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Production and Purification of Keratinolytic Protease From Bacillus Cereus for Poultry Waste Degradation

Bindhumol Ismail, Roja Chandran, Sanoopa M Mohan, A Jayakumaran Nair
Department of Biotechnology, University of Kerala

Abstract:- Natural by-product of agricultural waste can be turned into products of commercial interest. Much effort from scientists and researchers all around the world has been put to extend the full use of agricultural waste. Reports on keratinise enzyme production from the bioconversion of keratinous materials has much been made. However there is still much space to find the most suitable conditions. Keratinolytic microorganisms have a great importance in poultry waste degradation and its bioconversion to compost or animal feed. The goal of the study was to investigate the keratinase enzyme production ability of bacterial strain against keratinous bio-waste like chicken feather at environmental parameters of pH (7), temperature (37ºC) and incubation period (24-120h). Keratinase enzyme production was studied using submerged fermentation. Production of keratinase enzyme was analysed by protease and keratinase assays. In the keratinase method, maximum enzyme production of 95 IU/ml was achieved at temperature of 37ºC by *Bacillus* sp. in feather keratin with pH of 7.0 at 72 hr. Statistical optimization increased the keratinase production by eight fold. The organism was identified as *Bacillus cereus* which showed potent efficiency in feather degradation and its partial purification was done.

Keywords:- *Keratinase, Degradation, Response surface methodology, Box-Behnken, Fermentation, Fractional factorial, Statistics.*

I. INTRODUCTION

Presently, poultry feather wastes are one of the main reasons for environmental pollution. Billions of chicken are being killed annually for many purposes and as a result around 8.5 billion tonnes of poultry feather are produced. These poultry wastes are being cleaned up by dumping, landfilling, incineration or burying etc. but some of these methods can cause problems in storage, handling, emissions control and ash disposal. Discarded feather also become a serious cause of various human ailments including chlorosis, mycoplasmosis and fowl cholera [1].

Hydrolytic enzymes form a permanent solution for this waste problem by converting poultry waste to value-added products. Hydrolytic enzymes as proteases, amylases, lipases, cellulases occupy a major portion of our industrial market and out of these, proteases contribute a major share. Keratinases are a new addition to the group of proteases which are serine or metallo proteases able to degrade the recalcitrant, proteinase-K resistant proteins.

Microbial keratinases exhibit great diversity in their biochemical properties with respect to activity and stability in various pH and temperature ranges [2]. But bacterial keratinase are particular because of their action on a broad range of substrates [3]. Most of the keratinases reported were found to be monomeric enzymes with diversity in molecular weights ranging from 14-240 KDa.

The diversity in their properties makes them good candidates in various fields like food, feed, leather, fuel etc. [2]. The keratinolytic proteases lacking collagenolytic and having mild elastolytic activities are increasingly being used for the dehairing purposes. It helps in the selective breakdown of keratin tissue in the follicle. It will create easy to pull out intact hairs without affecting the tensile strength of leather [4].

Keratin treated feathers are a good source of dietary protein in food and feed supplements. Several bacteria are found to produce keratinolytic proteases including Streptomyces and *Bacillus* [5]. The production of the enzyme at an industrial aspect requires optimum cultural conditions, operational conditions and effective downstream processing. The present work discusses isolation and screening of microorganisms able to produce keratinolytic proteases, their biochemical characterization and process parameter optimization for enhanced enzyme activity, so that they can be used in poultry waste management in an eco-friendly manner.

II. MATERIALS AND METHODS

A. Microorganisms and Maintenance

Nutrient broth and nutrient agar were used for the isolation and culturing of keratinolytic protease producing microorganisms. The culture was maintained at 4 ºC by sub-culturing from the nutrient agar cultures. Glycerol stocks were prepared in nutrient broth for long term preservation at -80 ºC.

B. Sample Collection

Soil and feather samples were collected from poultry farms located at Kollam and Thiruvananthapuram. The samples were stored at 4ºC for use.

C. Preparation of Native Chicken Feathers

The collected feathers were washed in tap water for several times and dried in sun. The feathers were then boiled in water for 20 minutes and sun dried. The sun dried feathers were then pre-treated with chloroform and ethanol in a ratio 3:1. This was kept for two days and then drained the solution and sun dried. The sun dried chicken feathers were cut into small fine pieces.

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D. Isolation of Microorganism

Soil collected from poultry farm was used for the isolation of keratinolytic microorganisms. 1g of soil was inoculated into 100 ml feather meal broth (NH_4Cl-0.2g, NaCl-0.2g, K_2HPO_4-0.12g, KH_2PO_4-0.16g, $MgCl_2$-0.04g, yeast extract-0.04g and feather-4g) of pH 7.0 and was incubated on a rotary shaker (100 rpm) at 37ºC for 24-72 h. The 72 h old culture (0.1 ml) was plated on nutrient agar plates (Fig. 1) and incubated at 37 °C for 24 h to isolate microorganisms which could grow on feather meal broth.

E. Screening for Keratinolytic Protease Producing Bacteria

Selected colonies from nutrient agar plates were plated on to skim milk agar plates and incubated at 37 °C for 48 h. The positive colonies were plated on to feather meal agar and incubated at 37°C for 24-72 h. Microorganisms showing positive keratinolytic protease activity on skim milk agar (Fig. 1) and feather meal agar were further confirmed by morphological and microscopic examination and biochemical characterization.

F. Protease Assay

Proteolytic activity was assessed using 160 µl azocasein properly diluted in 2% TrisHCl (50 mM, pH 8.5) with 100 µl of supernatant (24 h old). The reaction mixture was incubated at 37 º C for three hours in a water bath. After incubation the reaction was stopped by adding 800 µl of 10% TCA and incubated at 4 º C for 10 minutes and centrifuged at 8000 rpm for 20 min to collect supernatant. 1200 µl of 1N NaOH was added to the supernatant and absorbance was measured at 440 nm.

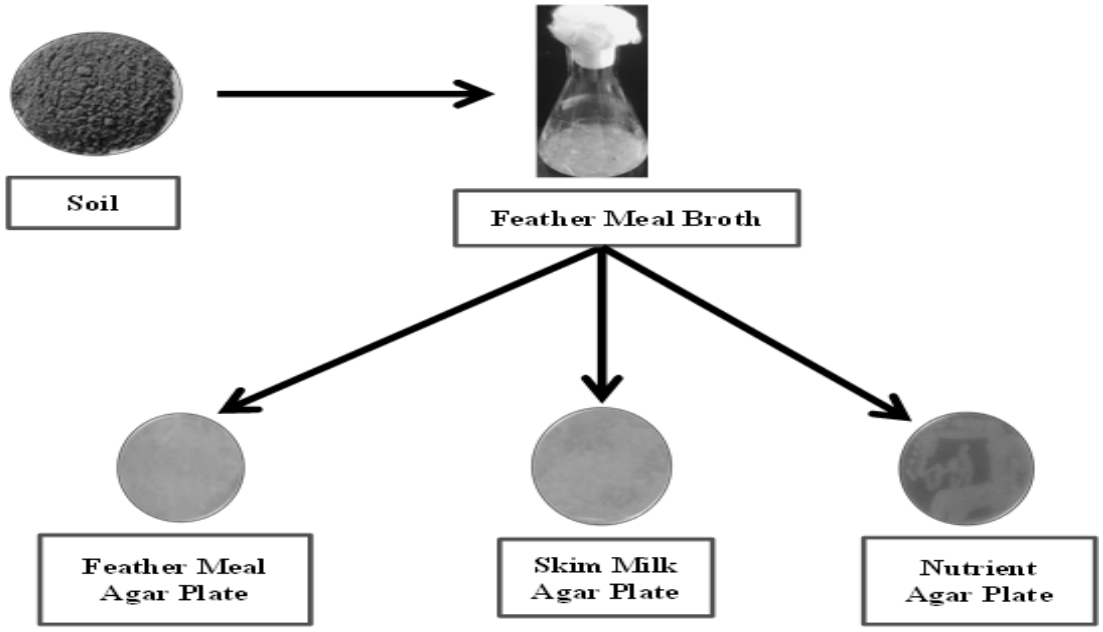

Fig 1:- Isolation of keratinase producing microorganism from soil

A blank was set with Tris-HCl alone and substrate and enzyme blanks were also set for the experiment. One unit (IU/ml) of protease activity was defined as the amount of enzyme required to bring an increase in absorbance (A440) of 0.01 under standard assay conditions [6].

G. Keratinase Assay

Keratinolytic activity was checked using 100 µl supernatant (24h old) with 1.9 ml keratin solution (0.5 % in phosphate buffer, pH 7.0). The reaction mixture was incubated at 50oC for 10 minutes in a water bath. After incubation the reaction was stopped by adding 2.0 ml of 0.4 M TCA and centrifuged at 8000 rpm for 20 min to collect supernatant. 5 ml of 0.4 M sodium carbonate was added to 1 ml of this supernatant. Then 0.5 ml of Folin's reagent was added and incubated under dark for 30 min. After incubation, the absorbance was measured at 660 nm. A blank was set with phosphate buffer alone and substrate and enzyme blanks were also set for the experiment. One unit (IU/ml) of keratinase activity was defined as the amount of enzyme required to bring an increase in absorbance (A660) of 0.01 under standard assay conditions [7].

H. Microscopical Examination

The positive isolate which gave maximum production in protease and keratinase activity was microscopically examined for cell morphology & cellular arrangement and Gram stain reaction. The result was observed under oil immersion using a Bright field microscope.

I. Biochemical Characterization

Biochemical characterization of the positive isolate selected was done according to the method of Fawole and Oso [8]. Catalase test, Oxidase test, Indole test, Methyl red test, Voges-proskaeur test and Citrate tests were done to biochemically characterize the positive isolates.

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J. Molecular Method of Identification

Genomic DNA of the selected isolate was extracted to perform molecular identification. The isolate was grown in nutrient broth and incubated on a rotary shaker (100 rpm) at 37 ⁰ C for overnight. Bacterial culture (FM4), 5ml was centrifuged at 8000rpm for 15 minutes at 4 ⁰ C. The pellet was collected and kept in 20 ⁰ C for 1 hour. The pellet was then thawed and washed in TES buffer (6.7 % sucrose, 50mM tris-HCl, 1mM EDTA, pH 8.0). Then re-suspended the pellets in 300µl of STET buffer (8% sucrose, 5% triton X- 100, 50mM tris- HCl, 50mM EDTA, pH 8.0) and cells were vortexed for 60 seconds. Then the cells were incubated at 37 ⁰ C for 10 minutes and again incubated at 65 ⁰ C for another 10 minutes. To this 100µl of TE buffer was added and the lysate was extracted with 1 volume of phenol: chloroform: isoamylalcohol (49:49:1). Then they were centrifuged at 8000 rpm for 15 minutes. The upper aqueous phase was collected and added 70µl of 5M sodium chloride and 1ml isopropanol. The tubes were kept in ice for 15 minutes for precipitating DNA. The DNA was collected by centrifuging at 8000 rpm for 15 minutes at 4 ⁰ C. Pellet was washed in 70% ice cold ethanol. The ethanol was air dried and pellets were suspended in 100µl of TE buffer and added RNase and incubated at 37 ⁰ C for 15 minutes.

The extracted DNA was used for performing 16S rRNA sequencing. Polymerase Chain Reaction (PCR) was performed in mixtures (25 µl) containing 50 ng of DNA, 1.5mM $MgCl_2$, the four deoxynucleoside triphosphates at 1.0 µM each, each primer (27F 5′ AGAGTTTGATCCTGGCTCAG 3′,1492R ′ TACGGTTACCTTGTTAC ACTT 3′) at 10 pM in Taq buffer, and 0.5 U of Taq polymerase. The PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) by following the programs as described. The amplification profile consisted of one cycle at 95 °C for 3 min, then 30 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min and finally, one cycle at 72 °C for 8 min. Amplicons were analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer. A 1 kb DNA ladder (Fermentas) was used to identify the molecular sizes of the bands. The amplicon was sent for sequencing and the results analysed.

K. Statistical Design for Media Optimization

A fractional-factorial design, Box-Behnken model was employed for the statistical optimization of production medium for protease and keratinase production using the selected isolate FM4. The experimental design consisted of seventeen runs and the independent variables were studied at two different levels, a high level and a low level. The high level is commonly coded as +1 and the low level as -1. It is necessary to include centre points as well (in which all factors are at their central values). The response variables were fitted by a second order model in order to correlate the response variables to the independent variables.

Based on the literature available, three factors which favour keratinase production were selected for the design. The three factors selected were glucose concentration, yeast extract concentration and inoculum volume. A statistical design (Table 1) was generated using 'Design Expert' software (version 6.0, Stat-Ease, Inc., Minneapolis, USA) with these factors, having both low and high levels. The low level for glucose and yeast extract was 1 % and the high level was 5 %. The low level for inoculum volume was 1 ml and a high level of 3 ml/100 ml of medium. The pH of all flasks was adjusted to 7.0 and the incubation time for production was kept as 72 h for keratinase and protease production. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability P(F), correlation coefficient R, determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's t value for the estimated coefficients and associated probabilities, P(t). Graphical analyses of the data were obtained.

Media was prepared in the above mentioned combinations along with other components of feather meal broth and was inoculated using 100 µl of inoculum and incubated on a rotary shaker (100 rpm) at 37 ⁰ C for 72 h. After incubation, the culture was centrifuged at 8000 rpm for 10 min and supernatant was collected and analysed for protease and keratinase activities.

Sl. No.	Glucose (%)	Yeast Extract (%)	Inoculum (%)
1	3	3	2
2	1	5	2
3	5	1	2
4	5	3	1
5	5	3	3

ISSN No:-2456-2165

6	3	3	2
7	5	5	2
8	3	5	3
9	1	3	3
10	3	1	3
11	1	1	2
12	3	3	2
13	3	3	2
14	3	1	1
15	3	5	1
16	1	3	1
17	3	3	2

Table 1. Experimental design for media optimization

L. Purification of Keratinase

The 72 h old FM4 culture was centrifuged at 8000 rpm for 10 min to remove cell debris and supernatant was collected. The supernatant so obtained was subjected to ammonium sulphate precipitation at different percentages 40, 60 and 80 %. The partially purified protein fractions were dialysed in separate dialysis bag of cut off 10 KDa with phosphate buffer (pH 7.0) and allowed to equilibrate at 4^{o}C overnight. The protein fractions were tested for the activity using Lowry method [9] and was analysed on SDS-PAGE.

III. RESULTS AND DISCUSSION

A. Isolation of Microorganisms

Nutrient agar plates (Fig. 2) plated with culture obtained from feather meal broth with soil sample incubated for 72 h showed several different colonies of microbes. Feather meal was used as a substrate for this isolation of microbes. All the obtained cultures were selected for preliminary screening for keratinolytic protease production.

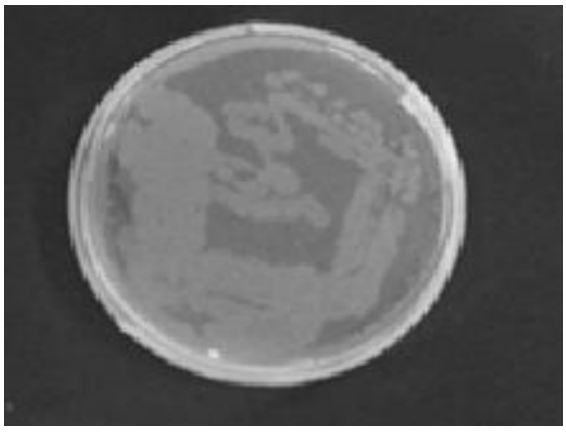

Fig 2:- Microorganism grown on nutrient agar plate.

B. Screening of Keratinolytic Protease Producing Microorganism

The selected isolates were grown on skim milk agar and feather meal agar plates to check the protease activity. The strains which were showing clear zone (Fig. 3) on skim milk agar plates were characterised by proteolytic activity as they could hydrolyse casein, the protein present in skim milk. It was mentioned by Mohanapriya et al [10] that when microbes with keratinase activity were grown on casein plates transparent zones were observed. The microorganisms

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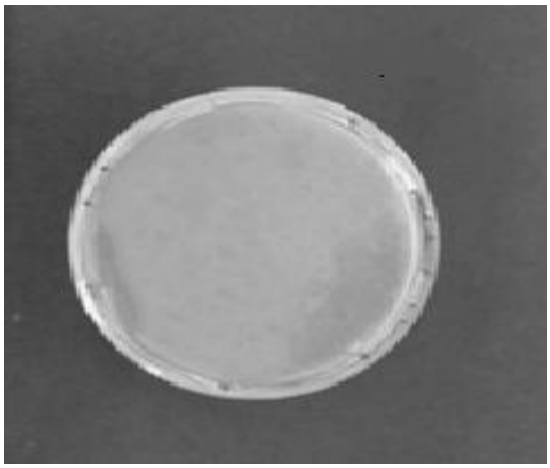

Fig 3:- Microorganism grown on skim milk agar plate

Which gave clear zone in skimmed milk agar were cultured in feather meal agar plates and the isolates which could hydrolyse keratin grow on the media giving a clear zone.

Out of the isolates, four of them were showing positive results in both skimmed milk agar and feather meal agar plates as they produced clear zone on the plates. This four isolates designated as FM1, FM2, FM3 and FM4 were selected for further characterization.

C. Protease Activity

The strains (FM1, FM2, FM3 and FM4) which showed positive results in the preliminary screening were subjected for analysing protease activity in feather meal broth. The studies showed that the isolates produced protease in a range of 200-2000 IU/ml (Fig. 4).

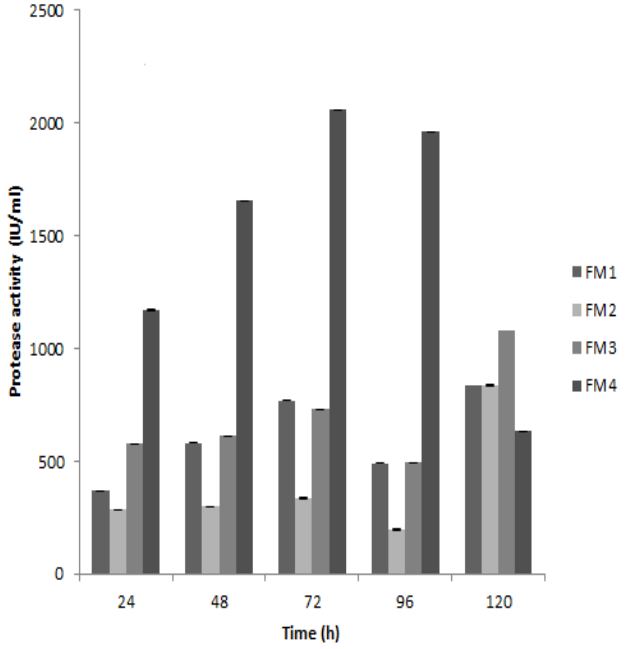

Fig 4:- Protease activity at different time intervals

Out of the four isolates, FM4 showed a maximum activity of 2060 IU/ml at 72 h. All the rest of the isolates were showing maximum activity at 120 h.

D. Determination of Keratinase Activity

The isolates FM1, FM2, FM3 and FM4 were analysed for keratinase activity and the studies showed the efficiency of all isolates in the production of enzyme. It was found that the isolates produced enzyme in a range of 10-150 IU/ml (Fig. 5). FM4 gave a maximum enzyme activity of 95 IU/ml. FM1 gave a maximum production at 48 h, but the enzyme activity was 77 IU/ml.

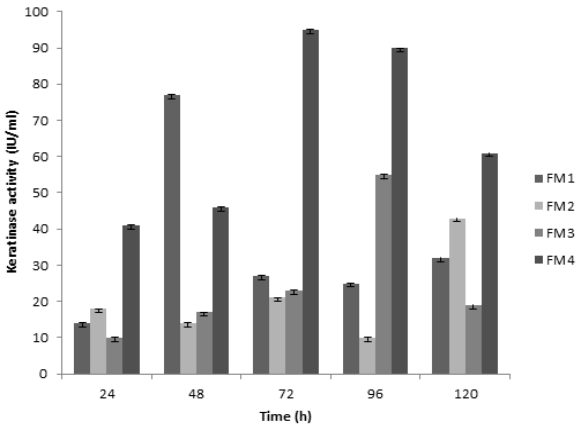

Fig 5:- Keratinase activity at different time intervals

FM3 was giving a maximum value of 55 IU/ml by 96 h but FM2 gave maximum production of 43 IU/ml only by 120 h. The degradation of feather started from 2nd day of incubation and complete degradation was observed on 8th day (Fig. 6). From the experiment, the strain FM4 was selected based on the enzyme activity produced by an incubation period of 72h which was having maximum activity in less time. FM4 was further identified and used for further experiments.

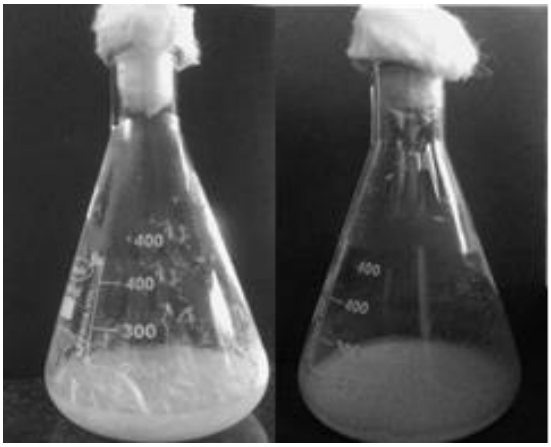

Fig 6:- Flask before and after incubation with keratin degrading organism

E. Microscopical Examination

The isolate FM4 was examined under microscope for its morphology and it was found to be Gram-positive bacteria upon Gram staining. FM4 was identified as Gram positive, rod shaped bacteria. FM4 was seen as Gram positive rods which were typical of *Bacillus* sp. nature.

ISSN No:-2456-2165

F. Biochemical Characterization

The positive isolate FM4 was further biochemically characterized (Table 2) and compared with Bergey's manual. FM4 was sporulating rods which produced gas bubbles in Durham's tubes and were catalase positive. According to Bergey's manual [11], the isolate FM4 was grouped under the genus *Bacillus*. Govinden and Puchooa [12] reported three isolates of *Bacillus* sp. with keratinase activity.

Characters	Response
Indole test	Negative
Methyl red test	Positive
VogesProskauer test	Positive
Simmon Citrate test	Positive
Catalase test	Positive
Oxidase test	Positive

Table 2. Result of biochemical characterization

G. Molecular Method of Identification

A band of size ~1508 bp specific for eubacterial 16SrRNA was obtained by PCR. The 16SrRNA sequence obtained was aligned and compared with other 16SrRNA gene sequences in the GenBank by using NCBI Basic Local alignment search tools BLAST n program. The BLAST results of the PCR amplicons showed 99 % identity with *Bacillus cereus.* The gene sequence was submitted in NCBI under the Gen Bank Accession Number: MF 668696. Several reports exist on *Bacillus* sp. with keratinolytic activity [13]and one of the prime producers among them is *Bacillus cereus* [14].

H. Statistical Design for Media Optimization

In order to search for the optimum combination of factors in the medium for a better protease and keratinase production, the Box-Behnken model of RSM was employed. A model was generated by the software Design Expert (version 6.0, Stat-Ease, Inc., Minneapolis, USA), which had total seventeen runs each in triplicates. The experimental data (Table 3) were statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA) (Table 4 & 5) and the 3D graphs were designed.

Sl. No	Glucose (%)	Yeast Extract (%)	Inoculum (ml)	Protease		Keratinase	
				Predicted Value (IU/ml)	Actual Value (IU/ml)	Predicted Value (IU/ml)	Actual Value (IU/ml)
1	3	3	2	250	250	165	165
2	1	5	2	37.5	160	-4.5	97
3	5	1	2	242.5	120	201.5	100
4	5	3	1	100	160	215	121
5	5	3	3	175	240	104	208
6	3	3	2	250	250	165	165
7	5	5	2	52.5	50	132	224
8	3	5	3	212	150	306	111
9	1	3	3	290	230	-87.25	7
10	3	1	3	502	560	69.25	67
11	1	1	2	507	510	138	46
12	3	3	2	250	250	165	165
13	3	3	2	250	250	165	165
14	3	1	1	477	540	615	811
15	3	5	1	107.5	50	165	168
16	1	3	1	235	170	206	103
17	3	3	2	250	250	165	165

Table 3. Statistical design for media optimization

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F	
Model	317488.2	9	35276.47	4.11	0.0379	Significant
A-Glucose	31250	1	31250	3.64	0.0981	
B-Yeast extract	217800	1	217800	25.37	0.0015	
C-Inoculum	8450	1	8450	0.98	0.3542	
AB	19600	1	19600	2.28	0.1746	
AC	100	1	100	0.01	0.9171	
BC	1600	1	1600	0.19	0.6790	
A^2	28657.89	1	28657.89	3.34	0.1104	
B^2	7605.263	1	7605.263	0.89	0.3780	
C^2	4447.368	1	4447.368	0.52	0.4950	
Residual	60100	7	8585.714			
Lack of Fit	60100	3	20033.33			

Table 4. ANOVA for Protease Enzyme Production

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F	
Model	472918.9	9	52546.54	10.19	0.0029	Significant
A-Glucose	25992	1	25992	5.04	0.0596	
B-Yeast extract	31250	1	31250	6.06	0.0434	
C-Inoculum	86944.5	1	86944.5	16.86	0.0045	
AB	812.25	1	812.25	0.16	0.7033	
AC	90.25	1	90.25	0.02	0.8985	
BC	187056.3	1	187056.3	36.27	0.0005	
A^2	88069.01	1	88069.01	17.08	0.0044	
B^2	28919.01	1	28919.01	5.61	0.0498	
C^2	31050.59	1	31050.59	6.02	0.0439	
Residual	36103	7	5157.571			
Lack of Fit	36103	3	12034.33			

Table 5. ANOVA for Keratinase Enzyme Production

The data explains both the models to be significant with a probability value <0.05 with a model F-value of 4.11 and 10.19 for protease and keratinase respectively. There is only a 3.79 % and 0.29 % chance that a "Model F-Value" this large could occur due to noise in both cases respectively. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 6.614 and 13.93 for protease and keratinase indicates an adequate signal. The goodness of fit of the model was determined by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.84 and R^2 = 0.92) indicates the significance of the respective models. The 3D surface plots showed the interaction of different factors on protease and keratinase production.

The quadratic effect of all the factors and the interaction between yeast extract and inoculum was found to be significant for keratinase production but the quadratic effect and interaction effect was not significant for protease production. Lakshmi et al [15] mentioned that yeast extract as nitrogen source was well supporting keratinase production. The study showed that a combination of glucose 3 %, yeast extract 1 % and inoculum of 3 ml at pH 7.0, gave a maximum production of protease of 560 IU/ml while maximum keratinase activity of 811 IU/ml was observed at a combination of glucose 3 %, yeast extract 1 % and inoculum 1 ml at the same pH. Even though keratin serves as the sole carbon and nitrogen source in the medium, additional carbon and nitrogen sources in the medium was seen to increase enzyme production [2, 16]. In some of the studies it was observed that glucose as carbon source stimulated keratinase production in *Streptomyces* sp., *Stenotrophomonas* sp., *Microbacterium* sp., and in some fungi [17]. Ningthoujam et al [18] reported that keratinase production was influenced by the use of yeast extract as nitrogen source in a statistical design. Gupta and Ramnani

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[19] have reported that an alkaline pH from 6 to 9 supports keratinase production in most microorganisms and Keratinases are also known to be generally active and stable over a wide range of pH from 5 to 13 [20]. The isolate, *Bacillus cereus* could attain a maximum production of keratinase of 811 IU/ml as the incubation temperature of 37 ⁰ C was also favourable as it is reported that an optimum temperature of most is in the range of 30 to 80°C [20].

The activity obtained for protease was less than that obtained in the initial study using feather meal broth while keratinase activity during statistical optimization was increased by eight fold.

Studies on keratinases using *Bacillus cereus* by several researchers showed wide range of enzyme activities. The maximum activity reported by Shankar et al [21] with *B. cereus* was 63.01 U/ml. Sivakumar et al [22] also reported a maximum keratinase production of about 60.67 U/ml by *B. cereus*. Another study by Lateef et al [23] showed a production 51.7 U/ml with *B. cereus* in feather containing medium. Mlaik et al [24] reported only a production of 283.8 IU/ml for protease using *B. cereus*. In the study conducted by Ahmadpour et al [25] with *B. cereus*, the maximum keratinase production attained was 350 U/ml. *B. cereus* strain genetically manipulated by Ahmadpour and Yakhchali [26] inorder to develop an asporogenic strain could produce only protease (450 U/ml) and keratinase (390 U/ml) against a production attained for protease (560 IU/ml) and keratinase (811 IU/ml) in the present study.

Purification Steps	Total activity (Keratinase)	Total activity (Protease)	Total protein (mg)	Specific activity (Keratinase)	Specific activity (Protease)	Yield Keratinase (%)	Purification Fold (Keratinase)	Yield Protease (%)
Crude Sample	2000	4000	100	20	40	100		100
After salt precipitation 40 % Precipitation	1000	1100	140	7.14	7.86	50	0.36	28
60 % Precipitation	1200	3780	40	30	94.5	60	1.5	95
80 % Precipitation	1600	3800	50	32	76	80	1.6	95

Table 6. Activity of enzyme before and after ammonium sulphate precipitation

I. Purification of Keratinase and Qualitative Analysis by SDS-PAGE

The specific activity of keratinase was found to be 7.14, 30 and 32 for 40%, 60% and 80% fractions respectively and the specific activity of protease was found to be 7.86, 94.5, 76 for 40%, 60% and 80% respectively (Table 6). The yield of fraction before precipitation was taken as 100 % and the yield values for keratinase and protease were calculated.

The yield for keratinase was found as 50%, 60% and 80% for 40%, 60% and 80 % fractions respectively and the yield percentage for protease was calculated as 28, 95 and 95 for 40%, 60% and 80% fractions respectively. As there is 80 % yield for keratinase and 95 % yield for protease with 80 % ammonium sulphate precipitation, this percentage of salt precipitation can be considered for purification of keratinolytic proteases. But the purification fold obtained was only 1.6 and 1.9 for keratinase and protease respectively at 80 % fractions.

The SDS-PAGE (Fig. 7) of ammonium sulphate fractions on analysis gave bands of high molecular weight around 100 KDa on comparison with a standard marker.

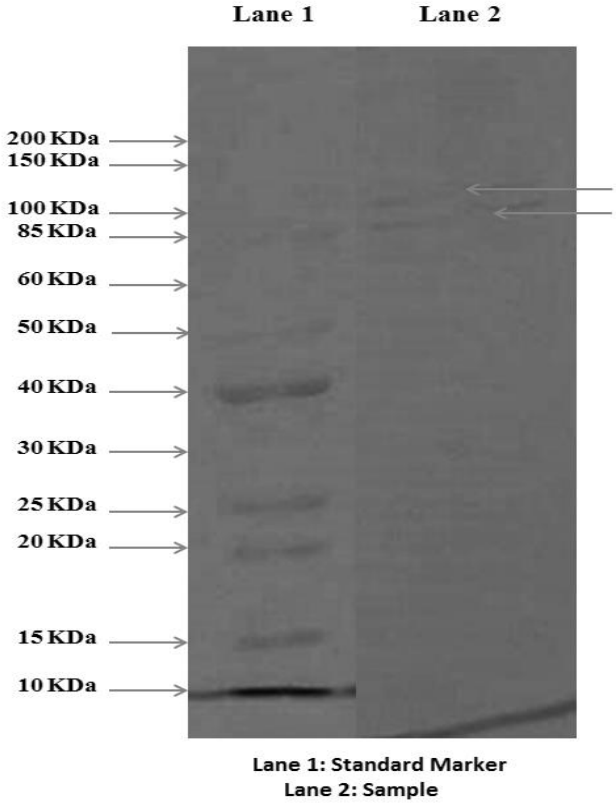

Fig 7:- SDS Page analysis of keratinolytic protease

Two bands of high molecular weight were found to be in the range 85-100 KDa.As single bands were not observed, further purification has to be done to check the homogeneity, eventhough Gupta and Ramnani [20] reported that molecular mass of keratinases range from 18 to 200 KDa. This has been supported by Friedrich &Antranikian [27] stating that keratinase with wide molecular mass range (18, 40 and 130 KDa) were studied from *Streptomyces albidoflavus*, *Streptomyces thermoviolaceus* and *Fervidobacterium pennavorans* respectively and at 69 KD akeratinase from *Chrysosporium keratinophilum*. Multiple bands were found after zymogram of keratinolytic protease produced by three strains of *Bacillus* sp. P6, P7, and P11 using feather degrading medium [28]. Balaji et al [29] has reported keratinases of molecular weight above 60 KDa from *Bacillus subtilis*. So the presence of multiple bands needs to be confirmed.

IV. CONCLUSION

Several different bacteria were isolated from soil. Out of this four bacteria were selected due to their efficiency for degrading keratin which was identified by clear zone on skim milk agar and feather meal agar plate after 24 – 72 h. The organisms were analysed for protease and keratinase enzyme activity. Based on the production efficiency one of the isolate FM4 was selected for production and purification studies. Statistical optimization of process parameters using Box-Behnken model gave maximum production of protease of 560 IU/ml and maximum keratinase activity of 811 IU/ml. An eight fold increase was observed in the activity of keratinase on comparison to the production in feather meal broth under unoptimized conditions. The best isolate was identified as *Bacillus cereus* based on Gram stain, biochemical characters and 16 S rRNA sequencing and sequence have been deposited in GenBank. The study also aimed at purification of keratinase enzyme by SDS- PAGE. SDS-PAGE revealed the presence of keratinase of molecular weight in the range 85 KDa to 100 KDa.

The isolation and characterization of promising strains for keratinase production becomes inevitable as they have large scale applications in various industries including detergent, leather, waste management etc. The factors in bioprocess media contribute largely to the production of enzyme. Hence optimization of these various parameters can increase enzyme production. The production pattern of enzymes should be in a way that it meets the demand of society. The development of a bioprocess method for production of adequate amount of keratinase can result in an eco-friendly approach of keratin waste degradation and emergence of value-added products from the process.

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