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Isolation and Screening of Keratinolytic Actinobacteria form Keratin Waste Dumped Soil in Tiruchirappalli and Nammakkal, Tamil Nadu, India

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Abstract: The aim of this study was to isolate keratinolytic Actinobacteria from feather dumping soil. Feather dumping soil was collected from several areas in Tiruchirappalli, Nammakkal, Tamil Nadu, and India. Twenty two isolates were selected after growth on Bennett's agar and they named as SD1 to SD22. All the twenty two isolates were subjected for primary screening on milk agar plates and among twenty two isolates ten were showing proteolytic activity in terms of making clear zone surrounding their colony on the Milk agar medium. The ten positive isolates were again subjected for the secondary screening on Feather Broth and three isolates, SD5, SD6 and SD7 were showing degradation of feather during their growth. Though the degradation process was taking long time, all these three isolates achieved complete degradation of feather between 20 to 25 days and they used feather as the sole organic source for carbon, sulfur and energy. These novel keratinolytic Actinobacterial isolates have potential biotechnological use in processes involving keratin hydrolysis.

Key words: Bennett's Agar, keratin hydrolysis, keratinolytic Actinobacteria, milk agar, modified basal liquid medium

INTRODUCTION

Feather waste, generated in large quantities as a by-product of commercial poultry processing, is nearly pure keratin protein (Moran *et al.*, 1966). Keratin in its native state is not degradable by common proteolytic enzymes such as trypsin, pepsin and papain. However keratin does not accumulate in nature and keratinolytic activity has been reported for species of *Aspergillus*, *Ctenomyces* (Gupta *et al.*, 1950), *Bacillus* sp. (Molyneaux, 1959) and *Streptomyces* (Noval and Nickerson, 1959). Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal feed stuffs. A current value-added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments. These methods can destroy certain amino acids and decrease protein quality and digestibility (Moritz and Latshaw, 2001; Wang and Parsons, 1997). The nutritional inferiority and insolubility of native feather protein derive from the composition and molecular configuration of constituent amino acids that ensure the structural rigidity of feathers (Parry and North, 1998). Resistance to proteolytic enzymes has been attributed to the complex structure of β-keratin filaments. In addition, disulfide cross-links produce a compact three-dimensional network (Bradbury, 1973), as a result of intermolecular disulfide bonds between rod domains and terminal domains of the constituent molecules (Parry and North, 1998). The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described. Feather meal fermented with *Streptomyces fradiae* and supplemented with methionine resulted in a growth rate of broilers comparable with those fed isolated soybean protein (Elmayergi and Smith, 1971).

The crude keratinase enzyme increased the digestibility of commercial feather meal and could replace as much as 7% of the dietary protein for growing chicks (Odetallah *et al.,* 2003). Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of feather keratin. They may have important applications in processing keratin-containing wastes from poultry and leather industries through the development of non-polluting methods (Onifade *et al.,* 1998). Generally, an increase in keratinolytic activity is associated with thermophilic organisms, which require high energy, inputs to achieve maximum growth and the decomposition of keratin wastes (Friedrich and Antranikian, 1996; Nam *et al.,* 2002). The Actinobacterial isolates can degrade raw feathers and therefore useful to develop efficient processes involving keratin substrates.

In this study, we described the collection of feather dumping soil from several areas, isolation of Actinobacteria from feather dumping soil and selection of keratinolytic Actinobacterial isolates by performing primary and secondary screening.

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Table 1: Collection of soil samples from different areas

		Collected sample		
S. No.	Location	Nature of the collection area	Soil nature	Soil Colour
1	Mathur, Tiruchirappalli	Feather dumping soil	Smooth, Dry	Brown
2	Mathur, Tiruchirappalli	Hair dumping soil	Smooth, Slightly wet	Dark brown
3	Vayaloor, Tiruchirappalli	Feather dumping soil	Smooth , Dry	Black
4	Balasubramani Poultry Farm, Thopur, Mohanur, Nammakkal	Dried faecal material under the chicken cage	Hard, Dry light	Brown
5	Siva Shakti Poultry Farm Thopur, Mohanur, Nammakkal	Soil + Faecal material under the chicken cage	Smooth, Wet dark	Brown
6	Chinaswamy Poultry Farm, Mohanur Road Nammakkal - 2	Soil sample under the elevated farm (30-40 ft from the ground level)	Smooth, Dry light	Brown
7	Vardhraj Farm Mohanur Road Nammakkal - 2	Soil surrounding the chicken cage very	Smooth in Nature, Dry	Brown

MATERIALS AND METHODS

Soil sample collection: Soil samples were collected from two different feather waste dumping areas respectively Mathur and Vayaloor, one hair dumping area Mathur in Tiruchirappalli and four poultry farms in Nammakkal, Tamil Nadu, India (Table 1 and Fig. 1). In case of feather waste dumping areas, soil samples were taken 30 cm depths from the surface of the soil and in poultry farms samples were taken from the surface soil. Samples were carrying to the laboratory in sterile plastic bags and followed by immediate processing. All the poultry farm soils, which were not collected from the feather dumping area, but collected from the farm surrounding area, were mixed with cleaned, white chicken feather in order to increase the keratinolytic microbes load and kept as such for one month period.

Medium: Bennett's agar was used for the isolation of Actinobacteria from the soil samples contained the following (in grams per liter): glucose, 10.0; casein, 2.0; beef extract, 1.0; yeast extract, 1.0; agar, 15. pH was adjusted to 7.3 and the media was supplemented with streptomycin and cyclohexamide at the concentration of 50 μg/ml. All the ingredients were obtained from Hi-media.

Preparation of soil suspensions: Soil suspensions were prepared by the following methods: Serial dilution of soil sample: 1 g soil sample from each different collection area, was vigorously shaking in 10 ml of sterile distilled water for 30 min on a shaker. Serial 1 in 10 dilutions were then made down to 10^{-6}. Centrifugation of soil sample: 1 g of each soil sample was mixed with 10 ml of sterile distilled water and centrifuge at 1600 rpm for 20 min (Rehacek, 1959).

Isolation of actinobacteria: Bennett's agar medium was prepared and sterilized at 121ºC temperature, 15-psi pressure for 15 min in autoclave. Medium was poured on sterile Petridishes once it's reached the tolerable temperature (45ºC) and allowed to solidify. Spread plate technique was followed to isolate the Actinobacteria. Each plate was received 0.2 ml of 10^{-4}, 10^{-5} or 10^{-6} dilutions of the inoculums. The plates were incubated at room temperature and examined the plates weekly for three weeks.

Maintenance of suspected actinobacterial isolates: Suspected Actinobacterial isolates were maintained in ISP1 (International Streptomyces Project) medium contained the following (g/l): tryptone, 5.0; yeast extract, 3.0; agar, 16.0 and the pH was adjusted at 7.3. ISP1 medium was sterilized and poured into sterile Petridishes. Inoculation of suspected Actinobacterial isolates was done on solid medium surface and incubated the plates at room temperature for 7-10 days.

Primary screening of keratinolytic actinobacteria: Milk agar medium was used for the primary screening of keratinolytic actinobacteria (Riffel and Brandelli, 2006), contained the following (in grams per liter): Peptone, 5.0; yeast extract, 3.0; dextrose, 1.0; skim milk Powder, 10.0, agar 15.0 and pH was maintained at 7.2. All the ingredients of Milk agar medium were sterilized in autoclave except skim milk powder. Skim Milk Powder was added separately once the medium reached the tolerable temperature (45ºC) and poured the medium in sterile Petridishes. Suspected Actinobacterial isolates, which already maintained in ISP1 medium, were inoculated in milk agar plates. The plates were incubated at room temperature and examined the plates for clear zone formation on the milk agar plate after 4 days.

Secondary screening of keratinolytic actinobacteria: All positive isolates obtained from the primary screening, were subjected to perform the secondary screening in order to isolate the feather degrading actinobacteria. Modified basal liquid medium supplemented with raw chicken feather was used for the secondary screening. $MgSO_4$, $7H_2O$ 0.2 g/l; K_2HPO_4 0.3 g/l; KH_2PO_4 0.4 g/l; $CaCl_2$ 0.22 g/l and Yeast extract 0.1 g/l were used to prepare the modified basal liquid medium (Mona, 2008). Whole fresh raw feather was collected from chicken processing shop. Feathers were washed properly

Feather collection area in Mathur, Trichy

Feather collection area in Vayaloor, Trichy

Hair collection area in Mathur, Trichy

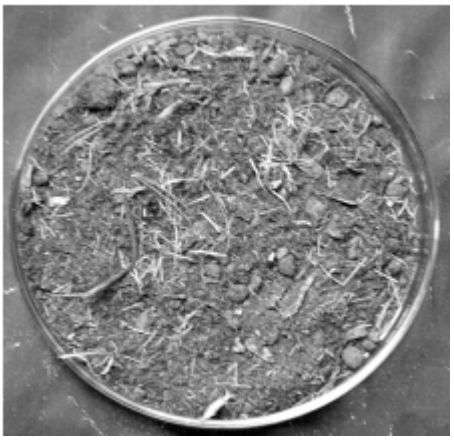
Fig. 1: Soil samples collection areas

with tap water to remove the blood and other dust particles from it and followed by washing with distilled water. Washed, cleaned, white chicken feather was dried in room temperature. Twenty five milliliter of Modified Basal Liquid medium was taken in each boiling test tubes and added one cleaned, dried medium size chicken feather to the each boiling tubes. Sterilized the medium and inoculated the isolates once the medium got cool. Selected isolates were chosen based on their zone forming capability on the milk agar medium. Incubated the boiling tubes at room temperature and examined the tubes weekly for four weeks.

Table 2: Isolation of actinobacterial isolates from different areas

Sample	Culturing type	Processing of isolates			
		Colony obtained	Colony identity	Medium	Nature
Feather dumping soil, Mathur, Trichy	Centrifuged sample	Four	SD1	Bennett's Agar	Initial Pale white, after maturation slight brownish and slimy appearance
			SD2	Bennett's Agar	Slimy white colony
			SD3	Bennett's Agar	Pale white, Slimy appearance
			SD4	Bennett's Agar	Yellowish White.
Feather dumping soil Mathur, Trichy	Serial dilution 10^{-5}	Five	SD5	Bennett's Agar	Pinkish, Clear Zone Observer around the colony in Bennett medium
			SD6	Bennett's Agar	Pale White, Clear Zone Observer around the colony in Bennett medium
			SD7	Bennett's Agar	Pale White, Clear Zone Observer around the colony in Bennett medium
			SD8	Bennett's Agar	Whitish
			SD9	Bennett's Agar	Whitish
Vardhraj Poultry Farm, Nammakkal	Serial dilution 10^{-6}	Three	SD10	Bennett's Agar	Pale White
			SD11	Bennett's Agar	White
			SD12	Bennett's Agar	Cream White
Chinaswamy poultry Farm, Nammakkal	Serial dilution 10^{-5}	Two	SD13	Bennett's Agar	Powdery White
			SD14	Bennett's Agar	Pale White
Hair dumping soil, Mathur, Trichy	Serial dilution 10^{-6}	Four	SD15	Bennett's Agar	Brownish
			SD16	Bennett's Agar	Yellowish Brown
			SD17	Bennett's Agar	Dark Yellowish
			SD18	Bennett's Agar	Brownish
Feather dumping Vayaloor, Trichy	Serial dilution 10^{-5}	Four	SD19	Bennett's Agar	Cream White
			SD20	Bennett's Agar	Cream White
			SD21	Bennett's Agar	Pale White
			SD22	Bennett's Agar	Pale White

Feather on the soil surface



Feather degraded on soil by Microbes

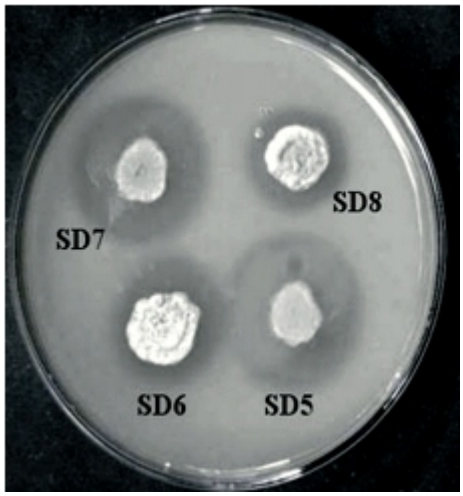
Fig. 2: Enrichment of Soil by adding Chicken feather

Microscopic examination of actinobacteria: All the positive keratinolytic Actinobacterial isolates were streaked on ISP1 medium plate and inserted one sterile cover slip at 45º angles on the medium. The plates were incubated at room temperature for 8-12 days. Cover slip was taken out carefully from the medium once the matured mycelial growth of Actinobacterial isolates were observed on ISP1 medium. Placed the cover slip on clean glass slide and observed under the Phase contrast microscope at 20X resolution.

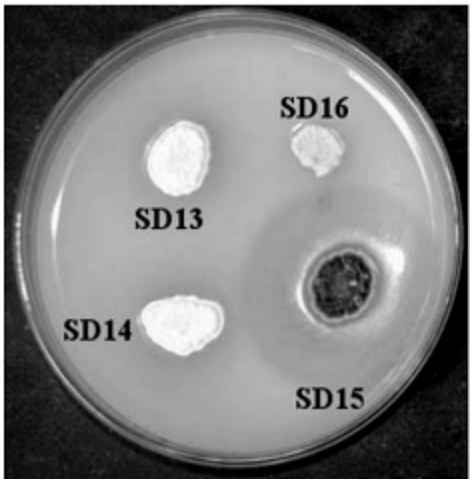
RESULTS AND DISCUSSION

Soil samples collected from the Nammakkal poultry farms, which were mixed with feather, after one-month period it was observed that feathers were completely, decomposed in the soil samples (Fig. 2). The addition of Streptomycin and Fluconazole to Bennett's agar inhibited the growth of certain bacteria and fungal contaminants. From the Bennett's agar plate total 22 dried, powdery, whitish, light brownish, slight pinkish suspected

SD1, SD2, SD3 with slimy appearance SD4 with clear zone



SD5, SD6, SD7 and SD8 with clear zone



SD13, SD14, SD15 with clear zone and SD16

Fig. 3: Isolates in skim milk agar plate

Actinobacterial isolates were selected and marked them as SD1 to SD22. SD1 to SD4 were obtained from centrifuged sample of Mathur feather dumping soil, where as SD5 to SD9 were obtained serially diluted soil sample from the same dumping soil. SD10 to SD 14 were obtained from Nammakkal poultry farm soil and SD15 to SD18 from Mathur hair dumping soil. Remaining SD19 to SD22 was obtained from Vayaloor feather dumping soil (Table 2). SD10 to SD22 were obtained from serially diluted respective soil sample. All the 22 isolates were subjected for primary screening on Milk Agar plate and among the 22 isolates SD4, 5, 6, 7, 8, 13, 14 and 15 were formed the clear zone, which supported the degradation and utilization of casein (Skim Milk Powder) by the respective isolates (Fig. 3). SD1, SD2 and SD3 were showing a distinct character as they produced slime (Fig 3). Secondary screening were done to find out the feather degradation Actinobacteria among the positive isolates and SD5, 6 and 7 were able to degrade the feather among the 8 isolates selected through primary screening. All these three, SD5, SD6 and SD7 isolates were found to degrade the whole chicken feather in Modified Basal Liquid Medium after 15-25 days of incubation period (Fig. 4). Isolate, SD15 was observed with different character in the same Modified Basal Liquid medium that it grown on the feather surface but unable to degrade the feather (Fig. 4). In case of remaining isolates, slight growths were observed in terms of turbidity in the liquid medium. SD5, SD6 and SD7, all these three isolates were grown on ISP1 medium where they have shown nice dried, brownish white growth along with visible substrate mycelium (Fig. 5). These three isolates growth also been observed under phase contrast microscope by cover slip technique. All the cases substrate and aerial mycelium were observed clearly and SD7 was observed along with spiral spores (Fig. 6).

Actinobacteria were isolated from feather dumping soil, hair dumping soil and poultry farm soil that owned keratinolytic activity and ability to degrade keratin wastes. Preliminary screening test indicated that isolate SD4, 5, 6, 7, 8, 13, 14 and 15 were capable to degrade and utilize the casein, which confirmed their proteolytic nature. Isolates grown on medium containing whole raw feather, could utilize feather as a unique carbon and nitrogen source and secondary screening indicated SD5, SD6 and SD7 were the best three isolate among the other isolates capable to degrade feather. Feathers are keratinous in nature and consist of high disulfide bonds, its make very hard to degrade the feather. Though SD5, SD6 and SD7 these three isolates took long time (15-25 days) for feather degradation, but they showed the significant property to degrade the feather that is difficult to achieve.

Considering that feather protein has been showed to be an excellent source of metabolizes protein

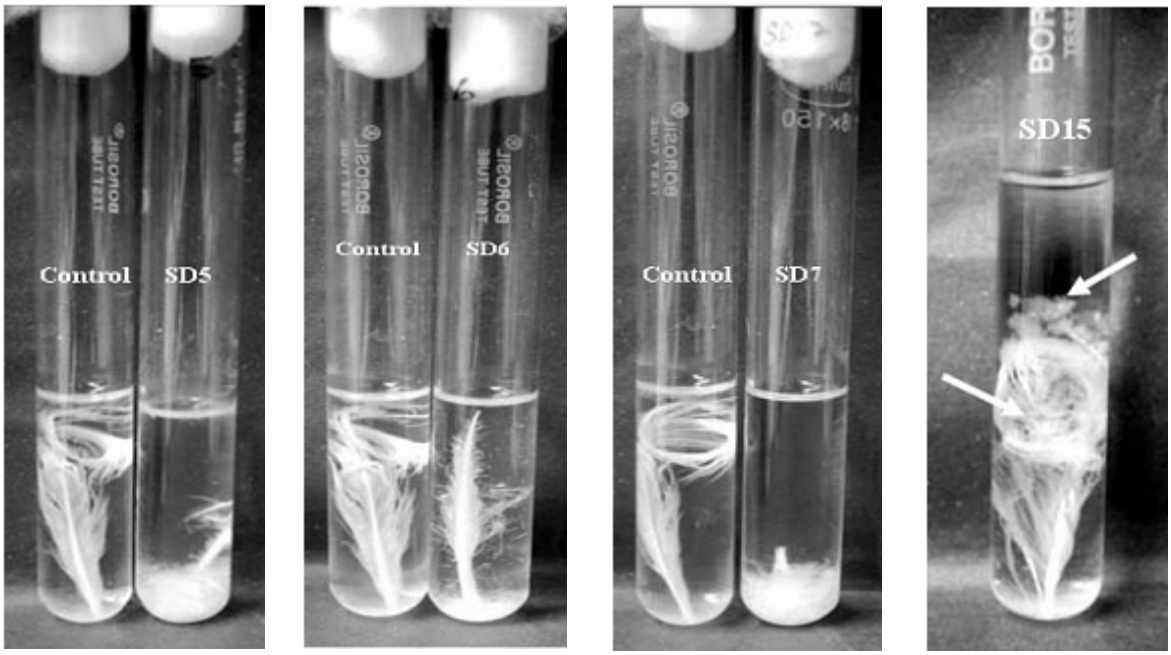
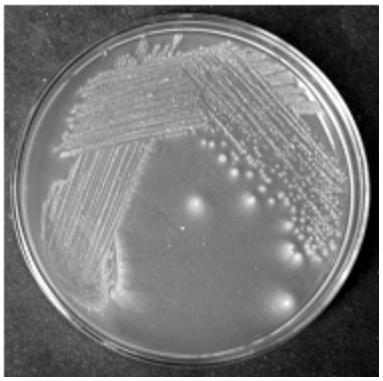
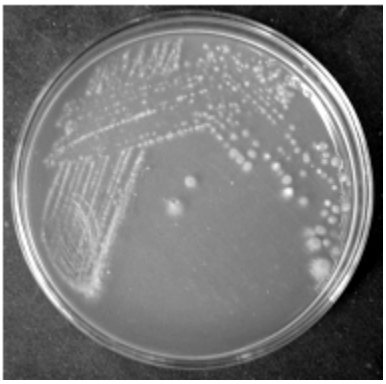


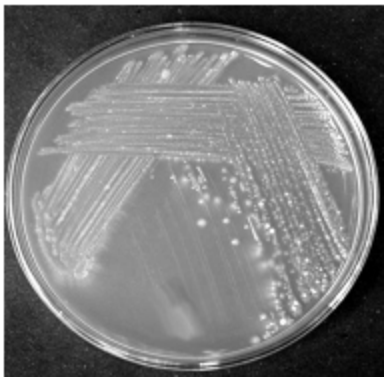
Fig. 4: Degradation of feather by SD5, SD6, SD7 and SD15 with growth on feather



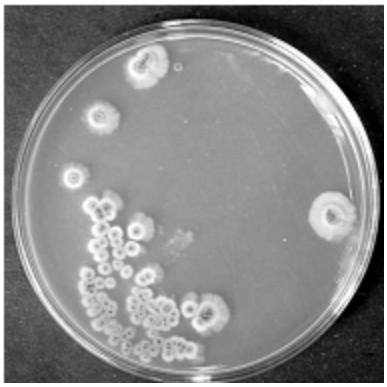
Isolate SD5 on ISP1, white powdery appearance with visible substrate mycelium



Isolate SD6 on ISP1, pale whitish appearance with visible substrate mycelium

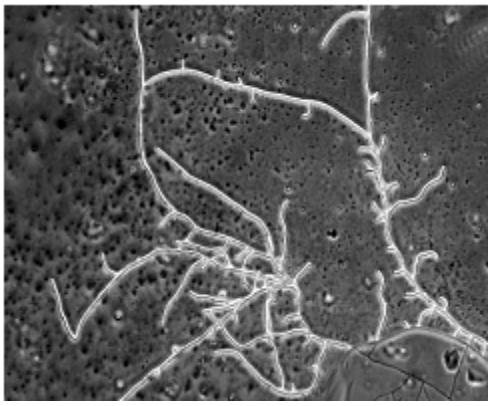


Isolate SD7 on ISP1, pale whitish appearance with visible substrate mycelium

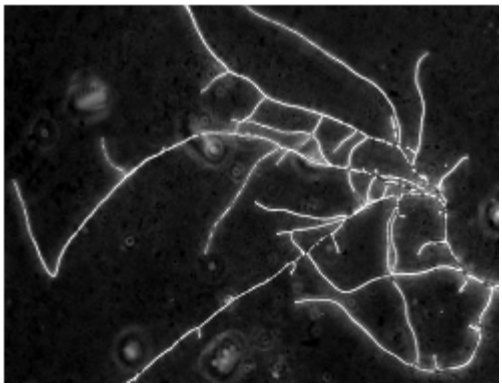


Isolate SD15 on ISP1, brownish centre, Whitish surrounding with visible substrate mycelium

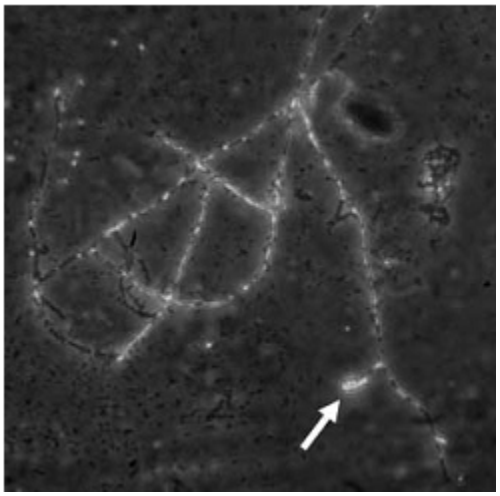
Fig. 5: SD5, SD6, SD7 and SD15 on ISP1 Medium



Isolate SD5



Isolate SD6



Isolate SD7 with spiral spore

Fig. 6: Phase Contrast Microscopic view of SD5, SD6 and SD7

(Klemersrud *et al*., 1998) and that microbial keratinases enhance the digestibility of feather keratin (Lee *et al*., 1991; Odetallah *et al*., 2003) these keratinolytic Actinobacterial isolates could be used to produce animal feed protein. In addition, the selected isolates were able to grow and display keratinolytic activity in diverse keratin waste (raw feather). This would be beneficial for the utilization of these residues. These isolates present potential biotechnological use in processes involving keratin hydrolysis.

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