Isolation and Identification of Pathogenic Bacteria from Brackish Waters of Chilika Lagoon, Odisha, India for Pharmaceutical Use

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ABSTRACT

Aims: The present investigation was undertaken in order to isolate bacteria from eighteen different water samples collected from three different sectors of 'Chilika' lagoon of India and to study the resistance against ten different antibiotics viz., norfloxacin, tetracycline, ciprofloxacin, neomycin, nalidixic acid, ofloxacin, chloramphenicol, nitrofurantoin, streptomycin and amoxicillin as well as their serological implications.

Methodology and Results: Four different pathogenic bacteria species viz., *Shigella dysenteriae*, *Streptococcus lactis*, *Bacillus cereus* and *Klebsiella pneumoniae* were isolated which showed a wide range of sensitivity to norfloxacin, tetracycline, ciprofloxacin, ofloxacin and nitrofurantoin. *S. dysenteriae* was sensitive to streptomycin where as other isolates were found to be resistant. Agarose gel electrophoresis failed to reveal plasmid DNA band indicating that the observed resistance was perhaps encoded by nucleotide sequences harboured on the chromosomal DNA. Bacterial isolates were used as antigen for the production of polyclonal antibodies in rabbits.

Conclusion, significance and impact of study: All the isolates exhibited strong antigenic character with specific serological relationship which can be implicated towards development of novel and pharmaceutically effective anti-bacterial products.

Keywords: 'Chilika', bacterial isolates, antibiotic resistance, serological test

INTRODUCTION

'Chilika' is the largest lagoon in the subcontinent which is situated along the east coast of India between latitude 19° 28' and 19° 54' N, and longitude 85° 05' and 85° 38'E. This is a shallow, brackish-water lake formed due to the silting action of the Mahanadi River, which drains into the northern end of the lake, and the northerly currents in the Bay of Bengal, which have formed a sandbar along the eastern shore (Arya and Lakhotia, 2006). It is the largest brackish water wetland complex in Asia, declared as a Ramsar site under the convention on "Wetlands of International Importance". This lagoon is presently under threat from both natural and anthropogenic pressures (Nayak *et al.*, 2004). The marine realm constitutes the major habitat of the biosphere covering 73 % of the earth surface which provides the largest inhabitable space for living organisms, particularly microbes. Marine microbes flourish not only in the surface water of the sea, but also in the lower and abyssal depths from coastal to the offshore regions and from the general oceanic to the specialized niches like blue waters of coral reefs to black smokers of hot thermal vents at the sea floor.

As the water being served as major solvent, it plays an important role in the pharmaceutical industries. It is

considered as the primary source of contamination for pharmaceutical products (Tamara *et al.*, 1998). Microbes present in the marine waters represent a potential source for commercially important bioactive compounds and their bioremediation capabilities are also remarkable. There is an increasing demand of biodiversity from natural resources for development of therapeutic drugs. Antibiotics are broadly used as chemotherapeutic agents which, albeit in a very low quantity, can inhibit the pathogenic activity of microorganisms. The potential contribution of marine organisms to the discovery of new bioactive molecules is increasingly challenging (Sponga *et al.*, 1999; Skulberg, 2000). The microorganisms have become a significant attraction as natural source of bioactive molecules with a broad range of biological activities, such as antibiotics, antivirals, antitumorals, antioxidant and anti-inflammatory (Okami, 1982; Kamei *et al.*, 1987; Nunez *et al.*, 2006; Uzair *et al.*, 2009; Shankar *et al.*, 2010). Evidence of phytochemical and pharmacological studies on microbes is available in the literature (Zeeshan *et al.*, 2010; Odeyemi *et al.*, 2010; Bragadeeswaran *et al.*, 2010).

Previously, there were a number of researchers that had worked on 'Chilika' regarding hydrological characterization, water quality variation, antimicrobial

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activity and physiochemical variation (Rao *et al.*, 1981; Nayak *et al.*, 2004; Patra *et al.*, 2009, Patra *et al.*, 2010). Unfortunately progress made on pharmaceutical studies of marine microorganism from 'Chilika' is inadequate. This underpinned the present study with a view to investigating the physiological, biochemical, and serological characterization of bacteria isolated from marine waters of 'Chilika' aiming at their exploitation for pharmaceutical benefits.

MATERIALS AND METHODS

Isolation and characterization of bacterial strains

The total study area is comprised of 18 sampling stations covering three different sectors of the 'Chilika' lagoon i.e. Central, Southern and Outer Channel (**Figure 1A, 1B**) during the period from January to March 2008. The exact sampling locations were fixed by using Global Positioning System (GPS). All the water samples were collected in the morning hours between 7:00 A.M-11:00 A.M. During sample collection necessary precautions had been taken to collect undisturbed water samples in the lake. Samples were collected in pre-sterilized polypropylene bottles (500 mL). Physical parameters like temperature and pH of the samples were determined on the spot of collection. Then the water samples were kept in the icebox and transferred to laboratory for further analysis and the samples were preserved at 4°C before isolation and identification in the laboratory. The experiments were carried out within 4 months of collection. Chemicals used for preparation of reagents in the present investigation were of analytical reagent grade and for preparation of solutions double-distilled water was used. One milliliter of water sample was subjected through serial dilution to obtain 10^{-8} dilution. After that 1mL of 10^{-8} diluted sample was mixed with 10 mL of sterilized Nutrient broth medium and then it was incubated at 37 °C in a BOD incubator for 24 h. After incubation a loop-full of microbial culture were picked up from different colonies and streaked separately on the different agar-gelled sterilized media and the plates were incubated at 37 °C for 24 h for isolation of pure culture. The colony characteristics such as colour, appearance, and shape of the isolate were recorded. Further, morphological, and biochemical tests were carried out in the laboratory by following standard microbiological methods described by Cappuccino and Sherman (2002) for identification and characterization of isolated microorganism. The isolated strains were also characterized through different physiological factors such as pH, temperature and salinity to observe their tolerance capacity in the stressed environmental conditions.

Collection and preservation of pre-immunized serum

For production of antisera 3-4 month-old white rabbit (2.5 - 3 kg) were selected and kept in separate cage and labeled properly. The pre-immunized sera of the rabbit used for the test were collected before 7 days of immunization with different antigens to confirm that the

A.

B.

Figure 1: Location map of 'Chilika' lagoon in the State of Odisha of India (**A**) and revealing different ecological sectors as sampling sites (**B**).

rabbit did not contain any antisera. The sterilized tube containing fresh drawn blood was kept at room temperature for 1-2 h for clot formation. The clots were stirred with glass rod and was kept overnight in refrigerator to allow the clot contraction. The plasma were centrifuged (3000 rpm, 15 min). The straw-coloured serum was withdrawn into a sterilized centrifuge tube which was labeled properly. For preservation of serum, it was added with 0.02 % sodium azide (NaNH_2) to give a final concentration of 1:5000 and kept in a deep freeze at -20 °C for further use.

Serological test

Preparation of whole cell antigen: The antigen was prepared from the freshly grown bacterial cultures. The bacteria were cultured on NSA medium in test tube at 27

°C for 24 h. The test tube was filled with PBS or saline water. The culture was scraped with sterilized inoculation needle. The scraped bacteria with saline water were transferred to the centrifuge tube. The bacterial growth was mixed thoroughly with the help of a vortex mixture to make it completely homogenized. The whole cell of bacteria was centrifuged (3000 rpm, 30 min, room temperature). The supernatant was discarded and the pellet was re-suspended with saline water and centrifuged. The process was repeated three times to make the bacteria free from any foreign material. After the last centrifugation bacteria were re-suspended in normal saline/butler saline, collected in sterilized specimen tube, labeled properly, preserved and stored in deep freeze at -20 °C for further use.

Preparation of antigen for agglutination test: The antigens were prepared by suspending live bacteria in buffer saline as described earlier except that the antigen prepared was more concentrated having an optical density of 0.5 using a Gallen Kamp colorimeter at 520 nm.

Immunization of rabbits for antisera production and harvesting of immunized serum: The rabbits were immunized by intra-muscular and intravenous injections. Both right and left legs were used for intra-muscular injection. In case of intravenous injection, marginal vein of ear was used. Three doses of antigen (0.5 mL, 1.0 mL, and 1.5 mL) were injected intra-muscularly at four days interval. Acetone was used to dilate the vein. The designed quantity was injected slowly into the blood system. A booster injection was given 4 days after the last injection to accelerate antibody production. Five or seven days after the booster injection, the rabbits were held by cardiac puncture for collection of antisera. The procedure of collection of blood separation and preservation of antiserum was the same as described earlier.

Antigen-antibody reaction tests: Mixture of antigen and antibody showing positive reaction resulted in the formation of precipitation due to binding of antibodies with their respective antigens. Two different serological tests were conducted to record the precipitations or positive reaction of antigen and antibodies. The gel diffusion test was performed following the method as originally described by Ouchterlony (1958, 1962) while the tube agglutination test was carried out for homologous and heterologous antigens using a national standard method (NHS, 2010).

Antibiotic sensitivity test

Antibiotic sensitivity test of the identified bacteria were repeated 3 times for each strain using 10 different antibiotics namely norfloxacin, tetracycline, ciprofloxacin, neomycin, nalidixic acid, ofloxacin, chloramphenicol, nitrofurantoin, streptomycin and amoxicillin (Hi-Media, Mumbai, India) by disc diffusion method (Bauer *et al.*,

1966). Antibiotic activity was measured in terms of zone inhibition (mm diameter).

Plasmid DNA isolation and agarose gel electrophoresis

The alkaline-lysis mini-prep method (Wizard Plus Minipreps DNA Purification Systems, Promega, USA) was used for plasmid extraction from all the four bacterial isolates. Overnight-grown bacterial cultures (3-5 mL) were pelleted by centrifugation (10,000 x g, 10 min), supernatant decanted and pellet re-suspended in 300 µL of Cell Re-suspension Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg/mL RNase A). The re-suspended cells were transferred to 1.5 mL micro-centrifuged tube to which 300 µL of Cell Lysis Solution (0.2 M NaOH, 1 % SDS) was added and samples thoroughly mixed. To the cleared lysate 300 µL of Neutralization Solution (1.32 M potassium acetate, pH 4.8) was added and mixed properly. The lysate was centrifuged (10,000 x g, 5 min) and plasmid purification was carried out using the vacuum manifold (Promega Vac-Man, USA). The purified samples were electrophoresed using 0.7 – 1.0 % agarose gel followed by ethidium bromide (0.5 µg/mL) staining (Sambrook *et al.*, 1989). Electrophoresis was carried out in 1 x Tris borate-EDTA (TBE) buffer at 120 V for 3 h or until the loading dye (Genie-Merck, Bangalore, India) neared the bottom of the gel. Gels were visualized and photographed under GEL DOC XR SYSTEM (Bio-Rad, USA).

RESULTS AND DISCUSSION

To identify the isolates obtained from 3 different sectors of the 'Chilika' lagoon each of them were tested for various morphological, biochemical, physiological and serological characters. The results of morphological and biochemical tests were presented in **Table 1**. The isolates are closely related to *Shigella dysenteriae*, *Streptococcus lactis*, *Bacillus cereus*, *Klebsiella pneumoniae* (**Table 1** and **Table 2**). In nutrient agar plates *Shigella dysenteriae* appeared as thin, even and greyish colonies measuring ca. 3-4 mm diameter. *Streptococcus lactis* colonies were thin, even-growths on nutrient agar plates ranging ca. 2-3 mm diameter. In comparison, *Bacillus cereus* colonies which were fermented on nutrient agar plates had abundant, opaque, white waxy growths measuring ca. 3-4 mm diameter. *Klebsiella pneumoniae* colonies were skimmy-white, raised growths with ca. 2-4 mm diameter. Each bacterial colony cultured on nutrient agar medium was then subjected to Gram staining and thereafter observed under light microscope. The shape and staining property of the bacteria were recorded. *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Bacillus cereus* were found to be rod-shaped bacteria whereas *Streptococcus lactis* had a spherical (coccus) shape. *Streptococcus lactis* and *Bacillus cereus* were found to be Gram positive bacteria whereas *Shigella dysenteriae* and *Klebsiella pneumoniae* was Gram negative.

Table 1: Morphological and biochemical tests for identification of the isolates

Test Organisms		<i>Shigella dysenteriae</i>	<i>Streptococcus lactis</i>	<i>Bacillus cereus</i>	<i>Klebsiella pneumoniae</i>
Agar Plate Culture Characteristics		Thin, even, grayish growth	Thin, even growth	Abundant, opaque, white waxy growth	Slimy, white, raised growth
Litmus Milk Reaction		Alkaline	Curd	Peptoniz-ation	Gas
Gram's stain		Rod (-)	Cocci (+)	Rod (+)	Rod (-)
Capsule stain		-	+	-	+
Acid-Fast stain		-	-	+	-
Fermentation	Lactose	-	A	-	AG
	Dexrose	A	A	A	AG
	Sucrose	A	A	A	AG
H₂S roduction		-	-	-	-
NO₃ Reduction		+	-	+	-
Indole Production		-	-	-	-
MR Reaction		+	+	-	-
VP Reaction		-	-	+	+
Citrate Utilization		-	-	-	+
Urease Activity		-	-	-	+
Catalase Activity		+	-	+	+
Oxidase Activity		-	-	-	-
Gelatin Liquefaction		-	-	+	-
Starch Hydrolysis		-	-	+	-
DNase Test		-	-	-	-
Triple Sugar Iron Test		Lactose	Glucose	Glucose	Glucose
Cholesterol Hydrolysis		-	+	-	-
Arginine Dihydrolase		-	-	-	-

+ = Positive; - = Negative; A = Acid; AG = Acid and Gas

Table 2: Physiological tests for identified microorganisms at optimum parameters

Test Organisms	pH	Temperature	Salinity
<i>Shigella dysenteriae</i>	7-8	40 °C	9 %
<i>Streptococcus lactis</i>	7-9	40 °C	9 %
<i>Bacillus cereus</i>	7-9	45 °C	8 %
<i>Klebsiella pneumoniae</i>	7-8	45 °C	9 %

In addition to morphological and biochemical characterizations the isolated strains were also characterized through different physiological factors such as pH, temperature and salinity to observe their tolerance capacity in the environmental stress conditions. The identified bacterial species were tested for their halotolerance nature on the basis of pH, temperature and salinity. The optimal physiological conditions of isolated strains are presented in **Table 2**. It was found that all isolated strains survived in conditions at a varying pH 8 - 9, temperature regime 40 °C – 45 °C, and salinity ranging 8 – 9 %, thus indicating that these isolates were halotolerant.

Antibiotic sensitivity tests were performed for each of the four identified bacterial species. Those cells which were completely inhibited and failed to resist the action of antibiotics against them became sensitive to the corresponding antibiotics. But partial inhibition happened when somehow some mutant cells emerged and they could successively resist the action of the antibiotics against them (Johnson *et al.*, 1995). In this study *Shigella dysenteriae* was the only one that showed sensitivity to streptomycin but the three remaining strains showed resistance to streptomycin (**Table 3**). Multi-drug resistance by *Shigella* sp. was not uncommon (Lien *et al.*, 1994). Although all the isolates showed resistance to neomycin and chloramphenicol; they showed a wide range of sensitivity to norfloxacin, tetracycline, ciprofloxacin, ofloxacin and nitrofurantoin; albeit intermediate sensitivity to nalidixic acid and amoxicillin.

It was interesting to note that in none of the extracted samples from resistant strains plasmid DNA was detectable in the agarose gel following electrophoresis regardless of agarose concentrations in the gel (data not shown). Perhaps, the observed antibiotic tolerance in

Table 3: Antibiotic sensitivity tests for identified microorganisms

Test antibiotic	Antibiotic (µg/disc)	Zone of inhibition Diameter (mm)			
		SD	SL	BC	KP
Norfloxacin	10	30(S)	25(S)	27(S)	27(S)
Tetracycline	30	19(S)	20(S)	22(S)	19(S)
Ciprofloxacin	10	35(S)	27(S)	33(S)	33(S)
Neomycin	30	- (R)	- (R)	- (R)	- (R)
Nalidixic Acid	30	18(I)	18(I)	16(I)	16(I)
Ofloxacin	5	30(S)	30(S)	30(S)	28(S)
Chloramphenicol	10	- (R)	- (R)	- (R)	- (R)
Nitrofurantoin	200	22(S)	20(S)	23(S)	23(S)
Streptomycin	25	28 (S)	- (R)	- (R)	- (R)
Amoxicillin	25	14(I)	14(I)	15(I)	16(I)

SD = *Shigella dysenteriae*; SL = *Streptococcus lactis*; BC = *Bacillus cereus*; KP = *Klebsiella pneumonia*
 S = Sensitive; R = Resistant; I = Intermediate

Table 4: Serological tests for identified microorganisms

Test Organisms	Gel Diffusion Test	Tube Agglutination Test
<i>Shigella dysenteriae</i>	+	+
<i>Streptococcus lactis</i>	+	+
<i>Bacillus cereus</i>	+	+
<i>Klebsiella pneumoniae</i>	+	+

+ = Positive

such strains isolated from the brackish waters of the 'Chilika' lagoon is due to phosphorylation/acetylation activity of enzymes encoded by gene loci borne on the chromosomal DNA instead of on plasmids. It was not uncommon to enteric bacteria among which the multi-drug resistance regulatory chromosomal locus, *mar* is widespread as was reported by Cohen *et al.*, (1993). Constitutive expression of *mar*-A leading to differential expression of over 60 chromosomal genes in *E. coli* has been reported by different investigators. According to Barbosa and Levy (2000), such kind of expression possibly conferred multiple antibiotic resistance on *E. coli*. Another gene namely *emr* harboured on *E. coli* chromosome has been identified that codes for protein products of membrane translocase family and accounts for multi-drug resistance (Lomovskaya *et al.*, 1992).

All strains isolated from the brackish water samples of 'Chilika' lagoon revealed a strong antigenic character basing on two different serological tests namely gel diffusion test and tube agglutination test respectively (Table 4). The present investigation strongly revealed that these four promising marine bacterial strains hold potential to be used for development of anti-pathogenic agents in pharmacology and pharmaceutical industries. Nevertheless, further validation is necessitated prior to

exploiting these strains aiming at development of novel and specific antibacterial compounds, antibiotics and enzymes which could offer considerable pharmaceutical value.

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