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Research Article

SCREENING OF POTENTIAL PROBIOTICS AGAINST FISH PATHOGENS UNDER AQUACULTURE CONDITIONS

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ABSTRACT

Probiotics, live cells with different beneficiary characteristics, have been extensively studied and explored commercially in many different products in the world. This study was aimed to isolate potential probiotic bacterial isolates from different sources and to study its inhibitory properties against fish pathogenic bacteria. Isolation of bacteria from curd and milk samples and diseased fishes were conducted on MRS agar, nutrient agar and BHI agar respectively. The identifications were carried out using, morphological and biochemical and molecular methods. The two bacterial isolates obtained were then checked for antagonism within them and against the fish pathogen. Optimization studies for pH, temperature, carbon and nitrogen sources were also conducted for these two probiotic strains. The isolates obtained from curd, milk and diseased fishes were morphologically and biochemically identified as *Bacillus subtilis*, *Lactobacillus acidophilus* and *Vibrio alginolyticus* respectively. *Bacillus subtilis* and *Lactobacillus acidophilus* showed no antagonism against each other whereas their bacterial consortium found to have excellent antagonism against *Vibrio alginolyticus*. Optimization of various parameters found to be associated with the increased production of *Bacillus subtilis* and *Lactobacillus acidophilus.* Probiotic properties were assayed including acid tolerance, pH tolerance, temperature tolerance and estimation of lactic acid produced. Thus the isolated bacteria can be further used in the development of probiotic therapy for fishes.

INTRODUCTION

Fish being a potent source of protein, makes it an important ingredient in food by the people globally. According to the United Nations Food and Agriculture Organization, the growth of aquaculture sector is higher than any other types of animal food production systems (www.fao.org).With the intensive hike in the production, this sector is also facing multifarious problems. Disease outbreaks are being progressively recognized as a significant constraint on aquaculture production and trade, disturbing the economic development of the sector in many countries. The conventional approaches, such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic disease (Maragkoudakis *et al.,* 2006; Subasinghe, 1997). Furthermore, there is a growing concern about the use and, particularly, the abuse of antimicrobial drugs not only in human medicine and agriculture but also in aquaculture. Several alternative approaches to the use of antimicrobials in disease control have been proposed and have already been applied very successfully in aquaculture (Hernandez and Zarate, 2005; Labeer*et al.,* 2008). Probiotics, as per definition, are live cells with exceptional beneficiary characteristics and had been significantly studied and explored commercially in many different products inside the global. They are blessings to human and animal health has proven in hundreds of scientific studies.

The rising cognizance of the health benefits of consuming microorganisms as probiotics has encouraged consumer's worldwide (Patel *et al.,* 2009). Over the last 10 years there has been collective public and scientific interest in the administration of these live micro-organisms to prevent or treat disease. Under natural conditions, a defensive gut microflora develops and there is no need for a bacterial supplement. But the changing food habits and routines force us to take processed and sterile food, which affects our access to, and colonization, by some type of bacteria. Lactic acid bacteria (LAB) are potentially promising because they produce bactericidal bioactive agents that are able to control the growth

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of the pathogens including the fish pathogens. Beneficial effects discoursed by LAB, including inhibition of gram negative and positive pathogenic bacteria described by Maragkoudakis *et al*., (2006) and Charlier *et al*., (2008). Confirming the antimicrobial activities of probiotics will affirm their use in the development of functional foods for the betterment of the health of the consuming health (Eduardo *et al.,* 2003).

The aim of the study was to provide a method for screening of potential probiotics against fish pathogens under aquaculture conditions. The isolated strains, *Lactobacillus acidophilus* and *Bacillus subtilis,* in the present study, exhibited very remarkable and noticeable antimicrobial activity against one of the fish pathogenic bacterium *Vibrio alginolyticus*. Antagonism studies have been done to check the antimicrobial activity against the fish pathogen. Consortium of the two microbial isolates was formulated to check the increase in efficiency against *Vibrio alginolyticus* which could be a potent innovative strategy that can be accomplished in the industry of aquaculture.

MATERIALS AND METHODS

Screening of Probiotic Bacteria

Collection of Samples

This study was conducted at Maharajas College, Ernakulam, Kerala. The different samples were collected that included curd samples and milk samples that are commercially available at neighbouring milk parlours. Infected fish samples were also collected from local markets for screening pathogenic bacteria. These samples were collected in clean, sterile, wide-mouthed containers, without disinfectant or detergent residue and tight-fitting leak-proof lids. Immediately after collection, the samples were transferred to the laboratory for microbiological analysis and stored aseptically in low temperature (4^0 C) refrigerator to protect from contamination and deterioration.

Isolation of Lactic Acid Bacteria (LAB)

Milk and curd samples were used for the isolation of LAB. The sample (1mL) were serially diluted upto 10^{-7} using 0.9% sterile saline. Pour plating was then carried out using their selective media, MRS agar From four dilutions (10^{-1},10^{-3}, 10^{-5}, 10^{-7}). All plates were then incubated at 25-30^{o}C for 2 days. The isolates were further subculture on to De Man, Rogosa and Sharpe (MRS) agar slants in order to obtain pure culture. Pure isolates were maintained at 4^{o}C in refrigerator for further studies. Further identification of LABs were done using dichotomous key of Bergey's Manual of determinative Bacteriology.

Screening of disease Pathogen from Fishes

Bacterial Culture

Five infected fishes with fin rot and tail rot, ulceration, haemorrhagicsepticaemia, and abdominal distention were collected from different areas of Ernakulum district and externally disinfected. The infected area was swab carefully and streak on to the brain heart infusion agar plate (BHI).The plate was incubated at 27^{o}C for 24 hours.

Morphological Identification of bacterial pathogens

Microscopic observation
Gram Staining

Into a clean glass slide, a loopful of bacterial suspension was taken and spread into a thin, uniform smear. The smear was air dried and heat fixed. It was first stained by primary stain, crystal violet for 1 minute and next by gram's iodine for 1 minute. Then decolorized by acetone and then counter stained with safranin for 20-30 seconds. The smear was then air dried and viewed under oil immersion objective.

Motility test

A drop of bacterial suspension was placed on a coverslip. Onto the four sides of the coverslip, apply petroleum jelly. A cavity slide with the concavity facing down was placed over the coverslip and then rotated upside down quickly. Then the slide was observed under 45 x objectives to examine for motility in the edge of the drop.

Biochemical tests

The picked colonies (isolates) were selected for further identification by standard procedures. All the isolates were subjected to the biochemical test to identify the isolates. IMViC Tests, catalase and oxidase tests and sugar fermentation tests are carried out for the biochemical analysis of the selected isolates.

Antagonism study

The initial screening of antagonism by the two types of Lactic acid bacteria was done by the agar well diffusion plate assay (Bauer *et al.,* 1966).

Antagonism between probiotics

Growth inhibition study was done on microbial isolatesby well diffusion assay. For the preparation of inoculum, a loopful of each organism was inoculated separately into 5mL peptone water. After 4-5 h of incubation of 37^{o}C, 1mL of each organism was inoculated into 10mL of their respective selective media (MRS broth and nutrient broth). Antagonism was screened by loan culture of isolated microorganismson the respective agar plates. Wells were cut into the agar and filled with 100 µL of bacillus and vice versa. The presence of antimicrobial metabolites produced by the isolates inhibited the growth of the other, producing a zone of inhibition around the well.

Antagonism of probiotics to pathogen

For the preparation of consortium, a loopful of each organism was inoculated separately in to 5mL peptone water. After 4-5 h of incubation of 37^{o}C, 1mL of each organism were inoculated into 25mL of their respective selective media (MRS broth and nutrient broth) and agitation at 120 rpm at room temperature for 48 h. Pathogenic isolate was cultured in brain heart infusion agar plate and incubated at 28^{o}C overnight after incubation, a single colony was inoculated in to 5mL peptone water for 4-6 h and transferred to loan culture on nutrient agar plate.Wells were cut into the agar and filled with 100 µL of consortium. After 48 h, zones of inhibition were recorded as being present or absent.

Optimization of culture conditions and culture medium for probiotics

Optimization of incubation period

The growth of both bacterial isolates was studied at various incubation periods at intervals of 24 hours and was determined for 96 hours. The isolates were inoculated into 50mL culture medium and were kept at 30°C. The growth was measured using Absorbance at600 nm.

Optimization of pH

Effect of pH on the growth of both isolates were studied by inoculating a single bacterial colony on to their respective medias like nutrient broth and MRS media varying a range of pH from 6 – 9.

Effects of Various Concentrations of Different Nitrogen Sources

Effect of various concentrations of different nitrogen sources 1-5% (beef extract, peptone, and yeast extract) on the growth of isolates was determined at 48 hours.

Effects of Different Concentrations of Carbon Source

MRS media with different concentration of glucose (1-5%) were prepared and inoculated with corresponding isolates and incubated at 30°C for 48 h on a rotary shaker at 120 rpm and growth was measured from the absorbance at A_{590} in a spectrophotometer to find out the optimal range.

Screening for Probiotic Properties of Lactic Acid Bacteria

Bile Tolerance Assay

Bile tolerance of bacterial isolates was screened by inoculating them in MRS (De Man, Rogosa and Sharpe) medium supplemented with bile salts of increasing concentrations: 1%, 4%, & 6%. The culture tubes were incubated at 37°C for 24 hours. After incubation, 1 mL aliquot of control (organism alone) and test (with varying concentrations of bile salt) were withdrawn and their optical densities were recorded at 600 nm. The highest concentration of bile salt supporting bacterial growth was defined as the tolerance level. The graph was plotted accordingly to represent the quantitative data.

Temperature Tolerance Assay

Temperature tolerance of bacterial isolates was screened by inoculating them in nutrient broth medium. The culture tubes were incubated at -20°C, 4°C, 25 °C & 40°C for 24 hours. After incubation, 1 mL aliquot of the control (organism alone) and test were withdrawn and their optical densities were recorded at 600 nm. The highest 'concentration of NaCl' to 'temperature' supporting bacterial growth was defined as the temperature tolerance level. The graph was plotted accordingly to represent the quantitative data.

pH tolerance assay

pH tolerance of bacterial isolates was screened by inoculating them in nutrient broth medium and adjusting the pH levels at 2,4 & 6 (acidic). The culture tubes were incubated at 37°C for 24 hours. After incubation, 1 mL aliquot of the control (organism alone) and test (with varying pH) were withdrawn and their optical densities were recorded at 600 nm. The highest pH that supports the bacterial growth was defined as the optimum pH. The graph was plotted accordingly to represent the quantitative data.

Lactic acid estimation

Titration of the bacterial culture supernatant against 1 N NaOH was done. Phenophthalein was used as indicator and the end point was determined by color change from yellow to pink. Lactic acid present in the supernatant was calculated as:
Lactic acid (in mg/mL) = 0.9V, where V=Volume of NaOH required till end point

Molecular Characterization of Lactic Acid Bacteria

Genomic DNA was isolated and purified (Ausubel *et al*., 1987). A portion of the 16S rDNA was amplified using a primer pair for 16S rDNA. The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al*., 1990).

RESULTS AND DISCUSSION

The aim of this study was to provide a method for the screening of potential probiotics that could be applied for fish pathogens under aquaculture conditions. To accomplish this, the growth of probiotics proved the antagonism effects against the fish pathogens and also has been suggested for enhancing the ability to stimulate the immune system.

Isolation and Identification

In accordance with the colony characteristics, the colonies were obtained from the milk was similar to *Bacillus sp*, the colonies obtained from the curd showed similarity to *Lactobacillus sp.* Similarly, coloniesobtained from infected fishes (Fig 1) matched to the characteristics of *Vibrio sp*. These were confirmed as the initial screening. *Bacillussp* and *Lactobacillussp* were Gram positive bacilli and *Vibriosp* was Gram negative comma shaped rods and the biochemical reactions of these isolates summarized in Table 1.Figure 1 demonstrate the infected fishes.

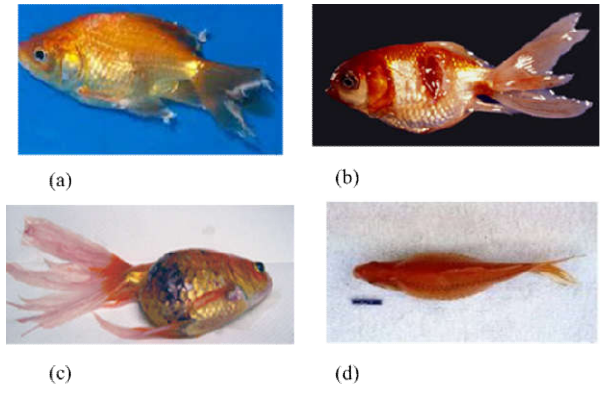

Figure 1(a-d) Clinical features of infected fish

Antagonism effects

Antimicrobial secondary metabolites produced by two probiotics were screened for antagonism using well diffusion method and no zones of inhibition (Fig.2) were observed. In this study, the probiotic modes of competition such as fast growth and ability to attach to the intestinal tract can be

considered together with the probiotics ability to produce substances antagonistic to pathogens.

Table 1 Biochemical reactions of isolates

Gram staining	+verod	-verod	+verod
Motility	-	+	+ve
Glucose	+	+	+
Lactose	+	-	+
Sucrose	+	+	+
Maltose	+	+	+
Indole	-	+	+
Methyl red	-	-	+
VP	-	+	-
Citrate	-	-	-
Urease	-	-	-
Catalase	-	+	+
Oxidase	-	+	-
TSI	A/K	K/K	A/K with H_2S production
Suspected Identity of Microorganism	***Lactobacillus sp***	***Vibriosp***	***Bacillus sp***

* +ve - Positive, -ve - Negative K/K, Alkali/Alkali, A/K: Acid /Alkali

The consortium of probiotics showed inhibitory zones or inhibitory activity against the growth of pathogen obtained from infected fishes (Fig.3).Antagonistic compounds are defined as chemical substances produced by bacteria that are toxic or inhibitory towards other microorganisms. These substances may be produced as either primary or secondary metabolites and therefore have different modes of inhibitory action.

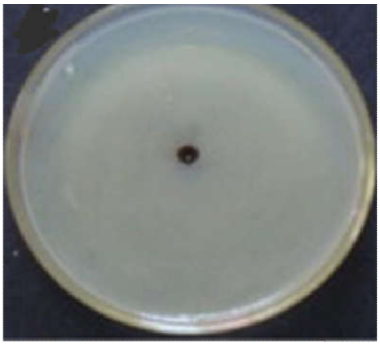

Fig 2 Antagonism between probiotics

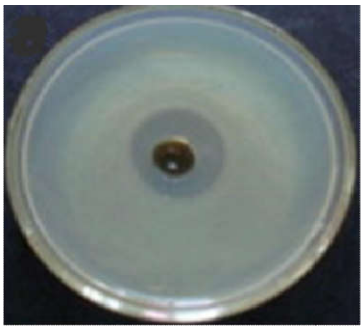

Figure 3 Antagonism of probiotics to pathogen

Optimization for the biomass production of probiotics

Optimization for the production of Bacillussp

The growth curve of the bacterium was determined by measuring the OD at 550 nm at 24 h interval for 96h.The growth increased till 72 h and after 96 h, it decreased. Hence maximum growth appears to be between 48 h and 72 h.

To find out the optimum range of each parameter such as pH and nitrogen source were suggested that the optimum pH was 8 and the optimum range was 7-8.Peptone was used for the optimum concentrations of nitrogen sources (0.1 % - 1%) (Figures 4, 5& 6).

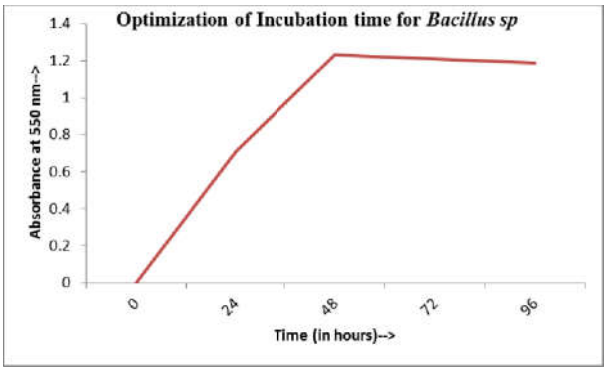

Fig 4 Optimization of incubation time for *Bacillus sp*

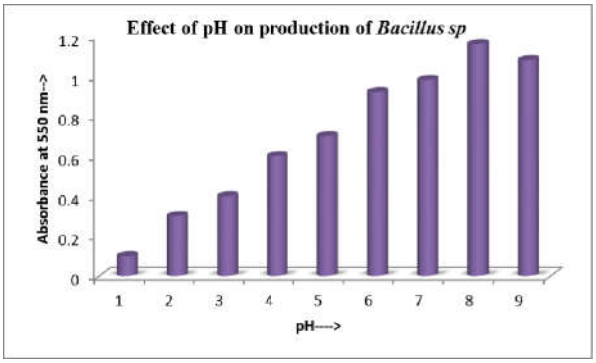

Fig 5 Effect of pH on *Bacillus sp* production

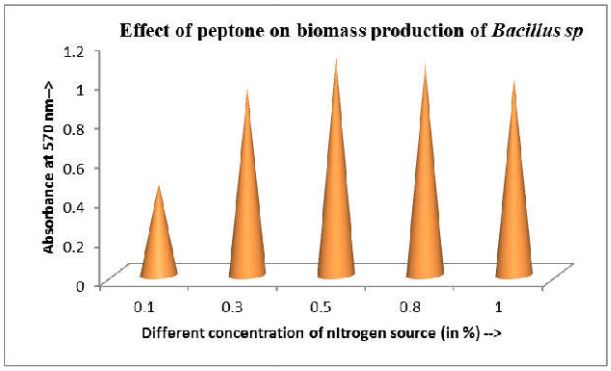

Figure 6 Effect of peptone on Biomassproduction of *Bacillus sp*

Optimization for the production of Lactobacillussp

The growth curve of the lactobacillus was determined by measuring the OD at 590nm at 24 hrs interval for 96hrs.The growth increased till 72hrs and after 96hrs it decreased. Hence maximum growth appears to be between 72hrs and 96hrs (Figures 7, 8, 9).

Optimal conditions and their performance in terms of contribution to achieving maximum yield for the production of lactobacillus .It can be seen from the table that factors such as pH and carbon source play more significant roles in product formation. To find out the optimum range of each parameter were suggested that the optimum pH was 5 and the optimum range was 5-6 in the case of carbon optimum was 1.5% the range was 1-1.5%.

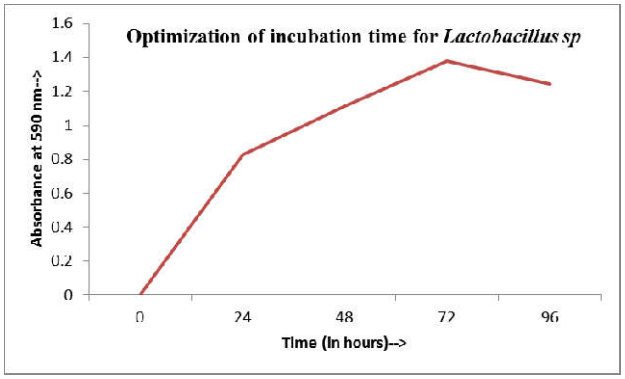

Figure 7 Optimization of incubation time for *Lactobacillus sp*

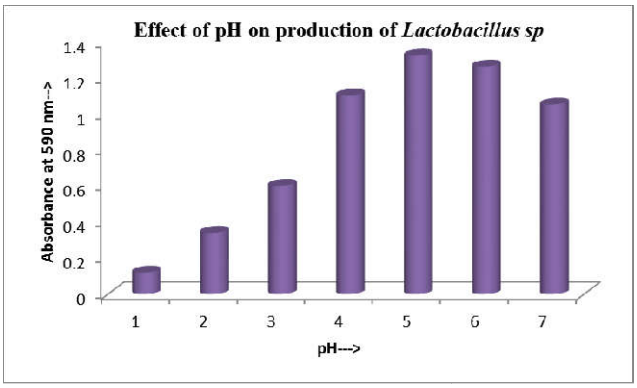

Figure 8 Effect of pH on *Lactobacillus sp* production

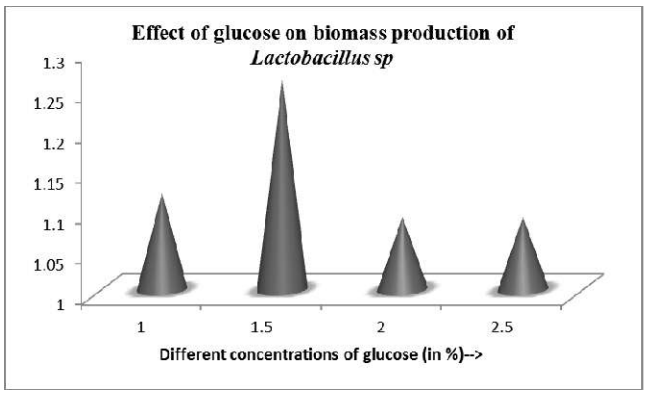

Figure 9 Effect of Glucose on biomass production of *Lactobacillus sp*

Screening for probiotic properties of Lactobacillus sp and Bacillus sp

Bile tolerance assay

Bile tolerance assay was carried out and the results showed that, for *Bacillus sp*, greater the bile concentration, greater the growth shown on nutrient agar (1% > 4% > 6%). On the other hand, *Lactobacillus sp* showed more growth in low bile concentrations (1%<4% < 6%)(Figures10 &11).

Temperature tolerance assay

Temperature tolerance assay was performed and found that the optimum temperature for *Bacillus sp* was 25°C and for *Lactobacillus sp*, it was 40°C, even though both of them showed growth in all temperatures assayed (Figures 10 &12).

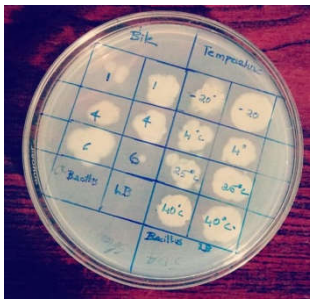

Figure 10 showing the growth of *Bacillus sp* and *Lactobacillus sp* after bile tolerance and temperature tolerance assays on nutrient agar

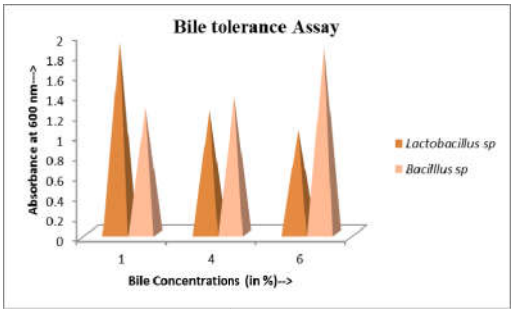

Figure 11 showing the growth of *Bacillus sp* and *Lactobacillus sp* after bile tolerance assay

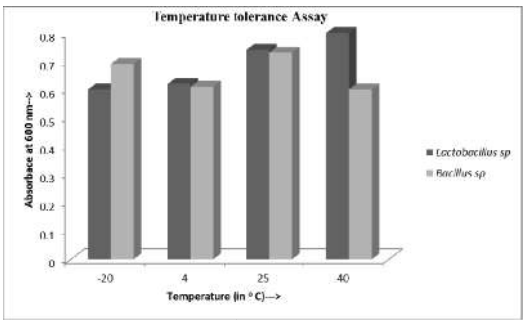

Figure 12 showing the growth of *Bacillus sp* and *Lactobacillus sp* after temperature tolerance assay

pH tolerance assay

On optimization of pH, it was found that *Lactobacillus sp* was able to grow in all acidic conditions (pH 2, 4 & 6) whilst for *Bacillus sp*, higher acidic conditions (6 > 4 > 2) favored the growth (Figures 13& 14).

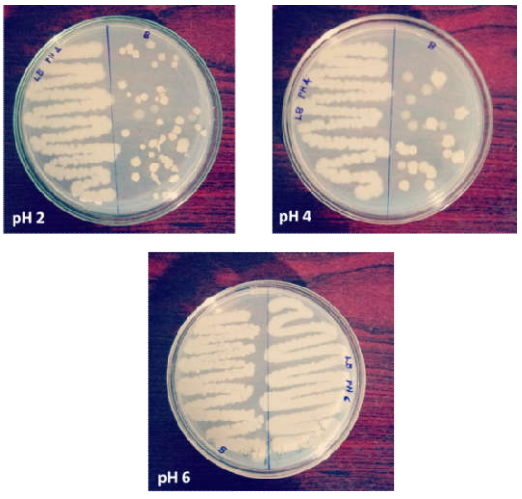

Figure 13 showing the growth of *Bacillus sp* and *Lactobacillus sp* after pH tolerance assay on nutrient agar

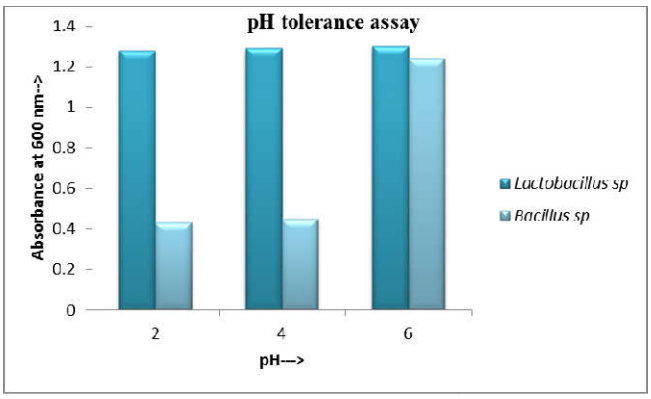

Figure 14 showing the growth of *Bacillus sp* and *Lactobacillus sp* after pH tolerance assay

Lactic Acid estimation

Lactic acid produced was found to be 2.89 mg/mL for *Lactobacillus sp* and 1.62 mg/mL for *Bacillus sp.*

Molecular Identification

Genomic DNA was isolated, purified and quantified. The agarose gel electrophoresis of genomic DNA isolated was performed is represented in figure 15.PCR based 16S rDNA amplification and sequence analysis thereafter, was used for molecular characterization of the biofilm formers. Following BLAST the identity of the biofilm formers was determined and the sequence data was submitted to the NCBI database. The 16S rDNA analysis revealed that the organisms were *Bacillus subtilis, Lactobacillus acidophilus and Vibrio alginolyticus.*

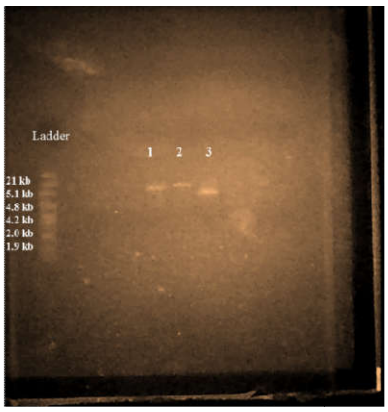

Figure 15 Agarose gel electrophoresis of genomic DNA isolated from 3 isolates Ladder- Lambda DNA / EcoR1/Hind III/ Double digest, 1- *Lactobacillus acidophilus,* 2- *Bacillus subtilis*, 3-*Vibrio alginolyticus*

Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. The prevention of disease transmission and enhancement of growth and feed efficiency are critical in modern animal husbandry, there has been widespread incorporation of antibiotics into animal feeds in many countries (Doyle *et al.,* 2006). The worldwide increase in bacterial resistance to antibiotics (Van der Waaij & Nord 2000) has stimulated investigations into the use of probiotics. In aquaculture, antibiotics are discharged into the environment causing the occurrence of resistant bacteria on fish farms or in the sediment below net cages (Miranda & Zemelman 2002). During the last few decades, the public has become increasingly alarmed by new scientific data that make their way into the popular media about the connection between the overuse of antibiotics in both medicine and the agriculture-agrifood industry and the emergence and spread of antibiotic-resistant bacteria. Microbial resistance to antibiotics is on the rise (Khachatourions, 1998). The increase in the anxiety about antibiotic-resistant microorganisms has led to suggestions of alternative disease-prevention methods, such as bacterial probiotics.

The production of inhibitory metabolites depends on the media upon which the bacteria are cultured (Olsson *et al.,* 1992), culture methods and media which eliminate or reduce the loss of these selective criteria need to be used. Nikoskelainen *et al.,* (2001b) found that the ability of three lactic acid probionts to produce the antimicrobialcompounds antagonistic towards fish pathogens diminished over time. Although it is possible that the lack of antimicrobial production was due to subculturing, the authors attributed it in part to different screening methods. Ring *et al.*, (2004) suggested that the ability of probiotics to produce metabolites which inhibit the growth of other microorganisms can be lost due to storage and sub culturing. Therefore, ongoing in vivo testing may ensure that the efficacy of the probiotic remains at a level beneficial to the larvae.

The probable mode of action of probiotics include competitive exclusion, i.e., the probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and/or space, alteration of microbial metabolism, and/or by the stimulation of host immunity. Probiotics may also stimulate appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet, and by the breakdown of indigestible components. Enhancement of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both in vitro and in vivo through several different mechanisms. The range of probiotics examined for use in aquaculture has encompassed both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts and unicellular algae. Aerobic Gram-positive endospore-forming bacteria, i.e. *Bacillus* spp., have been evaluated as probiotics, with uses including the improvement of water quality by influencing the composition of waterborne microbial populations and by reducing the number of pathogens in the vicinity of the farmed species (Wang *et al*., 1999). Thus, the bacilli are thought to antagonize potential pathogens in the aquatic environment. This is curious because it is generally accepted that laboratory cultures do not survive well when re-introduced into the natural environment; the cells being often outcompeted/antagonized by the natural microflora (Irianto & Austin, 1988). Nevertheless, a direct benefit to the use of the bacilli was the reduction in the use of chemicals in the aquatic environment and in enhanced growth of the farmed species (Wang *et al.,* 1999).

The lactic acid producing bacteria usually have no mobility and are nonsporulating bacteria that produce lactic acid. Some members of this group contain both rods (*Lactobacilli and Carnobacteria*) and cocci (streptococci). Different species of lactic acid bacteria (such as *Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Enterococcus, Vagococcus, Lactobacillus, Carnobacterium*) have adapted to grow under

widely different environmental conditions. They are found in the gastrointestinal tract of various endothermic animals, in milk and dairy products, seafood products, and on some plant surfaces. Gram-negative bacteria *Pseudomonas fluorescens* has been reported to inhibit *Saprolegnia* sp. and *A. salmonicida* in finfish culture (Gatesoupe, 1999).

Several researchers have reported on application of probiotics for aquaculture (Moriarty, 1998). The use of probiotics as farm animal feed supplements dates back to the 1970s. A widely accepted definition is taken from Fuller (1987), who considered that a probiotic is a cultured product or live microbial feed supplement, which beneficially affects the host by improving its intestinal (microbial) balance. The important components of this definition reflect the need for a living micro-organism and application to the host as a feed supplement. They were originally incorporated into feed to increase the animal's growth and improve its health by increasing its resistance to disease. The use of probiotics in animal nutrition is well documented and recently, they have begun to be applied in aquaculture (Verschuere *et al.,* 2000; Irianto and Austin, 2002; Bachere, 2003).

According to an earlier study (Usman & Hosono, 1999), the survival rate at pH 3.0 is considered as optimal acid tolerance for probiotic strains. All strains from this study were able to tolerate pH 2.0 – 6.0 with a survival percentage of more than 60%, and thus they can be considered as acid-tolerant LAB strains. This tolerance capacity of lactic acid bacteria justified the production of various antimicrobial substances which might create unfavorable conditions for the growth of different pathogens as well as toxigenic and spoilage organisms in humans and/or animals (van der Meulen *et al.,* 2007).

Bile salt tolerance is usually considered a basic property for LAB strains to survive in the small intestine. In the human gastrointestinal tract, the mean bile concentration is about 0.3% and is considered vital and high enough to screen resistant strains (Gilliland *et al.,* 1985). In this study, there was extreme variability of resistance to bile salts in *Lactobacillus sp* and *Bacillus sp strains*, which grow and metabolize in normal physical bile concentration, could survive in gastrointestinal tract (Sanders 1996). Both tested strains indicated a proportion of growth above 65% in the presence of bile salt concentrations of about 4 %, which demonstrated good bile salt tolerance. These results were reliable with a previous study (Turchi *et al.,* 2013).

Temperature tolerance was also studied. According to Ibourahema *et al.,* 2008, the bacterial capability to grow at higher temperatures is considered as one of the good characteristics, as it could be interpreted as indicating an increased rate of growth and lactic acid production. In this study, it was found that both the strains were able to grow at very low and high range of temperatures. Estimation of Lactic acid produced was also determined in the study and demonstrated that both the strains were moderate producers.

Thus the present study is a start up for the development of probiotics with a consortium of two isolates *Bacillus subtilis* and *Lactobacillus acidophilus* which is found to have antagonistic effect on fish pathogen, *Vibrio alginolyticus*. Further characterization of the isolates for probiotic properties and studies on pathogenic genes would add flavor to the future prospects in the field of probiotics in aquaculture.

CONCLUSION

The aim of this study was to provide a method for the screening of potential probiotics from curd and milk that could be applied as consortium against fish pathogen. To achieve this, two probiotics such as *Bacillus subtilis* and *Lactobacillus acidophilus* were swabbed separately on different plates and then using well diffusion method and no zones of inhibition observed. Hence, the consortium of two probiotics showed inhibitory zones or inhibitory activity against the growth of fish pathogen *Vibrio parahemolyticus* obtained from infected fishes. Bacteria that produce various vitamins as secondary metabolites have been isolated from fish intestine. Probiotics may provide their larval host with digestive enzymes. Even though care must be exercised in the choice of probiotic, because it is essential to ensure that the organism is harmless to the host. The optimum concentration of process parameters showed significantly increases the biomass production of *Bacillus subtilis* and *Lactobacillus acidophilus*. The optimization study concluded that the optimal conditions and their performance in terms of contribution to achieving maximum yield for the production of consortium for probiotic. Bile, Temperature and pH tolerance assays were performed to confirm the probiotic effect. Probiotic treatment of fish against pathogens like Vibriosthus offers a very promising alternative to the use of antibiotics and chemotherapy.

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