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Research Article

UNDERSTANDING PROBIOTIC POTENTIALS OF *BACILLUS* BACTERIAL POPULATION ISOLATED FROM *CHITALA CHITALA* (OSTEOGLOSSIFORMES; NOTOPTERIDAE) BY COMPARING THE ENZYME ACTIVITY *IN VITRO*

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ABSTRACT

In the present investigation, three types of *Bacilli* were isolated from the intestine of *Chitala chitala* larvae. After the morphological, physiological and biochemical characterization the isolates were identified as *Bacillus subtilis, B. pumilus and B. licheniformis*. A varied extracellular enzymatic (amylolytic, proteolytic and lipolytic) activity was observed among these three isolates during the qualitative and quantitative method of in vitro culture. The temperature optimum for growth of the three isolates was ranged between 30 to 40°C with an optimal pH range of 8-10 and salinity 7-9%. Among the three isolates the proteolytic and lipolytic activity was found to be the highest in *B. licheniformis* (P<0.05) and the amylase producing ability was found to be the highest in *B.subtilis* (P<0.05). Thus the present preliminary study will be useful in future to develop the cost-effective formulated probiotic feeds for a sustainable culture practice of this valuable species by incorporating these enzyme-producing *Bacillus* isolates.

Key words: *Chitala chitala, Bacillus subtilis, B. pumilus, B. licheniformis.*

INTRODUCTION

The featherback *Chitala chitala* (Hamilton, 1822) constitutes an important part of riverine fisheries in India, commands high market demand and has been prioritized as a new candidate for freshwater aquaculture system. Due to high demand and overexploitations, this species has been categorized as endangered (EN) in the Conservation Assessment and Management Plan, 1998 (Ayyappan *et al*., 2001). Therefore development of a suitable culture practice for this potentially important species has immense relevance and large scale farming of this species will ensure effective resource utilization, biodiversity conservation and widening consumer's choice for this new species. The success of effort to introduce and cultivate any new species depends on the adequate knowledge of the functional development of their digestive system and nutritional requirements. Now-a-days it is becoming increasingly apparent that the microenvironment of intestinal microflora associated with the gastrointestinal tract of the fish and its metabolic activities can be an important contributing factor in nutrition, physiology and animal welfare. Thus proper identification and characterization of the microorganisms are essential to determine their specific functions in fish nutrition, effect on gut enzyme activity and their role as probiotics in enhancement of food digestibility. Numerous studies have reported diverse microbial communities in the GI tract of carnivorous, herbivorous and omnivorous fish species (Nayak, 2010). However, surprisingly, no information on the composition of bacterial communities

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associated with the gastrointestinal tract of this important species *C. chitala* is available. Thus, the understanding of these intestinal microfloras could be useful for the mass-scale rearing by developing probiotic feed to get the optimum growth of this 'endangered' species.

MATERIALS AND METHODS

The featherback eggs were obtained from a 2years old brood stock (weight=2.31±0.6 kg, total length=86±5.5cm) from local fish farm and incubated at 27-28°C, pH 7.81±0.12 and water flow 0.3 liter/sec. Incubation period lasted about 8 days. After hatching, larvae reared in a recirculating system at a density of 45,000 larvae m^3 in each tank (450 L). The photoperiod was maintained on a 12 L: 12 D cycle. Temperature and pH ranged from 30±2.2°C and 7.4±0.6, respectively throughout the experiment. Dissolved oxygen was maintained above 8.0±2.5 mg/L by constant aeration. At 12 dph the whole yolk sac was completely absorbed and feeding started. From 12 dph to 30dph the fishes were fed slightly in excess on newly hatched *Artemia* nauplii three times daily. 30 dph (weight = 1.389±0.334 gm, total length = 30.45±0.546 mm.) larvae were randomly netted from recirculating tank. Larvae from two different tanks were used to avoid a possible tank effect. Before being dissected the larvae were starved for 48 hours. The larvae were anaesthetized in an ice bath for 5–10 min and the ventral surface of the fish was thoroughly scrubbed with 1% iodine solution (Trust and Sparrow, 1974). The fishes were dissected on ice within laminar flow and their alimentary tracts were removed and cleaned with sterile chilled physiological saline (0.89% NaCl in PBS buffer, pH 7.2). Subsequently, the intestine of all the fishes was homogenized with sterilized 0.9% NaCl solution (1:10; weight/volume) to remove non-adherent bacteria.

Microbial culture

The Homogenate of the intestinal mucosa of each of the test fish was used for microbial culture after five serial 1: 10 serial dilutions (Beveridge *et al*., 1991). Samples (0.1 ml) were taken from each dilution and poured aseptically under laminar air flow on Tryptone Soya Agar (TSA) plates, in triplicate. These plates were incubated at 37°C for 48 hours and then examined for the development of bacterial colonies. The well-separated colonies with apparently different morphological appearance were streaked separately on above media plates to obtain pure cultures. By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate were determined (Rahmatullah and Beveridge, 1993).

Characterization of the bacterial isolates

Morphological tests: Colony morphology of all the isolates formed on TSA plates was studied visually by hand magnifying lens for determining their configuration, margin, elevation, surface, density, texture and color. Bacterial staining was performed by using crystal violet, Iodine solution, absolute alcohol and safranin (Gram's Method). Size of all the bacterial isolates was determined by stage and ocular micrometry. Capsular staining was done by using India ink and Carbol fuchsin (Gin's method). Endospore formation by the isolates was studied by spore staining technique using 5% aqueous malachite green and 0.5% aqueous safranin solution (Wirtz's modified method).

Biochemical tests: The biochemical characterization of the isolated bacteria include indole production, methyl red test, citrate utilization, casein, starch and urea hydrolysis, nitrate reduction, H_2S production, cytochrome oxidation test, catalase production and sugar fermentation.

All these biochemical tests were performed following prescribed methods of Society of American Bacteriologists (1957).

Impact of pH, temperature and salt tolerance: Selective isolate was tested for growth at different pH of 4.0 - 10.0 and temperatures 10°-40°C was tested using Nutrient Broth. Each condition was analyzed in triplicate and incubated under aerobic condition for 1-3 days. Then the inoculated cultures were spread on Nutrient agar medium and bacterial counts were made after 24-48 hours incubation. Salt tolerance

(5-10% NaCl) was determined for viability and growth. Isolates were grown in broth cultures and studied after 24 hours of incubation at 37°C for the physiological characteristics. The bacterial population was expressed as number of Colony Forming Units (CFU) per ml of sample.

Screening of isolates for extracellular qualitative and quantitative enzyme production

The α-amylase production on solid medium was tested by streaking bacterial culture on starch agar (Nutrient agar with 1% soluble starch). Plates were incubated at 37°C for 3 days after which they were flooded with iodine solution (0.3% w/v I_2 in 3% w/v KI). Amylase activity was indicated by a clear zone surrounding the colony. Crude enzyme preparation was obtained by centrifuging the broth culture at 5000 rpm for 15 minutes in a refrigerated centrifuge and the supernatant was used for enzyme assay. The α-amylase activity was determined in triplicate by using 1% soluble starch, as substrate, with 3,5-dinitrosalicyclic acid (DNS) at 550 nm (Bernfeld, 1951). Specific α-amylase activity is reported as U = µg of maltose min^{-1} mg^{-1} of protein present in the enzyme extract tested at 37°C.

For extra-cellular protease production, bacterial isolates were streaked on peptone-gelatin-agar medium and incubated for 48 h at 37ºC. The appearance of a clear zone around the colony after flooding the plate with 15% $HgCl_2$ indicated the presence of proteolytic activity. Quantitative activity of protease was measured by using 1% azocasein as substrate in 50 mM Tris–HCl, pH 7.5 (Garcia-Carreño, 1992). Specific protease activity is reported as U = µg of tyrosine min^{-1} mg^{-1} of protein present in the enzyme extract tested at 37°C.

Glycerol tributyrate agar was used to determine the lipolytic activity of the isolates. It was autoclaved at 121°C for 15minutes poured and allowed to solidify and cool to 45°C. Isolates were streaked on the surface of the agar, and the plates were incubated at 37°C for 7 days and observed at two days interval. Lipolytic activity was observed by a clear zone around the bacterial streak on the medium. Quantitative estimation of lipase was assayed based on measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan, 1990). Specific activity of lipase is reported as U= µg of fatty acid $min^{-1}mg^{-1}$of protein present in the enzyme extract tested.

Soluble protein in the crude enzyme was measured by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard.

Qualitative extracellular enzyme activity was assessed based on the measurement of a clear zone (halo) around the colony as follows; + (low, 4 - 6 mm halo diameter), ++ (moderate, 7 - 9 mm halo diameter), +++ (high, 10-12 mm halo diameter) and ++++ (very high, 13-16mm halo diameter).

Statistical analysis

Mean values of enzyme activities among the different isolates were analyzed using one way ANOVA test. Results that were significantly different were analyzed by Duncan's multiple range test with 0.05 α. All the specific activities of the enzymes were carried out in triplicate and results were given as mean ± SE.

RESULT AND DISCUSSION

The larger yellow colonies were selected and on the basis of characters depicted in Table 1 and 2, the isolated bacteria from *C.chitala* gut were identified using the guidelines described in the Manual of Determinative Bacteriology (Bergey, 1957). The genus of the isolates has been identified as *Bacillus*. The spores were terminal and subterminal in position having ellipsoidal shape. All three organisms were rod shaped, short chained forming gram positive and capable of forming moderate colonies. Temperature optimum for growth was 30-40°C with an optimal pH range of 8-10 and salinity 7-9% (Figure 1) and this indicates the optimum culture conditions for these isolated strains. Isolate I showed positive MR, positive starch hydrolysis, positive citrate utilization, positive casein hydrolysis, negative H_2S production, positive catalase, positive sugar fermentation with glucose, fructose, maltose. Hence it was similar

to the species of *Bacillus subtilis*. The isolate II was positive for MR test, citrate utilization and casein hydrolysis, glucose, fructose, xylose fermentation. Negative for nitrate reduction, starch hydrolysis and indole production. Hence it was similar to *Bacillus pumilus*. The isolate III showed positive to MR test, starch hydrolysis, citrate utilization, H_2S production, fructose, maltose, sucrose, mannose, lactose fermentation and identified as *Bacillus licheniformis*.

Extracellular enzyme production by bacterial strains isolated from the digestive tract of the investigated fish was assayed qualitatively (Table 3) and quantitatively (Figure 2).The isolates were found to produce varied range of extracellular enzymes such as amylase, protease and lipase. The α-amylase activity was found to be the highest in *B. subtilis* (P<0.05) and the amylolytic activity was absent in *B. pumilus*. The highest protease producing ability was found in *B. licheniormis* followed by *B. pumilus* and *B. subtilis*(P<0.05).But *B. licheniformis* among the three isolates showed the highest lipolytic activity (P<0.05).

Generally bacteria are abundant in environment in which fish lives and it is therefore rather important to avoid them being a component of their diet (Hansen *et al*.,1999) and these population of endogenous microbiota may depend on age, genetic, nutritional and environment factors (Olafsen, 2001). Resident intestinal bacteria in fish are known to accelerate the digestion process by producing extracellular enzymes (Sugita *et al.*, 1998). Assessment of the substrate degrading ability of gut microflora is important in understanding the nutrition and physiology of the host organism and may help in formulating appropriate feeds. The composition of enzyme producing bacterial flora in the fish digestive tracts are correlated to their feeding habits. To test this hypothesis, Kar *et al.* (2008) isolated maximum population of proteolytic bacteria in carnivorous *Channa punctatus* compared with herbivorous *Labeo rohita*. From this observation, it appears that protease activity may dominate in carnivorous fish. But on the contrary a good amount of endogenous protease producing *Bacillus* strains were also isolated from the gastrointestinal tract of the herbivorous *L. rohita* fingerlings and *L. bata* (Ghosh *et al*., 2002; Mondal *et al*. 2010).In the present experiment, from the invitro studies of enzyme producing capacity it was evident that the bacterial flora present in the gastrointestinal tract of *C. chitala* are good producer of proteolytic enzymes and this can be correlated with the feeding habit of *C. chitala,* which is commonly carnivore and insectivore in nature (Alikunhi,1957). Among the fish, endogenous amylase activities in the intestine of herbivorous carps are much more intense than in carnivorous species and it was evident with the findings of Ray *et al.* (2010),who detected a huge population of amylase-producing *Bacillus* in the GI tract of like *Catla catla*, *Cirrhinus mrigala* and *Labeo rohita*. Mondal *et al.* (2010) also isolated amylase producing *B. licheniformis* and *B. subtilis* from the digestive tract of *L. bata*. But amylolytic bacteria were not detected in the carnivorous climbing perch, carnivorous catfish and murrel (Bairagi *et al.*, 2002; Mondal *et al.*, 2010). In the present study, a moderate amylolytic activity was observed in the larvae of *C.chitala* which was similar to the findings of Kar *et al.* (2008) where higher densities of amylolytic bacteria was detected in the digestive tracts of carnivorous murrels. The result of presently reported study also indicates a good amount of lipolytic bacterial strains are also exist in digestive tract of *C. chitala* larvae and it supports the hypotheses that gut bacteria may be beneficial for larval growth and survival (Ringø and Birkbeck,1999).Colonization of amylolytic, proteolytic and lipolytic *Bacillus* strains in such a varied intensity in *C. chitala* may suggest that there is a symbiotic (mutual) relationship between these bacterial flora and the fish species (Kar *et al.,* 2008), which help the fish to adapt towards the changes in the amount and composition of the available diet during ontogenetic shift.

Table 1. Physiological tests for Isolate I *(Bacillus subtilis)*, IsolateII (*Bacillus pumilus)* and Isolate III (*Bacillus licheniformis)* isolated from *C. chitala* gut.

Parameters	Characteristics		
	Isolate I **(*Bacillus subtilis)***	Isolate II **(*Bacillus pumilus)***	Isolate III **(*Bacillus licheniformis)***
Colony morphology			
Configuration	Irregular, lobate	Round	Irregular
Elevation	Flat	Convex	Convex
Surface	Dull	Smooth	Rough
Pigments	ND	ND	ND
Gram's reaction	+	+	+
Shape	Rod shaped Short chained	Rod shaped Short chained	Rod shaped Short chained
Size	Moderate	Moderate	Moderate
Spore			
Endospore	+	+	+
Shape, Position	Ellipsoidal Subterminal	Ellipsoidal, Terminal	Ellipsoidal, Subterminal

+ = Positive, ND = Not detected.

Table 2. Biochemical Tests for Isolate I *(Bacillus subtilis)*, IsolateII (*Bacillus pumilus)* and Isolate III (*Bacillus licheniformis)* isolated from *C. chitala* gut.

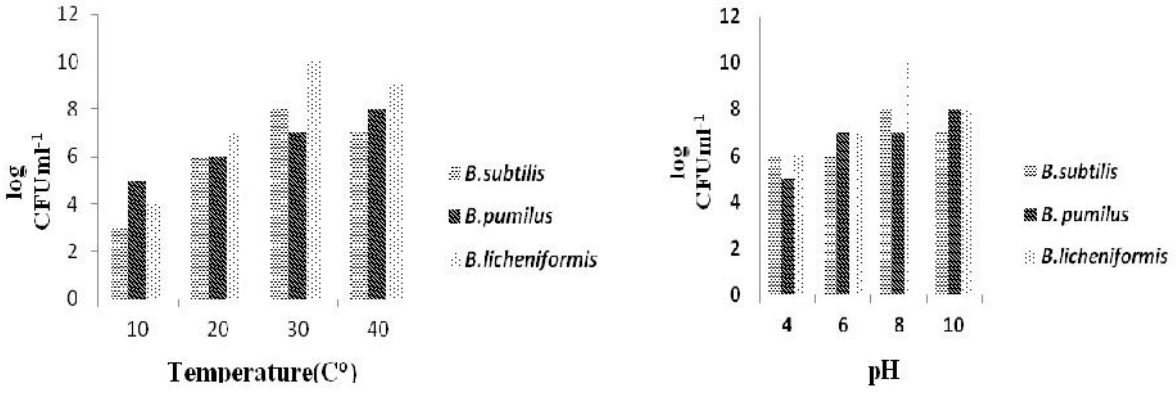
Tests	Characteristics		
	Isolate I **(*Bacillus subtilis*)**	Isolate II **(*Bacillus pumilus)***	Isolate III **(*Bacillus licheniformis)***
Indole production	ND	ND	ND
Methyl Red Test	+	+	+
Citrate utilization	+	+	+
Casein hydrolysis	+	+	+
Starch hydrolysis	+	ND	+
Urea hydrolysis	ND	ND	ND
Nitrate reduction	+	ND	ND
H_2S production	ND	ND	+
Cytochrome oxidase test	+	ND	+
Catalase test	+	+	ND
Sugar fermentation:			
Glucose	+	+	+
Fructose	+	+	+
Xylose	ND	+	ND
Maltose	+	+	+
Mannitol	+	ND	ND
Sucrose	+	ND	+
Lactose	+	ND	+
Mannose	+	ND	+

+ = Positive, ND = Not detected.

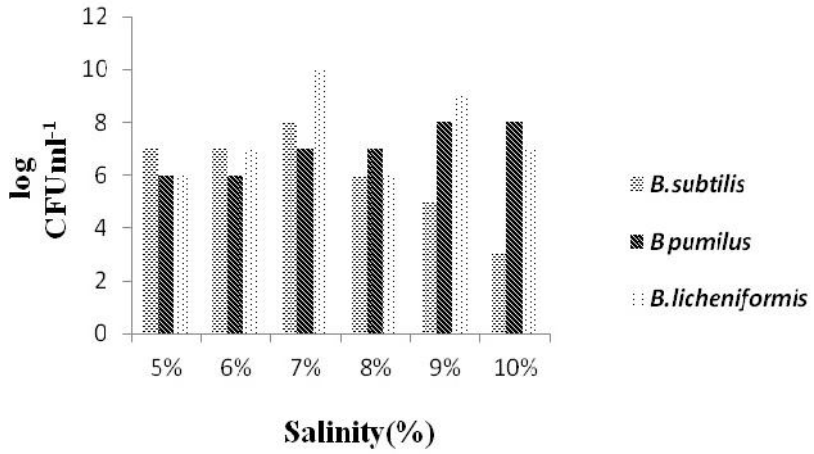
Table 3. Qualitative extracellular enzyme production by the Isolate I *(Bacillus subtilis)*, IsolateII (*Bacillus pumilus)* and Isolate III (*Bacillus licheniformis)* isolated from *C. chitala* gut.

Enzymatic activity	Isolate I (***Bacillus subtilis***)	Isolate II (***Bacillus pumilus***)	Isolate III (***Bacillus licheniformis***)
Amylase	+ + +	absent	+
Protease	+	+ + +	+ + + +
Lipase	+ +	+ +	+ + + +

++++, very high (13-16mm halo diameter); +++, high (10-12mm halo diameter); ++, moderate (7-9mm halo diameter); +, low (4-6mm halo diameter).



(a) (b)



(c)

Figure 1. Effect of (a) Temperature, (b) pH and (c) salinity on the growth of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* isolated from *C. chitala* gut.

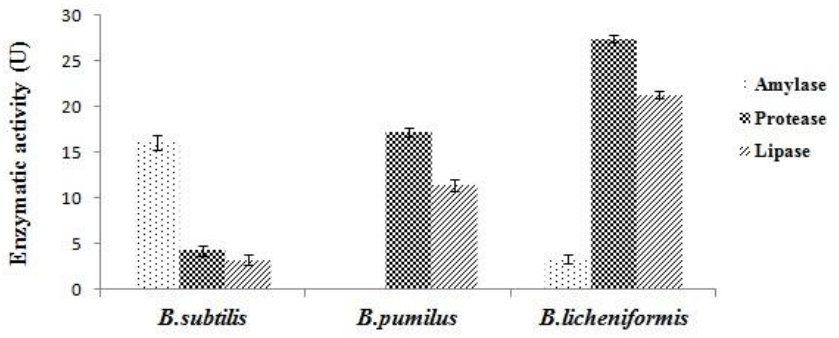


Figure 2. Specific activities of α-amylase, protease and lipase activity of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* isolated from *C. chitala* gut. (Values are mean±SE). Statistical analyses are subjected to the one way ANOVA test by SPSS 21 software.

CONCLUSIONS

This study presented the first data on the isolated gut *Bacillus* of *C. chitala.* In future the result of the present experiment may help to develop the cost-effective formulated probiotic feeds for a sustainable culture practice of this valuable species by incorporating these enzyme-producing *Bacillus* isolates especially for the larval stages when the enzyme system is not efficient.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests associated with this article.

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