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CHARACTERIZATION OF SNAKE SCAT FLORA FOR PRODUCTION OF PROTEASE, KERATINASE AND ESTERASE ENZYMES

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Abstract

Snakes are reptiles found in diverse geographical conditions and are known to ingest their prey lacking the step of chewing. The indiginous microbiota of snake must be elevating its digestive efficiency through secretion of various enzymes which may prove significant for industrial applications as well. In present study, scat samples of 12 snakes were collected from Rajiv Gandhi Zoological Park, Katraj, Pune for isolation of snake scat flora. Samples were spread plated on nutrient agar, trypticase soya agar, yeast peptone dextrose agar, brain heart infusion agar, salmonella shigella agar and ravan agar. 371 morphologically distinct isolates were obtained and screened qualitatively for protease, keratinase and esterase using skim milk agar, feather meal agar and tributyrin agar respectively. Among the isolates, 46% were positive for protease, 22% were positive for keratinase and maximum isolates i.e. 85%, were positive for esterase. 20% of total isolates showed production of all three enzymes. The first five isolates showing largest zone of clearance in qualitative assays were characterized quantitatively for protease and keratinase. Results obtained indicate that snake scat flora is a large untapped reservoir of industrially important microbial enzymes and can be a potential resource for degradation of animal tissue waste generated from slaughter house and poultry industries.

Keywords *– Snakes, snake scat flora, keratinase, esterase and microbial enzymes.*

Introduction

Snakes are reptiles belonging to suborder serpentes of kingdom animalia (Smith, Smith & Sawin 1977). There are around 3000 species of snakes found worldwide of which about 270 species are found in India which includes both venomous and non venomous snakes (Khaire 2014, Indian snake database). These incredible reptiles can thrive on variety of prey such as termites, rodents, birds, frogs, small deer and other reptiles (Rodríguez-Robles, Bell & Greene 1999; Gregory 1984). Snakes ingest their prey as a whole and undergo an extensive digestive exercise to assimilate it. Thus they do not get advantages of chewing the prey, as in case of other carnivores, where digestive enzymes in the form of saliva are mixed with food properly and also fragmentation of food by chewing aids in fast digestion (Arroyo, Bolaños & Muñoz 1980). Previous reports indicate that the normal gut flora of the host like humans and other mammals helps host in digestion through secretion of degradative enzymes (Tremaroli & Bäckhed 2012; Ley *et al* 2008) and this must be occurring in case of snakes as well but has never been investigated.

Flora of snake oral cavity (Fonseca *et al* 2009, Blaylock 2001), gut (Costello *et al* 2010) and venom (Goldstein *et al* 1979) have been reported earlier by different authors. Bacteria belonging to genera *Escherichia, Bacillus, Aeromonas, Edwardsiella, Salmonella, Shigella, Staphylococcus* and *Clostridium* are known to normally inhabit snake oral cavity and intestinal tracts (Ferreira *et al* 2009; Blaylock 2001; Arroyo, Bolaños & Muñoz 1980, Iveson 1971), but lack of data beyond identification and diversity of cultures prompted us to undertake present study. Although the normal flora of snakes has always been looked at through the lenses of clinical importance, the functional significance remains to be probed. Production of various degradative enzymes such as proteases, keratinases and esterases by these microorganisms underlines their ability to degrade animal tissue effectively, which will aid in digestion of prey. It will also prove to be of significant utility in industries like leather, tanning, biofertilizer and waste management of slaughter house and poultry industries (Sahni, Sahota & Gupta Phutela 2015; Sawant and Saraswathy 2014; Kulkarni *et al* 2013).

In present work, we have focused on study of protease, keratinase and esterase production by the isolates from scat samples of different venomous and non venomous snakes. Proteases are a class of degradative enzymes which catalyze cleavage of peptide bonds in proteins and represent one of the largest groups of industrial enzymes. Bacteria

belonging to *Bacillus, Alteromonas, Microbacterium, Pseudomonas* are known to produce protease and find wide applications in leather, food and pharmaceutical industries (Sawant and Saraswathy 2014; Aurachalam & Saritha 2009, Gupta R, Beg QK and Chauhan B 2002). Keratinases are specific proteases capable of keratin degradation, produced by organisms of genera *Streptomyces, Bacillus, Microbacterium* and other (Sharma, Verma & Gupta 2013; Daroit, Correa & Brandelli 2009; Mabrouk 2008). Poultry farm, leather, biofertilizer, biohydrogen production are the industries requiring extensive use of keratinases (Sahni, Sahota & Gupta Phutela 2015; Brandelli 2008, R. Gupta and P. Ramnani, 2006; Onifade, A. A. 1998). Esterases are hydrolases that catalyze the formation or cleavage of ester bonds of variety of substrates and are known to be produced by *Bacillus, Pseudomonas, Burkholderia* and other genera (Kumar *et al* 2015). They are required in paper industries for deinking, antibiotic production, synthesis of optically pure compounds and a various catabolic processes. (Kulkarni et al 2013, Bornscheuer 2002). Thus, isolation of bacteria from snake scat having ability to produce these enzymes will prove important and open new resource for bioprospecting.

Research methods

Collection of samples

Fresh scat samples of 12 snakes were collected in sterile containers from Rajiv Gandhi Zoological Park, Katraj, Pune. These include five venomous snakes, *Naja naja* (Indian cobra), *Bungarus caeruleus* (common krait), *Echis carinatus* (saw scaled viper), *Trimeresurus gramineus* (bamboo pit viper), *Boiga trigonata* (common cat snake) and seven non venomous snakes, *Python molurus* (Indian rock python), , *Coelognathus helena* (common trinket), *Argyrogena fasciolata* (banded Racer), *Eryx johnii* (earth boa), *Lycodon aulicus* (common wolf snake), *Boiga forsteni* (forsten's cat snake) and *Eryx conicus* (common sand boa). Samples were transported to laboratory within one hour for further processing.

Isolation of bacteria from scat samples

Scat sample of each snake was serially diluted using sterile biological saline. Appropriate dilutions were spread on sterile nutrient agar (NA), brain heart infusion (BHI) agar, tryptone soya agar (TSA), yeast peptone dextrose (YPD) agar, ravan agar and salmonella shigella (SS) agar. Plates were observed after incubation at 37 ^{0}C for 24 hours and

colonies showing distinct morphologies were purified onto respective media. Pure cultures were preserved at 4 ^{0}C until further use.

Qualitative screening of protease, keratinase and esterase production

Dehydrated skim milk agar (HiMedia) (Dalal 2015), feather meal agar (Muthusamy, Selvankumar & Arunprakash 2011) and tributyrin agar (Kumar *et al* 2015) were used for screening of isolates for protease, keratinase and esterase activity respectively. Freshly grown culture of each isolate was spot inoculated on these media and occurrence of zone of clearance around the colony after incubation indicated positive result. Protease positive isolates were taken for screening of keratinase production. For this purpose, chicken feathers were used for preparation of feather meal (Mazotto *et. al.* 2011, Agrahari & Wadhwa 2010). Tributyrin was purchased from SRL and was used to prepare tributyrin agar as per manufacturer's instructions. Diameter of zone of clearance was measured for isolates producing these enzymes.

Quantitative estimation of protease and keratinase production

Isolates for quantitative estimation of enzymes were selected based on qualitative assays. First five isolates showing largest zone of clearance on skim milk agar and feather meal agar were selected for estimation of protease and keratinase enzyme activity (Beynon & Bond 2001). For protease, isolates were inoculated in protease specific broth. After incubation, broth was centrifuged at 10,000 rpm at 4 ^{0}C and clear supernatant was recovered. The protease activity in crude culture supernatant was determined by the method of Alnahdi (2012), by using casein as substrate. For keratinase, isolates were inoculated in keratinase specific broth; after incubation, broth was centrifuged at 10,000 rpm at 4 ^{0}C and clear supernatant was recovered. The keratinase activity in crude culture supernatant was determined by the method of Mazotto *et. al.* (2011) by using feather meal as substrate.

Results

Isolation of bacteria from scat samples

Total 371 morphologically distinct isolates were obtained from various snake scat samples. Highest bacterial morphological diversity of was observed in sand boa (Figure 1).

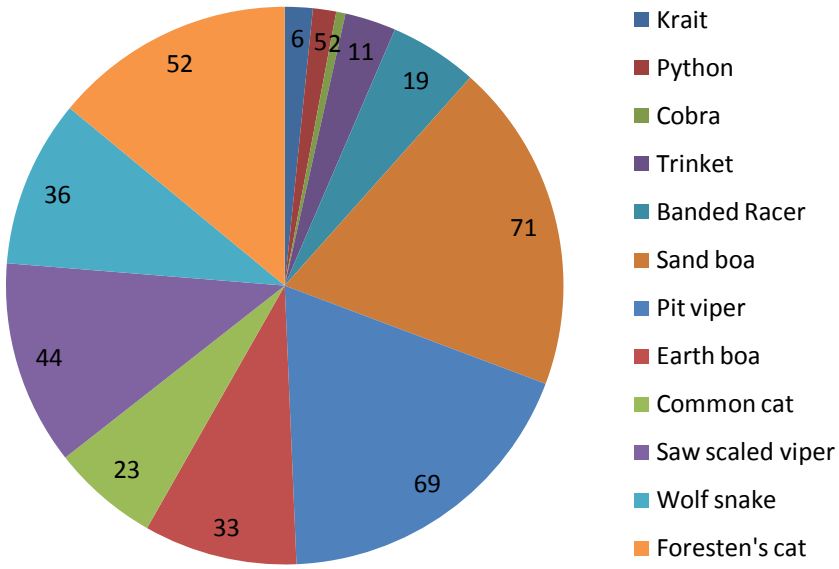


FIGURE 1: DISTRIBUTION OF MORPHOLOGICALLY DISTINCT BACTERIAL ISOLATES OBTAINED FROM SCATS OF VARIOUS SNAKES (NUMBERS IN SECTIONS OF PIE DIAGRAM INDICATE NUMBER OF MORPHOLOGICALLY DISTINCT ISOLATES FROM SNAKE SCATS).

Qualitative screening of enzyme production

Zone of clearance on skim milk agar, feather meal agar and tributyrin agar were observed for protease, keratinase and esterase respectively (Figure 2). Total 172 isolates (46%) showed production of protease while 81 isolates (22%) were positive for keratinase production. Most of the isolates i.e. 317 (85%) showed esterase production (Table 1). Figure 3 represents numbers of enzyme producing isolates obtained from different snakes.

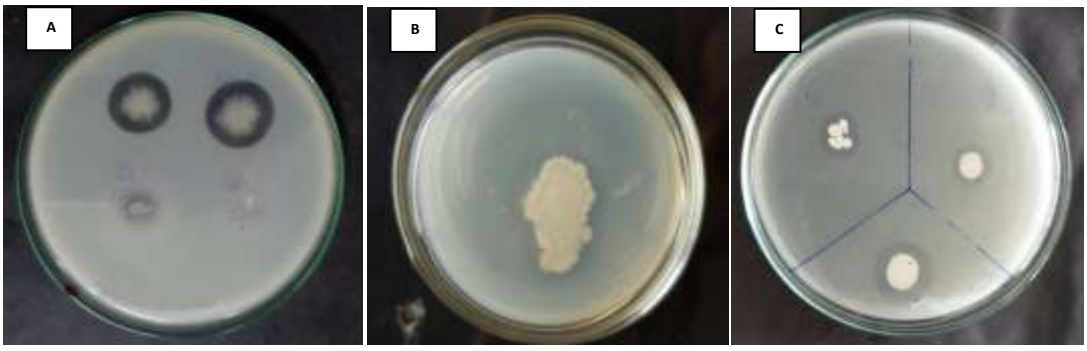
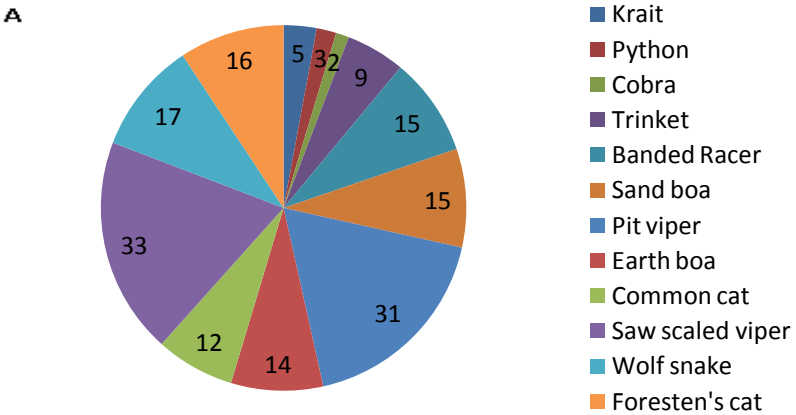
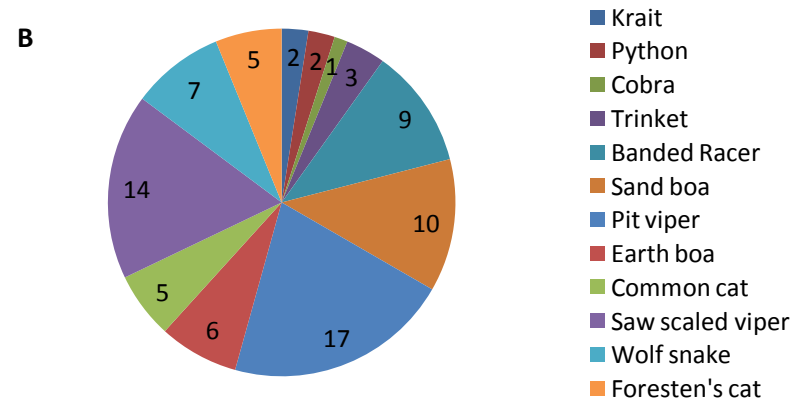


Figure 2: Zone Of Clearance On (A) Skim Milk Agar Plate For Protease (B) Feather Meal Agar Plate For Keratinase And (C) Tributyrin Agar Plate For Esterase Were Observed.

Table 1: Results Of Isolation And Qualitative Screening For Enzymes Production (Numbers In Bracket Indicate Percentage Of Isolates Positive For Given Enzyme).

Snake	Total isolates	Number of Protease positive isolates (overall percentage)	Number of Keratinase positive isolates (overall percentage)	Number of Esterase positive isolates (overall percentage)
Krait	6	5 (83%)	2 (33%)	6 (100%)
Python	5	3 (60%)	2 (40%)	5 (100%)
Cobra	2	2 (100%)	1 (50%)	2 (100%)
Trinket	11	9 (82%)	3 (27%)	10 (91%)
Banded Racer	19	15 (79%)	9 (47%)	17 (89%)
Sand boa	71	15 (21%)	10 (14%)	70 (99%)
Pit viper	69	31 (45%)	17 (25%)	60 (87%)
Earth boa	33	14 (42%)	6 (18%)	28 (85%)
Common cat	23	12 (52%)	5 (22%)	14 (61%)
Saw scaled viper	44	33 (75%)	14 (32%)	39 (89%)
Wolf snake	36	17 (47%)	7 (19%)	28 (78%)
Forsten's cat	52	16 (31%)	5 (10%)	38 (73%)
Total	**371**	**172 (46%)**	**81 (22%)**	**317 (85%)**





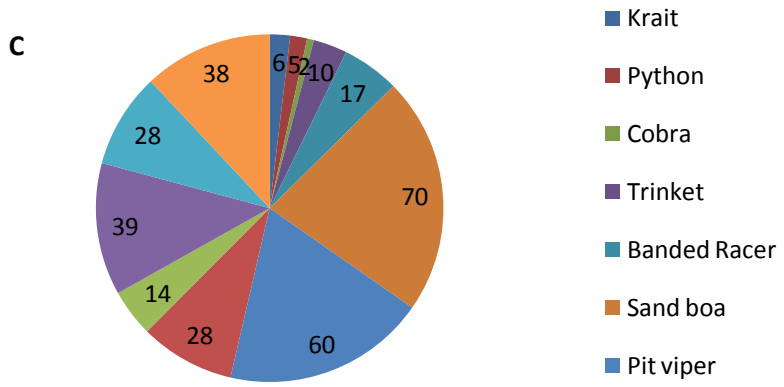


Figure 3: Distribution Of Isolates Positive For (A) Protease (B) Keratinase And (C) Esterase Obtained From Scats Of Different Snakes (Number On The Sections Of Pie Diagram Indicate Number Of Isolates From Snake Scats)

Quantitative estimation of enzyme production

Quantitative assays were performed for protease and keratinase for the first five isolates which showed largest zone of clearance on plates of qualitative assays. The enzyme activity of crude culture supernatant was expressed as unit activity per ml of crude supernatant per minute (U/ml/min). Isolate FY123, isolated from Forsten's cat snake, produced highest amount of protease using skim milk as substrate and isolate CR124, isolated from common cat snake, produced highest amount of keratinase using feather meal as substrate (Figure 4).

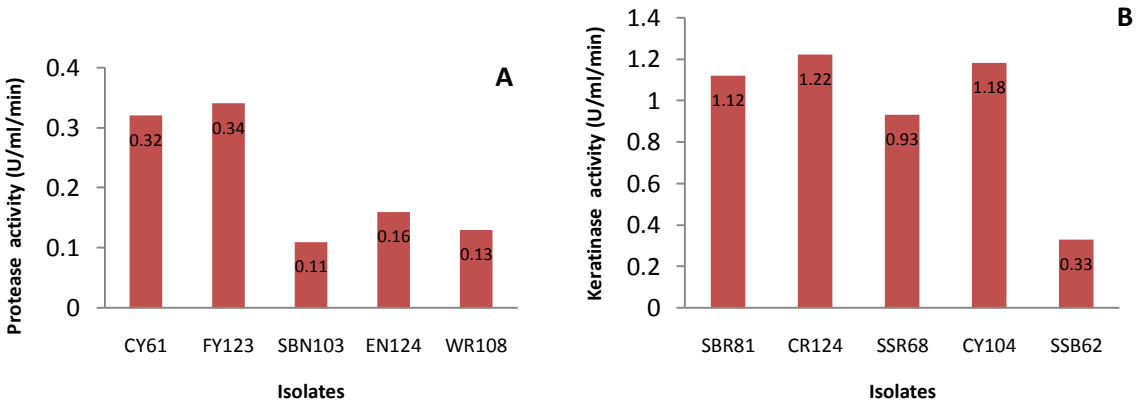


Figure 4: Quantitative Estimation Of Enzyme Activity From Crude Culture Supernatant For (A) Protease (B) Keratinase.

Discussion

Bacteria capable of producing different enzymes have been isolated from variety of habitats; however use of snake scat samples for this purpose was unexplored. Previous reports on gut flora of snakes were limited to isolation and identification of cultures (Costello *et al* 2010, Fonseca *et al* 2009, Blaylock 2001, Goldstein *et al* 1979). Our results indicate that snake scat flora is a rich reservoir of bacteria producing industrially important enzymes. Most of the isolates were capable of producing esterase (85%) indicates a strong relation between bacterial composition of snake gut and diet of snakes. Though esterase producers were found to be more abundant than protease producers (46%), the activity of protease was more prominent than esterase which was evident from early and higher production of protease on qualitative assay plates as compared to esterase. We report a novel and potential resource of keratinase producers with 22% abundance. Very few morphologically distinct isolates from scats of krait, cobra and python could be recovered on culture media indicating need for modification of routine media and simulation of natural habitat. 20% of the total bacterial isolates showed production of all three enzymes, which may prove to be extremely important in slaughter and poultry industry waste management and this facet needs to be explored in detail. Scat flora of both venomous and non venomous snakes was studied and presence of enzyme producers was found in all of them. Further, it would be interesting to explore these isolates for production of other enzymes and also to identify these isolates and find the difference, if any, in the bacterial diversity of venomous and non venomous snakes.

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