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PRODUCTION OF DIFFERENT ENZYMES BY GUT MICROFLORA

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ABSTRACT
Microorganisms in particular have been regarded as treasure sources of useful enzymes. They are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch. Enzymes are among the most important bio products and are being utilized in a large number of processes in the areas of industrial, environmental and food biotechnology. Considerable information are available concerning the intestinal microflora of homeotherms and also the role of those intestinal microflora in digestion; but very little information is available concerning the bacterial population in the gastrointestinal tract of poikilotherms. As shrimps are not adequately studied in this respect, the present study on the production of amylase, protease, lipase, cellulase and chitinase production was undertaken. All the identified bacterial strains showed amylolytic, proteolytic, lipolytic, cellulolytic and chitinolytic positive activities, except the *Pseudomonas* species which did not show any cellulolytic activity respectively.

KEYWORDS: Amylase, protease, lipase, cellulase, chitinase, Screening, gut bacterial flora.

INTRODUCTION

The bacterial flora of the gastrointestinal tract in general represents a very important and diversified enzymatic potential, and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal (Bairagi *et al.*, 2002). Some fish species acquire many of their intestinal enzymes from the microflora inhabiting their guts (Hamid *et al.*, 1979; Cachil, 1990; Amit Kumar Sinha *et al.*, 2007; Ringo *et al.*, 2010). Enzyme producing micro organisms were directly indicated by the formation of distinct clearing zones on the substantially darker background of the medium within two to three days of incubation (Vaidya *et al.*, 2003). Bairagi *et al.* (2002) employed the bacterial species isolated from gut for the qualitative detection of different enzymatic activities. Amylases are among the most important industrial enzymes and are of great significance in present-day biotechnology (Selvakumar *et al.*, 1996; Pandey *et al.*, 2000; Qader *et al.*, 2006). Amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands (Pandey, 1999 & 2000; Kathiresan and Manivannan, 2006). Almost all microorganisms of the *Bacillus* genus synthesized amylase, thus this genus has the potential to dominate the enzyme industry (Pretorius *et al.*, 1986). Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale (Brown and Yada, 1991; Escobar and Barnett 1993; Manjeet Kaur *et al.*, 1998; Dutta and Banerjee, 2006; Shankar *et al.*, 2011). Most of the available proteases produced commercially are of microbial origin (Oskouie *et al.*, 2007). They are important enzymes, which due to their ability to catalyze a number of reactions, are receiving considerable interest from both academia and industry (Gilbert, 1993; Hernández Sámano et al.2012). A relatively smaller number of bacterial lipases have been well studied when compared to plant and fungal lipases (Saxena *et al.*, 1993).Different fungi and bacteria have been used for cellulase production (Bahkali, 1996; Magnelli and Forchiassin, 1999; Shin *et al.*, 2000;Sunita and Sumit,2012; Nashima *et al.*, 2012). Chitinases are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea and viruses (Suzanne *et al.*, 2001). It is believed that stomach chitinases have an indirect digestive function, helping to breakdown the exoskeleton of prey, which allows other digestive enzymes access to soft inner tissues (Fange and Grove, 1979; Lindsay, 1984; Clark Quayle *et al.*, 1988). Considerable information are available concerning the intestinal microflora of homeotherms and also the role of those intestinal microflora in digestion; but very little information is available concerning the bacterial population in the gastrointestinal tract of poikilotherms. As shrimps are not adequately studied in this respect, the present study was undertaken.

MATERIALS & METHODS

Penaeus monodon is an important marine crustacean which inhabits in marine forms and is endemic in Peninsular India and other countries. It was collected from the Rajakkamangalam estuary at Rajakkamangalam, Kanyakumari District, Tamilnadu. The collected shrimps were aseptically transferred to the laboratory for further study.

PLATE 1. *Penaeus monodon*

Isolation and identification of gut bacterial flora

In the laboratory, the weight of the whole shrimp was noted and the gut sample was aseptically dissected out and serially diluted upto 10^{-5} dilution. From each dilution, 0.1 ml of sample was taken and spread plated on sterile nutrient agar medium. The plates were then incubated at 37°C for 24 to 48 h. The total viable count (TVC) of the colonies was finally noted. The isolated cultures were purified individually by streaking on nutrient agar plates and were sub cultured. Then the bacterial cultures were identified by performing biochemical tests.

Screening of enzyme producing bacteria

Amylase activity

Starch agar (Components (g/l): Starch (soluble): 20.0 g; Peptone: 5.0 g; Beef extract: 3.0 g; Agar: 15.0 g) plates were prepared and streaked with the individual test organisms. The plates were incubated at 37°C for 24-48 hours. After incubation, the plates were flooded with iodine solution. Amylase activity was indicated by a clear/white zone around the colonies.

Protease activity

Skim milk agar (Components (g/l) : Skim milk powder : 100 g ; Peptone : 5.0 g ; Agar : 15.0 g ; pH : 7.2) plates were prepared and streaked with test organisms. They were then incubated at 37°C for 24-48 h. After incubation, the plates were flooded with $HgCl_2$ solution and were observed for zone formation.

Lipolytic activity

The microbial isolates were single streaked individually on Spirit blue agar plates (Components (g/l): Hiveg hydrolysate : 10g ; Yeast extract : 5g ; Spirit blue : 0.15g ; Agar : 17g ; pH : 6.8 ± 0.2) and tributyrin agar plates (Components (g/l) : Tributyrin : 10 g ; Peptone : 5 g ; Agar : 15 g ; Rhodoamine B : 0.01 g ; pH : 7) with fluorescent dye Rhodomine B (0.001%) and they were incubated at 37°C for 48 h. Lipase positive cultures showed opaque zones around them and when exposed to UV light of 254nm, an orange fluorescent halo appearance around the colonies was observed.

Cellulase activity

The bacterial colonies were single streaked individually on Carboxy Methyl Cellulose (CMC) agar (Components (g/l): CMC: 10.0 g; KH_2PO_4: 4.0 g; Na_2HPO_4; 4.0 g; Tryptone: 2.0 g; $MgSO_4.7H_2O$: 0.2 g ; $CaCl_2$: 0.001g; $FeSO_4.7H_2O$:0.004 g; Agar: 15.0 g; pH : 7.0) plates and were incubated at 37°C for 24-48 h. The plates were flooded with 0.1% aqueous conjured solution and were allowed to stand for 20 min. Then they were thoroughly washed with 1 M NaCl solution and were observed for zone formation against a dark background.

Chitinase activity

The chitinase detection agar (CHDA) (Components (g/l) Colloidal chitin: 10.0 g ; Agar: 20.0 g; Soya bean powder: 20.0 g; Starch: 3.0 g ; Peptone: 3.0 g; Yeast extract: 2.0 g; $CACO_3$: 1.0 g: M9 medium: Na_2HPO_4: 0.65 g; KH_2PO_4: 1.5g; NaCl:0.25 g; NH_4Cl: 0.5 g ; $MgSO_4$: 0.12 g; $CaCl_2$: 0.005 g; pH: 6.5) plates were prepared. The isolated gut microbes were single streaked individually into the CHDA plates and were incubated at 37°C for 72 h. They were then observed for zone formation. The colonies which formed a zone around them were the chitinase positive strains. The positive cultures were then sub cultured regularly for further study.

RESULTS

Isolation and identification of gut microflora

The total viable count of bacterial colonies recorded in the gut sample of the shrimp *P.monodon* was $43 \pm 0.16 \times 10^2$ CFU/ml in 10^{-1} dilution and 2 ± 1.13 CFU/ml in 10^{-5} dilution.

Total viable count of bacterial colonies at different dilution

Sl. No.	Dilution factor	Number of colonies (cfu/ml)
1.	10^{-1}	$43 \times 10^2 \pm 0.16$
2.	10^{-2}	$8 \times 10^3 \pm 1.32$
3.	10^{-3}	$19 \times 10^4 \pm 0.08$
4.	10^{-4}	$2 \times 10^5 \pm 1.13$
5.	10^{-5}	Nil

Each value is a mean ± SD of triplicate analysis.

Based on the morphological, physiological and biochemical characteristics, seven bacterial strains were identified. The five strains *Bacillus cereus, B. polymyxa, B. stearothermophilus, B. circulans* and *B. mycoides* belong to Gram positive group and the two strains *Pseudomonas alcaligenes* and *P. anguilliseptica* belong to Gram negative group.

Enzymatic characterization of identified bacterial species

All the identified bacterial strains showed amylolytic, proteolytic, lipolytic, cellulolytic and chitinolytic positive activities, except the *Pseudomonas* species which did not show any cellulolytic activity.

Different enzymatic activities of identified bacterial strains.

Sl. No.	Bacterial strains	Amylolytic activity	Proteolytic activity	Lipolytic activity	Cellulolytic activity	Chitinolytic activity
1.	*B. cereus*	+	+	+	+	+
2.	*B. polymyxa*	+	+	+	+	+
3.	*B. stearothermophilus*	+	+	+	+	+
4.	*B. circulans*	+	+	+	+	+
5.	*B. mycoides*	+	+	+	+	+
6.	*P. alcaligenes*	+	+	+	-	+
7.	*P. anguilliseptica*	+	+	+	-	+

+ = Positive enzyme activity – = Negative enzyme activity

Morphological, physiological and biochemical characteristics of identified bacterial strains.

S. No.	Culture number	Grams staining	Spare staining	H_2S production	Motility	Citrate test	Methyl red	VP test	Urease	Gelatinase	Indole	Oxidase	Carbohydrate fermentation										Catalase	Nitrate	Triple sugar from test	Identified organisms
													Lactose	Inositol	Maltose	Glucose	Fructose	Sucrose	Treh	Xylose	Mannitol	Mannose	Salicin				
1.	Sh. 1	G (+) rods	+	-	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	A/A	*Bacillus cereus*
2.	Sh. 2	G (+) rods	+	-	+	-	+	+	-	-	-	-	+	-	+	+	+	-	+	+	+	+	-	+	+	A/A	*Bacillus polymyxa*
3.	Sh. 3	G (+) rods	+	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+	+	-	+	+	-	+	+	K/A	*Bacillus stearothermophilus*
4.	Sh. 4	G (+) rods	+	-	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	-	+	-	+	+	K/A	*Bacillus circulans*
5.	Sh. 5	G (+) rods	+	-	-	+	+	+	-	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	K/K	*Bacillus mycoides*
6.	Sh. 6	G (-) rods	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	K/A	*Pseudomonas alcaligenes*
7.	Sh. 7	G (-) rods	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	K/A	*Pseudomonas anguilliseptica*

+ = Positive; – = negative

A/A = Acid slant / acid butt; K/A = Alkaline slant / acid butt; and K/K Alkaline slant / alkaline butt

DISCUSSION

The digestive tract of aquatic organisms is colonized by a great number of heterotrophic bacteria and they are responsible for the production of certain digestive enzymes (Preetha and Palavesam, 2002). Bairagi *et al.* (2002) reported that a distinct microbial source of digestive enzymes such as, amylase, cellulase, lipase and protease were found in the gut of Tilapia and Carp. To know much about gut microflora and their enzyme production capabilities, the present study was undertaken.

Moriarty (1999) stated that *Bacillus* sp. are generally present in the sediment, on which shrimp are feeding and they colonize in the gut of shrimp. Similarly Hoshino *et al*. (1997) isolated a *Pseudomonas* sp. from the intestine of fish which was capable of producing protease enzyme. In the present study also, different *Bacillus* sp. (*B. cereus, B. polymyxa, B. stearothermophilus* and *B. mycoides*) and *Pseudomonas* sp. (*P. alcaligenes* and *P. anguilliseptica*) were isolated and identified from the gut of shrimp *P. monodon* and their ability on production of five different enzymes namely amylase, protease, lipase, cellulose and chitinase were studied. In accordance with these, a wide variety of gram negative and gram positive bacterial species were reported to produce different gut enzymes (Gilbert, 1993; Gupta *et al.*, 2004; Vandana and Bera, 2005). The amylase enzyme of gut plays an important role in promoting the digestion, even the growth of shrimp (Chen and Lin, 1992). Amylase help in increasing their host's (shrimps) digestive efficiency (Dempsey and Kitting, 1987). Ozcan *et al.* (2010) also studied about the amylase enzyme production by *Bacillus* sp. These microbes produce different enzymes needed for the breakdown of numerous dietary constituents in the gut and they have been regarded as treasure sources of useful enzymes. From the results, it is found that all the *Bacillus* sp. showed amylolytic, proteolytic, lipolytic, cellulolytic and chitinolytic activities except the *Pseudomonas* sp., which was not cellulolytic positive.

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