Isolation of *Bacillus* Strains from Gastrointestinal Tract of Fish and Screening for certain Probiotic Properties

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Abstract: **Probiotics are most commonly defined as viable microorganisms which, after sufficient oral intake, lead to beneficial effects for the host by modifying the intestinal microbiota. The objective of present study was to isolate and characterize different *Bacillus* strains having probiotic properties from gastrointestinal tract (GIT) of fish. The presence of probiotic bacteria in the digestive tracts of fish was subjected to several factors such as their ability to adhere to the surface of the intestinal epithelium and the production of substances that antagonise pathogenic microorganisms. *Bacilli are* spore forming bacteria which are heat resistant, that gives stability to microorganism in different environmental conditions this characteristics makes this organism most suitable to be used as probiotics strain. The selected 11 bacterial strains from GIT of fish were investigated for bile salt tolerance (1% to 8% concentrations), lipolytic activity, protease activity, haemolytic activity, catalase activity, hydrophobicity and autoaggregation. Among these 11 isolates, however, there was only 1 isolate, named as F10 which showed the haemolytic activity. Based on various activities results suggest that isolated strain F3 is considered to be suitable for probiotic applications.**

IndexTerms: Probiotic, *Bacillus* strain, Fishgut, Bilesalt tolerance, Hydrophobicity, Autoaggregation

I. INTRODUCTION

The term probiotic is derived from the Greek and literally translates as 'for-life'. The probiotic concept was born at the end of 19th century by Elie Mechnikoff at the pasture institute in Paris. The fermentive capabilities of the GIT microflora also contribute towards large bowel digestive function, acting on recalcitrant compounds of dietary origin and on host secretions, allowing recovery of nutrients otherwise lost to the host [1]. The gastrointestinal microbiota presents a significant barrier that must be overcome for a pathogen to initiate an infection. The concept of preventing or ameliorating intestinal infections through dietary interventions designed to manipulate commensal bacteria, or as a means of introducing transiently colonizing probiotic strains, has received much attention in recent years. Further, an increase in stress and modern day living which makes a consequential demand on the immune system can disturb the homeostasis of the GIT. Consumption of pharmaceutical compounds, particularly antibiotics which kill bacteria, can alter the gut microflora in such a way that numbers of beneficial microorganisms in the gut decrease and numbers of pathogenic bacteria increase leading to disease [2]. Therefore it is of considerable benefit to the host to maintain a good community structure in the gut through increasing the levels of beneficial bacteria.

The scientific interest in *Bacillus species* as probiotics though, has only occurred in the last 15 years and three principal reviews have covered the field [3][4][5]. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier [6][7]. A specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will then be targetted to small intestine in its intact form. Spore probiotics are being used extensively in humans as dietary supplements, in animals as growth promoters and competitive exclusion agents and lastly in aquaculture for enhancing the growth and disease-resistance of cultured shrimps, most notably the Black Tiger shrimp *(Penaeus monodon)*.

I. MATERIALS AND METHODOLOGY

Isolation of *Bacillus* from the Fish Sample

Fish gut were collected and chopped in saline water in aseptic condition and then it was taken in to 2ml sterile distilled water tube and mixing was carried out by vortexing for 5min. After that, 1 ml of supernatant was added in to the 4 ml saline water tube. The suspension was then diluted up to 10^{-6} dilution factor and 0.1 ml from last 3 dilutions was spreaded on Hicrome Bacillus Agar plates. The plates were incubated at 37 °C for 24hr. Different *Bacillus sp.* gave different coloured colony on Hicrome Bacillus agar medium. Also Gram staining was carried out from each plate

Screening for probiotic properties

Bile salt tolerance

The tolerance of vegetative cells and spores of these isolates to bile salts was checked which were subjected to partially simulated gastrointestinal conditions was assayed as per [8], with certain modifications. Initially, 1 ml of 48-h old culture, bearing organisms inoculated in to Hicrome Bacillus broth with bile salt (1% to 8% concentration) and incubated at 37 °C with agitation. After 24hrs, observed the growth in the tubes [9].

Catalase test

The catalase activity of the isolates were detected by resuspending the culture in a 3% solution of hydrogen peroxide. Catalase is an enzyme that converts hydrogen peroxide into water and oxygen. If the bubbles appear it means that this test gives positive result [9].

Haemolytic test

Haemolysis was determined on brain heart infusion agar supplemented with 5% human blood after incubation at 37 °C for 24 hrs [9]. Isolates were streak on to the blood agar plate and incubated at 37 °C for 24hrs. After incubation, Zone formation was checked around the colonies. Zone around colonies indicate positive test.

Hydrophobicity test

The degree of hydrophobicity of the isolates was determined by employing the method described by[10], based on adhesion of cells to organic solvents. The cultures were grown in 10 ml of Hicrome Bacillus broth, centrifuged at 6000rpm for 5 min for cell separation [9]**.** The pellet was washed, resuspended in 10 ml of Phosphate buffer solution and absorbance of this aqueous phase at 600nm as A_0 was measured. Cell suspension was then mixed with equal volume of solvent and mixed thoroughly by vortexing for 2 minutes wherein xylene (an apolar solvent), chloroform (a monopolar and acidic solvent) and ethyl acetate (a monopolar and basic solvent) were added to the suspension in different tubes. After 30 minutes two phases were separated and absorbance at 600 nm of non aqueous was recorded as A_1. The hydrophobicity of strain adhering to solvent was calculated as:

$$\text{Hydrophobicity } (\%) = (1 - A_1/A_0) \times 100$$

Autoaggregation assay

The assay was performed according to [11], with certain modifications. Overnight grown *Bacillus* culture at 37 °C in nutrient broth was pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get absorbance 0.5 at 595 nm (Anil, 2009). Cell suspension (4 ml) was mixed by vortexing for 10 s followed by incubation at 37 °C for 1 h. Then A_{595} of upper layer was measured [12]**.** Autoaggregation percentage was expressed as: $1 - (At/A_0) \times 100$, where At represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0.

III RESULT AND DISCUSSION

Gram's staining and Colony characteristics

All 11 isolated organisms obtained were Gram positive.

Isolated Organisms	Source	Colony characteristics	Gram's staining
F1	Fish	Small, round, opaque, flat, green color colony	Gram positive, rod in shape occurred in single.
F2	Fish	Medium, round, translucent, convex, light pink color colony.	Gram positive, rod in shape occurred single
F3	Fish	Large, round, translucent, flat, light green color colony.	Gram positive, rod in shape occurred single
F4	Fish	Large, round, translucent, flat, yellow color colony.	Gram positive, rod in shape, occurred in chain.
F5	Fish	Small, round, opaque, convex, yellow color colony.	Gram positive, rod in shape, occurred single.
F6	Fish	Small, round, translucent, convex, pink color colony.	Gram positive, cocci in shape, occurred in cluster.
F7	Fish	Large, round, flat, light green color colony.	Gram positive, rod in shape, occurred in single.
F8	Fish	Large, round, translucent, flat, Light green color colony.	Gram positive, rod in shape, occurred in chain.
F9	Fish	Small, round, opaque, convex, green color colony.	Gram positive, cocci in shape, occurred in cluster.

F10	Fish	Small, round, opaque, yellow color colony.	Gram positive, rod in shape, occurred in chain.
F11	Fish	Small, round, law convex, opaque, blue color colony.	Gram positive, rod in shape, occurred in single.

Table no. 1 Result of Gram staining and Colony characteristics of F1 to F11 organisms.

Catalase and Haemolytic assay

Some probiotic *Bacilli* strains produce catalase enzyme. In Catalase test, isolated strains F2, F3, F4, F5, F6, F9, F10, and F11 gave positive result. After the addition of hydrogen peroxide, it leads to bubble formation which indicates that organisms produced catalase enzyme and cleaved hydrogen peroxide in to water and oxygen.

In Haemolytic test, isolated organism F10 gave positive result by producing haemolyase enzyme. Enzyme cleaved the RBCs and gave clear zone around the colonies indicates that organism was pathogenic for the host and not applicable as probiotic

Isolated Organisms	Source	Catalase	Haemolytic activity
F1	Fish	Negative	Negative
F2	Fish	Positive	Negative
F3	Fish	Positive	Negative
F4	Fish	Positive	Negative
F5	Fish	Positive	Negative
F6	Fish	Positive	Negative
F7	Fish	Negative	Negative
F8	Fish	Negative	Negative
F9	Fish	Positive	Negative
F10	Fish	Positive	Positive
F11	Fish	Positive	Negative

Table no: 2 Isolates showing Catalase and Haemolytic activity

Hydrophobicity and Autoaggregation assays

The mean percentages for cell hydrophobicity of 9 probiotic *Bacilli* isolates are shown in Figure 1. In this study, isolated organisms F1, F2, F3, F6, and F7 showed highest hydrophobicity and this isolates were found to possess higher than 40% hydrophobicity and they were regarded as hydrophobic while the least hydrophobicity was found in isolates F4, F5, F8, and F10. Hydrophobicity is one of the property of probiotics. The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics bacteria because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization to exert the beneficial effects to the host [13].

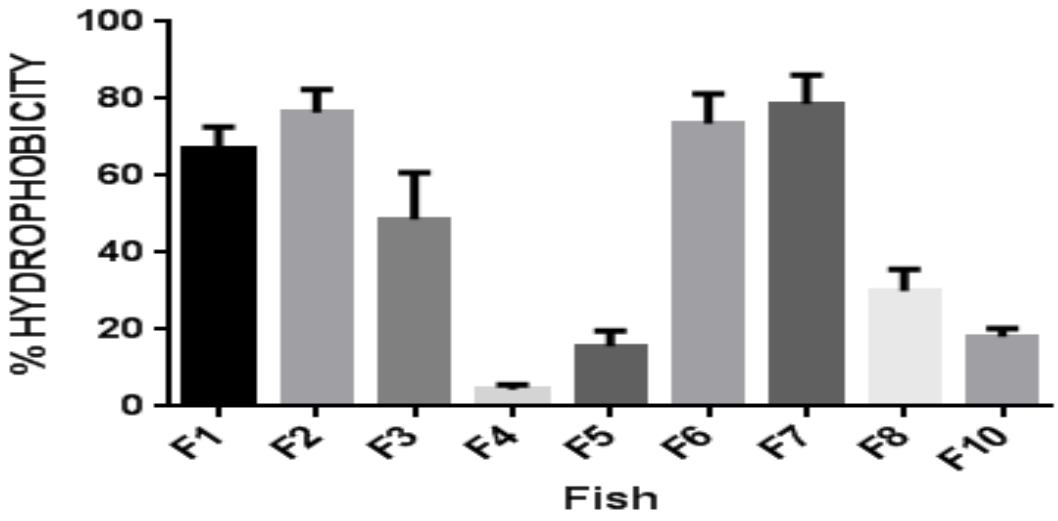

Fig.1. Hydrophobicity assay

The mean percentages for autoaggregation of 11 probiotic *Bacilli* isolates are shown in Figure 2. In this study, isolated organisms F1, F2, F3, and F7 showed a greater autoaggregation percentage compared to F4, F5, F6, F9, F10 and F11. Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. The ability of probiotic bacteria to form cellular aggregates is considered a desirable characteristic, as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or coaggregation with commensal organisms on the intestinal mucosa or by direct coaggregation with the pathogens to facilitate clearance [14][15].

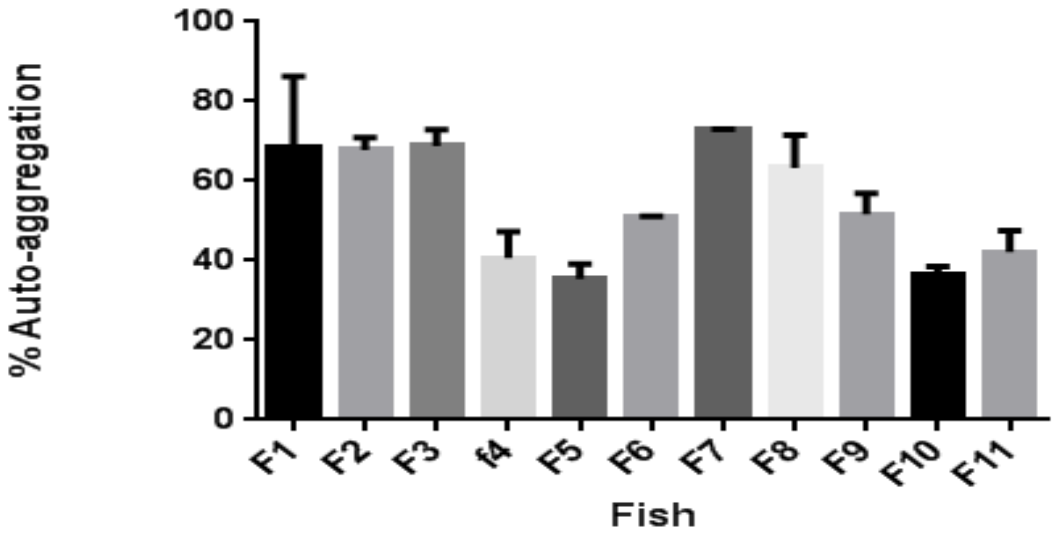

Fig.2. Autoaggregation assay

Bile salt assay

Bile salt tolerance is the probiotic trait. Isolated organism F1 tolerated the 2% concentration of bile salt and F3 - 5%, F4 - 3%, F6 - 1%, F7 - 1%, F8 – 1%, F9 – 2%, F10 – 3% concentration of bile salt was tolerated. probiotic organisms survive at maximum 15% concentration of bile salt in gut. Resistance to bile salt is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Acidity, presence of bile salts, and pancreatic enzymes in the gastrointestinal tract (GIT) are some of the major stresses that an orally taken probiotic encountered in the GIT. It is essential that a potential probiotic strain is able to tolerate these stress conditions in order to survive in the GIT. Detailed studies showed that bile salt tolerance of some probiotics is likely due to the presence of BSH and some transporter proteins, which are functionally related to each other to resist efficiently to the bile stress. Bile salt hydrolase (BSH, EC 3.5.1.24), which is an enzyme that catalyses the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and deconjugated bile salts [16]**.**

In this experiment, the most important probiotic property of desirable bacteria is their ability to remain viable in bile conditions of gastrointestinal tract. Among them only one isolated organism F3 tolerated 5% concentration of bile salt.

Isolated Org.	**Time Hrs.**	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**7%**	**8%**
F1	24hr	+++	+++	-	-	-	-	-	-
F2	24hr	-	-	-	-	-	-	-	-
F3	24hr	+++	+++	+++	++	+	-	-	-
F4	24hr	+++	+++	+++	-	-	-	-	-
F5	24hr	-	-	-	-	-	-	-	-
F6	24hr	+++	-	-	-	-	-	-	-
F7	24hr	½+	-	-	-	-	-	-	-
F8	24hr	++	-	-	-	-	-	-	-
F9	24hr	+++	++	-	-	-	-	-	-
F10	24hr	+++	++	++	-	-	-	-	-
F11	24hr	-	-	-	-	-	-	-	-

Table no. 3 Result of Bile salt tolerance assay.

- = No growth, + = Good growth, ++ = Very good growth, +++ = Excellent growth.

IV CONCLUSION

Bacillus sp. named as F3 was only one isolate of *Bacillus* probiotic species isolated from gastrointestinal tract of fish produced catalase enzyme, tolerate bile salt concentration up to 5% and also showed good Hydrophobicity along with auto aggregation activity. So, F3 was considered to be suitable for probiotic applications.

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