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SCREENING OF MARINE MICROORGANISMS AS PROBIOTICS FOR PRODUCTION OF BACTERIOCIN

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ABSTRACT

The aim of present study was to select potential probiotic from marine environment. A total of 203 strains isolated from six different coastal area of India were screened for bacteriocin production. Out of 203 strains there are 107 bacteria, 37 Yeastand 59actinomycetes. The 10 isolates from bacteria, 2 from yeast and 5 from actinomyceteshas shown antibacterial activity against human pathogens brought from MTCC culture collection. The isolates has shown maximum antibacterial activity against *Klebsiella sp., Escherichia coli and Pseudomonasaeruginosa*. The optimum pH and bile salt concentration required for enhanced growth of screened isolates were found to be pH 3.0 and0.3% bile salt.

INTRODUCTION

The term Probiotic means "for life" is derived from the Greek language. A probiotic is a live microbial feed supplement, which beneficially affects in the host animal by improving its intestinal microbial balance. Penetration of biotechnology into marine environment has opened up unexpected new horizons for finding novel organisms for trapping their potential resources. However, culturally independent methods have demonstrated that marine sediments contain wide range of unique microorganisms (Salam *et al*. 1999; Stach *et al.* 2003). Lactic acid bacteria (LAB) are the biological basis for the production of a great multitude of fermented foods (Lasagno *et al*., 2002). This inhibition may be due to the production of many metabolites such as organic acids. (Lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Ennahar *et al*., 2000; Lasagno *et al.*, 2002). Some bacteriocins kill only bacteria belonging to the same species as producer whereas other bacteriocins kill a broad range of Gram positive bacteria (Conventry*et al*., 1997; Ennahar*et al*., 2000; McAuliffe *et al*., 2001; Garneau *et al*., 2002). They have attracted considerable interest in recent years and several works have focused on the isolation and development of new strains of bacteriocin-producing bacteria.

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Streptomyces scopuliridies and Streptomyces pluripotent are novel a bacteriocin producing sreptomycetes (Faris *et al*., 2011; Lee *et al*., 2004). These shown to produce a broad spectrum bacteriocin. The strains of actinobacteria belonging to the genus Streptomyces might be promising probiotics in aquaculture because they produce compounds with potential bioactivity against pathogens of fish and shellfish. Marine yeasts are ubiquitous in the marine environment. They are frequently found in the digestive tract of marine organisms and in seawater and beach sand (van Uden and Branco 1963, Taysi and van Uden 1964, Kawakita and van Uden 1965, Fell 1967, Vogel *et al*. 2007; Kutty and Philip 2008). It is therefore considered that the factors affecting the distribution of marine .yeasts include currents, migration of marine organisms, and contamination from terrigenous sources (van Uden and Branco 1963, Fell 1967, Vogel *et al*., 2007, Kuttyand Philip 2008). Saccharomyces cerevisiae and Saccharomycesboulardii are work as probiotics, but no one work done for production of bacteriocin. Inpresent study, isolation of marine microorganisms as probiotic.

MATERIALS AND METHODS

Collection of marine samples

Marine water and sediment samples we recollected from six different coastal areas of India, name as Calangute beach Goa, Dadarchaupathy Mumbai, Gopalpur Orissa, Thirumullavaram

beach Kerala, Elliot's beach Chennai, Angellar beach. Samples were collected from 5-15 cm depth and stored in dark during transport tolaboratory, stored for further study.

Isolation of marine microorganisms

MRS broth (de Man Rogosa Agar), SCA (Starch casein Agar) broth, PD (Potato Dextrose) broth was used for isolation and enumeration of marine microorganisms viz., bacteria, actinomycetes, yeast respectively for its probiotic ability. 1ml of each marine water sample was inoculated in 10 ml sterile broth prepared in sea water and incubated for48 hrs. for bacteria, 7 days for yeast and actinomycetes. The broth culture was serially diluted and subjected for isolation by spread plate method on MRS agar prepared in sea water using 0.1 ml of last three dilutions (10^{-4}, 10^{-5},10^{-6}) and incubated at30^0C for 3 days. 1 gm. of each surface soil from sediment sample was mixed with 9 ml of sterile saline(0.85% NaCl), homogenized by incubating on rotary shaker (150 RPM) for 10 min. These homogenized samples were inoculated at 10 %(v/v) level in broth prepared in sea water and incubated at 30^0C for 3 days. The enriched samples were subjected for isolation on agar containing sea water and incubated at 30^0C under anaerobic conditions (in anaerobic jar using gas pack) for bacteria. The plates were observed and good isolates were procured and identified further.

Screening for probiotics

Screening of probiotics amongst 203 marine microorganisms was carried out on the basis of potential antibacterial activity against human pathogens. Pathogens used for this activity were isolated from human urine and fecal samples as well as pathogens brought from MTCC Centre were used. The isolated human pathogens were subjected for Antibiotic sensitivity test to determine response of antibiotic towards isolated pathogens. Antibacterial activity of marine microorganisms against pathogens was determined by agar diffusion method. A lawn ofindicator strains including, *Escherichia coli, Klebsiellasp. pneumonia, Pseudomonas aeruginosa*, *Staphylococcussciuri*, *Enterococcusfacaelis,* and MTCC pathogens name as *Escherichia coli (1687), Pseudomonas aeruginosa,(1688), Salmonella typhi (531), Bacillus subtilis (8960), Proteus vulgaries(742), Klebsiella pneumonia (535), Staphylococcus aureus (96*) was made over the surface of MullerHinton Agar plates. After solidification and drying of plates wells wereprepared on surface of agar plates. Cell free extract ofisolates was prepared by growing them overnight in broth, cells were separated by centrifugation at 10,000xg for15 minute and supernatant was collected and neutralized upto pH 7 using 0.1 N NaOH and then used for bacteriocinassay. The crude extract at 100ul quantity was incorporated in respective well and plates were incubated at 37^0C for 24hours. Similarly for fugal isolates potato dextrose agar with pH 3.0 and 0.3% bile salt was used and incubation cycle is of 7 days. For actinomycetes, Starch casein broth of pH 3.0 and 0.3% bile salt concentration was used and incubated upto14 days for observation of growth (Melagro *et al*.) The results were recorded by observing and measuring zone of inhibition (Kanagaraj Nithya *et al.,* 2012). Amongst isolates those were selected showing broad spectrum antibacterial activity.

Optimization of pH and Bile salt concentration

Based on probiotic potential two selected strains of bacteria, one fungal and one actinomycetes were further characterized for their growth at low pH and bile salt concentration on growth.

Resistance to low pH

For this purpose, 24 hrs. old active selected marineprobiotics were used. Intact cells were prepared by growing them in MRS broth and harvesting cells by centrifugation at5000rpm at 4 C for 10 minutes. Pellet was washed using phosphate- buffer saline (pH 7.2) and then suspended insane buffer of pH 7.2. Further to study the resistance to low pH the viability of these cells was checked by incubating the cells in phosphate buffer of different pH viz. 1, 2, 3 and 4 at 37^oC for variable period of time from 1 to 4 hours. Viability was measured every after one hour of incubation by measuring turbidity at OD620nm (Prasad J. *et al*. 1998). Potato dextrose broth was used for yeast/fungi and starch casein broth was used for actinomycetes.

Tolerance against bile salt

The prepared intact cell suspension at 1% (v/v)level was inoculated in MRS broth containing 0.2%, 0.3%,0.4% bile and incubated for variable period of time. Periodically viability was measured every after one hour of incubation by measuring optical density at 620 nm (Prasad J. *et al*. 1998).

RESULT AND DISCUSSION

Isolation of microorganisms

In our study total 203 microorganisms were isolated from coastal area of India belonging to three different groups viz. Bacteria (107), fungi (37) and 59actinomycetes(Table 1). KhosroIssazadeh also worked on marine microbes isolated from marine environment of the Guilan province in north of Iran (Caspian Sea) and identified strains of *L. acidophilus* and *L. Plantarum as potential probiotics producing bacteriocin.* Mohan Remya, Ramasamy Vijayakumar isolated 64 actinomycetes from West Coast of India (Ernakulum to Kannur) out of which 24 isolates were from mangrove soil, 40 from sea shore soil having antimicrobial property. Several decades ago Cheng and Lin 1977 reported isolation of marine habitat yeasts from estuarine and coastal sediments in western Taiwan.

Primary screening

Out of 203 marine isolates only 21 had shown antibacterial activity. The efficiency of isolates as probiotics amongst diverse groups was 13% bacteria, 2% yeast/fungi and 9% of actinomycetes as recorded in table 2 and shown in figure 1. Our results reported that isolate L43 and B25 has shown 100% antibacterial activity against pathogens. Further specially these strains viz., Lb43 and Bf25 had shown highest antibacterial activity against *K. pneumoniaessp pneumonia.* The Actinomycetes strain A17 and F1 fungal strain were showing highest antibacterial activity against *Enterococcus casseliflavus* and MTCC Pathogen *Escherichia coli* (1687). Similarly Singh *et al.,* (2013) has reported that isolated strain of Lactobacillus fermentum is probiotic in nature as it has shown antibacterial activity towards *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli and Klebsiella pneumonia*. The strain MUSC 135(T) exhibited a broad specrum bacteriocin against the pathogen MRSA, ATCC BAA -44, *Salmonella typhi* ATCC 19430(T) and

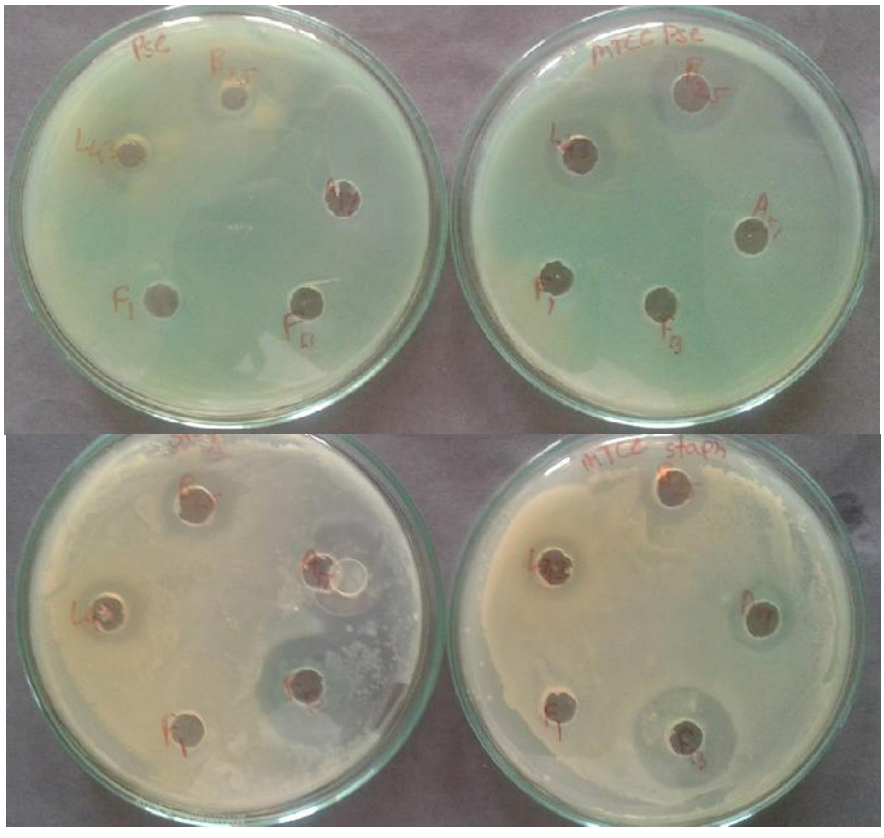
Table 1. Isolation of microorganisms

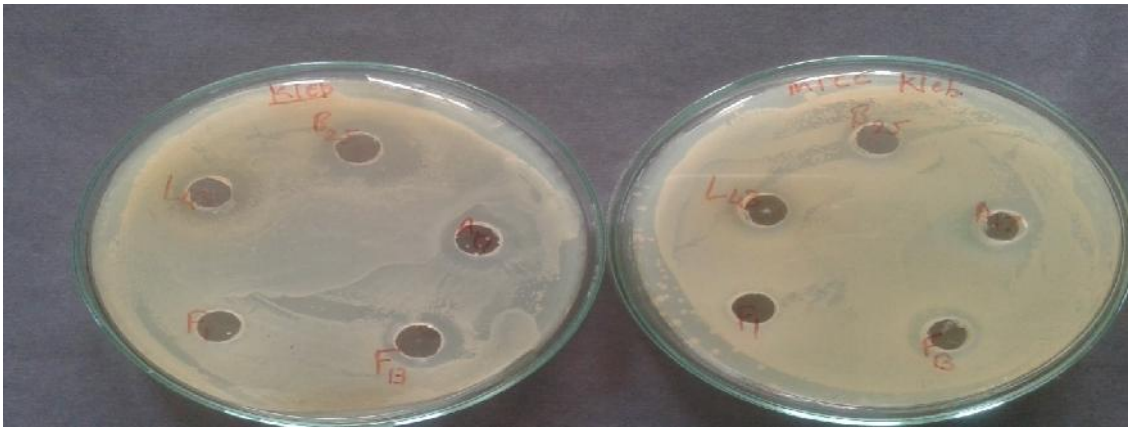
No.	Marine samples	No. of bacteria	Probiotic bacteria	No. of yeast	Probiotic yeast	No. of actinomycetes	Probiotic actinomycetes
1.	Elliots beach, Chennai (water)	05	01	-	-	-	
2.	Elliots beach, Chennai (Sediment)	07	-	-	-	12	02
3.	Calangute beach, Goa (water)	21	03	-	-	-	
4.	Calangute beach, Goa(Sediment)	09	-	05	-	17	01
5.	Thirumullavaram beach, Kerala(water)	22	02	-	-	-	
6.	Thirumullavaram beach, Kerala(Sediment)	12	03	08	01	09	01
7.	Gopalpur, Orrisa(water)	11	01	02	-	-	
8.	Gopalpur, Orrisa (Sediment)	08	01	07	-	09	-
9.	Dadarchaupati, Mumbai(water)	-	-	-	-	-	
10.	Dadarchaupati, Mumbai (Sediment)	07	01	11	01	12	01
11.	Angell beach(water)	05	01	04	-	-	
12.	Angell beach(Sediment)	-	-	-	-	-	
	Total	107	14	37	02	59	05
	% Probiotic property		13		2		9

Table 2. Antibacterial activity of isolated microorganisms against identified pathogens and MTCC pathogens

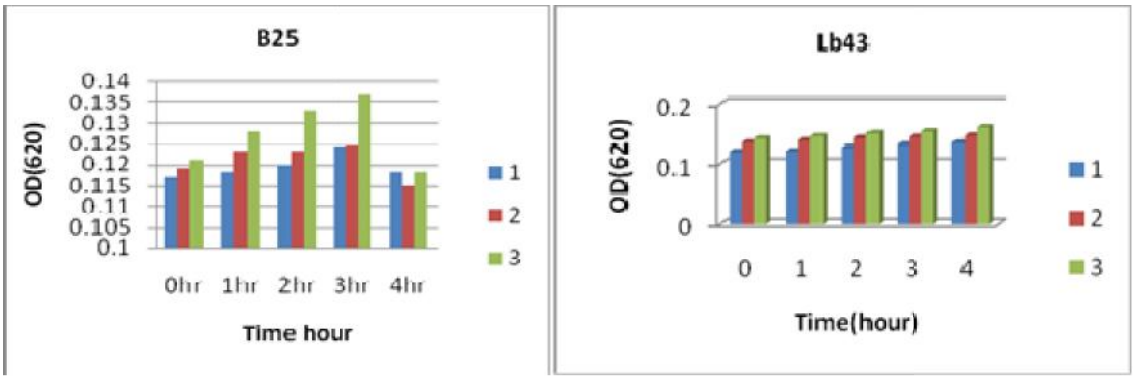
Sr.no	Name of Pathogens	Lb43	Bf25	F1	F13	A17
	Isolated in Lab.	Diameter of zone of inhibition(mm)				
1	*K. pneumoniaessp pneumonia*	16	15	R	R	R
2	*Escherichia coli*	13	13	10	R	13
3	*Pseudomonas aeruginosa,*	13	12	R	15	13
4	*Enterococcus casseliflavus*	15	R	R	13	14
5	*Staphylococcus sciuri*	13	12	R	16	13
	Efficacy of probiotic	100%	80%	20%	60%	80%
	MTCC Pathogens					
6	*Escherichia coli(1687)*	12	13	09	R	14
7	*Pseudomonas aeruginosa,(1688)*	15	18	R	R	R
8	*Salmonella typhi(531)*	16	15	11	R	R
9	*Bacillus subtilis(8690)*	12	14	R	11	13
10	*Proteasvulgaries(742)*	16	13	R	10	13
11	*Klebsiella pneumonia(535)*	17	16	R	13	12
12	*Staphylococcus aureus(96)*	13	13	R	16	13
	Efficacy of Probiotic	100%	100%	22%	55%	77%

Method

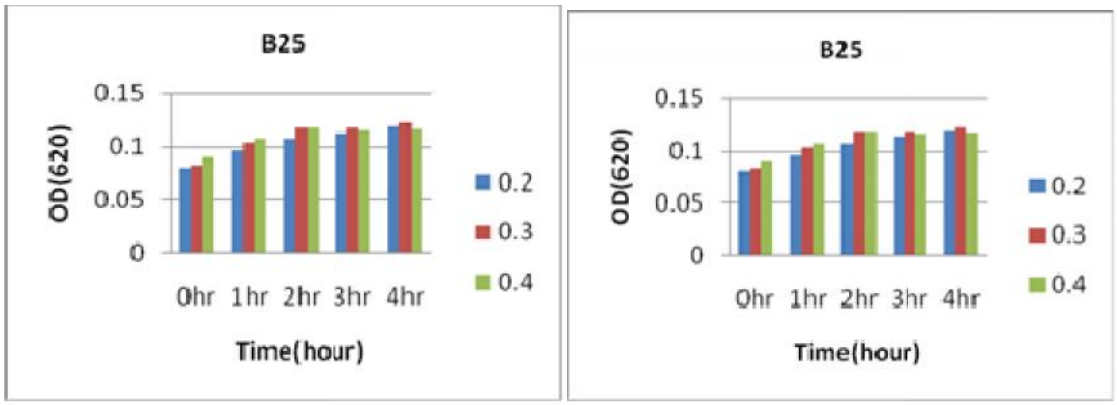




Legend: L43 & B25- *Lactobacillus* sp., F1 and F13-Yeast, A17- Actinomycete



Resistance to low pH



Tolerance against bile salt

Fig. 2. Probiotic properties: Resistance to low pH and Tolerance against bile salt of bacteria

Table 3. Probiotic properties: Resistance to low pH and Tolerance against bile salt of Yeast and Actinomycetes

Sr. No.	Growth of Yeast	Resistance to low pH(Days)	Tolerance against bile salt(Days)
1	F1	07	14
	Growth of actinomycetes		
2	A17	03	03

Legends: F- yeast and A- Actinomycetes.

Aeromonas hydrophilla ATCC 7966(T) (Lee *et al*. 2004). Our results revealed the presence of the compound bacteriocin have been reported to be inhibitor against several other bacteria.

Probiotic Properties

Resistance to low pH: Viability of bacterial strains was observed at pH 3.0. As it is seen from graphs in figure 2, bacterial strain L43, B25 are very stable in pH3 which means these isolates are able to survive in this pH values. Prasad *et al*., (1998) reported that a significant decrease in the viability of strains is often observed at pH 2.0 and below. The F1 strain of yeast-mold had grown at pH 3 upto 7 days and A7 strain of actinomycetes was growing that pH nearly 3-7 days. All actinomycetes strains had a similar behaviour to different pH exhibiting no growth at pH 1-3, but growing at pH higher than 3(Milagro *et al*.2015). It has been estimated that the survival

rate of traditional probiotics in human gut is 20-40%, acidity may be one of the main obstacles (Bezkorovainy, A. 2001).

Tolerance against bile salt: Strains were found to be viable in 0.3% bile during 4 hrs. According to the results, all of the isolates were resistant to 0.3% bile salt as shown in figure 3.Compare to all strains, sp. L43 and sp. B 25 has shown high tolerance. (Clark *et al.,* 1994) have reported that *B. Aldocentis* and *B. Infantis*survived in two per cent oxgall but at a lesser extent than *B. longum*. The growth of *B.aldocentis*was decreased substantially in four percent oxgall while did not survive in two or four per cent oxgall during 12 hrs. of incubation. Fungal strain F1 was showing viability upto 14 days and A7 strain of actinomycetes upto 3 days in presence of 0.3% bile salt.

Conclusion

Present work shows that the marine microorganisms isolated from different coastal areas of India may play a good role as a Bio control agent as probiotics and bacteriocin producers. The marine isolates from marine environment showed broad spectrum antibacterial activity against pathogens. Isolation and characterization of bacteriocin producing strains from extreme environment like marine would provide lead to approaches like bio preservatives.

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