Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ANTAGONISTIC ACTIVITY OF *BACILLUS CEREUS* AGAINST HUMAN AND FISH PATHOGENIC BACTERIA

S.VIJAYARAM* AND S.KANNAN.

Dept of Environmental Studies, School of Energy Environment and Natural resources, Madurai Kamaraj University, Madurai.

ABSTRACT

The most commonly used probiotic is usually characterized as gram positive, non motile, non sporulating bacteria that produce lactic acid as their main by product due to fermentation. The Morphological, Biochemical, Acid and Bile salt tolerance, Haemolytic test are used for identification and conformation of probiotic bacteria. Isolated *Bacillus cereus* was analyzed using the agar diffusion method, in terms of their general inhibition effects against few human/fish pathogens. The inhibitory effects of *Bacillus cereus* isolates against human and fish pathogenic bacteria *Eshcherichia coli* (7mm), *Klebsiella* spp(8mm), *Bacillus* spp (5mm), *Proteus mirabilis* (7mm), *Serratia marcescens* (12mm), *Staphylococcus aureus* (7mm), *Vibrio harveyi* (20mm), *Vibrio parahaemolyticus* (10mm). In this research, antagonistic activities of *Bacillus cereus* species isolated from kudukunda (*Punitus melanampyx*) fish sample were studied.

KEYWORDS: Probiotics, Lactic acid bacteria, *Punitus melanampyx*, *Bacillus cereus*, Antibacterial activity , Periyar lake.

S.VIJAYARAM
Research Scholar, Dept of Environmental Studies,School of Energy Environment and Natural resources Madurai Kamarai Universitv. Madurai.Tamilnadu.India

***Corresponding author**

INTRODUCTION

The most commonly used probiotic are usually characterized as gram positive, non motile, non sporulating bacteria that produce lactic acid as their main by product due to fermentation[1]. The use of probiotic for aquatic animals is increasing with the demand for environment friendly sustainable aquaculture. Earlier studied, various strategies modulate the composition of the gut microbiota for better growth, digestion, immunity, disease resistance of the host and have been investigated in various livestock as well as in human beings[2] .The increased practice of aquaculture have led to a higher number of disease outbreaks with an increasing range of pathogens. Consequently, the extensive use of broad-spectrum antibiotics in aquaculture has led, as in other fields, to drug resistance problems [3]. In recent years, many studies have emerged with regard to the antimicrobial properties of strains of *Bacillus* [4-8]. Probiotics can provide an alternative way to reduce the use of antibiotics in aquaculture and simultaneously may avoid the development of antibiotic-resistant bacteria. Most probiotics that are proposed as biological control agents in aquaculture belongs to the lactic acid bacteria family [9]. Most studies have reported that the different strains of *Bacillus* sp. and lactic acid bacteria are found in fish and fish products [10-13].

MATERIALS AND METHODS

Sample collection

Lived Fish samples were collected at Periyar Lake, Kerala from the catchers (Tribal mannarkudi fishermen). The samples were randomly collected in pre-sterilized polyethylene bags containing the habitat water and transported to the laboratory. The fishes were identified using standard reference manuals [3].

Isolation of probiotic bacteria from fish gut

The *Punitus melanampyx* were washed with sterile distilled water to remove any undesired dusty matters. Then the fishes were dissected to remove the gastro-intestinal tract under sterilized conditions. The gastro-intestinal tract was homogenized using sterile distilled water and centrifuged at 13000 rpm for 10 min. After centrifugation, the supernatent was taken and serially diluted using sterile distilled water and plated on Nutrient Agar plates. Inoculated plates were incubated at room temperature for 24 hours. Selected individual colonies were inoculated in Nutrient Broth for further studies[4].

Table 1
Antibacterial activity for human and fish pathogens

Prothebiotic bacteria	Human pathogens and its codes	Fish pathogens
Bacillus cereus	*Escherichia coli*(MTCC1303)	*Vibrio harveyi*
	Klebshiella spp, (MTCC3384)	*Vibrio parahaemolyticus*
	Bacillus spp, (MTCC6428)	
	Proteus mirabilis (MTCC9493)	
	Serratiamarcescens (MTCC7103)	
	Staphylococcusaureus (MTCC7405)	

Test Micro-organisms

The probiotic bacteria isolated from the intestinal tracts of Kudukunda fish (*Punitus melanampyx*) were used in this study. It was done in the Department of Environmental Studies in Madurai Kamaraj University. The names and codes of the test bacteria are presented in Table 1. The human and fish pathogenic bacteria were activated through incubation in a NB for 24 h. The lactic acid bacteria were cultured in NB broth for 24 hours.

Gram stain

The Gram stain has been in existence for more than 100 years, and remains a key starting

point to identify microbial species. The stain makes use of the differing membrane structures between Gram positive (single cell membrane with a tough outer cell wall of peptidoglycan), and Gram negative organisms (have two layers of membranes, with a thin layer of peptidoglycan sandwiched between them).

Catalase Test

Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h of agar and fresh broth cultures were used for catalase test by dropping 3% Hydrogen peroxide (H_2O_2) onto randomly chosen colony and 1ml of overnight broth cultures. The isolates , which did not give gas bubbles, were selected as catalase negative [11].

Oxidase Test

A well-isolated colony was picked from a fresh (18- to 24-hours culture) bacterial plate. The filter paper which are coated by 1% Kovács oxidase reagent and dried. The bacterial plate was rubbed onto treated filter paper and observed for colour changes[10].

Simmons Citrate

The Simmons Citrate agar was inoculated by making a streak on the surface of the slant with an 18 to 24 hr old pure culture and incubated for up to four days at 20˚C - 24˚C. The results were observed when the growth medium changed the colour to blue-green or royal blue as positive whereas little or no growth and no colour change for negative [10] .

Indole Test

Tryptone broth was inoculated with a light inoculum from 18 to 24 hours pure culture and incubated for 24 to 48 hours at 20˚C -24˚C. At the end of 24 hours incubation, 2ml of sterile media was placed in an empty sterile test tube. The extra tube was stored for 48 hours incubation, if necessary. Five drops of Kovac's reagent was added to one of the tubes.

At the surface of media, cherry red ring was formed within 1-2 minutes and no colour change are considered as Positive and Negative results [10] .

Nitrate Reduction

The Nitrate broth was inoculated with 18-24 hour old pure culture and incubated for 24-48 hours at 20°C -24°C aerobically. After incubation about five drops of α-napthylamine and sulfanilic acid was added into the medium and shaken gently to mix reagents. When there was no colour developed added α napthylamine and sulfanilic acid and then added a small amount of zinc dust[10].

Arginine Hydrolysis Test

Arginine broth medium was dispensed in test tubes and autoclaved at 115°C for 10 minutes. Cultures were inoculated in arginine broth and incubated at 37°C for 48 hour. Few drops of Nessler's reagent were added. Brown color indicated the presence of enzyme arginine hydrolysis[10].

Amylase Test

Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 48 hours. After incubation iodine solution was flooded with dropper for 30 seconds on the starch agar plate[10].

Carbohydrate Fermentations

Isolates were characterized according to their fermentation profiles of ability to ferment 5 different carbohydrates. — carbohydrate solutions were prepared separately MRS broth without glucose and containing pH indicator chlorophenol red. Each sugar solutions were prepared at a final concentration of 2%. Also positive and negative controls were used to indicate the contamination. Inoculated 1% of cuture in tubes at incubated at 37˚C, the turbidity and the color change from purple to yellow recorded was positive fermentation results compared with the control [13].

Probiotic characterization

Not all the lactid acid bacteria posses the ability to confer health benefits for the host. Thus, it becomes necessary to screen and chacterize numerous strains to obtain ideal probiotics. For the determination of probiotic

properties of isolates these major selection criteria were chorused: Resistence to low p^H, tolerence against bile salt and the antimicrobial activity.

Acid tolerence

Resistence to low pH is one of the major characteristic feature of probiotic bacteria [14] since,the bacteria cope up with the presence of food stress in the stomach. In most in vitro assaypH 3.0 is conducive for the bacteria which even matche the stressful condition of pH 3.0. pH 3.0 in the stomach. Purpose of acid tolerenc is of prime factor for the probiotics to tolerate the high acidity levels in the stomach. Probiotic can tolerate the pH range of 1.5 to 3.0 in the gastro-intestinal tract[15].

Bile tolerence

Bile salt was secreted in small intestine. Presence of this bile salt creates a stressful condition for bacteria. Bile salt reduce the survival of bacteria by impairing the bacteria cell membrane which bears lipid and fatty acid as the major components. Blocking by the bile salts in the cell membrane of the bacteria affects the permeability physiology of the cell wall system[16].

Haemolytic assay

Haemolytic assay will be screened as per the procedure described[17]. Isolates were screened on blood agar plates containing 5% sheep blood and incubated at 37°C for 48 hours. Hemolytic activity was scored as the presence of a clear zone around bacterial colonies

Inhibitory Effect by Agar-disc Diffusion Method

The inhibitory effects of isolates on test bacteria were determined by agar-disc diffusion method[14]. Similarly, the inhibitory effects of the lactic acid bacteria on the *Bacillus* isolates were analyzed by using the same method. All the bacteria were incubated at the appropriate temperature and medium for 24 hours. Nutrient agar media (20 ml) were poured into each sterile petri dish (100 mm diameter). Hundred microlitre suspensions of target strain cultured for 24 hours were spread on the plates, and discs of 0.5 mm diameter were placed.The probiotic culture was poured into the disc. The inoculated plates were incubated for 24 hours at their optimum growth temperatures and the zone of inhibition was measured with calipers in mm. The measurements were done from the edge of the zone to the edge of the wall.

RESULTS AND DISCUSSION

Bacillus cereus species were isolated from Kudukunda fish sample. In the identification tests, *Bacillus cereus* strains were identified as 16s rRNA gene sequencing. This study examined the antagonistic activities of the *Bacillus cereus* strains against the test bacteria. *Klebshiella* spp*, Escherichia coli, Bacillus* spp*,Proteus mirabilis, Serratiamarcescens, Staphylococcus aureus* and fish pathogenic bacteria *Vibrio harveii* and *Vibrio parahaemolyticus*. This study revealed that the isolates used have no inhibitory effects regarding *Escherichia coli* (MTCC1303), *Klebshiella* (MTCC3384), *Bacillus (*MTCC6428), *Proteus mirabilis (*MTCC9493), *Serratiamarcescens (*MTCC7103), *Staphylococcus aureus (*MTCC7405). *Bacillus cereus* strain isolated from fish was active against most Gram-positive bacteria but inactive Gram-negative bacteria. The inhibitory effects of *Bacillus cereus* isolates against human and fish pathogenic bacteria *Eshcherichia coli* (7mm), *Klebsiella spp* (8mm), *Bacillus spp* (5mm), *Proteus mirabilis* (7mm), *Serratiamarcescens* (12mm), *Staphylococcus aureus* (7mm), *Vibrio harveyi* (20mm), *Vibrio parahaemolyticus* (10mm). In previous findings it was reported that bacteria were isolated and characterized the Lactobacillus from two species of sturgeon fish inhabiting Caspian Sea. These fishes were highly valuable species for fisheries and aquaculture management in Iran. Presumptive lactobacilli species found in this study were relatively similar to the species described by Bucio Galindo *et al*. (2006). These authors reported *L. alimentarius, L. coryneformis, L. casei, L. sakei, L. pentosus, L. plantarum, L. brevis* and

L. oris, as *lactobacilli* presented in the intestinal content of studied fish. This study was aimed to investigate the intestinal Lactobacillus in fish; with the goal of selecting a strain to be used as a feed supplement for warm freshwater fish. Consequently, the discovered *lactobacilli* in this study can be used as efficient probiotic bacteria. They should resist processing and storage conditions and be alive and active beyond the gastrointestinal passage in the different organ system of the body through vascular system. It's notable that selection of probiotic strains is achieved by screening procedures for several characteristics in vitro, such as resistance to gastric secretions, bile tolerance and growth in faecal matter. The bile tolerance ability of isolates was checked with various concentrations such as 0.3%, 0.5%, 1.0% of bile salt. It showed not only viable but also proliferation in all three concentrations for all the incubation periods. As bile salt concentration increased, the growth rate of LAB decreased significantly. *L. mesenteroides sp*. Mesenteroides showed not only viable but also proliferation in all three concentrations for all the incubation periods[18]. (A similar trend with more proliferation was observed at 4 and 8 h incubation periods). Furthermore, the strain *Bacillus cereus* showed a better ability to survive and grow in bile salt. Bile salt tolerance is essential for probiotic bacteria to grow and survive in the fish intestine[16 17] Determined that *L. plantarum* as a probiotic could survive in 0.3% of bile salt. [19]reported the growth of *L. plantarum* pH 0.4 for bile salt ranging from 0 to 0.4%. In addition *Lactobacillus fermentum* and *L. plantarum* isolated from intestine of rainbow trout were examined for growth at 2.5 to 10% extracted bile from the fish gall bladder. They tolerated bile concentration for 1.5 and no significant changes which is evident from the viable counts as observed by [20] Perusal of literature reveals that all the bacterial cells were killed at 0.2% and even in higher percentage[21]. Comparing to this study, our experiments showed much more resistance by bacteria to the detrimental actions of bile salts where the viability of strains seemed to improve with increase in bile concentration. The probiotics isolated could tolerate low pH and bile salt means they not only can transit through stomach and be active in intestine but also have tendancy to survive in stress conditions. Moreover the protective effect of food matrix may also prevent the bacteria from bile exposure and hence increase the resistance of the bacterial strains. The results of the present study showed that the isolated strains *Bacillus cereus* are suitable to act as probiotics however, extensive study is required to further characterize the efficiency of the isolated strains by invitro. Review of literatere cited highlighted that the probiotic applications in the animals studies which guide the researchers towards the implementation of the isolated bacterial strains as supplementary food in the field of aquaculture.

Gram staining for microscopic images

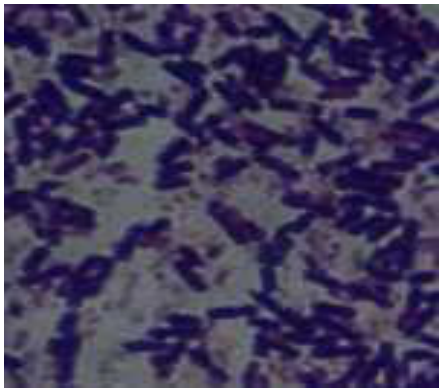

Table 2
TEST MICROORGANISMS

Probiotic bacteria	Human pathogens	Zone of inhibition (mm)	Fish pathogens	Zone of inhibition (mm)
Bacillus cereus	*Escherichia* coli MTCC(1303)	7mm	*Vibrio harveyi*	20mm
	Klebsiella spp MTCC(3384)	8mm	*Vibrio parahaemolyticus*	10mm
	Bacillus spp MTCC(6428)	5mm		
	Proteus mirabilis MTCC(9493)	7mm		
	Serratiamarcescens MTCC(7103)	12mm		
	Staphylococcus aureus MTCC(7405)	7mm		

Table 3
Growth at various temperatures

Test	*Bacillus cereus*
Growth at 10 ºC	No growth
Growth at 15 ºC	No growth
Growth at 25 ºC	Growth seen
Growth at 37 ºC	Growth seen
Growth at 45 ºC	Growth seen
Growth at 50 ºC	No growth
Growth at 65 ºC	No growth

Table 4
Physiological and morphological characterization of probiotic bacteria

Test	*Bacillus cereus*
Gram Staining	Gram positive
Morphology	Rod shaped
Motility	Non-motile

Table 5
Biochemical characterization of Bacillus cereus

Test	*Bacillus cereus*
Catalase	Negative
Oxidase	Negative
Gas production	Negative
Nitrate Reduction	Positive
Arginine Hydrolysis	Negative
Citrate	Negative
Indole	Negative
Methyl Red	Negative
Vogues Proskauer	Positive
Amylase	Positive
Glucose	Positive
Mannitol	Negative
Lactose	Positive
Fructose	Positive

Acid tolerance

The acid tolerance test is used for survival of the bacterial growth in acidic condition. Survival in the extreme low pH is one of the major selection criteria for probiotic strains. Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays pH 3.0 has been preferred.

Organisam name	1.5	2.5	3.5	4.5
Bacillus cereus	.025	.036	.047	*.053*

Tolerance against Bile

The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v. strains that are able to tolerate bile salt was checked by growing them in concentration of bile salts 0.3% for the growth period of bacterial strain was observed at 560nm. The results of the present study reveals that the growth of both the isolates were more in 0.3% of bile salt where as growth of bile salts were less. According to the result *Bacillus cereus* isolate were resistant to 0.3% bile salt.

Organisam name	0 hours	2 hours	4hours
Bacillus cereus	.044	.030	.015

Antimicrobial activity

Among the bacteria used as probiotics, lactic acid bacteria have an important role because they are beneficial to human and animal health [15, 16]. Probiotic strains should have a desirable antibiotic resistance and sensitivity patterns, be antagonistic towards potentially pathogenic microorganisms, and have metabolic activities beneficial to the well fare of the fish species and the human intestine[22].In the present study, the different strains of microbes such as *Escherichia coli* (MTCC1303), *Klebshiella* (MTCC3384), *Bacillus (*MTCC6428), *Proteus mirabilis (*MTCC9493), *Serratia marcescens (*MTCC7103), *Staphylococcus aureus (*MTCC7405). *Vibrioharveii*, *Vibrio parahaemolyticus* cultures were used to investigate the antibacterial activity against the human and fish pathogens *(*Microbial Cultures are Collected From Centre For Marine Science and Technology, Rajakamangalam,Tamilnadu,India).The other lactic acid bacteria inhibited the growth of the *Bacillus* strains at different levels of inhibition zones. It is concluded that *Bacillus cereus* isolated from the intestinal tracts of kudukunda fish showed antimbacterial activity against some human pathogen/fish pathogenic bacteria. Also, it has been found that lactic acid bacteria isolated from the intestinal tracts of fish have inhibitory effects against *Bacillus cereus*.

ACKNOWLEDGEMENT

This study was funded by UGC-MRP scheme for the financial assistance and also the laboratory facilities obtained through DST-PURSE programme of Madurai Kamaraj University.

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