Isolation and molecular identification of keratinase hydrolyzing microorganisms isolated from feather samples of Gulbarga region

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Abstract

Enzymes are delicate protein molecules necessary for life. Among various different enzymes, protease are the enzymes which are found to play an important role in the metabolism of almost all organisms. Among proteases, Keratinase (EC 3.4.4.25) is able to hydrolyze insoluble keratins more efficiently than other proteases and their action are very specific i.e. they act only on keratin substrates. Keratins are insoluble fibrous proteins found in hair, wool, feather, nail, horns and other epithelial covering which are rich in beta helical coil linked through cysteine bridges. Keratinases are widely used not only in chemical and medical industries but also in food in dustries.

In respect to this, present study aimed to isolate keratin hydrolyzing micro-organisms from the feathers samples collected from various butcher shops and poultry farm in and around Kalyan-Karnataka region where we have isolated around 46 isolates from 20 different types of samples among which KLHR-30 was found to be potent keratinase producer showing activity of 90 U/ml found to be an actinomycetes from molecular analysis and deposited in gene bank with an accession number of OR841343.

Keywords: Feathers, Keratin, Actinomycetes, Keratinase

Introduction

Nowadays with the increasing use of chickens for the propose of food, the feathers of the chicken have been dumped in large amount as a by-product. As feather signifies around 5-7% total mass of the adult chicken which is rich in pure keratin protein, is a naturally occurring protein, which can be utilized as a good source of protein. Other than chicken feathers, it is also present abundantly in in hairs, nails, wools, horns, scales, beaks, feathers, hoofs, claws and epithelial cells in the outermost layers of the skin. The main interest in feather-degrading bacteria recently originated from waste disposal.

The total worldwide consumption of chicken makes the tons of feathers a waste, which produces around 18,500 lakhs tons of poultry feather waste, where alone produced India around 3500 tons. Proteases are the single class of enzymes which play an important part in the metabolism of almost all organisms (Plants, Animals, Fungi, Bacteria and Viruses). Investigation of proteases is a central issue in enzymology due to its wide applications in laundry detergents, pharmaceutical, leather products, photography, food, agricultural products and bioremediation process. Among proteases class, Kertainases are found to be extracellular in nature which can be commonly produced by growing micro-organisms in a basal medium containing keratinous substrates[8].

It is believed that in the future, microbial keratinase will occupy a special place among protease class of enzyme as a important enzyme in the eco-friendly processing of keratinous wastes, which are released as huge amounts into the environment due to human activities[7,19].

Because of their higher specificity, Keratinase nowadays emerged as a potential source for the generation of pollution-free waste which can replace proteases in leather and detergent industry[5]. We aimed to isolate potent keratinase producing bacteria isolated mainly from poultry samples in and around Gulbarga region.

Material and Methods

Chemicals and Reagents: All the chemicals and reagents used in this experimental design were procured from HiMedia Ltd., Mumbai, India.

Materials: Chicken feathers were collected immediately after slaughtering of the chickens and extensively washed with tap water until the effluent became very clear and then washed finally with distilled water. The washed feathers were dried under sunlight and further dried at 60^0C for 48h. After drying, the large feather stocks were cut by hand into smaller pieces to fit to the culture flask. They were stored at 40^0c until used[12].

Sample Collection and Isolation of keratinolytic Microbes: A total of 25 different soil samples were collected from different regions of Kalaburagi, India. Rotted feather samples and soil were collected from a local poultry plant's waste site in and around Gulbarga and Bidar region.

Primary screening: It was done on skimmed milk agar medium. The serial dilution plate culture method was employed to isolate the actinomycetes from pretreated soil samples. Adequate serial dilutions (up to 10-5) were prepared in sterile tap water from the soil samples. 0.1 mL of the inoculum from respective dilutions was inoculated on starch casein agar (Soluble Starch - 10.0 g; K_2HPO_4 - 2.0g; KNO_3 - 2.0g; NaCl - 2.0g; casein – 0.1g, $MgSO_4$-0.05 g;

$CaCO_3$-0.02g; $FeSO_4.H_2O$ - 0.01 g; Agar-20g; Distilled water-1000 mL). The protease producing organisms were identified by the presence of zone around the colonies on skimmed milk agar medium.

Secondary screening: The organisms which showed positive for protease activity, were further screened for keratinolytic activity. The selected organisms were grown in agar medium (g/L); NH_4Cl-5g, NaCl-5g, K_2HPO_4-3g, KH_2PO_4—3g, $MgCl_2.6H_2O$-1g, feather meal-10g, pH-8.0[14,15] and plates were incubated at 40°C for one week. The initial pH of the medium was adjusted to 7.5. Growth of colonies of actinomycetes on the medium was observed at every 24 h for a week. Based on large CZ/CS ratios, potent keratinolytic microbial strains were selected for the further quantitative screening.

Enzyme assay: Keratinase assay was performed as per the method given by Rayudu and Jayaraj[16]. 2 ml of reaction mixture contained 1 ml of keratin solution and 1 ml of supernatant as crude enzyme. The reaction mixture was incubated for 10 minutes at 40° C in water bath. To the cooled mixture, 2ml of 10% chilled TCA was added and kept for 20 minutes incubation. The mixture was later centrifuged at 5000 rpm for 10 minutes. 0.1 ml of the supernatant was added to 0.9ml of distilled water and 0.5ml 500mM sodium carbonate was incubated at 35°C for 10 minutes. 2ml Folin ciocalteu reagent (1:3 v/v) was added and incubated for 20 minutes. A blue color developed at the end of the reaction and the absorbance was measured at 660nm. A standard graph was generated using standard tyrosine solution of 10-100ug/ul. One unit of keratinolytic activity is defined as the amount of enzyme that liberates 1ug of tyrosine.

Identification of actinomycetes: The colonies grown on modified starch feather meal agar were identified preliminarily as actinomycetes based on the typical features of colony (size, shape, surface), pigmentation (aerial mycelium, substrate mycelium diffusible pigment) and microscopic characters (Gram staining and mycelia branching) as per the standard criteria. The identified colonies of actinomycetes were subcultured on SCA and preserved at 40ºC for further studies.

Characterization: Microscopic characterization of isolates was done by Gram staining. Biochemical characterization of isolates was performed as per the regular IMVIC test.

Molecular Characterization: Gene sequencing technology (16S rRNA) was used for the molecular identification. A DNA extraction kit was used to obtain the genomic DNA of the strain. The amplification of the 16S rRNA gene was accomplished using the primers 785F (5'GGATTAGATACCCTGGTA) and 907R (5'CCGTCAATTCMTTTRAGTTT). Sequencing of the isolated DNA was done. In order to receive accession numbers, the sequence was eventually submitted to GenBank. The nucleotide sequence was compared to the reference sequence using the basic local alignment search tool (BLAST) available on the NCBI website.

Phylogenetic Analysis: The sequence analysis was carried out using Bioinformatics tool BLAST of NCBI. Multiple sequence is aligned used Clustal W. The phylogenetic tree was constructed using Mega11 software. The obtained 18s sequences are compared to the sequences obtained in the gene bank using BLAST program on the NCBI website.

Results

Isolation of the micro-organism: Isolation of micro-organisms from different types of samples was done by serial dilution method i.e. 1gm of the samples is serially diluted using sterile distill water. For the bacterial isolation, 10^{-5} and 10^{-6} are taken so as for fungi isolation, 10^{-3} and 10^{-4} should be taken and for actinomycetes isolation, 10^{-2} and 10^{-3} should be taken. 0.1ml from the dilution test tube was taken on above mentioned media which is supplemented with skimmed milk. The microbial strains used in the present study were isolated from the nearby non agricultural felid soils, feather dump/poultry soil, slaughter house soil and several faecal samples.

The culture showing good results are transferred to the fresh media. After 3 more transfers, the pure culture of the micro-organism was obtained. The pure culture was maintained in the skimmed milk agar media for further investigation

Primary screening: Screening plays an important role was differentiating microorganisms according to their enzymatic activities. Primarily, proteolytic bacterial strains were isolated from respective samples on skimmed milk agar (SMA) plates qualitatively by rapid plate assay method. Pure colonies with distinct halo around the growth on SMA plates have been assigned respective isolate lab codes with HRKL followed by strain number. A total of 45 isolates have been isolated with duly designated codes from KLHR1 to KLHR45. Based on high zone to colony ratios (i.e. ≥ 3.5) on SMA plates, out of 45 isolates in the present study 23 proteolytic bacterial strains have been selected for further identification and their taxonomic studies.

Secondary screening: Among all isolates, comparatively actinomycetes showed highest enzyme activity than bacterial and fungal isolates. Comparatively, isolate KLHR-30 found is highest enzyme producer under SmF. Further optimization studies for the enhanced keratinase production will be continued with KLHR30 as shown in images plate 1 and plate 2.

Microscopic Observation and Biochemical test: The microbial isolate KLHR30 was identified as Actinomycetes on the basis of cultural characteristics and microscopic observations. The isolate was produced as powdery small sized round colonies with rhizoidal margin on starch casein agar media.

Table 1
Zone of casein hydrolysis by various isolates:

S.N.	Isolates	Zone of casein hydrolysis(mm)	S.N.	Isolates	Zone of casein hydrolysis(mm)
1	KLHR1	10	26	KLHR26	10
2	**KLHR2**	**29**	27	KLHR27	5
3	KLHR3	9	**28**	**KLHR28**	**19**
4	KLHR4	10	29	KLHR29	15
5	KLHR5	5	**30**	**KLHR30**	**34**
6	KLHR6	10	31	KLHR31	10
7	KLHR7	10	32	KLHR32	17
8	KLHR8	8	33	KLHR33	5
9	KLHR9	10	34	KLHR34	8
10	KLHR10	7	35	KLHR35	17
11	KLHR11	15	**36**	**KLHR36**	**22**
12	KLHR12	17	**37**	**KLHR37**	**24**
13	KLHR13	9	38	KLHR38	10
14	KLHR14	10	39	KLHR39	16
15	KLHR15	5	**40**	**KLHR40**	**24**
16	KLHR16	23	41	KLHR41	12
17	KLHR17	9	**42**	**KLHR42**	**26**
18	KLHR18	17	43	KLHR43	10
19	KLHR19	9	**44**	**KLHR44**	**28**
20	KLHR20	6	45	KLHR45	17
21	KLHR21	7			
22	KLHR22	23			
23	KLHR23	5			
24	KLHR24	9			
25	KLHR25	10			

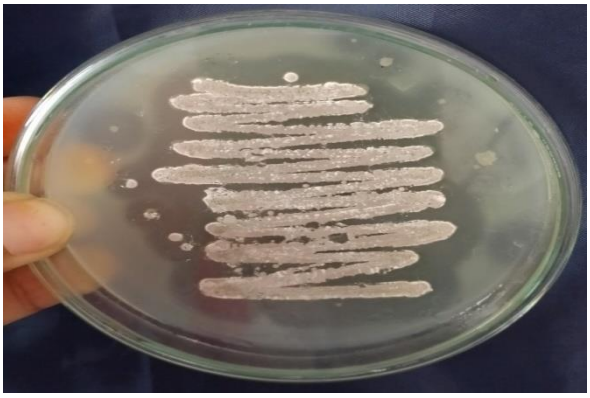

Plate 1: Casien hydrolysis by actinomycete KLHR30

Plate 2: Zone on feather meal media by the isolate KLHR30

Plate 3: *In situ* feather degradation. After incubation of 72hrs, the feathers got completely degadraded.

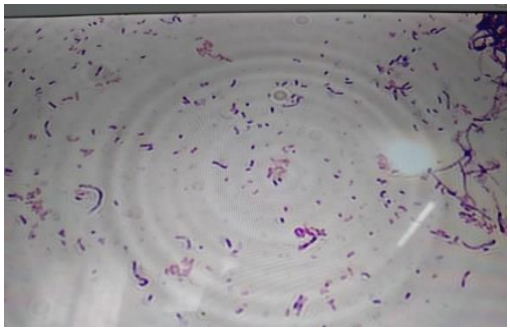

Plate 4: Gram's nature of KLHR30

The colonies were found with white coloration up to five days of incubation, thereafter the colonies imported with gray color pigmentation. Gram's staining of the isolate revealed that it is Gram positive organism with branched structures as shown in Petri plate 3. Table 2 shows biochemical test.

Table 2
Biochemical test

S.N.	Test	Result
1	Indole	Positive
2	Methylene blue	Negative
3	Vogues proskers	Negative
4	Citrate	Positive

Phylogenetic tree: The phylogenetic analyses of the selected Keratinase producing actinomycetes are closely related to the *Streptomyces* genera. In this study, the isolated culture showed 100% sequence similarity with *Streptomyces diastaicus*. Based on nucleotide homology and analysis and accession number of OR841343 is obtained for the given culture

GGCTCACCAAGGCGACGACGGGTAGCCGGCCTGA
GAGGGCGACCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACC
TGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTG
TCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCG
GGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGT
AGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAA
TGCGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGATCTCTGGGCCGATACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGGTGGGCACTAGGTG
TGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAA
CGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC
AAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGCTTAATTCGACGC
AACGCGAAGAACCTTACCAAGGCTTGACATACAC
CGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTC
GGTGTACAGGTGGTGCATGGCTGTCGTCA GCTCGT
GTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGTCCCGTGTTGCCAGCAACTCTTCGG
AGGTTGGGGACTCACGGGAGACCGCCGGGGTCAA
CTCGGAGGAATGGTGGGGGACGACGTCGAGTCAT
CATGCCCCTTATGTC TTGGGCTGCACACGTG CTA
CAATGGCCGGTACAATGAGCTGCGATACTGCAAG
GTGAAGCGGAATCTTCAAA AA.

Discussion

Nowadays the use of keratinases has been highly studied and explored in the field of microbiology. There is a need for an active keratin degrader as Keratinase producing micro-organisms are specific towards there substrates like hair, wool, nails and feathers. In the present study, we have collected various feather samples from in and around Gulbarga Region, in which we have isolated 42 isolates among which isolates KLHR-30 has showed highest keratinase activity of 90U/ml which on molecular identification was found to be actinomycetes. Similar results are obtained from Keratinase isolated from soil samples along with decaying feathers collected from poultry farms at Vita region in Sangli showing highest activity of 76U/ml at a room temperature of 37^0C and at an rpm of 130 within 72hrs.

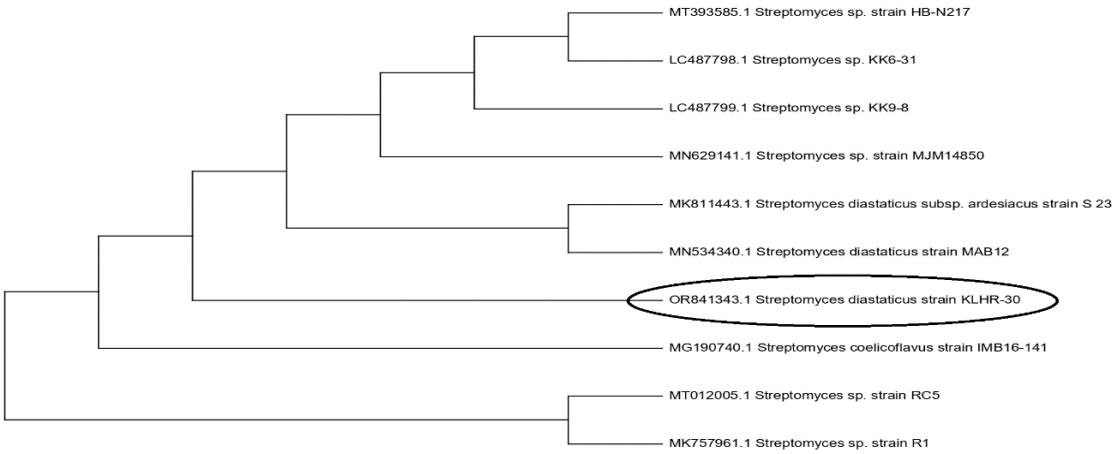

Figure 1: Phylogenetic analysis of the Keratinase

Around 52 keratinases were isolated producing micro-organisms from soil sample of Thailand region which showed a keratinase activity in the range of 0.7-2.6U/ml, among which isolate FK28 showed good activity related to *Bacillus* species.

Conclusion

In the present study we have tried to explore the potential of indigenous micro-organisms (isolated from poultry samples) for the production of industrial important keratinase, where in we have isolated around 42 isolates among which isolate KLHR-30 showed highest Keratinase activity of 70U/ml of actinomycetes species of *Streptomyces diastaicus* which can degrade the feathers completely in 92hrs, which can be further used for the poultry waste management as an eco-friendly technique. Further optimization can be done in future to get a maximum keratinase activity from the isolate.

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