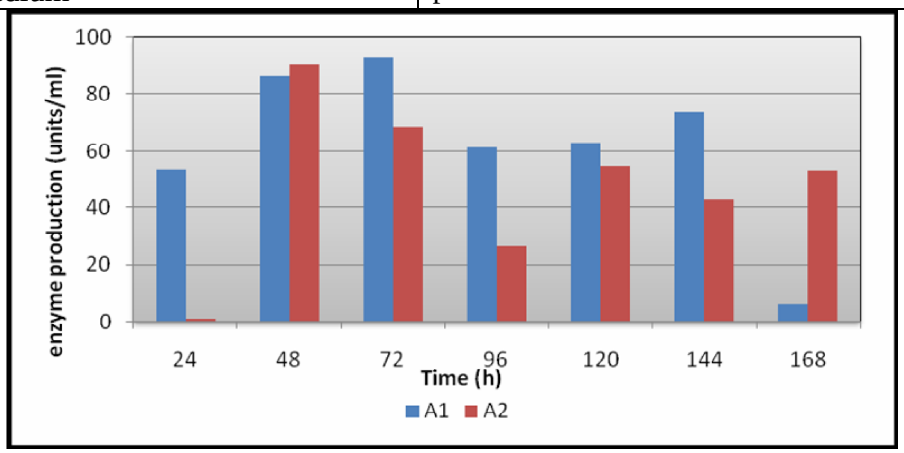



**Figure.8** Effect of Incubation time for keratinase production by *Streptomyces* sp.(A1)and *Saccharothrixxinjiangensis*(A2)

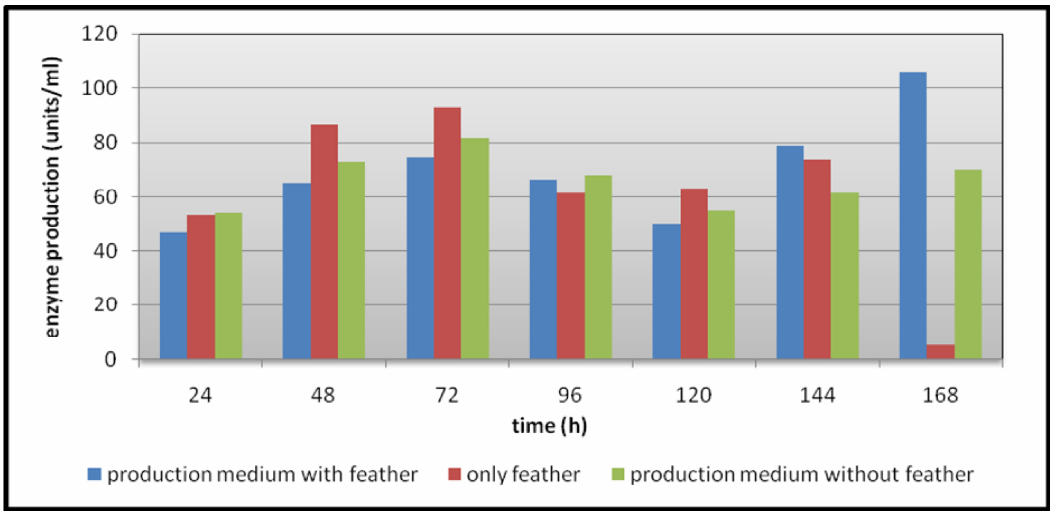
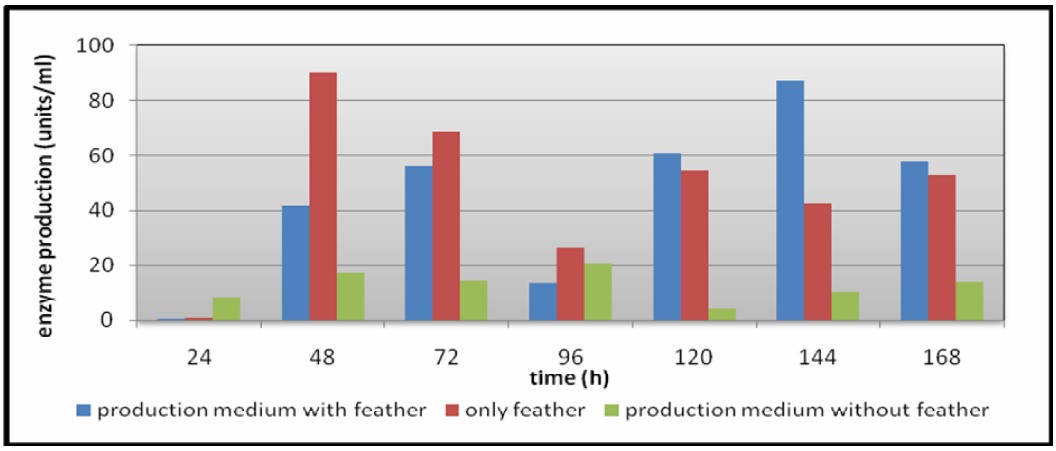



**Figure.9** Feather as an inducer of keratinase production by *Streptomyces* sp. (A1)

**Figure.10** Feather as an inducer of keratinase production by *Saccharothrix xinjiangensis* (A2)



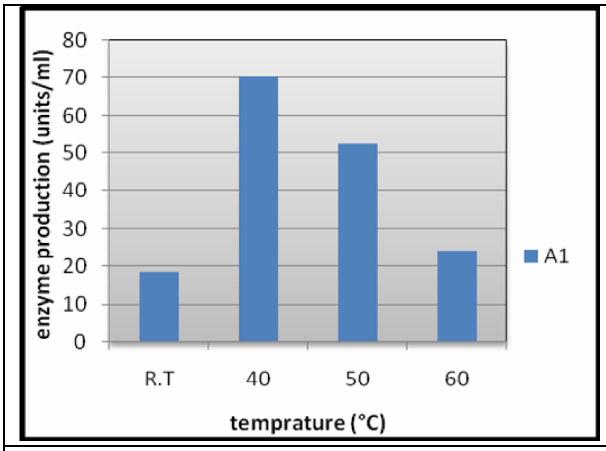




**Figure: 11a**, Effect of incubation temperature ($^{0}$C) on keratinase production by Streptomyces sp. (A1).

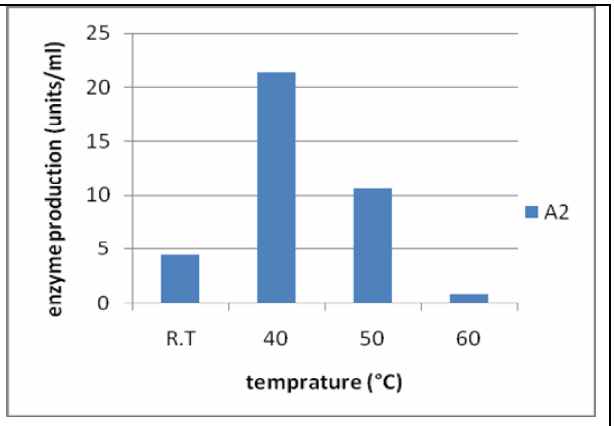



**Figure: 11b** Effect of incubation temperature ($^{0}$C) on keratinase production by Saccharothrix xinjiangensis (A2).

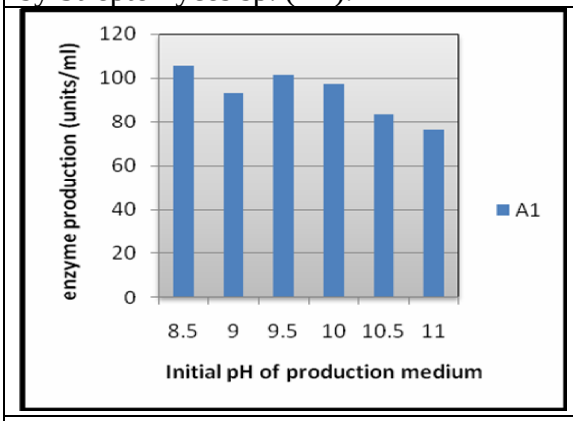



**Figure12a** Effect of initial pH on keratinase production by *Streptomyces* sp. (A1)

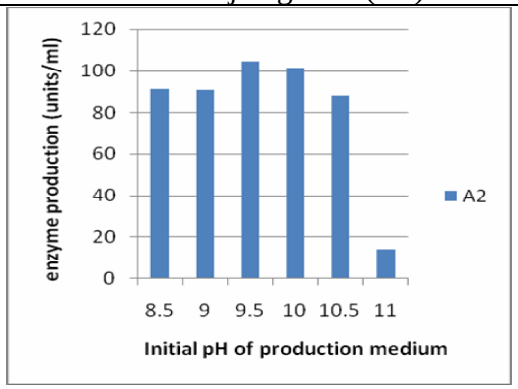



**Figure12b** Effect of initial pH on keratinolytic protease production by *Saccharothrix xinjiangensis* (A2)

**Figure.13** Effect of static and shaking condition of keratinase production by *Streptomycetes*sp. (A1) and *Saccharothrix xinjiangensis*(A2)

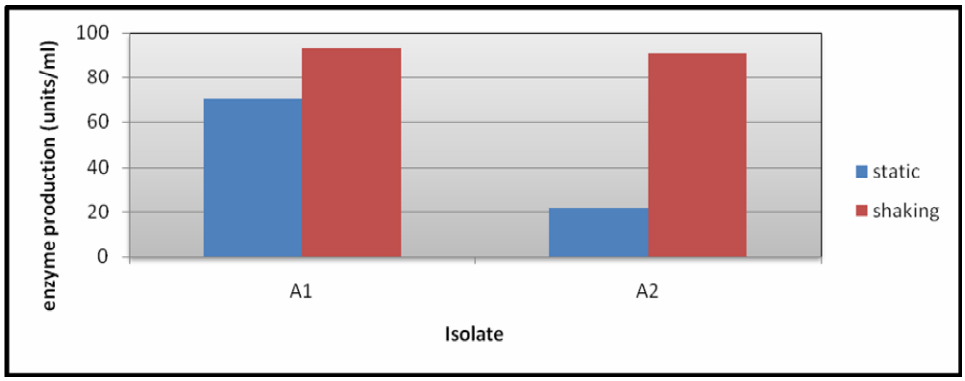



**Figure.14** amino acids profile of degrades feathers by Thin Layer Chromatograph (TLC).

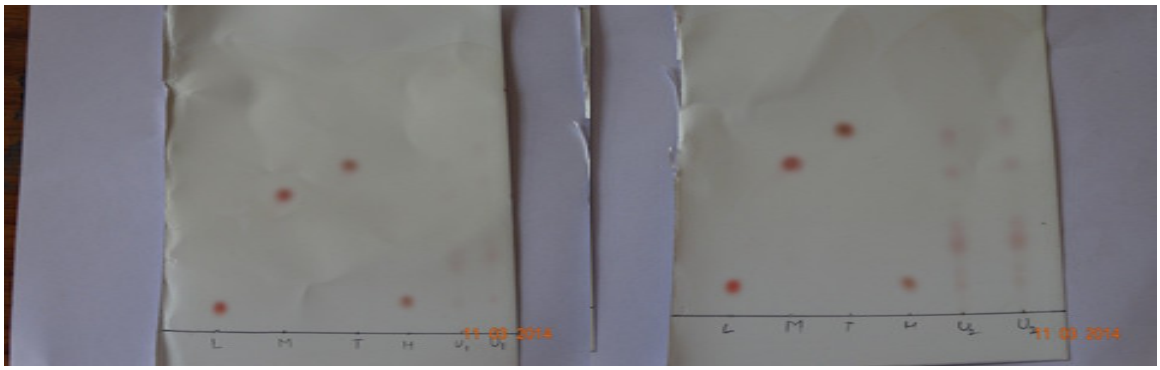


**Table.1**

| peptone | meat extract | NaCl | agar | sterilized skim milk/ casein | pH |
|---|---|---|---|---|---|
| 0.5 gm% | 0.3 gm% | 0.5 gm% | 3.0 gm%, | 10% v/v or1.0gm% | 8.5 |

**Table.2** Composition of production medium [Rao and Narasu 2007]

| **solution** | Glycerol | $K_2HPO_4$ | $KH_2PO_4$ | $MgSO_4$ | $CaCl_2$ | casein | Peptone | $NaNO_3$ | Distilled water |
|---|---|---|---|---|---|---|---|---|---|
| **amount** | 0.5% | 20mg | 20mg | 10mg | 10mg | 200mg | 200mg | 100mg | 100ml |

**Table.3** Feather degradation efficiency of potent isolates

| **Isolate** | **Feather degradation efficiency** |
|---|---|
| **A1** | **+++ within 72h** |
| **A2** | **+++ within 72h** |
| **A3** | **_** |
| **A4** | **+ after one week** |
| **A5** | **+** |
| **A6** | **_** |
| **A7** | **+ after4 days** |

**Table.4** Colony characteristics of potent protease producing actinomycetes

| Isolate | Size | Shape | Margin | Texture | Elevation | Opacity | Colony Colour |
|---|---|---|---|---|---|---|---|
| A1. | Big | Irregular | Uneven | Rough | Raised | Opaque | White |
| A2. | Small | Irregular | Uneven | Rough | Raised | Opaque | White |
| A3. | Big | Irregular | Uneven | Smooth | Raised | Opaque | Yellow |
| A4. | Big | Round | Even | Rough | Raised | Opaque | White |
| A5. | Small | Round | Even | Smooth | Raised | Opaque | Pink |
| A6. | Big | Irregular | Uneven | Smooth | Raised | Opaque | Creamish |
| A7. | Medium | Round | Even | Rough | Raised | Opaque | Light Pink |

**Table.5** Morphological characteristics of potent protease producing actinomycetes.

| Isolates | Reverse side Pigment | Spore morphology | Morphological characteristics |
|---|---|---|---|
| A1 | Greenish White | spiral | Gram positive filamentous with spore |
| A2 | Brown(soluble) | Single | Gram positive filamentous with spore |
| A3 | Yellow | warty | Gram positive filamentous with spore |
| A4 | GreyishWhite | hairy | Gram positive filamentous with spore |
| A5 | Pink | single | Gram positive filamentous with spore |
| A6 | Creamish | Spiral | Gram positive filamentous with spore |
| A7 | Yellow | warty | Gram positive filamentous with spore |

**Table.6** Biochemical activity of isolates A1 and A2:

| Name of Biochemical test | Strain A1 | Strain A2 |
|---|---|---|
| V.P test | Positive | Negative |
| Methyl red test | Negative | Negative |
| $H_2S$ production | Negative | Negative |
| Indole production | Negative | Negative |
| Citrate utilization | Positive | Positive |
| Catalase test | Positive | Positive |
| Nitrate reduction | Negative | Negative |
| Urease Production | Positive | Positive |
| Growth pattern in N-broth | Surface growth | Growth with brown soluble pigment |
| Gelatin hydrolysis | Positive | Positive |
| Carbohydrate utilization | | |
| Glucose broth | Positive | Positive |
| Xylose broth | Only gas | Only gas |
| Manitol broth | Only gas | Positive |
| Arabinose broth | Negative | Only acid |

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