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Isolation, Identification and Characterization of Keratin degrading microorganisms from Poultry soil and their Feather degradation Potential

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***Abstract*—** *Keratinolytic microorganisms have a great importance in poultry waste degradation and its bioconversion to compost or animal feed. The aim of this study was to isolate keratin degrading bacteria and fungi from poultry farm soil, and to study their ability to degrade chicken feathers. The poultry farm soil samples were added in basal medium with feathers as a source of carbon and nitrogen. Five bacterial cultures were isolated. Bacteria were grown in basal media with feathers meal and showed feather degrading capacity. Bacterial strains were identified as Aeromicrobium spp., Exiguobacter spp., Marinococcus spp. and Bacillus spp. 1 & Bacillus spp 2.These bacteria showed keratinolytic enzyme activity in the cell free culture supernatants. The highest biodegradation of feathers was obtained using Aeromicrobium (KD1-72.55%), among the isolated cultures. Two fungal cultures (F1 and F2) were also isolated by Hair Bait Technique, out of which F1 showed good keratinolytic activity. The good ability of selected microorganisms to degrade feathers can be utilized for their potential biotechnological application in processing of feather waste from poultry industry.*

Keywords*— *Feather degrading Bacteria,Characterization, Identification, Keratinase, Poultry waste.

I. INTRODUCTION

The day by day increase in consumption of meat received from chicken is causing harsh effect to environment, as the waste from the chicken birds, more particularly the feather are not properly treated. While in nature, the deterioration of feather is slow, generating sulphureous compounds, causing environmental problem. Feathers, which are almost pure keratin proteins, are produced in large amounts and constitute a waste by product at poultry processing plant. A total 5-7 % weight of mature chicken comprises of feathers. Feather waste is generated in large quantities as a byproduct of commercial poultry processing. Feathers are made up primarily of keratin which is resistance to common proteolytic enzyme such as pepsin, trypsin and papain [1] .World-wide poultry processing plants produce millions of tons of feathers as a waste product annually, which consists of approximately 90% keratin; the keratin is largely responsible for their high degree of recalcitrance if remain untreated.

Keratin is a major component of hair, feathers and wool and is the most complex of the cytoskeletal intermediate filament proteins of epithelial cells [2]. Keratin is an insoluble protein macromolecule with very high stability and low degradation rate. Keratin is mainly present in hair, feather, nails, wool and horns. High protein content of keratin waste can be used as a good source of protein and amino acids by systematic recycling. The prospective use of keratinases is in diverse applications where keratins should be hydrolyzed, such as the leather and detergent industries, textiles, waste bioconversion, medicine etc. Recycling of feathers can provide a cheap and alternative protein feed stuff. Further this can be used for animal feed and for many other purposes. However, poor digestibility of keratin is a problem in recycling.

Keratinase is an extracellular enzyme used for the bio degradation of keratin. Keratinase is produced only in the presence of keratin substrate. Keratinase attacks the disulfide bond of keratin to degrade it. Some microbes have been reported to produce keratinase in the presence of keratin substrate. Keratinase producing microorganisms have the ability to degrade chicken feather, hair, nails, wool etc. Keratinolytic enzymes are widespread in nature and are produced by several microorganisms including bacteria such as *Bacillus sp.*[3-8], *Fervidobacterium islandicum* [9], *Elizabethkingia meningoseptica* KB042 [10], *Pseudomonas aeruginosaKS1*[11] and Actinomycetes such as *Streptomyces sp*. [12-14] and fungi such as *Chrysosporium tropicum* [15], *Trichoderma atroviridae* [16], *Doratomyces*

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microsporus [17]; *Paecilomyces marquandii* [18]; *Scopulariopsis brevicaulis* [19]; *Alternaria, Paecilomyces, Penicillium, Curvularia* and several *Aspergillus sp*. [20]. Diversity of keratinolytic fungi in soils have been studied and reported [21, 22]

Keratinophilic fungi are generally considered as soil saprophytes. Soil that is rich in keratinous material is most conducive for the growth and occurrence of keratinophilic fungi. Keratin decomposition in soil leads to an increase in carbon, and nitrogen ratio in soil. They are therefore fast growing nonpathogenic keratinophilic fungi which can be utilized for the recycling of keratin in soil and may be exploited for their biotechnological potential in industry.

Keratinase which are produced by these keratinolytic organisms could be used to degrade feather waste and further the digested products could be an excellent material for producing animal feed, fertilizers or natural gas [23]. Use of keratinolytic organisms for feather degradation is an economical, environmentally friendly alternative. Keratinolytic proteases offer considerable opportunity for a low cost technology for biotechnology of poultry feather from pollutant to nutritionally upgraded protein feed for a livestock [24]. Most feather waste is land filled or burnt which involves expense and can cause contamination of air, soil and water. Utilizing poultry feathers as a fermentation substrate in conjunction with keratin degrading microorganisms and enzymatic degradation may be better alternative to improve nutritional value of poultry feathers and reduce environmental waste [25].It would also solve the waste disposal problem of poultry waste and recycling of keratinacious waste would be beneficial financially and environmentally.

Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into soluble protein or polypeptide[26].These protein byproduct may be used as animal and livestock feed, and as leather filling agents[27]. Keratinase has also emerging application in de-hairing process in leather industry instead of sodium sulphides [28].

In view of above, the present study was aimed to isolate, identify and characterize the kearatin degrading microorganisms from poultry farm soil and study their feather degradation potential.

II. MATERIALS AND METHODS

2.1 Chemicals: All Chemicals required for experimental work were of analytical grade, pure and purchased from Hi-media laboratory.

2.2 Sample collection: Soil samples were collected from regular feather dumping site of poultry processing farms from outskirts of Pune, in sterilized sampling bottles. The samples were taken from 30 cm depth from the surface of the soil. The samples were brought to the laboratory and processed for isolation of microorganisms.

2.3 Processing of chicken feathers and Preparation of feather meal broth

Chicken feathers were washed thoroughly with tap water and dried. The dried feathers were defatted by soaking in diethyl ether for 24 hrs, and washed thoroughly with tap water and distilled water, air dried and cut into small pieces before autoclaving, the processed feathers referred as Feather meal. The medium used for keratinase production contained the following constituents (g/100ml.) - Feather meal 5gm, NaCl 0.005, K_2HPO_4 0.038, KH_2PO_4 0.04 $MgCl_2.6H_2O$ 0.02, Yeast extract 0.01, pH 7.5

2.4 Isolation and screening of keratinolytic bacteria

Soil suspensions were made with 0.9% saline and inoculated in feather meal broth and incubated on rotary shaker at room temperature. After visible turbidity was observed, serial dilutions of the culture suspensions were spread on skimmed milk agar plates for selection of protease producing bacteria, as per the method described earlier [29]. The petri plates were incubated at 30ºC for 24 hours. The isolated bacterial colonies showing zone of clearance on skimmed milk agar were selected for further studies. The isolates were characterized for colony characteristics, morphological characteristics and biochemical characteristics and identified with Bergey's manual of determinative bacteriology.

2.5 Isolation of keratinolytic fungi

The keratinophilic fungi were isolated using 'hair baiting techniques' [30]. In this technique sterile Petri plates were half filled with soil and short strand of sterilized chicken feathers were spread over the surface of soil. About 10-12 ml sterile water was added to Petri plates for the facilitation of fungal spores to germinate. Petri plates were incubated at 30˚C for 3-4 weeks. After 3-4 weeks the colonies were observed on surface of feathers, were picked up and grown on Potato Dextrose Agar, for purification and identification.

2.6 Production of keratinase Enzyme and feather degradation by bacterial and fungal isolates

Cultivation of the isolated cultures was performed using 250 ml Erlenmeyer flask containing 90 ml of Feather meal broth medium.10ml of overnight grown culture of each isolate were inoculated and incubated on rotary shaker at 150 rpm for 7 days. Control was feather meal broth without any inoculum. Growth was observed for visible turbidity

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and recorded a6 600 nm, degradation of feathers was visually observed.

2.6.1Extraction of Enzyme

The culture medium was filtered through WhatmannNo.1 Filter paper to remove un-degraded residues. The filtrate was then subjected to centrifugation at 10,000 rpm for 10 min to remove bacterial residue. After centrifugation keratinase activity was determined in supernatant.

2.6.2 Determination of feather degradation

The feather degradation was studied according to the method described by Kumar et. al[31]. The five bacterial isolates namely KD1, KD2, KD3, KD4 and KD5 were inoculated in feather meal broth with 1% feathers as the sole source of carbon and incubated on rotary shaker for one week. After one week the residual feathers remained was determined gravimetrically by filtering the culture broth and taking the weight of filter paper before and after filtration. Percent reduction of feathers was calculated from the difference in the initial weight and weight obtained after one week of incubation.

2.6.3 Assay for keratinase activity:

Keratinase activity was assayed according to the method of [32].Each culture filtrate was Centrifuged at 5000 rpm for 30 min.20 mg feather meal +3.8 Tris HCl buffer+0.2 ml supernatant of culture filtrate were taken ; control was kept where 0.2ml cultures supernatant was replaced with distilled water. the tubes were incubated at 30^0C for 1 hour then chilled in ice water for 10 minute, filtered and OD was taken at 280 nm.O.D. values were converted into enzyme unit/ ml Enzyme Units per ml was calculated by using following formula:

Enzyme Units per ml= Optical Density x 4 x dilution rate /0.01 x T

Where T = incubation time, 4= total volume used.

2.6.4 Effect of incubation time on growth of isolates

The effect of incubation time on growth of isolates was determined in feather meal broth for 7 days. Culture samples were added namely KD1, KD2, KD3, KD4, KD5, F1 and F2 and with one control flask which was without culture. Feather meal broth 100 ml with 1 gm feathers was added with 2 ml overnight culture in each flask ,incubated on rotary shaker 150 rpm. OD was taken at 600 nm, every 24 hours, up to 7 days for monitoring growth.

2.6.5 Effect of different temperatures on keratinase enzyme activity

The optimum temperature for keratinolytic protease activity was determined by performing the enzyme reaction at incubation temperatures between 30ºC to 90º C.40 mg feathers+7.5 ml Tris HCl buffer+0.4 ml supernatant of culture filtrate of each isolate were taken in sterile test tubes, and tubes were incubated at different temperatures (30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC, and 90ºC) for 1 hour. After incubation at different temperatures all tubes were chilled in ice water for 10 minute. Filtered & OD was taken at 280 nm.O.D. values were converted to enzyme unit/ ml.

2.6.6 Effect of different pH on keratinase enzyme activity

Keratinolytic protease activity was studied in the pH range of 4 to 9 using 0.2 M Tris- HCl buffer. 40 mg feathers + 7.5 ml of bufferhaving different pH from 4-9, to which 0.4 ml supernatant of culture filtrate of each isolate was added. All tubes were incubated at 30^0C for 1 hour, and then chilled in ice water for 10 minute to stop the enzyme reaction, then filtered & OD was taken at 280 nm. O.D. values were converted to enzyme unit/ ml.

III. RESULTS AND DISCUSSION

Soil samples were inoculated in feather meal broth to obtain bacterial isolates which are feathers degrading and were capable of producing extra cellular keratinase, using feather (keratin) as sole carbon source. After a week of incubation the flasks showed turbidity and disintegration of feathers were observed, (fig 1).

Feather meal Broth

Growth observed

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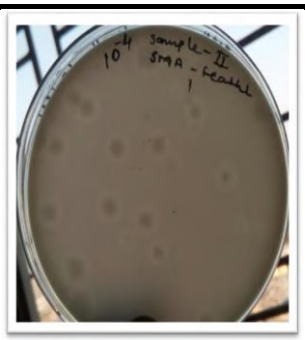

Bacterial Isolates with zone of Clearance on Skim Milk Agar

Fig 1: Growth on Feather meal broth and isolates on Skim milk agar

The same culture suspension was serially diluted up to 10^{-8} with normal saline and plated on skim milk agar for, selection of keratinolytic bacteria. The colonies showing zone of clearance were further selected and characterized. Fig 2 shows the fungal isolates by bait technique.

Feathers with poultry soil

Fungal Growth observed on feathers after 5 weeks

Fig 2: Bait Technique for isolation of keratinolytic Fungi

Colonies showing zone of clearance on skim milk agar were counted. Table 1 shows the bacterial counts of soil samples 1 and 2 on Nutrient agar plates and Skim milk agar plates. The colonies showing zone of clearance were selected and observed for colony characteristics, morphological characteristics and biochemical characteristics. Based on colony characteristics five types of bacterial isolates were obtained, these isolates were designated as KD1, KD2, KD3, KD4 and KD5.

Table.1: Bacterial Count on Nutrient Agar (NA) and Skim Milk Agar (SMA)

		NA Plates CFU/ml			**SMA plates CFU/ml**	
Dilutions	**10^{-4}**	**10^{-6}**	**10^{-8}**	**10^{-4}**	**10^{-6}**	**10^{-8}**
Bacterial Count, Soil sample1	**21 x 10^5**	**12x 10^6**	**4 x 10^8**	**4 x 10^4**	**1 x 10^6**	**1 x 10^8**
Bacterial Count, Soil sample 2	**14 x 10^5**	**8 x 10^6**	**2 x 10^8**	**3 x 10^5**	**2 x 10^6**	**1 x 10^8**

Table 2 shows the colony characteristics of the bacterial isolates. The morphological characteristics of the isolates are shown in Table 3.The results of biochemical characterization of the bacterial isolates are shown in Table 4.On the basis of colony, morphological and biochemical characteristics and reference to Bergey's manual of determinative bacteriology the five cultures KD1, KD2, KD3 KD4 and KD5 were identified up to genus level and were identified as *Aeromicrobium Sp*., *Exiguobacter*Sp., *MarinococcusSp*,*Bacillus Sp1 and Bacillus Sp2*.Most of the studies on isolation of keratinolytic organisms have resulted in isolation of Bacillus spp, however in the present study the isolated bacteria *Aeromicrobium*, *Exiguobacter* and *Marinococcus* have been reported for the first time.

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Table.2: Colony and Morphological Characteristics of the Bacterial Isolates

Colony characteristics	Shape	Size	Colour	Margin	Opacity	Consistency	Elevation
Isolates KD1	Irregular	Pin point	Cream	Irregular	Opaque	Sticky	Flat
KD2	Irregular	Pin point	Orange	Irregular	Opaque	Sticky	Flat
KD3	Circular	Pin point	Orange	Regular	Opaque	Sticky	Convex
KD4	Circular	2-4 mm	Cream	Regular	Opaque	Sticky	Flat
KD5	Circular	3-5 mm	White	Regular	Opaque	Sticky	Flat

Table.3: Morphological characteristics of the isolates

Morphological Characteristics of the isolates	KD1	KD2	KD3	KD4	KD5
Gram Reaction	**Gram + ve short rods**	**Gram + ve long rods**	**Gram +ve Coccobacilli**	**Gram +ve rods**	**Gram +ve rods**
Motility	**Non-motile**	**Motile**	**Actively motile**	**Actively motile**	**Motile**
Endospore Staining	**Non spore forming**	**Non spore forming**	**Non spore forming**	**Spore forming**	**Spore forming**

Table.4: Biochemical Characterization of the Bacterial Isolates

Sugar	KD1	KD2	KD3	KD4	KD5
Glucose	+	+	+	+	+
Cellulose	-	-	-	-	-
Fructose	-	+	+	+	+
Raffinose	-	-	-	-	-
Galactose	-	+	-	-	-
Maltose	+	+	+	+	+
Sucrose	-	+	-	+	+
Mannitol	-	-	-	-	-
Arabinose	-	-	-	-	-
Lactose	-	-	-	-	-
Xylose	-	-	-	-	-
Oxidase	+	-	-	+	+
Catalase	+	+	+	+	+
Citrate utilization	-	-	-	-	-
Gelatinase	-	+	-	-	-
Nitrate reduction	-	+	-	-	-
Caesin hydrolysis	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Identified organism	***Aeromicrobium* spp**	***Exiguobacter* spp**	***Marinococcus* spp.**	***Bacillus* spp 1**	***Bacillus* spp.2**

Out of five bacterial isolates, two isolates have been identified as *Bacillus spp*. as also reported by others[24,35]. In our study all the bacterial isolates have been found to be gram positive, whereas others have reported keratinolytic activity by gram negative bacteria [33]. Two fungal cultures, F1 and F2 were also isolated by bait technique and were found to grow on feather meal broth. However they could not be identified. Many researchers have worked on isolation and characterization of bacteria from poultry processing wastes and shown to degrade feathers, hair or

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wool, but the optimization for industrial production of keratinases still remains to be done.

Table 5 shows the results of feather degradation experiment.

The result shows that KD1 was the most effective and showed 72.55% feather degradation , it is followed by KD2- 70.59%, KD5-60%, KD4- 58.82% and KD3- 55%.

Table.5: Feather degradation by the isolated bacterial cultures

Culture sample inoculated in Feather Meal Broth (FMB)	Initial weight of filter paper with feathers(gm)	Final weight of filter paper with feathers(gm)	% reduction of feathers
KD1 (*Aeromicrobium* spp.)	1.02	0.28	72.55%
KD2 (*Exiguobacter* spp.)	1.02	0.3	70.59%
KD3 (*Marinococcus* spp.)	1.02	0.45	55.88%
KD4(*Bacillus spp. 1*)	1.02	0.42	58.82%
KD5(*Bacillus spp. 2*)	1.02	0.40	60.78%

Effect of incubation time on growth of microbial isolates is shown in figure 3. KD4 and KD5 isolates entered stationary phase after 48 hrs, KD3 and KD1 on 3rd day, while KD 2 on 4th day. After day 5 all isolated entered the decline phase. The highest keratinase activity was shown by KD1(18 U/ml), followed by KD 2, KD3, KD5, and KD4, as shown in fig 4.

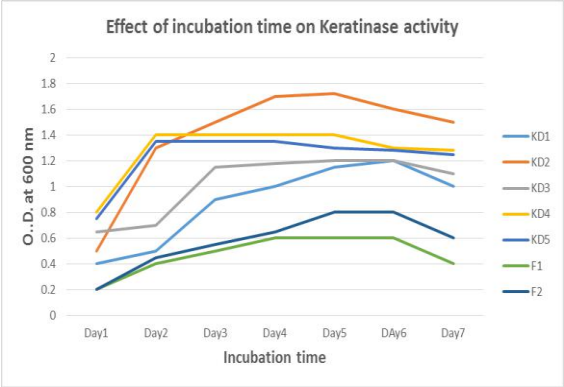

Fig.3: Effect of Incubation Time on Growth of Isolates

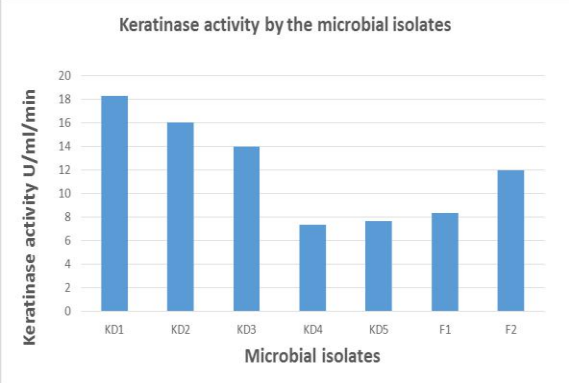

Fig.4: Keratinase Activity with The Isolated Cultures

Whereas other workers have reported maximum keratinase activity of 13.6U/ml and 8.8 U/ml[34]. F2 fungal isolate showed Keratinase activity of 12U/ml.

Fig.5 shows the result of the effect of different pĤ on keratinase activity, it was observed that the optimum pĤ for the bacterial isolates KD1, KD3, KD4, KD5 and fungal isolates F1& F2 was 9, whereas for KD2 it was 7.Most of the isolates in this study have shown higher activity at alkaline pH as also reported by Inamdar et.al [36]. Keratinase from the most of the bacteria, actinomycets and fungi have pH optima in neutral to alkaline range. Enzyme with optimum activity at alkaline pH has definite advantages in application, both in degradation of feathers as well as in leather industry.

The effect of temperature on keratinase activity is shown in figure 6. It was observed that the optimum temperature was 30°C for all the bacterial isolates, while others have reported optimum temperature of 40±2oC [14]. For F1 and F2 optimum temperature was found to be 50°C.

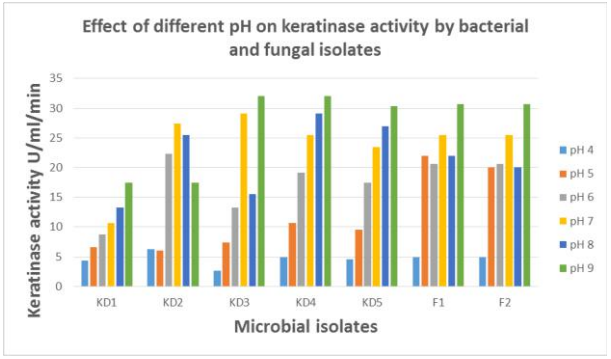

Fig.5: Effect of pH on Keratinase Activity

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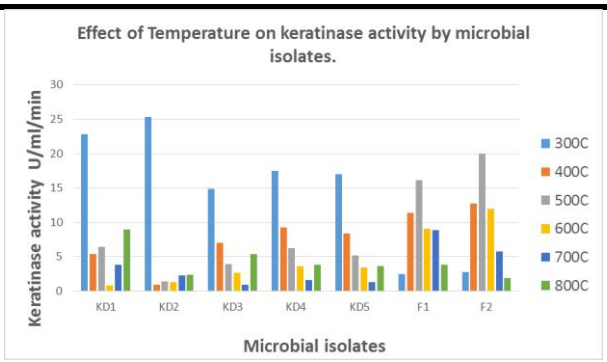

Fig.6: Effect of Temperature on Keratinase Activity of The Isolates

Feather degradation to the range of 70-72% by newly isolated *Aeromicrobium* and *Exiguobacter spp.* even under unoptimised condition can be further exploited for efficient degradation of feathers under optimised conditions.

In soil feathers are degraded by a consortium of bacteria and fungi, which act in synergy or compete for keratin.

Biodegradation by microorganisms possessing keratinolytic activity represents an alternative attractive method for improving the nutritional value for keratin wastes, as it offers cheap and mild reaction conditions for the production of valuable products there have been some reports on microorganism capable of degrading keratinous wastes.Further optimization of keratinase production and characterization of the keratinase would be helpful in application of keratinases on a large scale for degradation of keratin containing wastes.

IV. CONCLUSION

In the present study five bacterial cultures were isolated producing keratinase from habitats where keratin containing substrate were disposed in natural conditions. The five bacterial isolates were characterized and identified based on colony morphology, growth characteristics and biochemical characteristics. They were identified as belonging to genera *Aeromicrobium spp., Exiguobacter spp., Marinococcus spp., Bacillus spp1, Bacillus spp,* respectively. The isolate *Aeromicrobium spp*. (KD1) shows the highest feather degradation of 72.5%.The optimum temperature was 30°C for all the bacterial isolates, whereas for F1 and F2 it was 50°C.The optimum pH for bacterial and fungal isolates found to be 9except for KD2 it was 7.Feather degradation to the extent of 70-72% by newly isolated *Aeromicrobium* and *Exiguobacter spp.* even under unoptimised condition can be further exploited for efficient degradation of feathers under optimized conditions.

The ability of newly isolated bacteria to degrade feathers can be utilized for their potential biotechnological application in processing of feather waste from poultry industry. For the evaluation of biotechnological application of keratinase, however require more detailed understanding of the factors that enable this enzyme for complete degradation of native keratinase substrate. Therefore additional research will need to done for purification, characterization of keratinase, studying kinetics of enzymes, testing from various range of substrate, effect of inhibitors, and inducer on enzyme activity, submerged state fermentation for large scale production of keratinase. Further studies can be focused on whether consortium of bacteria and fungi can be utilized for feather degradation, rather than individual cultures for enhanced keratinolytic activity.

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