Enzymatic Activities of Aerobic Bacteria Isolated from Gastrointestinal Tract of Toad, *Bufo bufo* (L)

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Abstract

Bacterial ecology of gastrointestinal tract of toad *Bufobufo* has been studied to investigate the enzymatic activity of intestinal bacteria in physiology of digestion. Aerobic bacteria were isolated from the GI tract of *Bufobufo*. The total colony count of aerobic microflora was found to be 0.95 x 10^5 CFU/ml. Two morphologically different colonies of Gram positive bacilli were observed. Enzyme assays of the two bacterial strains were carried out to test cellulase, amylase, caseinase and gelatinase activities. Both isolates demonstrated amylase, caseinase and gelatinase activities; however, cellulase activity was absent. This indicates that the aerobic bacterial isolates do not play role in cellulose digestion in case of *Bufobufo*. The presence of caseinase and gelatinase activities indicates that these bacteria may have significant role in protein digestion. Amylase activity in the isolates indicates that starch can be a significant part of toad's food.

KEY WORDS-Gastrointestinal tract, Cellulase, Caseinase, Gelatinase, Amylase

Introduction

Bacteria harbor in almost all the body parts of an organism. Many are harmful causing infection and many are beneficial and play several significant roles such as helping in digestion and enhancing immunity. Those bacteria harboring in the gut include many beneficial species. The bacteria residing in the gut play important roles.

Bacterial isolates from gut have been reported to degrade p-nitrophenyl-β-N-acetylglucosaminide, chitin and collagen in Dover sole, *Soleasolea* (MacDonaldet al., 1986).

Amylolytic, cellulolytic, lipolytic and proteolyticmicroflora were identified from gastrointestinal tract of freshwater teleosts, namely Catla, Rohu, Mrigal, Silver carp, Grass carp, Common carp, Tilapia, Walking catfish and Murrel (Bairagi et al., 2002). It is likely that such organisms may contribute to nutritional processes. The intestinal bacteria prevent implantation of pathogens by producing antibacterial substances (Gorbach, 1996). Gut bacterial flora are a significant source of a range of vitamins (Hill, 1997). Gut microbiota also produces short chain fatty acids that help as important molecular signals (Morrison and Preston, 2016). Intestinal bacteria stimulate cell repair of intestinal mucosa (Hullar, 2014) and affects absorption of key minerals (Kau, 2011).They play a fundamental role on the induction, training and function of the host immune system (Belkaid and Hand, 2014). Probiotic bacteria have been reported to reduce cholesterol (Kumar et al., 2012).

Bacteria play major role in the physiological processes like digestion. Enzymes producing intestinal bacteria

are important in the metabolism of several vitamins, carbohydrates, proteins and other substrates. Beneficial bacteria have found role in food supplements in the form of Probiotics. These bacteria are thought to be growth promoters that could improve the health of the animal.

The importance of intestinal bacteria in the nutrition and well–being of their hosts has been established for homeothermic species, such as birds and mammals (Floch et al., 1970). Endogenous digestive enzymes in fish have been studied by several workers (Dhage, 1968; Kawai and Ikeda, 1972; Das and Tripathi, 1991). However, limited information is available about the gut microflora of frogs. Intestinal flora of leopard frog, *Ranapipiens* is similar to that of mammals and birds and that this flora can be maintained at temperatures close to freezing. Bacteria repeatedly isolated at high dilutions are strict anaerobes (Gossling et al., 1982). Similar observations have been reported in *Hyla japonica*(Benno et al., 1992). However, bacteria from the intestine of toad *Bufobufo* have not been investigated in details.

*Bufobufo*or common toad is an inconspicuous animal. It is cosmopolitan but is found mainly in forests, woodlands and marshes. It can reach about 15 cm in length. Females are normally stouter than males. It remains active at dusk and hunts during night. It feeds mainly on the invertebrates such as insects, larvae, spiders, slugs and worms. It is voracious and it catches prey with the help of sticky, prehensile tongue.

Due to lack of reports on the intestinal bacteria from toad *Bufobufo*, it is of interest to study the bacterial isolates from the intestine of *Bufo spp*. Aerobic bacteria from the intestine of *Bufobufo* and their enzymatic activities have been studied in the present work.

MATERIALS AND METHODS

Isolation of bacteria from Intestine:

Toad, *Bufobufo* was collected from village Chandanapuri, of tehsil Sangamner, Maharashtra, India. *Bufospp.*was identified using identification key (Frog and Toad identification, n.d.). The animal was kept under examination for 48 hours and was fed on bread sticks. Before the experiment, the toad was anaesthetized using diethyl ether. Weight and snout-vent length of the toad was determined. The skin was wiped with 70% alcohol using cotton ball to disinfect the skin. The intestine was removed aseptically and was washed with sterile saline twice. The intestine was homogenized in sterilized mortar-pestle using sterilized chilled saline. 1ml of homogenized extract was serially diluted up to dilution 10^4.

0.1 ml of each of the dilutions was spread plated on Luria Agar plates (1gm Tryptone, 0.5 gm Yeast extract, 1 gm Sodium Chloride, 2gm Agar powder, 100ml distilled water) in triplicate. Agar plates (PD85, Laxbro) were incubated for 48 hours at 37^oC. Bacterial colonies were observed, counted and used for enzyme assays.

Gram Staining:

Morphology of the isolated bacteria was observed using the Standard Gram staining method. The slides were observed at 100x magnification under oil immersion.

Qualitative enzymatic assay:

Congo red assay for cellulase activity:

For Cellulase production the isolates were spot inoculated on CMC Agar (0.1 gm Ammonium dihydrogen

Phosphate, 0.02 gm Potassium Chloride, 0.1 gm Magnesium sulphate, 0.1 gm yeast extract, 2 gm Carboxymethyl Cellulose, 2gm Agar powder, 100 ml distilled water) and incubated at 37^{o}C for 48 hrs. The culture plates were flooded with Congo red for 15 minutes. The plates were destained by 2% NaCl solution. The appearance of clear zone indicated the presence of cellulase activity.

Iodine assay for Amylase activity:

The isolates were spot inoculated on Starch agar plates (1gm Tryptone, 0.5 gm Yeast extract, 1 gm Sodium Chloride, 2 gm Starch, 2 gm Agar powder, 100 ml Distilled water) and incubated at 37^{o}C for 48 hrs. After incubation, the plates were flooded with Iodine solution. Iodine solution was washed off to observe zone of clearance.

Caseinase activity:

Casein agar plates (1 gm Tryptone, 0.5 gm Yeast extract, 1 gm Sodium Chloride, 10 ml Skimmed milk, 2 gm Agar powder, 90 ml Distilled water) were prepared. The isolates were spot inoculated and incubated at 37^{o}C for 48 hrs. Zone of clearance was observed and the diameter of the zone was measured.

$HgCl_2$ assay for Gelatinase activity:

Gelatin agar plates (1 gm Tryptone, 0.5 gm Yeast extract, 1 gm Sodium Chloride, 2 gm Gelatin, 2 gm Agar powder, 100 ml Distilled water) were prepared. The isolates were spot inoculated and incubated at 37^{o}C for 48 hrs. After incubation, plates were flooded with Mercury Chloride solution. Mercury chloride was washed off to observe the zone of clearance.

RESULTS

Bacterial count and isolation:

The total heterotrophic bacterial count was 0.95 x 10^5 CFU/ml in the intestine of *Bufobufo*. Two morphologically different colonies (BBL and BBS) were observed. Both the isolates were found to be Gram positive bacilli.

Screening of enzyme producing strains:

The bacterial strains were screened for Cellulase, Amylase, Caseinase and Gelatinase activities. The activities are summarized in the Table No. 1. The zones of clearance are presented in Plate No. 1.

Table No. 1: Enzymatic activity of bacterial isolates from *Bufobufo*.

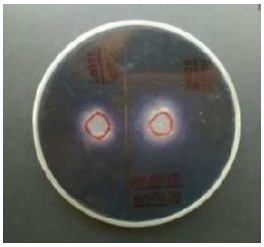
Enzymatic activities	Isolate BBL		Isolate BBS	
	Presence	Zone of clearance (mm)	Presence	Zone of clearance (mm)
Cellulase	-	-	-	-
Amylase	+	26±2.13	+	32±1.17
Caseinase	+	17.3±1.13	+	21.6±0.97
Gelatinase	+	35.4±0.87	+	32.8±1.11

The zone of clearance was observed for all the enzymes tested except cellulase. Maximum zone of clearance was observed for Gelatinase activity, followed by Amylase and Caseinase activity.

Plate No.1: Zone of clearance with respect to enzymatic activities in two isolates from GI tract of *Bufobufo*.

Cellulase activity: Isolate BBL

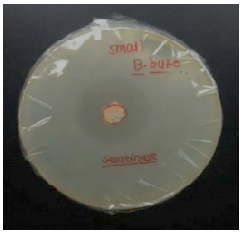
Cellulase activity: Isolate BBS



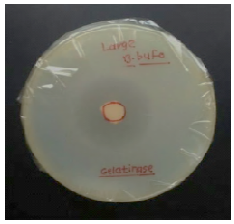
Amylase activity: Isolate BBL and BBS

Caseinase activity: Isolate BBL

Caseinase activity: Isolate BBS



Gelatinase activity: Isolate BBL



Gelatinase activity: Isolate BBS

DISCUSSION

The total heterotrophic bacterial count of the GI tract of *Bufobufo* was 0.95 x 10^5 CFU/ml. It reveals that moderate to dense population of bacteria occurs in the GI tract of *Bufobufo*. There are no reports of bacterial counts from the intestine of *Bufobufo*.

Microflora of many endotherms have been previously studied (Barnes et al., 1979; Holdeman et al., 1977; Hussong et al., 1979; Savage, 1977). Characteristics of frogs which distinguish them from these endotherms and influence their microflora are: i) lower temperature and slower metabolism (Reeder, 1964) ii) carnivorous habit with short intestine (Reeder, 1964) iii) diverse intestinal protozoal fauna (iv) presence of a larval stage with different mode of life (Reeder, 1964) v) hibernation. The space taken up by the protozoan may cause lesser number of bacteria in frog as compared to other endotherms. The hibernating frogs have lower counts as compared to active bacteria (Gossling et al., 1982).

The variation in bacterial count may be a result of varied bacterial load of the habitat as observed in the study carried out on *Labeorohita* and *Channapunctatus*(Kar and Ghosh, 2008).

The reports of enzyme producing bacteria from frogs are fewer, however extracellular enzyme producing bacteria in fish *Labeorohita* and *Channapunctatus* have been studied (Kar and Ghosh, 2008).Proteolytic and cellulase activities in fish *Gambusiaaffinis* have also been reported (Pingle et al., 2016). The presence of enzyme producing capacity can be correlated with the digestive physiology. Absence of the cellulase activity in the two isolates and predominant amylase activities along with protein digesting activities support the carnivorous habit of toad *Bufo*.

The information generated from the present investigation might contribute to commercial aquaculture in the form of supplement in aquaculture feed. However, further studies are needed to

identify these bacterial strains and to evaluate their efficacy under actual farm conditions.

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