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ISOLATION AND SCREENING OF MARINE BACTERIA AS PROBIOTICS AND EVATUATION OF ANTIBATERIAL ACTIVITY AGAINST HUMAN PATHOGENS

BHARTI PRAKASH WADEKAR[a1] AND SMITA M. DHARMADHIKARI[d]

[a]Research Student, Microbiology Department, Government Institute of Science, Aurangabad, Maharastra, India
[b]Department of Microbiology, Government Institute of Science, Aurangabad, Maharastra, India

ABSTRACT

A total 107 bacteria were isolated from coastal marine environment at six different location of India using MRS agar. Isolates were identified at genus level by growing them on selective media. Out of 107 isolates, 45% are observed to be Lactobacillus sp., 22% are *Enterococcus* sp., and 33% are *Bifidobacterium* sp. Five different human pathogens isolated in laboratory showing multiple antibiotic resistance were used as indicators for screening of probiotics. Amongst isolated marine bacteria only 11% isolates had shown antibacterial activity. The broad spectrum activity has been shown by four probiotics belonging to group of *Lactobacillus* sp. and *Bifidobacterium* sp. Higher stability at low pH up to 3 hours of incubation has shown by two strains of probiotics viz. *Bifidobacterium* sp. B25 and *Lactobacillus* sp. L43. Similarly they had shown tolerance to 0.3% bile salt up to 4 hours, which supports them as probiotics. Further based on bacteriocin assay the potency of probiotics were expressed in terms of LD50 value and that was found to be 0.76 mg/ml for Strain L 43 and 0.43 mg/ml for strain B 25.

KEYWORDS : Probiotics, Bacteriocin, Antibacterial Activity, LD_{50}, *Lactobacillus* sp., *Enterococcus* sp., and *Bifidobacterium* sp.

The marine environment harbors a wide range of microbes capable of exhibiting bacteriolytic and antibiotic activity. The term probiotic used in 1965 by Lily and Stillwell to describe substances which stimulate the growth of other microorganisms. Thus, Probiotic is a live microbial supplement which affects hosts health positively by improving its intestinal microbial balance. Microorganisms applied in probiotic products are *Lactobacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp., *Lactococcus* sp. etc Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceutical products.

Bacteriocins have extensively been studied with reference to microbiology, biochemistry and molecular biology, because of their applied importance in medicine, pharma-agro and food preservation industries. It has been evidenced that Bacteriocin Like Inhibitory Substances (BLIS) producing marine bacteria and their use in the control of undesirable bacterial infections with reference to their broad inhibitory spectrum against human, food spoilage and food borne pathogens.

The bacteriocins of many LAB are attractive in for being active against pathogenic and spoilage bacteria (Sarika et al., 2010). However, only limited wok had focused on bacteriocins of marine origin. Bacteriocins are ribosomally synthesized peptides, that exert their antimicrobial activity against either strains of the same species as the bacteriocin producer (narrow range), or to more distantly related species (broad range).The probiotic has been intensively studied as probiotics in mammals and in fresh water fishes (Sica et al., 2010). Their diversity in coastal marine environments and in marine fishes is still unknown. Hence, the study was aimed at the isolation of different LABs from the water and sediments of coastal marine environment and assessing their antimicrobial activity against pathogenic bacteria. Further, the characterization of the potent strain which produce bacteriocin had been attempted.

In present investigation work has been carried out to isolate marine bacteria and screening for selection of probiotic producers amongst isolates based on antimicrobial activity against isolated human pathogens as well as tolerance to low pH and bile salt, LD_{50} (mg/ml).

MTERIALS AND METHODS

Collection of Marine Samples

Marine water and sediment samples were collected from six different coastal areas of India, name as Calangute beach Goa, Dadar chaupathy Mumbai, Gopalpur

[1]**Corresponding author**

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

Isolation of Marine Bacteria

MRS broth was used for isolation and enumeration of marine bacteria for its probiotic ability. 1 ml of each marine water sample was inoculated in 10 ml sterile MRS broth prepared in sea water and incubated for 24 hrs. 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 at 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 MRS broth prepared in sea water and incubated at 30^0C for 3 days. The enriched samples were subjected for isolation on MRS agar containing sea water and incubated at 30^0C under anaerobic conditions (in anaerobic jar using gas pack). The plates were observed and good isolates were procured and identified further.

Identification at Genus Level

These marine bacterial isolates were identified at genus level by growing them on selective media like MRS agar for genus Lactobacillus, MRS Agar containing L-cysteine and *Bifidobacterium* modified media (Hi media) for confirmation of genus *Bifidobacterium*, Pfizer selective *Enterococcus* agar (Hi media) for *Enterococcus*. Identification has been carried out by observing characteristic growth pattern. All these identified cultures were stored for further study at -20^0C in nutrient broth /MRS broth with 20 %(v/v) glycerol (Khosaro Issazadeh et al., 2012).

Screening for Probiotics

Amongst 107 isolated marine bacteria further screening has been carried out for their probiotic nature on the basis of antibacterial activity towards distinct human pathogens isolated in our laboratory and showing multiple antibiotic resistance by agar diffusion method. A lawn of indicator strains including *E. coli*, *K. pneumoniae* ssp *pneumonia*, *Pseudomonas* sp., *Staphylococcus* sp., *Enterococcus* sp. was made over the surface of Muller Hinton Agar. The plates allowed to dry and wells were prepared on surface of agar plates. Cell free extract of isolates was prepared by growing them overnight in MRS broth, cells were separated by centrifugation at 10,000xg for 15 minute and supernatant was collected and neutralized up to pH 7 using 0.1 N NaOH and then used for bacteriocin assay. The crude extract at 50ul quantity was incorporated in respective well and plates were incubated at 37^0C for 24 hours. 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.

Resistance to Low pH

For this purpose, 24 hrs old active selected marine probiotics were used. Intact cells were prepared by growing them in MRS broth and harvesting cells by centrifugation at 5000rpm at 4^0C for 10 minutes. Pellet was washed using phosphate- buffer saline (pH 7.2) and then suspended in same 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 37^0C for variable period of time from 1 to 4 hours. Viability was measured every after one hour of incubation by measuring turbidity at OD 620 nm (Prasad J. et al., 1998).

Tolerance Against Bile

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.

Folin-Lowry Method for Estimation of Protein

The protein content of bacteriocin produced from strains of marine probiotics L43 and B25 was estimated by Folin Lowry's method 1951.

Bacteriocin assay and LD_{50}

Bacteriocin assay was set by preparing different dilutions of crude extract (0.1 to 1 ml) and mixed with 0.1 ml of active culture of multi drug resistant strain of laboratory isolated K. pneumoniae ssp pneumoniae. A volume was made up to 10 ml using nutrient broth and incubates at 37^0C

for 24 h. The antibacterial activity of bacteriocin was measured in terms of LD_{50}, calculated in terms of amount of bacteriocin needed to inhibit 50% growth (LD_{50}) of indicator strains. (Cabo et al., 1999).

RESULTS AND DISCUSSION

Isolation of Marine Bacteria

Total 107 bacteria were isolated from different marine water samples as shown in Table 1.

Identification at Genus Level

Based on growth on selective media and typical characteristics as shown in Figure 1 and Table 2, they were identified at genus level and found to be belonging to major groups viz. *Lactobacillus* sp. (45%), *Enterococcus* sp. (22%) and *Bifidobacterium* sp. (33%). *Enterococci* and *Bifidobacterium* were isolated mostly from sediments and Lactobacilli from surface sea water. Most of the scientist also reported that the detection of *Enterococci* and *Bifidobacterium* from marine water is associated with fecal contamination of sea water. Similarly Sica et al., in (2010) also reported the occurrence of *Enterococcus* sp. in the sediments of marine estuary. The frequency of the *Enterococcus* sp. in the sediments might be due to the exposure of the sediments to the land and the terrestrial run off of fecal matter from land to oceans. It was reported by De Oliveira in 2008 that the tendency of occurrence of *Enterococci* in sediment is more due to exposure of sediments to the land and terrestrial run off of fecal matter from land to the oceans.

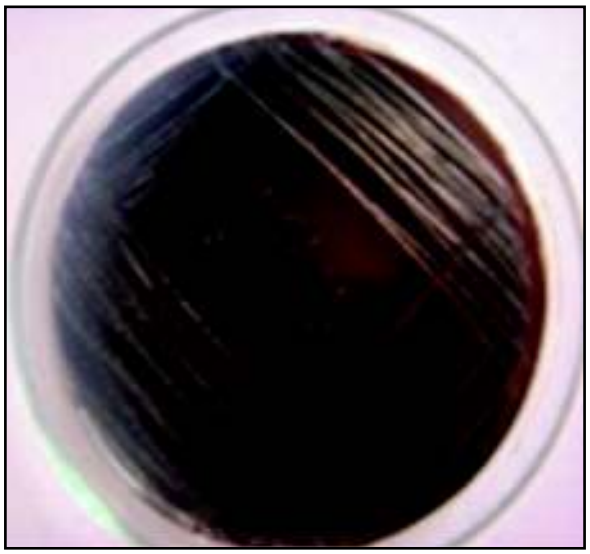
Screening for Probiotics

Five different human pathogens isolated in laboratory showing multiple antibiotic resistance viz. *Escherichia coli*, *K. pneumoniae* ssp *pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus sciuri*, *Enterococcus casseliflavus*, were used as indicator organisms for screening of probiotics. Amongst isolated marine bacteria only 9% isolates had shown antibacterial activity belonging to genus *Lactobacillus* sp. (03), *Enterococcus* sp. (03) and *Bifidobacterium* sp. (04). The strains of *Lactobacillus* sp. L4 and L 43 and *Bifidobacterium* sp. B25 has shown 100% antibacterial activity. The highest spectrum of inhibition (80%) was observed towards *Klebsiella pneumoniae* species *pneumoniae*. The data is presented in table number 3 and figure number 2. 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*. For *Enterococcus* our results reported about 14% *Enterococci* were showing antibacterial activity. Sarika et al., 2011. also reported about antibacterial potential of lactic acid bacteria and *Enterococci* isolated from surface sediments of Vizhinjam coast against indicator organisms. Our results revealed the presence of the compound bacteriocin have been reported to be inhibitor against several other bacteria.

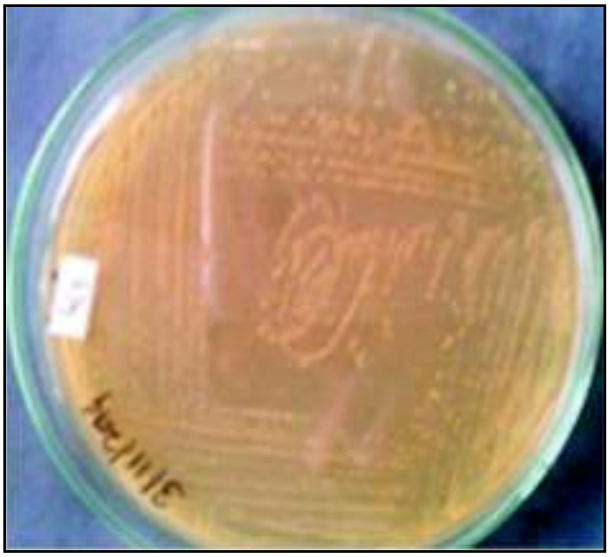
Table 1 : Isolation of Marine Bacteria

Sr. No.	MARINE SAMPLES	NO.OF ISOLATES
1.	Elliot's beach, Chennai (water)	05
2.	Elliot's beach, Chennai (Sediment)	07
3.	Calangute beach, Goa (water)	21
4.	Calangute beach, Goa(Sediment)	09
5.	Thirumullavaram beach, Kerala(water)	22
6.	Thirumullavaram beach, Kerala(Sediment)	12
7.	Gopalpur, Orissa(water)	11
8.	Gopalpur, Orissa (Sediment)	08
9.	Dadar chaupati, Mumbai(water)	-
10.	Dadar chaupati, Mumbai (Sediment)	07
11.	Angellar beach(water)	05
12.	Angellar beach((Sediment)	-
	Total	107

Figure 1 : Colony Characterization on Selective Media for Identification of Marine Bacteria



Pfizer selective Enterococcus agar
(*Enterococcus* sp.)



MRS Agar
(*Lactobacillus* sp.)

Bifidobacterium Modified Media
(*Bifidobacterium* sp.)

MRS+L-cystine
(*Bifidobacterium* sp.)

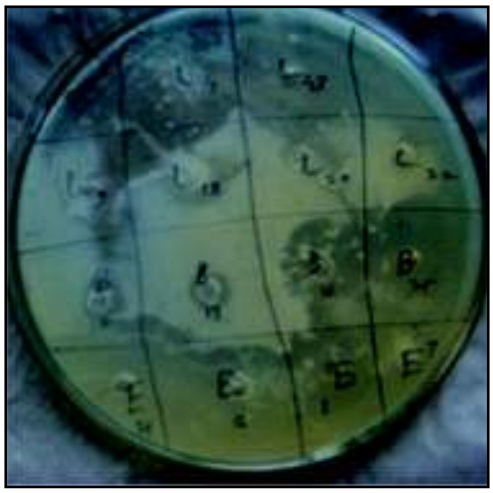
Resistance to Low pH

As shown in figure 3 both the selected cultures were capable of growing at low pH up to 1.0. The higher stability was observed up to 3 hours at pH 3.0. Amongst the two strains the strain *Bifidobacterium* sp. B 25 was found to be more stable. Prasad et al., (1998). reported that a significant decrease in the viability of strains is often observed at pH 2.0 and below. According to this experiment isolates were resistant to low pH. As it is seen in the graphics, L43 and B25 are very stable in pH 3.0 which means that these isolate are able to survive in this pH value. The conditions in stomach were simulated by using pH 15 phosphate-buffered saline.

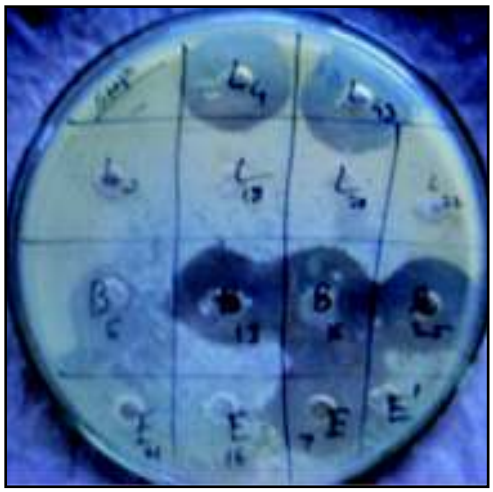
Figure 2 : Antibacterial Activity for Screening of Probiotics

Escherichia coli

K. pneumoniae ssp pneumonia



Pseudomonas aeruginosa



Staphylococcus sciuri

Enterococcus casseliflavus

Table 2 : Colony Characterization on Selective Media for Identification of Marine Bacteria

Name of Media	Colony Characteristics	Identified Genus	Total Isolates (%)
Pfizer selective Enterococcus agar	Black round colony	*Enterococcus*	22
MRS Agar	Creem,smooth,sticky colony	*Lactobacillus*	45
Bifidobacterium modified media	Concave round colonies, acid odour	*Bifidobacterium sp.*	33
MRS+L-cystine	Watery colonies	*Bifidobacterium sp.*	

Table 3 : Antibacterial Activity for Screening of Probiotics

	Zone of Inhibition (mm)										% Inhibition
Sr. No.	**L4**	**L20**	**L43**	**E1**	**E7**	**E16**	**B6**	**B13**	**B16**	**B25**	
Escherichia coli	19	_	23	12	_	14	17	15	_	21	70
K,pneumoniae ssp pneumonia	25	16	27	23	21	_	_	15	27	27	80
Pseudomonas aeruginosa	25	_	23	17	_	16	-	13	25	21	70
Staphylococcus sciuri	20		21	_	19	_	14	20	24	25	70
Enterococcus casseliflavus,	20	17	23	11	_	_	15	20	20	12	80
% Inhibition	100	40	100	80	40	40	80	100	80	100	

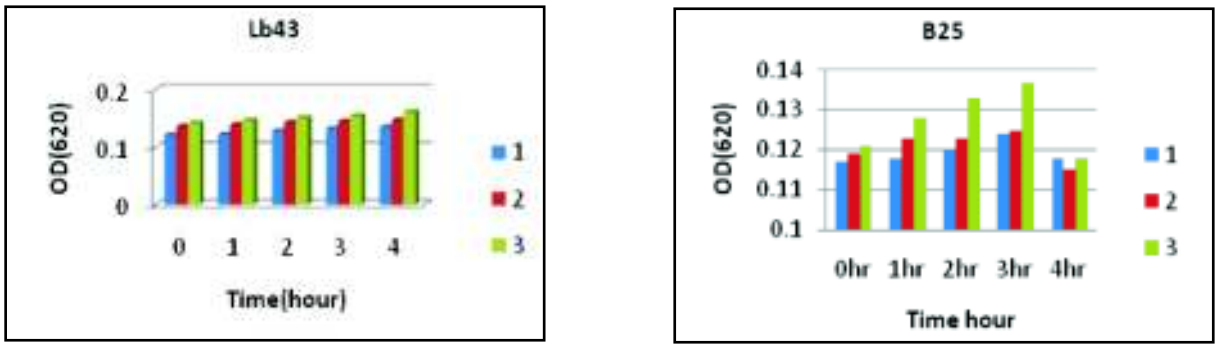


Figure 3 : Acid Resistance Studies of Probiotics

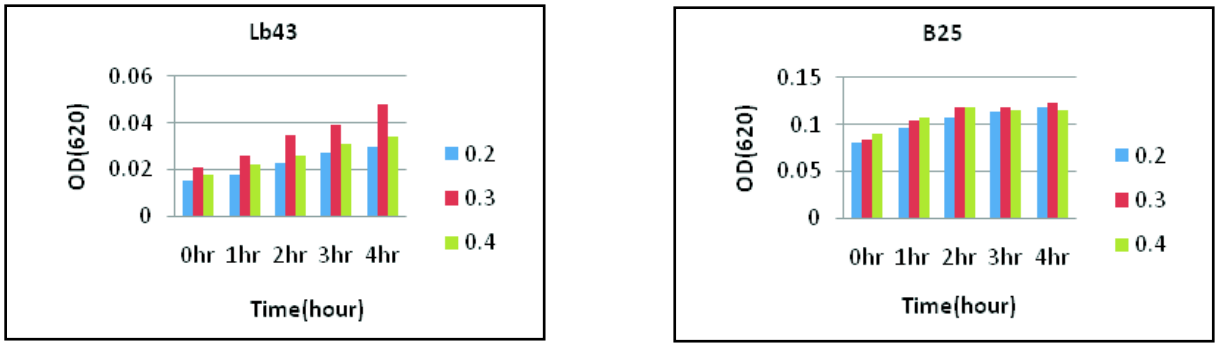
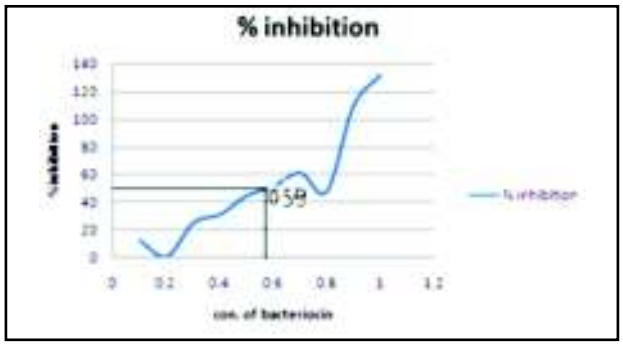
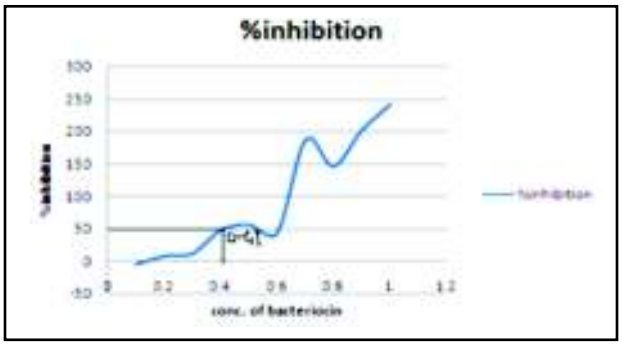


Figure 4 : Bile Salt Tolerance e Studies of Probiotics



Assay of L43



Assay of B25

Figure 5 : Bacteriocin Assay and LD_{50}

Tolerance Against Bile

Similar to low pH resistance the bile salt tolerance has been also studied for these two cultures. As per data shown in figure 4 both strains can tolerate higher concentration up to 0.4% but the optimum bile salt concentration was found to be 0.3% up to 4 hours of incubation. Compare to strain *Lactobacillus* sp. L43 *Bifidobacterium* sp. B 25 has shown high tolerance. Clark et al., (1994) have reported that *B adolescentis* and *B. infantis* survived in two per cent oxgall but at a lesser extent than *B. longum*. The growth of *B. adolescentis* was decreased substantially in four percent oxgall while *B. infantis* did not survive in two or four per cent oxgall during 12 hrs of incubation.

Folin-Lowry method for estimation of protein: The protein content of extracted bacteriocin was estimated by standard Folin-Lowry's method and found to be 1.32 mg/ml for L 43 and 1.66 mg/ml for B25.

Bacteriocin Assay and LD_{50}

Further based on bacteriocin assay the potency of probiotics were expressed in terms of LD_{50} value and that was found to be 0.76 mg/ml for Strain L43 and 0.43mg/ml for strain B25 as per Figure 5. Similarly Cabo et al., (1999). quantify bacteriocin by turbidometric method and express the potency in terms of ID 50 that is inhibitory dose where there is 50 % inhibition. The bacteriocin produced from *Bifidobacterium* B 25 was found to be more potent as lethal dose was found to be low to kill 50% organisms. The isolation and characterization of bacteriocin producing strains from extreme environment like marine would provide lead to approaches like biopreservative.

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