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RESEARCH ARTICLE

A NOVEL AND EFFECTIVE *CLOSTRIDIUM BIFERMENTANS* CPSS2 AGAINST SELECTED FISH PATHOGENIC BACTERIA.

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Abstract

Several bacterial species induces a variety of fish diseases, resulting in a severe reduction of aquaculture output. However, the current fish disease control is mainly dependent on the environmental friendly chemical formulations. Thus, the present work aimed to isolate an effective antagonistic microorganism against common fish pathogenic bacteria. A total six isolates were isolated, among them one isolate showed antibacterial efficacy in in-vitro conditions. The isolated bacterium was identified as *Clostridium bifermentans* (GenBank accession No. KT367517). The inhibitory activity of *C. bifermentans* CPSS2 was evaluated at 25, 50 and 100 µl (OD_{600}= 1.0; 1 x 10^8cfu/ml) against pathogenic strain of *Flavobacteriumcolumnare*ATCC 49513, *Staphylococcus aureus* JQ429749, *Aeromonashydrophila*JQ687063 and *EdwardisellatardaJX280148* (2.6±0.5 x 10^9cfu/ml) in agar well diffusion method and was validated by a broth co-culture assay with their kinetics plots. Extracted intracellular protein of *C. bifermentans* CPSS2 exhibited significantly ($p<0.05$) high antagonistic effect against *S. aureus* JQ429749 followed by *F. columnare*ATCC 49513and *A. hydrophila* JQ687063 and was least effective against *E. tarda*JX280148.The results of co-culture plate counts infer increasing count of *C. bifermentans* CPSS2 colonies positively antagonistic against the fish pathogenic bacteria. The result of phylogenetic study revealed that the isolated strain of *C. bifermentans* is closely related to *C. bifermentans E079*. The screening of inhibitory compounds from bacterium have clearly demonstrated that whole live bacteria and intracellular protein of *C. bifermentans* CPSS2 would be one of promising bio-control agent against common fish pathogen in freshwater aquaculture sector.

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Introduction:-

Aquaculture is one of the fastest growing food producing sector, and has grown tremendously during the last decades and contributed significantly in agricultural and total Gross Domestic Product (GDP) of India (Subasinghe, Soto &Jia2009; MPEDA 2014). The progress of fish farming is assisted with relevant management practices; however the concerns like deteriorating the environmental qualities and emergence of new infectious diseases are not to be denied. Indeed, in all animal industries, intensification generates high involvement of component of triad (host, pathogen and environment) and unbalances the system that initiates disease processes and fish mortality (Seenivasan, SaravanaBhavan, Radhakrishnan&Muralisankar 2012). Mostly, bacterial diseases are responsible for severe economic losses and huge mortalities in aquaculture industries worldwide (Bondad-Reantaso, Rohana, J, Kazuo, Supranee, Robert, Zilong& Mohamed 2005; Desriac, Defer, Bourgougnon, Brillet, Le & Fleury 2010). However, numerous chemotherapeutants and preventive measures are effectively used and well documented to control the bacterial pathogens in aquaculture. In addition to it, continuous use of antibiotics to control pathogenic microorganisms result in alterations in the normal micro biota of the aquaculture systems, increasing resistance to common antimicrobials, residual effect, environmental issues and many more including consumer safety (Ding & He 2010; Resende, Silva, Fontes, Souza-Filho, Rocha, Coelho &Diniz 2011). Therefore, alternative strategies to be developed to prevent opportunistic infections in aquaculture. The development of biocontrol agents appears to be one of the most promising ways to reach this goal (Merrifield, Arkadios, Andrew, Simon, Remi, Baker, Mathieu &Einar 2010). Bio-control agents are live cell preparations having beneficial effects like improving its feed conversion ratio, enzymatic contribution to digestion, inhibition of pathogenic microorganism's growth promoting factors and increase immune responses of the host (Verschuere, Rambaut, Sorgeloos&Vestracte 2000). Currently, prevention of fish diseases by the application of live pathogen-antagonistic bacteria has received widespread importance and application of probiotics are gaining much popularity (Vine, Leukes& Kaiser 2004;Dhanasekaran, Subhasish, Thajuddin, Panneerselvam 2008;Authira, Kirithik, Venkatesan, Ganesan&Muthuchelian 2011;Lara-Flores 2011; Tabak, Maghnia&Bensoltane 2012). The common probiotics that are used for aquaculture practices include *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanell, Bacillus, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Clostridium*, and *Saccharomyces* species.

Aeromonashydrophila, *Flavobacteriumcolumnare*, *Edwardisellatarda*and *Staphylococcus aureus* are the commonest fish pathogenic bacteria in the mainly in the tropical freshwater aquaculture system. Previously work has been carried out on antagonistic activity of cellular components of *Pseudomonas* species against *A. hydrophila* and its effect on *Cirrhinusmrigala*(Das, Samal, Samantaray, Sethi, Pattanaik& Mishra 2006). Similarly heat treated *Bacillus subtilis* was exhibited antagonistic activity against bacterial pathogens which resulted in significantly decreasing the fish pathogenic bacteria under in vitro condition (Das, Neha, Roy, Muduli, Swain, Mishra &Jayasankar 2014). Beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium bifidum*, have the ability to kill other bacteria by secreting small quantities of antibiotic-like substances, including lactic-acid, acetic acid, benzoic-acid, hydrogen peroxide acidolin, lactocidin and acidophilin (Vidya&lyer 2010). However, the information on bio-control agents those are isolated from natural resources like water or soil is scanty. Moreover, the selection criteria for effective biocontrol agent mainly depend on its visible antagonistic efficacy under *in vitro* condition. Keeping this backdrop information, the present study was conducted to isolate and identify biocontrol agent (bacteria) on biochemical and molecular basis and to investigate the *in vitro* antibacterial efficacy against selected fish pathogenic bacteria.

Materials and Methods:-

Collection of samples:-

Pond mud samples were collected from semi intensive aquaculture ponds of ICAR-CIFA, Bhubaneswar and kept in sterile zip locked polythene bags (Hi Media, India). Soil samples (1 gram each) were dissolved in 10 ml of sterile distilled water and filtered through Whatman Filter paper No. 40 in vertical laminar flow clean air work station (Klenzflo^{TM}) at room temperature. Filtered sample was serially diluted in normal saline (0.85% NaCl) for 24 hours at 28^0C. Individual colonies based on the morphology were picked, purified and stored at 4^0C for further identification (Joseph, Sasidharan, Nair& Bhat 2013). The pathogenic bacterial strains viz., *Flavobacteriumcolumnare*ATCC 49513, *Staphylococcus aureus* JQ429749, *Aeromonashydrophila*JQ687063 and *Edwardisellatarda*JX280148 maintained in soft agar (1% Agar) media were used in the study for the antagonistic study against identified *C. bifermentans* CPSS2. Further enriched bacterial populations were inoculated in the nutrient agar at 24^0C for 24 h..

Molecular identification of bacterial isolates:-
Genomic DNA of purified colonies (small, spherical and elevated blackcolour) was extracted (Leja, Myszka, Olejnik-Schmidt, Juzwa&Czaczyk 2014) with partial modification of (Joseph, Sasidharan, Nair & Bhat 2013). In brief, bacterial cells from a 24 hours old broth culture were subjected to centrifugation (8000 x *g* for 10 min) and pellets were harvested, re-suspended in TE (Tris-EDTA) buffer (10 mMTris/HCl, 1mM EDTA, pH 8.0), treated with 10% (w/v) SDS and proteinase K (20mg/ml)(Sigma) and incubated at 37^0C for 1 hour 30 minutes. After incubation, 10% cetyltrimethyl ammonium bromide (CTAB) in 0.5 M NaCl was added and the mixture incubated at 65^0C for 20 min. The DNA pellet was washed with chilled ethanol and suspended in TE for downstream processing. The concentration was estimated spectrophotometrically at 600nm and appropriate dilutions (50- 100ng) were used as template for PCR reactions.

16S rRNA gene sequencing of the isolates:-
The 16S rRNA gene was amplified using the Forward primer (5'-AAGAGTTTGATCCTGGCTCAG-3') and Reverse primer (5'-GGTTACCTTACGACTT-3'). Each PCR reaction containing 0.2 µM dNTP (BangloreGenei),10 picomole each primers, 1.25 mM of $MgCl_2$, 1X assay buffer and 0.5 U of Taq DNA polymerase (BangloreGenei) with 20 ng of DNA template. The procedure of PCR amplification was as follows: 95°C for 5 min initial denaturation followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min. and a final extension step at 72°C for 7 min. that carried out in an automatic thermal cycler (Applied Biosystem). PCR products (~1,500 bp fragments) were electrophoresed through 1.2% agarose gel and visualized by UV transillumination after staining with ethidium bromide. Products after PCR amplification were purified by gel extraction kit (Sigma Aldrich). Positive PCR products were sent to Sci Genome Laboratory Pvt. Ltd for DNA (Sanger Di-doxy) sequencing. The 16S rRNA gene sequence of bacterial isolate was submitted to the National Centre for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov) for BLAST search. Sequences were imported into Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 software (Tamura, Peterson, Stecher, Nei& Kumar 2011), with which a sequence alignment and phylogenetic tree were created on the basis of the neighbour-joining method.

Screening for antagonistic effect in agar diffusion assay:-
The bacterial inhibitory activity of selected isolate designated as *Clostridium bifermentans* CPSS2 was evaluated against *Flavobacteriumcolumnare*ATCC 49513, *Staphylococcus aureus* JQ429749, *Aeromonashydrophila*JQ687063 and *Edwardisellatarda*JX280148 with the agar well diffusion method (Harris, Daeschel, Klaenhammer 1989). The fish pathogenic bacteria at 2.6±0.5 x 10^9cfu/ml (100 µl) after adjusting to the colonies as per the OD value was seeded over the Tryptone Soya Agar (HiMedia Laboratories, India) plates (Gram &Melchiosen 1996). Each well filled with 25, 50 and 100 µl of *C. bifermentans*CPSS2. The plates were incubated at 28 °C for 24 h. The antibacterial activity of *C. bifermentans* CPSS2 was defined as the diameter of the clear inhibitory zone (mm) formed around the 4 mm diameter well. One solvent blank (broth solution) was used per plate, and each test was run in duplicate (Spanggaard, Huber, Nielsen, Sick, Pipper, Martinussen, Slierendrecht& Gram 2001).

Screening for antagonistic effect in broth co-culture assay:-
The antagonistic effects of *C. bifermentans* CPSS2 was observed in the diffusion agar assay and were validated by a broth co-culture assay with their kinetics. *C. bifermentans* CPSS2 (OD_{600}= 1.0, 1.6 x 10^{10} cells) were added to 1 mL of nutrient media (HiMedia Laboratories, India) in competition with different fish pathogenic bacteria (OD_{600}=1.0x10^{10} cells) and designated as:

T1 = 1.0 ml of fish pathogenic bacteria with 0.5 ml of *C. bifermentans*CPSS2
T2 = 1.0 ml of fish pathogenic bacteria with 1.0 ml of *C. bifermentans*CPSS2
T3 = 1.0 ml of fish pathogenic bacteria with 1.5 ml of *C. bifermentans*CPSS2 and
T4 = 1.0 ml of fish pathogenic bacteria with 2.0 ml of *C. bifermentans* CPSS2

Co culture was performed with the fixed volume of pathogenic bacteria corresponding to the fixed colonies with variable quantity of *C. bifermentans*CPSS2 as described in the treatments (T1-T4). These co-cultures were made in triplicates and incubated for 96 h at 28 ºC. The incubated co-cultures were streak on nutrient agar for colony count and corresponding OD at 600 nm were measured on 0, 24, 48, 72 and 96 h. The incubated cultures were diluted and growth of both *C. bifermentans* CPSS2 and pathogens was observed by spreading the co-culture on nutrient agar plate along with morphological identification and counting. Further a kinetic graph was plotted.

Extraction of extracellular and intracellular protein and in vitro efficacy of antibacterial activity

Extracellular and intracellular protein of the bacterial cell was obtained as followed by standard method (Austin & C 1981). In brief, An 18 h post incubated log phage culture of *C. bifermentans*CPSS2 bacteria was centrifuged at the rate of 8000 x *g* for 45 minutes, the supernatant was collected and filtered through 0.45 µm membrane . Further, the bacterial cell pellet was washed twice with 2 ml of PBS pH 7.4, and re-suspended in Phosphate Buffered Saline (PBS) in 1.5 ml. The different treatment viz., heat killed (60ºC for 10 min), sonicated (50 Hz for 15 min) of *C. bifermentans* CPSS2 bacteria and intracellular protein of *C. bifermentans*CPSS2 were respectively tested for antagonistic efficacy and performed well diffusion assay for evaluation of antibacterial activity.

Results:-

Screening of micro-biota from aquaculture pond:-

The six types of bacterial colonies were grown from the soil sample where *Clostridium* sp. and *Bacillus* sp. were found to be predominated. The isolates identified from pond soil as biocontrol agent against fish pathogenic bacteria, formed small, spherical and elevated black color colonies on TSA plates and were Gram positive motile rods. Biochemical tests revealed that they were negative for urease, inuline fermentation, trehalose and rhamnose fermentation and positive for mannose fermentation, indole, mannitol assimilation, citrate utilisation and glucose fermentation (Table 1). The amplified 16S rRNA gene from the isolate was sequenced and BLAST analysed and the results revealed 98% homology with *C. bifermentans*. The new isolate submitted as Accession No. **KT367517**. The phylogenetic analysis of isolated *C. bifermentans* CPSS2was revealed to close with *C. bifermentans* E079 strain (Figure 5).

Screening for antagonistic effect of selected strain in agar diffusion assay:-

The identified *C. bifermentans*CPSS2 were screened for antagonistic affects against four fish pathogenic bacteria *F. columnare*ATCC 49513, *S. aureus* JQ429749, *A. hydrophila*JQ687063 and *E. tarda*JX280148 by an agar diffusion assay. In all assays, zone of inhibition (in mm) were observed against selected fish pathogenic bacteria (Figure 1). *Clostridiumbifermentans*CPSS2 was showed significantly ($p<0.05$) high zone of inhibition against *S. aureus* JQ429749 (15.33±0.35 mm) followed by *F. columanare* ATCC 49513(14.00±0.30 mm) and and*A.hydrophila*JQ687063 (12.33±0.35 mm) however, significantly less zone of inhibition was observed against Gram negative *E. tarda*JX280148. In the present study, results revealed that with the increase of concentration of *C. bifermentans*CPSS2 reflected to higher antagonistic activity against said fish pathogenic bacteria i.e. 100µl inoculum has more antagonistic activity compare to 25 and 50 µl of *C. bifermentans*CPSS2 (Figure 2).

Validation of antagonistic effect in broth co-culture assay:-

In order to validate the presence of a competitive effect, the *C. bifermentans* CPSS2 exhibiting antagonistic effects were tested in broth co-culture assays with the fish bacterial pathogens. In most of cases, counts of *C. fermentans* CPSS2 were found higher when compared to fish pathogenic bacteria. The specific co-cultures of *Clostridium* and fish pathogenic bacteria were found significantly competitive against the gram positive *S. aureus* JQ429749 at 48 hours of incubation, whereas less effective against gram negative *E. tarda* JX280148 and *A. hydrophila*JQ687063 during 72 h of incubation (Table 2). The results of co-culture plate counts (x 10^{10} CFU ml^{-1}) showed that increasing the count of *C. bifermentans* CPSS2 colonies positively antagonistic against the fish pathogenic bacteria. Interestingly the results showed that T4 treatment group (1ml pathogenic bacteria with 2 ml *C. bifermentans*CPSS2) have less CFU ml^{-1} count when compare T1 treatment group (1ml pathogenic bacteria with 0.5 ml *C. bifermentans*CPSS2). The growth kinetic plots of co-culture of different treatment group of pathogenic bacteria and *C. bifermentans* CPSS2 were reflected that with increase in sampling time period OD at 600 nm was positively higher, the same trends were observed against the all the co-culture samples (Figure 3). Moreover, positive relationship between growth kinetic curves of high peak associated with more colonies of *C. bifermentans*CPSS2 compared to pathogenic fish bacteria in co-culture plate count with increasing time periods signifies the antagonistic activity of *C. bifermentans*CPSS2.

Efficacy of antibacterial activity of Extracellular protein and Intracellular protein under *in vitro* condition:-

Intracellular protein (ICP) derived by sonication of *C. bifermentans*CPSS2exhibited the significantly ($p<0.05$) high antagonistic effect against *S. aureus* JQ429749 followed by *F. columnare*ATCC 49513and *A. hydrophila*JQ687063 and less effective against *E. tarda*JX280148. The higher concentration (100 µl) of ICP of *C. bifermentans* CPSS2 was found more effective compare to less (50 µl) of inoculums as the highest zone of inhibition was found 16mm in 100 µl against *S. aureus* JQ429749 and the lowest value of 7mm in 50 µl against *E. tarda*JX280148 (Figure 4). However, there was no antagonistic antibacterial activity was observed in extracellular protein and whole cell.

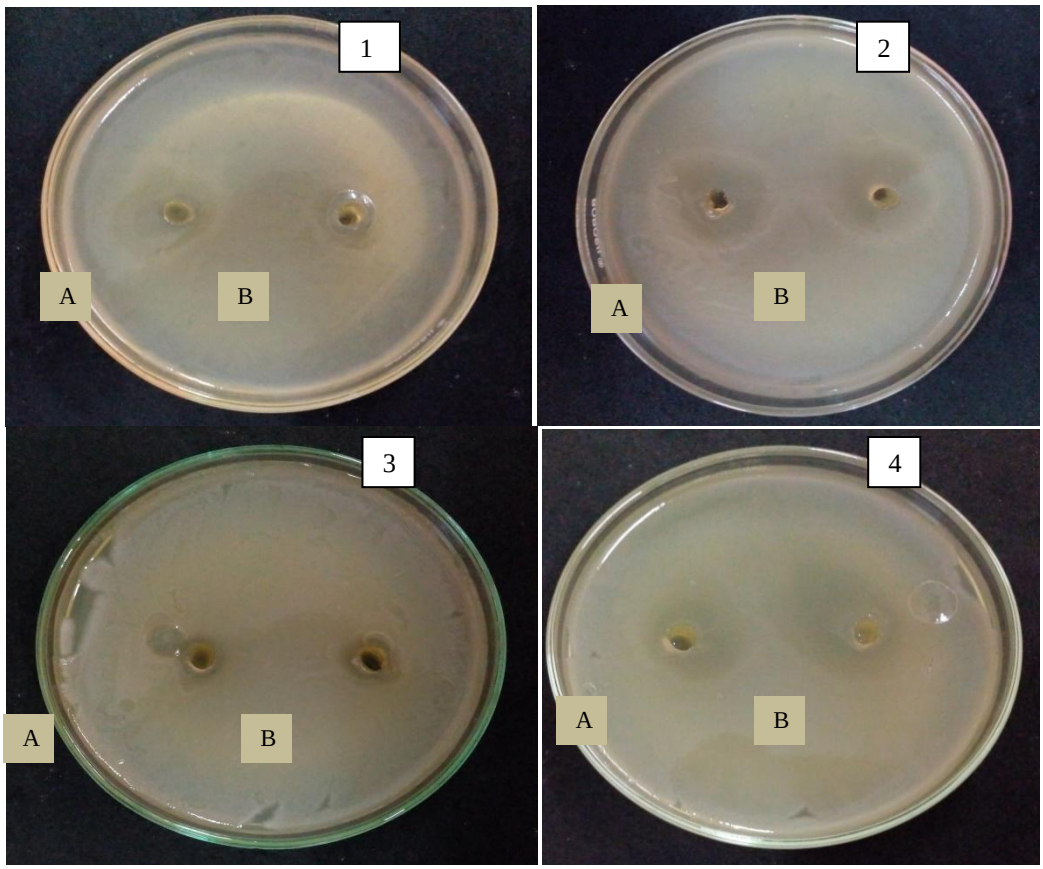
Table 1:- Results of the biochemical tests of *C. bifermentans*CPSS2.

N.B: "+" and "++ infers "positive" and " highly positive" respectively.

Name of the Test	Results (+/-)
Indole	+
Methryl Red(MR)	-
Voges–Proskauer (VP)	++
Citrate utilization	+
Oxidase	++
Catalase	+
Mannitol motility	+
Triple Sugar Iron(TSI)	
a. Yellow butt and red slant	++
b. Yellow butt and yellow slant	-
c. Red butt and red slant	-
d. Cracking of TSI agar	+

Table 2:-Plate count (x 10^{10} CFU/ml) of different Co-culture ratio of **A**. *E. tarda* with *C.bifermetans* CPSS2 **B**. *A.hydrophilia* with *C.bifermetans* CPSS2 **C**. *S.aureus* with *C.bifermetans* CPSS2 **D**. *F. columnare* with *C.bifermetans* CPSS2

A								
Time periods	0 h		24 h		48 h		72 h	
Treatments	*E.tarda*	*Clostridium*	*E.tarda*	*Clostridium*	*E.tarda*	*Clostridium*	*E.tarda*	*Clostridium*
T1	3.26	1.64	7.55	12.25	1.3	4.5	0.8	2.5
T2	3.23	1.7	9.1	11.3	0.5	2.55	0.2	1.2
T3	3.2	1.74	9	10.45	0.5	0.8	0.3	0.6
T4	3.15	1.78	7.05	4.45	0.5	1	0.2	1
B								
Time periods	0 h		24 h		48 h		72 h	
Treatments	*A. hydrophila*	*Clostridium*	*A. hydrophila*	*Clostridium*	*A. hydrophila*	*Clostridium*	*A. hydrophila*	*Clostridium*
T1	3.8	1.8	14.25	18.3	1	5.35	0.8	2.05
T2	3.65	1.98	9.5	20.1	1	3.7	0.75	1.59
T3	3.8	2	5	11.45	2.15	3.5	0.4	1.8
T4	3.6	2.2	5.9	10.35	2.3	1.05	0.1	0.8
C								
Time periods	0 h		24 h		48 h		72 h	
Treatments	*Staphylococcus*	*Clostridium*	*Staphylococcus*	*Clostridium*	*Staphylococcus*	*Clostridium*	*Staphylococcus*	*Clostridium*
T1	2.7	1.64	1.75	8.85	0.84	9.65	0.2	5.68
T2	2.65	1.8	1.75	8.95	0.78	10.55	Nil	6.9
T3	2.72	1.88	0.3	26.5	0.1	28.5	Nil	8.5
T4	2.65	1.9	0.2	40	Nil	45	Nil	15.8
D								
Time periods	0 h		24 h		48 h		72 h	
Treatments	*Flavobacterium*	*Clostridium*	*Flavobacterium*	*Clostridium*	*Flavobacterium*	*Clostridium*	*Flavobacterium*	*Clostridium*
T1	3.6	1.8	7.55	12.25	2.4	1.4	0.4	6.84
T2	3.4	2	9.1	10.8	1.5	6.4	0.8	10.55
T3	3.8	1.9	9.5	10.45	1.85	25.45	Nil	8.54
T4	3.2	2	8.1	14.2	3.2	20.85	Nil	10.25



A: 100 µl and B: 50 µl of inoculum

Figure 1:- Zone of inhibition (in mm) formed antagonistic activity of live *Clostridium bifermentans*CPSS2against 1. *Flavobacteriumcolumnare* ATCC 49513 2.*Staphylococcus aureus* JQ429749 3.*Edwardsiellatarda* JX280148 and 4.*AeromonashydrophilaJ*Q687063

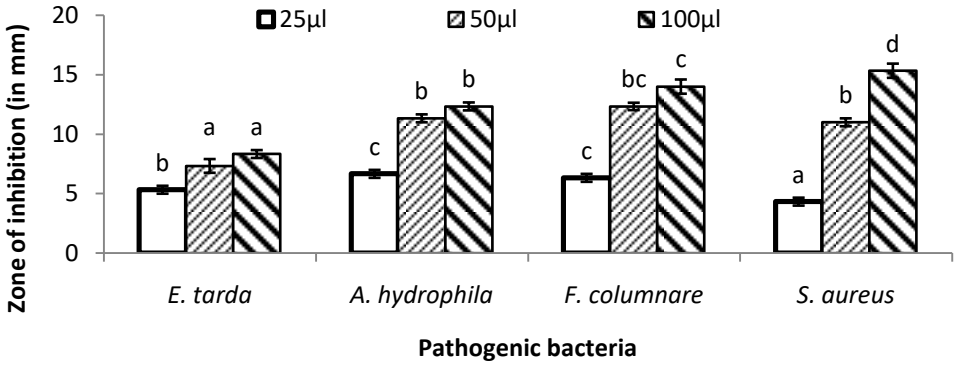


Figure 2:- Antagonistic effect measured in zone of inhibition (in mm) of varied amount of live *Clostridium bifermentans* CPSS2 against some selected fish pathogenic bacteria. The data is represented with different superscript differ significantly ($p<0.05$) and express as mean±SE.

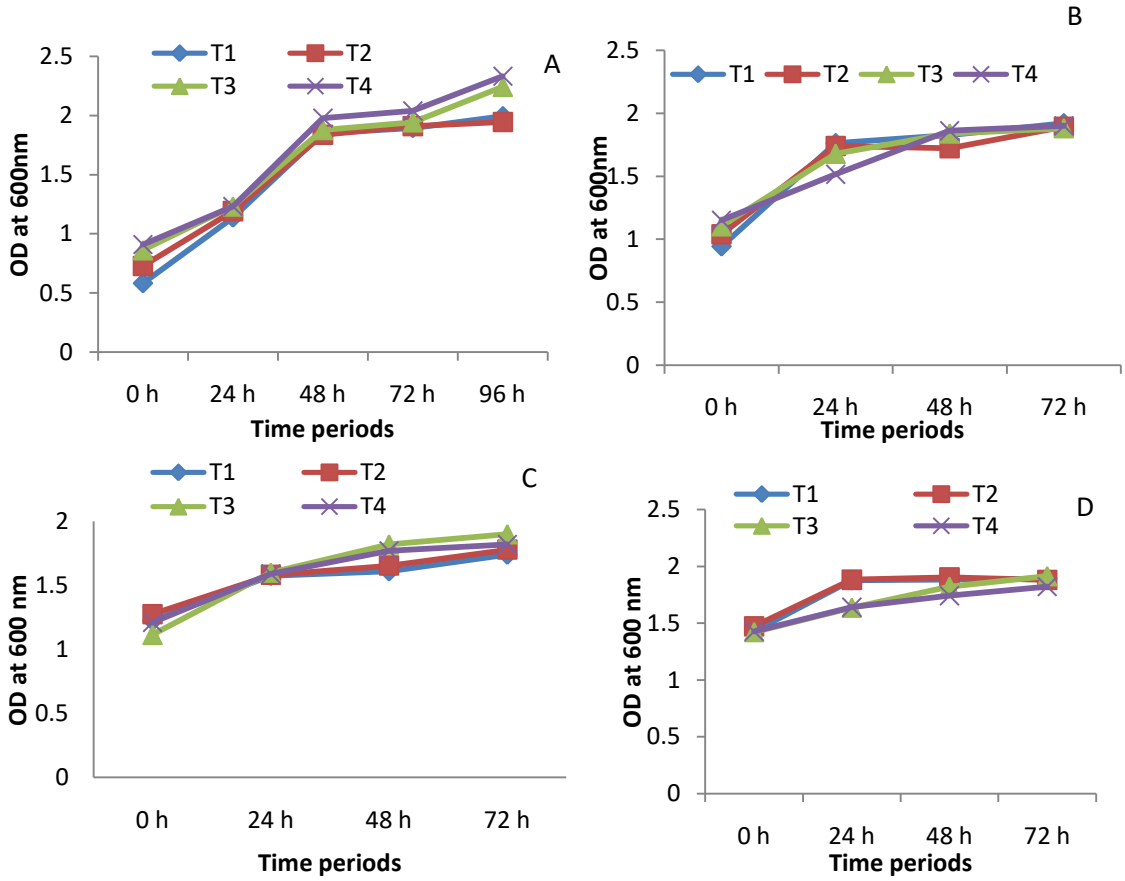


Figure 3:- OD at 600nm of co-culture of varied ratio of A. *Flavobacteriumcolumnare* and *Clostridium bifermentans* CPSS2 B. *Staphylococcus aureus* and *Clostridium bifermentans*CPSS2 C. *Aeromonashydrophila* and *Clostridium bifermentans* CPSS2 D. *Edwardisellatarda* and *Clostridium bifermentans* CPSS2 at different time periods.

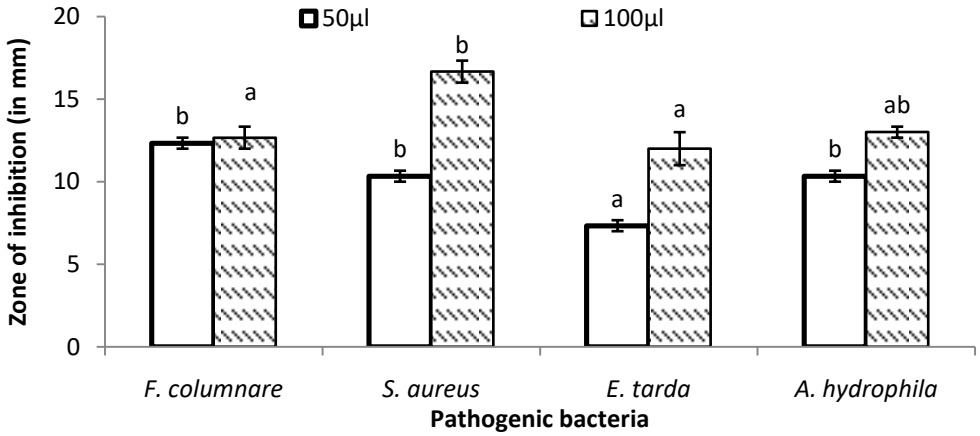


Figure 4:- Zone of Inhibition (in mm) formed by varied amount of intracellular protein of *Clostridium bifermentans* CPSS2 against some selected fish pathogenic bacteria. The data is represented with different superscript differ significantly ($p<0.05$) and express as mean±SE.

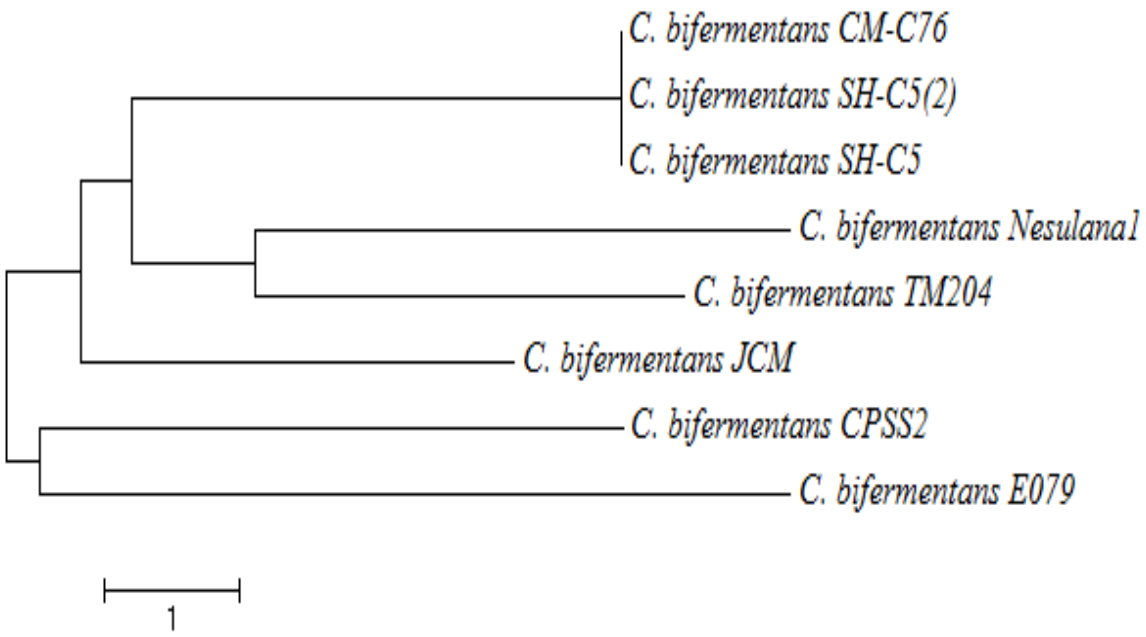


Figure 5:- Neighbor-joining tree showing the phylogenetic relationships between the isolates and the type strains based on 16S rRNA gene sequences, only some representative isolates were indicated.

Discussions:-

Presently chemotherapeutic agents are being used to control the majority of bacterial diseases. However, several limitations in their application and negative have impact on environment; attract to search an alternative to biocontrol agents. This possibility has already been highlighted by several workers (Austin,Stuckey, Robertson, Effendi & D 1995; Robertson, O'Dowd, Burrells, Williams & Austin 2000; Ringo, Lodemel, Myklebust, Kaino, Mayhew & Olsen 2001). In the present study, biocontrol agent was investigated under *in vitro* condition as potential competitor against selected fish pathogenic bacteria.

Among six bacterial isolates, one isolate of *C. bifermentans* CPSS2 has been evaluated for biocontrol agent. Indeed, not more than 1% of the environmental bacterial communities are cultivable (Amann, Binder, Olson, Chisholm, Devereux & Stahl 1990). Moreover, in the present study, the predominant bacterial group isolated from soil of aquaculture ponds was composed of Gram positive bacteria *Clostridium* and *Bacillus* spp. Similarly, 12 strains belong to genus *Clostridium* were isolated and identified from soil and composts by sequencing as *C. bifermentans* and *C. butyricum* (Leja, Myszka, Olejnik-Schmidt, Juzwa&Czaczyk 2014). Further, they reported the morphology and biochemical characteristics of *C. bifermentans* strains in TSA medium having opaque, black, circular, low convex colonies with slightly undulated margins. The result of our findings confirms the characteristics of *C. bifermentans* strains (Prevot&Malgras 1950; Regan & Crawford 1994).

Further, the results of biochemical characterization showed that isolate is negative for urease, inuline, trehalose and rhamnose fermentation and positive for indole, mannitol assimilation, citrate utilisation, glucose and mannose fermentation. *C. bifermentans* strains was reported as indole positive (Regan and Crawford 1994; Nachman, Kaul, Li, Slim, Filippo & Horn 1989). Similar results were documented for *Clostridium* which showed indole positive and urease negative (Brooks, Moss & Dowell 1969). The ability of *C. bifermentans* to saccharide fermentation along with fermented glucose, fructose, maltose, glycerol, and sorbitol were reported by (Brooks & Epps 1958).

Results from agar gel diffusion assays clearly indicate that *C. bifermentans* CPSS2 have antagonistic effects against selected fish bacterial pathogens. It shows that the mechanism involved in antagonistic effect is a competitive exclusion or a synthesis of non-diffusible antimicrobial which is validated by co-culture inhibition assay. Further, *in vitro* antibacterial efficacy of intracellular protein (IP) strengthens the finding that *Clostridium* has specific antagonistic antibacterial activity. In this respect, zone of inhibition was observed on agar plates and growth kinetic plot was drawn which confer the antibacterial activity in co-culture. *Clostridium butyricum* has been evaluated as a

probiotic candidate and showed antimicrobial activity against *A. hydrophila* and *Vibrio anguillarum* under *in vitro* condition (Pan, Wu, Zhang, Song, Tang & Zhao 2008). Similarly, antagonistic activity of cellular components of *Pseudomonas* species was identified as biocontrol agent against *A. hydrophila* and its effect on *Cirrhinusmrigala*(Das, Samal, Samantaray, Sethi, Pattanaik& Mishra 2006). Further heat stable *B. subtilis* isolate derived from mangrove forest was exhibited antagonistic activity against fish pathogenic bacteria (Das, Neha, Roy, Muduli, Swain, Mishra&Jayasankar 2014).

The concept of inhibition of pathogens by the growth of environmental or resident bacteria was first suggested in birds (Nurmi&Rantala 1973). The competitive exclusion exerted by biocontrol against pathogens was also reported in fish, crustaceans, and other aquatic organisms (Balcazar, DeBlas, Ruiz-Zarzuela, Vendrell&Muzquiz 2004). Competitive exclusion is the most promising mode of probiotic action because it involves many different processes and factors which are very important in microbial dynamics (Smith 1993). The mechanism related of nutrient competition, extracellular protein like bacteriocin production, intracellular protein or alteration in medium chemical properties are widely responsible for inhibit the another group of bacteria. In order to become more competitive, pathogens need to evolve and to gain new functions. Each of these new functions facing a single process implied in the competition. On the contrary, when competitors inhibit growth by secretion of a single antimicrobial agent, the pathogen needs to acquire only one specific resistance gene to this specific antimicrobial agent (Moriarty 1998).

The antibacterial activity of *C. bifermentans* CPSS2 was observed against selected fish pathogenic bacteria but at different level in agar gel diffusion and co-culture inhibition assay. The result suggests that Gram positive and Gram negative bacterial morphological differences may play a critical role in their protection. In the present study, the whole live *C. bifermentans*CPSS2 and intracellular protein have showed more antibacterial activity against Gram positive *S. aureus* JQ429749 compared to Gram negative *E. tarda* JX280148 and *A. hydrophila* JQ687063. Similar mechanism was reported for selection of probiotic bacteria and highlighted the importance of a non-diffusible antimicrobial compound (Oliveira, Oliveira & Gloria 2008). The target of bacteriocin is the cytoplasmic membrane, but Gram-negative bacteria like *Flavobacterium* do have a protective barrier provided by the lipopolysaccharide of the outer membrane. However, some circumstances can disrupt the integrity of this barrier and increase the effectiveness of bacteriocin against gram-negative bacteria (Stevens, Sheldon, Klapes&Klaenhammer 1991; Mortvedt-Abildgaard, Nissen-Meyer, Jelle, Grenov, Skaugen&Nes 1995). In the conclusive remarks, the study was conducted to isolate and characterize the biocontrol bacteria *C. bifermantan* CPSS2 which showed in-vitro antagonistic activity against number of fish pathogenic bacteria viz., *Flavobacteriumcolumnare* ATCC 49513, *Staphylococcus aureus* JQ429749, *Aeromonashydrophila* JQ687063 and *Edwardisellatarda* JX280148. Agar diffusion assay combined with broth co-culture assays and the screening of inhibitory compounds from bacteria, have clearly demonstrated that live bacteria and intracellular protein isolated from *C. bifermentans* CPSS2 could be a possible means of biocontrol agent which could compete with fish pathogenic bacteria. Thus, the present study opens a new idea to evaluate antibacterial efficacy of *C. bifermentans* CPSS2 under in vivo condition and its role as biocontrol agent. So, it would be a promising bio-control agent in near future in the freshwater aquaculture sector as well as the strain isolated in this study would be a valuable resource deserving future detailed studies.

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