2.3 Antibacterial activity assay of bacterial probiotic metabolites

The antibacterial activity of bacterial secondary metabolites extracted with different solvent was tested by agar diffusion assay and disc diffusion assay method. The plates were incubated at 37oC for 24h during which activity was evidenced by the presence of a zone of incubation surrounding the well and disc. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the incubation zone (mm) produced by the secondary metabolite when compared to control.

3. Results and Discussion

In this study the role of enriched probiotic bacteria such as *Streptococcus faecalis* (30 millions), *Clostridium butricum* (2 millions), *Bacillus mesantericus* (1 million) and *Lactobacillus sporogens* (50 millions) from commercial probiotic capsule (Becelac PB,) are potential competitors of the pathogenic bacteria was investigated. The use of probiotics in aquaculture is a new potential and natural prevention mechanism against fish disease caused by pathogenic bacteria.

The enriched probiotic bacteria exhibited a marked antagonistic activity against all the bacterial pathogens (Table 1 and 2). The Toluene extract (25 µl,50 µl and 75 µl) obtained from the probiotic mixture showed high significant antimicrobial activity against selected gram positive and Gram negative fish pathogens in disc diffusion method, *Aeromonas hydrophilus* (19mm), *Bacillus* sp (23mm), *E.coli* (24mm), *Pseudomonas aerogenosa* (20mm), *Streotococcus* sp (22mm), *Vibrio harveyi* (20mm)*Staphylococcus* sp (23mm) and Vibrio sp (20mm) when compared with the standard streptomycin (22mm) for 25ug/disc (Table 1). The antibacterial activity exhibited by the solvent extract was equivalent to that of the activity of streptomycin. The ability of consortium probiotic bacteria to suppress pathogen in vitro conditions suggests that it is a promising probiotic bacteria. Among the three solvent used in these study, ethyl acetate extract have given less antibacterial activity (Table 1and fig 1). Nestle yogurt probiotic were bactericidal for *S.aureus* and *P.aeruginosa*, but were inhibitory for *S.typhi*. Neslac probiotic s killed the test organisms *E.coli* and *S.typhi*. They were only inhibitory for *S.aureus* and *C.albicans*. Results of the study showed the antimicrobial activity of the probiotic enriched from the commercial probiotic capsule. This may be due to the production of acetic acid and lactic acid that lower the pH of the medium, and stimulate the production of hydrogen peroxide and bacteriocins (13).

The solvent extracted bacterial supernatant of mixed culture of *Streptococcus faecalis, Clostridium butricum, Bacillus mesantericus* and *Lactobacillus sporogens* exhibited greater inhibitory activity against eight bacterial fish pathogens in well diffusion method (Table 2 and fig 2). The highest inhibitory zone was observed in the ethyl extract of probiotic metabolites around the well against pathogen *Streptococcus sp* was 11-13 mm in diameter. In toluene extract, the highest zone inhibition was 18-30 mm against *Vibrio harveyi*. The highest zone of inhibition of benzene extracts was 18-29 mm against *Pseudomonas aerogenosa*. In our finding, inhibitory activity has vary from solvent to solvent, pathogens to pathogens and concentrations (25µl, 50µl and 75µl). The *B.subtilis* UTM 126 inhibited growth of *V.alginolyticus, V.para haemolyticus* and *V.harveyi*. The inhibitory mechanism is production of volatile and non volatile compounds. *Bacillus* produces polypeptide antibiotics such as bacitracin,gramicidin and polymyxin, which are active against a wide range og gram positive and gram negative bacteria (14).

Balcazar and Rojas Luna, (15) reported that the fish food supplementation with probiotic for shrimp culture, the reduction of shrimp mortality was found from *V. harveyi* infection. Vaseeharan and Ramasamy,(2003) found that growth of pathogenic V.harveyi in tiger shrimp was controlled by the probiotic effect of *B.subtilis* BT23 in vitro and in vivo. Kanatani et al(16) has stated that a bacteriocin from *L.acidophilus* TK9201 had inhibitory effect on closely related lactic acid bacteria and food born pathogens including *L.monocytogenes*. Itoh et al., (17) indicated that *L.gasseri* LA39 was one of the most active bacteriocins for use against enteric pathogens.*L.plantarum* strains give an inhibition diameter of 20mm for *S.aureus*,11mm for *Bacillus* sp and 10mm for *E.coli* (18).Generally the medium composition values recorded for production of metabolites by measuring the diameter of the high inhibition zone, show that the Gram positive bacteria than Gram negative bacteria. Todorov and Dicks (19) observed that a high level of bacteriocin was produced when the cells were grown in the presence of K_2HPO_4. The antimicrobial activity (mm) was measured and the data represented as mean +- SE (including disc diameter), all the pathogens were inhibited significantly at probability level ($P<0.05$).

Out results suggest that the growth and production of secondary metabolites of mixed culture of *S.faecalis, Cl.butyricum, B.mesentericus* and *L.sporogens* can take into account their interactions with pathogens. Future challenge experiments in secondary metabolites could provide valuable insight into its potential probiotic effect in situation directly relevant to aquaculture conditions.

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Table 1: Antibacterial activity of bacterial Probiotic metabolites extracts against fish pathogens (Disc diffusion method)

Fish pathogens	Zone of Clearance (mm)								
	Ethyl acetate extract			Toluene extract			Benzene extract		
	25µl	50µl	75µl	25µl	50µl	75µl	25µl	50µl	75 µl
Control for Dc **(5%-DMSO)**	-	-	-	-	-	-	-	-	-
Control for Tc/Bc	-	-	-	-	-	-	-	-	-
Aeromonas hydrophilus	9	11	12	14	16	19	13	18	18
Bacillus sp	7	12	12	18	19	23	14	17	16
E.coli	6	7	8	18	19	24	14	17	18
Psedomonas aerogenesa	6	9	11	13	19	20	14	17	18
Streptococcus sp	11	12	13	19	19	22	13	16	17
Vibrio harveyi	9	8	10	14	16	20	15	17	19
Staphylococcus sp	8	8	10	19	20	23	16	20	20
Vibrio sp	6	8	10	16	16	20	13	16	17

SD of zone inhibition was calculated from three replicates of each pathogen

Signification; **P<0.05**

Table 2: Antibacterial activity of bacterial Probiotic metabolites extracts against fish pathogens (well diffusion method)

Fish pathogens	Zone of Clearance (mm)								
	Ethyl acetate			Toluene extract			Benzene		
	25 µl	50 µl	75 µl	25 µl	50 µl	75 µl	25 µl	50 µl	75µl
Control for Dc **(5%-DMSO)**)	-	-	-	-	-	-	-	-	-
Control for Tc/Bc	-	-	-	-	-	-	-	-	-
Aeromonas hydrophilus	9	11	12	16	16	20	19	23	26
Bacillus sp	7	12	12	17	22	25	16	20	23
E.coli	6	7	8	21	23	25	20	22	28
Psedomonas aerogenesa	6	9	11	12	18	22	18	26	29
Streptococcus sp	12	11	13	21	26	27	21	25	28
Vibrio harveyi	9	8	10	18	27	30	20	27	26
Staphylococcus sp	9	8	10	20	20	22	19	22	25
Vibrio sp	6	8	10	22	24	25	22	23	29

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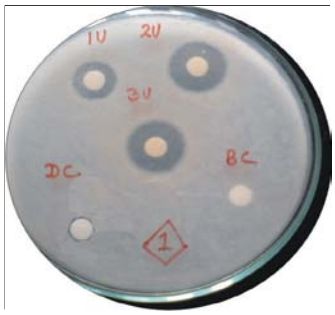
Fig-1 Benzene mixer

Dics diffusion method

Aermonas hydrophilus



Bacillus.ssp

E.coli

Psedomonas aerogenesa

Streptococcus

Vibrio.harviyi

Staphylococcus.ssp

Vibriio.ssp

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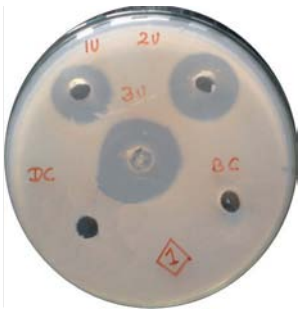
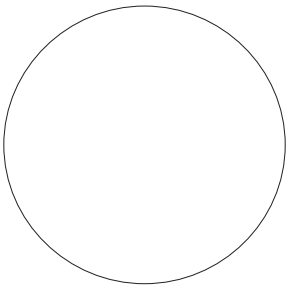
Fig-2 Benzene mixer

Welldiffusion method

Aermonas hydrophilus

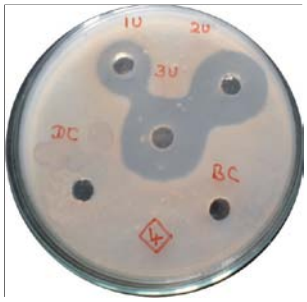


Bacillus.ssp

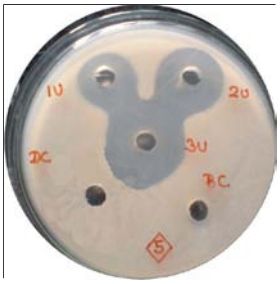


E.coli

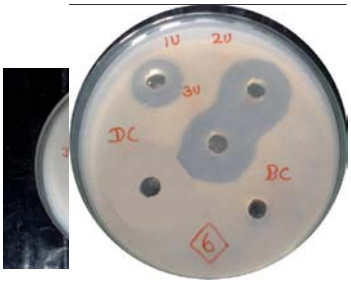
Psedomonas aerogenesa



Streptococcus



Vibrio.harviyi



Staphylococcus.ssp

Vibrio.ssp

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