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Characterization, antagonistic effect, resistant pattern and adaptation capability of halophilic bacteria isolated from Inani Beach, Cox's Bazar, Bangladesh

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Abstract

The ocean, with its rich salt tolerant microbial biodiversity, continues to serve as a source of potential salt tolerant gene. In this study, bacteria are isolated from marine water collected from the Inani Beach, Cox's Bazar, Bangladesh. Bacterial strains were inoculated in standard Luria-Bertani (LB) medium with 2% of NaCl. Two types of bacterial strain A and B were isolated according to the morphology and nature of colonies. Morphological and biochemical properties of bacteria indicated that strain A was cream colored, gram negative, round shaped, non-motile catalase positive and showed negative result in methyl red test. Strain B was creamy white colored, gram positive, rod shaped, non-motile and showed positive results in both methyl red and catalase test. The adaptability of bacterial samples were examined on different laboratory conditions and also observed their potential as a sources of antimicrobial agents. The optimum physiological conditions for both isolates were at pH 8, temperature 35°C and 2gm/l of NaCl. Both of the strains were highly resistant to Tetracycline, Cefuroxime, Erythromycin, Doxycycline, and Carbenicillin. Bacterial strain A and B both have the ability to utilize glucose, sugar, yeast extract and glycerol as their sole source of carbon. Among these yeast extract was proved as the best source of carbon. The present investigation also revealed that the isolated bacteria B have antagonistic effects on the human pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli*. Both of the isolates A and B were cultured in LB media without addition of NaCl. The growth rates of isolate A was significantly higher than isolate B in the medium and growth rates were increasing in every subcultures. Therefore, the study concluded that the strain has strong adaptation capability in normal environmental condition.

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Introduction

Ninety-seven percent of the earth's water is saline in nature and it accounts for approximately 71 % of the earth's surface, which generates 32 % of world's net primary production (Alexander, 1999). Although bacterial communities have been frequently studied, recent studies show that lots of bacterial communities need to be still characterized in marine environment (Gontang *et al.,* 2007).

Only recently has intensive investigation been initiated with marine bacteria concerning those properties which distinguish them physiologically from terrestrial bacteria. ZoBell (1946) summarized much of the research done to that date, showing that the ability to grow in and requirement for seawater as a base for media characterized these bacteria; solution mixtures of the major mineral components were shown to be substitutes for natural seawater for many isolates. Studies of mineral nutrition and osmotic relations have been reported for several marine bacteria (MacLeod *et al.,* 1954; Pratt *et al.,* 1954).

Marine bacteria usually require sodium and potassium ions for their growth and to maintain osmotic balance of their cytoplasm (MacLeod and Onofrey, 1957). This requirement for Na+ ion is an exclusive feature of the marine bacteria which is attributed to the production of iodole from tryptophan (Pratt and Happold, 1960), oxidation of L-arabinose, mannitol, and lactose (Rhodes and Payne 1962) as well as transport of substrates into the cell (Hase *et al.,* 2001). Other physical characters imputed to marine bacteria include facultative psychrophilicity (Bedford 1933), higher tolerance to pressure than their terrestrial counterparts (Zobell and Morita1957). These characteristics are likely to be crucial for their ability to fine-tune and rapidly respond to the changing environmental conditions like sudden nutrient influx ordepletion (Lauro *et al.*, 2009). The potential scope is enormous, by 2006 more than 14,000 novel chemicals had been identified by marine bioprospecting and 300 patents registered on marine natural products. Besides, naturally occurring saline environment, over utilization of ground water is increasing the saline area and about 25% global cultivated area shows excessive salinity (Chowdhury *et al.*, 1993). Therefore, investigation on bacteria of saline environment has significant roles.

In recent years, it has been found that several products of these bacteria such as exopolysaccharides (EPSs), halophilic enzymes and compatible solutes, may have very useful applications in biotechnology (Ventosa, 2004). Nowadays, the study of bacteria from marine origin and their potential role in the production of bioactive compounds is becoming a new topic for research (Faulkner, 2001). The emergence of resistance of bacteria to antibiotics is a common phenomenon. Therefore, there has been a great concern from scientists to investigate marine microorganisms as new source of antibacterial compounds (Pabba *et al.,* 2011).

Marine environment is a vast but represents a largely untapped source for isolation of new microorganism having ability to produce novel bioactive compounds (Kim *et al*., 2006). Marine microbes are new sources of novel bioactive compounds that may have application as pharmaceuticals (Dalmaso *et al.,* 2015). Potential applications for marine microorganisms in ameliorating environmental degradation also exist.

The bacterial diversity in marine water is little known, which is an inexhaustible resource that has not been properly exploited. The full potential of this domain remains largely unexplored as the basis for biotechnology, particularly in Bangladesh. The cox's bazar coastal region has diverse marine habitats such as seashore, hyper saline lakes, estuaries, saltpans and a variety of soil habitats (Nagasathya and Thajuddin, 2008).

The present study was designed with an aim to discover and identify marine bacterial strains, to observe their optimal growth rates in different laboratory conditions, to observe their response on different antibiotics, to observe their antagonistic

effects, and to investigate their adaptation capability in normal environmental conditions.

Materials and methods

Sample collection

The sample (marine water) was collected from Inani Beach, Cox's Bazar. Inani beach is an 18-kilometer-long sea beach in Ukhia Upazila of Cox's Bazar district, Bangladesh.

Enrichment of marine bacteria

Then one ml of marine water sample was suspended to individual 250 ml conical flasks each containing 100 ml of LB liquid medium. Control flasks without an inoculum were also prepared as control. The primary enrichment was incubated for several days at with shaking at 160 rpm (revolution per minute) on a shaking incubator. Cultures, which were found to be turbid after a period of up to 4 days were used as an inoculum in subsequent experiments.

Isolation of bacterial strains from mixed bacterial culture

Plating of mixed bacterial culture

Plating is essential to get single colony from mixed culture. Solidify the agar media and make the mixed culture diluted up to 10 times following dilution techniques. Then Spread the different diluted suspension on surface of different solid agar medium with glass rod spreader and marked with their dilution value.

Culture of Bacteria with LB Agar Medium (Streaking)

When the agar medium became solid, a single colony was taken from the previous agar plate with the help of a sterilized loop and streaked on the agar plate. The plates were incubated at 37°C for 24 hours.

Morphological and biochemical characterization of isolated bacteria

Morphological and biochemical tests were used for specific identification of bacteria. Isolated bacteria was characterized by several morphological (gram staining and motility) and biochemical tests (catalase and methyl red test).

Effect of temperature and pH on bacterial growth

Temperature and pH influenced on the bacterial growth. For the effect of pH, culture medium was adjusted to pH 5.0, 6.0, 7.0 and 8.0. Incubation temperature was varied at, 25°C, 28°C and 37°C. Bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter.

Carbohydrate utilization

In order to find out the ability of the isolates to utilize different carbohydrates, the cultures were inoculated in MS medium containing different carbohydrates *viz.* glucose, sucrose, yeast, and glycerol. The final concentration of the carbohydrates is 1%. The tubes were incubated at 35°C for 3-5 days and observed for any growth.

Antibiotic sensitivity test

Antibiotic sensitivity test was performed with Disc Diffusion method (Bauer, 1966). The isolated bacterial strain was grown overnight in nutrient broths through shaker at 35°C temperature and 160 rpm for the antibiotic sensitivity test. Nutrient agar plates were dried at 35°C.

The overnight grown LB culture (O.D. = 0.5) was poured onto nutrient plate and dried. Antibiotic discs were placed centrally on the respective plates and incubated overnight at 35°C. After overnight incubation the zone was observed on the plate and measured with the help of mm scale. Five commercially available antibiotic discs were used including tetracycline, cefuroxime, erythromycin, doxycycline, carbenicillin.

Growth optimization test with different nacl concentrated media

LB media (100 ml) were prepared with the suppliments of 1% to 10% of sodium chloride in 10 individual conical flasks and labeled them. Then 1ml of liquid bacterial culture was added from the sub culture of the bacteria.

Antagonistic test

The isolated bacterial strains were grown overnight in nutrient broths through shaking at 37°C temperature and 120 rpm for the antagonistic test. The *S. aureus and E.coli* were also sub cultured in nutrient media and kept on a shaker for 24 hours at 37°C temperature at 120 rpm. Sub cultured *S. aureus and E.coli* strain was poured onto nutrient agar plate and dried. Disks for antagonistic test were made by cutting the filter paper using a hole puncher.

Then using bacterial stain A and B, 8 disks were made in different bacterial concentrations. Then dried disks were placed on the respective plates and were incubated overnight at 35°C. After overnight incubation, the zone on the plates was observed and measured the zone with the helping of mm scale.

Test of adaptability of marine bacteria in lb media without the addition of NaCl

The LB media were prepared without the suppliment of NaCl or any other salts. Then the bacterial isolates A and B were innoculated. The both isolates were subcultured several times in the same types of meadia. Then the growth rates were measured and compared.

Results

Isolation of bacteria

spreading of bacteria from marine water

Plating is necessary to isolate bacterial strains and to know about the morphology of the bacteria. Two different types of bacterial colonies were detected based on the morphology and the nature of the colony. They were named strain A and strain B.

Table 1. Summary of morphological, physiological and biochemical test results.

Agar plate	Characters	Results for strain A	Results for strain B
LB agar plate	Size	(1-2) mm	(2-3) mm
	Shape	Round	Sphear
	Colour	Cream	Creamy white
	Consistency	Sticky	Sticky
Nutrient agar slant	Abundance of growth	Moderate	Moderate
	Colour	Cream	Creamy White
Nutrient broth culture		Uniform with fine turbidity	Uniform with fine turbidity
Microscopic observation	Gram straining	Gram-Negative	Gram-Positive
Biochemical test	Motility test	Non-motile	Non-motile
	Methyl Red test	Negative	Positive
	Catalase test	Positive	Positive

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm.

Table 2. Antibiotic sensitivity tests for strain A.

Antibiotics	Range of antibiotics	R	S and I
Tetracycline	13 mm	-	I
Cefuroxime	6 mm	R	-
Erythromycin	6mm	R	-
Doxycycline	6mm	R	-
Carbenicillin	6mm	R	-

Streaking of isolated strains

In this study two isolates, strain A and strain B were isolated from the mixed culture plates using streak plate method. The results were observed after 48 hours of streaking.

Morphological and biochemical characterization of isolated bacterium

Morphological tests indicated that isolated bacterial strain A was non- motile, gram negative, round shaped while and strain B was gram positive and sphear

shaped (Table 1.). Biochemically the isolate A was catalase positive and negative result was found for methyl red test. On the other hand the isolate B was catalase and also methyl red positive (Table 1.).

Table 3. Antibiotic sensitivity tests for strain B.

Antibiotics	Range of antibiotics	R	S and I
Tetracycline	6 mm	R	-
Cefuroxime	6 mm	R	-
Erythromycin	14mm	-	I
Doxycycline	6mm	R	-
Carbenicillin	6mm	R	-

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm.

Table 4. Growth inhibition activity of isolated bacteria (A and B) against two pathogenic bacteria.

Name of Test Bacteria	Dose (µl/disc)	Zone of inhibition (mm)		Resistant pattern	
		Isolate A	Isolate B	Isolate A	Isolate B
S. aureus	50	7	9	Resistant	Resistant
	100	10	12	Intermediate resistant	Resistant
	150	14	15	Intermediate resistant	Resistant
	200	17	18	Susceptible	Intermediate resistant
E. coli	50	6	6	Resistant	Resistant
	100	7	8	Resistant	Resistant
	150	11	13	Intermediate resistant	Resistant
	200	16	16	Intermediate resistant	Resistant

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm.

Growth characteristics of isolated strains

effect of ph on bacterial growth

The bacterial growth depends on pH. The optimum pH for the growth of the both isolates was 8.0 and extreme pH 5.0 and 9.0 restricted the growth of both isolates (Fig.1. and 2.).

Effect of temperature on bacterial growth

The media temperature is an important factor for bacterial growth. The optimum temperature was 35°C for the growth of bacterial strain A and strain B and extreme temperature 25°C and 40°C restricted the growth of both isolated bacterial strain (Fig.3. and 4.).

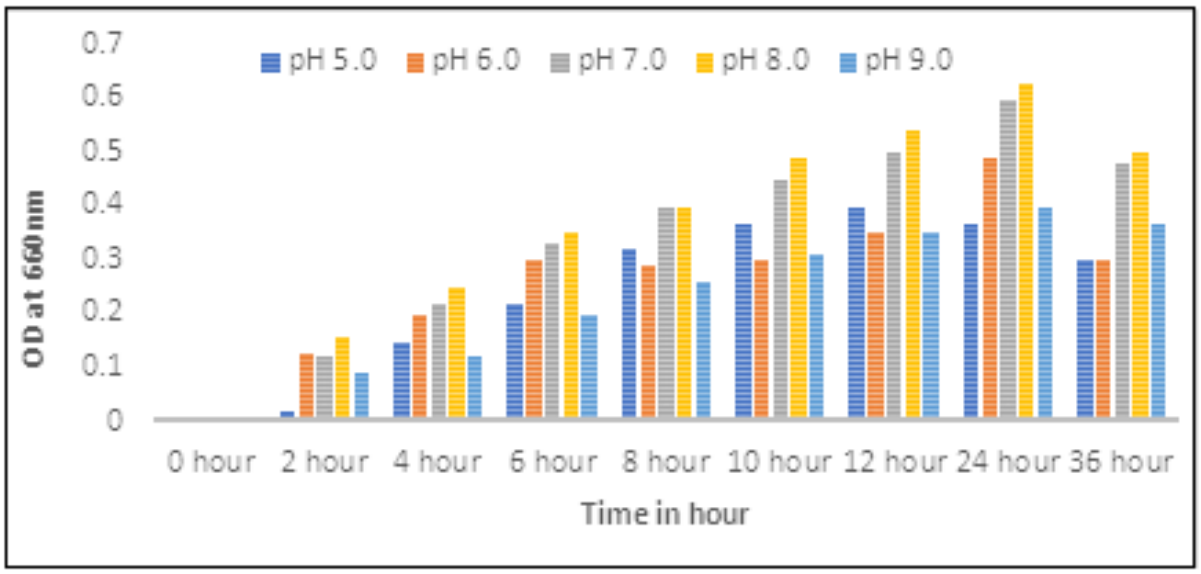

Fig. 1. Effect of pH on growth of strain A.

Carbon source utilization tests

Different carbon Sources such as Glucose, Sugar, Yeast Extract, and Glycerol are utilized by the isolated bacterial strain A and strain B. Optical Density of different carbon sources at different time interval are measured. The utilization of Yeast Extract by the both bacterial strains showed highest value (Fig. 5. and 6.).

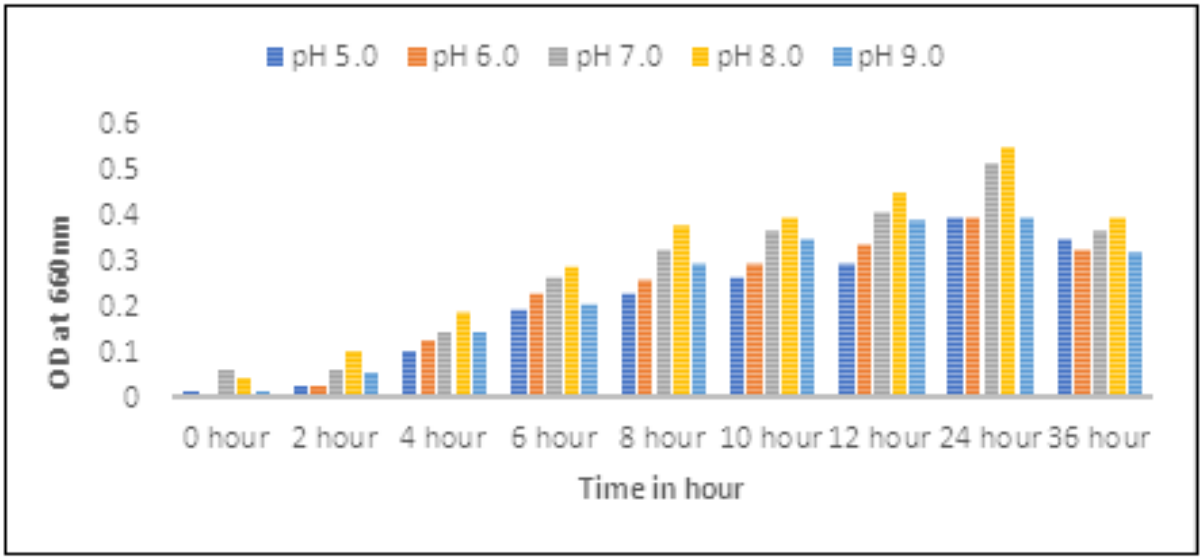

Fig. 2. Effect of pH on growth of strain B.

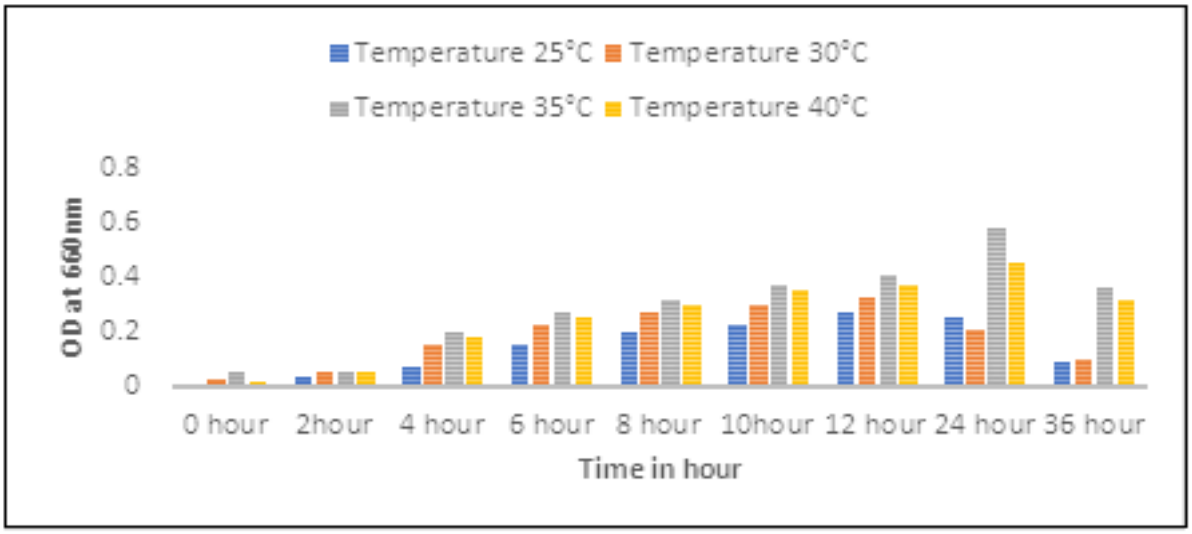

Fig. 3. Effect of temperature on growth of strain A.

Antibiotic sensitivity test

The patterns of sensitivity and resistance of isolating bacterial cultures to five different antibiotics were tested by the disc diffusion method using LB broth medium.

The diameter of the inhibition zone was measured after incubation overnight at 35°C. Antibiotic sensitivity is presented in the. From the table it is evident that strain A is resistant to 4 antibiotics out of five antibiotics tested, and susceptable to only antibiotic tetracyclin (Table 2.). On the other strain B is resistant to 4 antibiotics out of five antibiotics tested and susceptable to only antibiotic erythromycin (Table 3.).

Growth inhibition activity of isolated bacteria against pathogenic bacteria (Antagonistic test)

The present study explored the growth inhibition activity of isolated bacterial strains against

pathogenic bacteria with four different doses like 50, 100, 150 and 200 µl/disc. In case of isolate A, *S. aureus and E. coli* were susceptible at only 200 µl/disc (Table 4.). On the other hand, in case of isolate B, *S. aureus and E. coli* was also susceptible at the dose of 200 µl/disc (Table 4.).

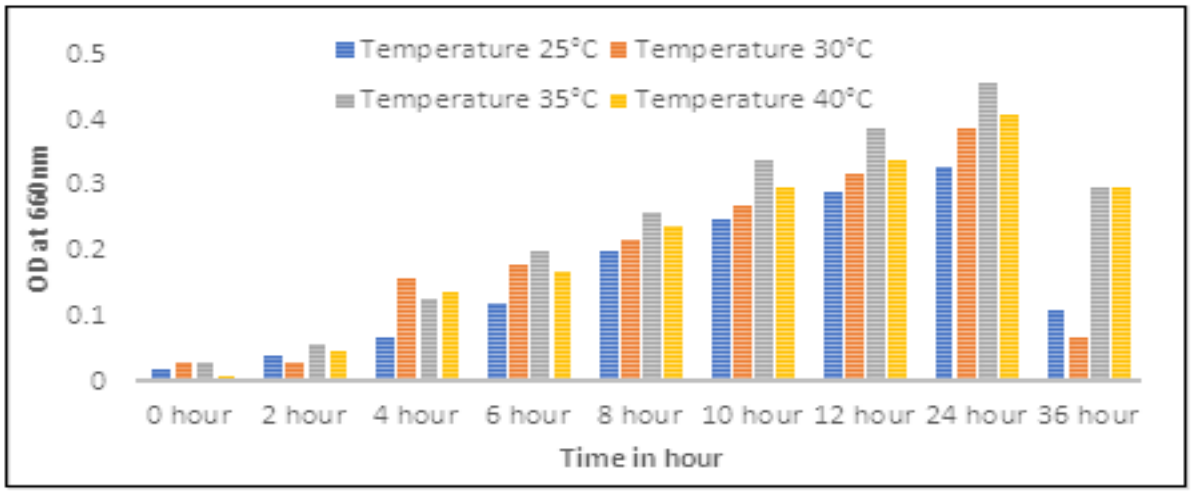

Fig. 4. Effect of temperature on growth of strain B.

Adaptability test of isolated halophilic stains in salt free media

Adaptability of marine bacterial strain A and B in fresh water was investigated. For this purpose, strain A and B were grown in LB medium without suppliment of NaCl and optical density was measured for the observation of bacterial growth. Both of the strains were capable to grow in salt free media.

The growth rates of strain A was significantly higher than strain B in the medium and growth rates were increasing in every subcultures (Fig. 7.).

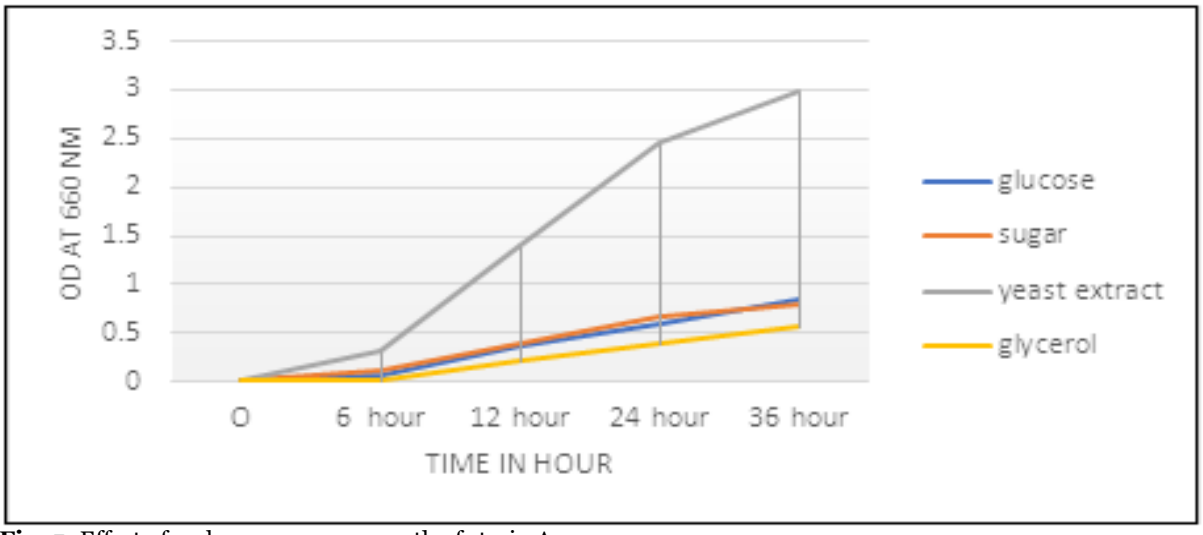

Fig. 5. Effect of carbon source on growth of strain A.

Discussion

Marine environment is a huge resource of marine organisms. Marine bacteria face most diverse condition (Takizawa *et al.,* 1993). So their adaptability to many diverse conditions like extreme saline condition has immense fundamental and applied importance. Halophiles thrive in salt concentrations. Halophilic bacteria are important for numerous industrial, clinical trial and drug processes (Newman *et al.,* 2004). So, investigation on bacteria of marine environment has significant roles.

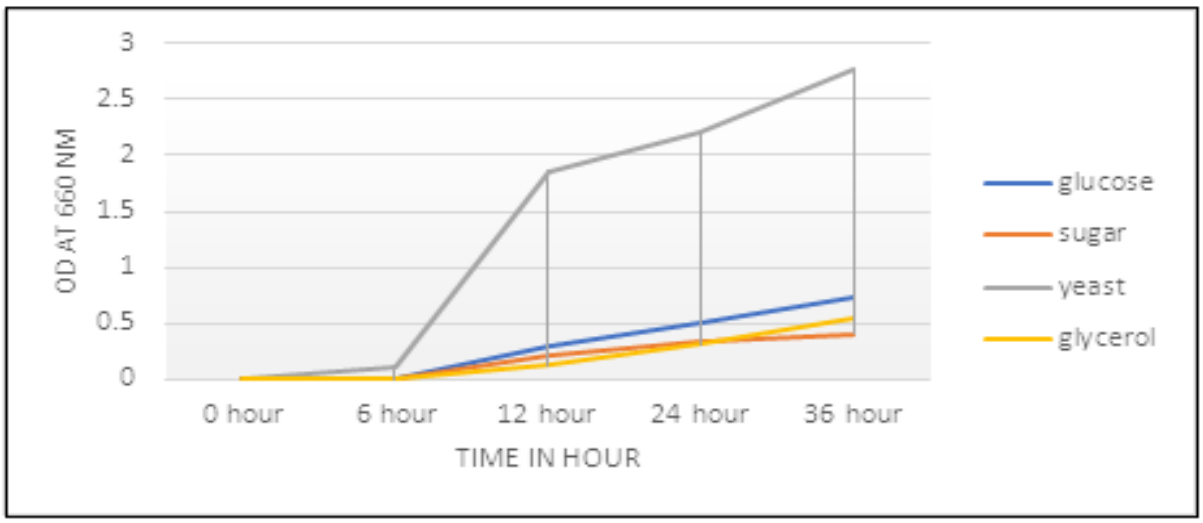

Fig. 6. Effect of carbon source on growth of strain B.

In this present study, the salt tolerant two bacterial stain A and stain B were isolated from the marine water collected from Inani Beach, Cox's Bazaar and different investigation has been done to observe their optimal growth rate in different laboratory conditions. The Gram's staining indicated that the isolated strain A is Gram negative, round shaped and stain B is gram positive, rod shaped. The additional information from Gram staining was in the form of cell morphology and arrangement. The growth pattern of this isolate A and B on LB agar media was small round and sphear shaped respectively.

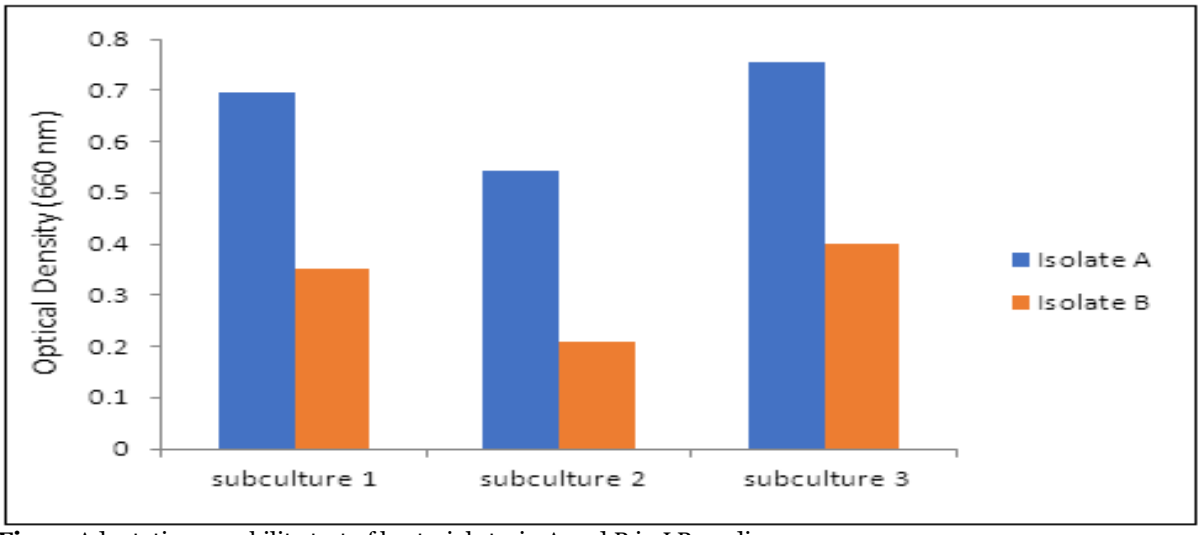

Fig. 7. Adaptation capability test of bacterial strain A and B in LB media.

The effect of pH and Temperature on the growth of isolated bacterial strains A and B was also evaluated. Both the isolate A and B exhibited optimum growth under aerobic conditions at temperature 35 ℃ and pH at 8.0 and sodium chloride level at 2 % (w/v). So, the strains were considered as mesophillic, alkaliphillic and moderate salt tolerant strains in nature (Ventosa *et al.,* 1998). The Biochemical tests were also done for both of the strain. The methyl red test for stain A was positive and for strain B was negative. Catalase activity test was positive for both of the isolated bacterial strains. The motility test indicates that both stains are non-motile. The carbon sources utilization by the bacteria on different time intervals were also examined. The optimum salt level in LB liquid medium for marine bacterial growth was identified by observing their growth pattern in different salt concentrated media at different time intervals.

The isolated bacterial strain A from the marine water sample indicates that they are naturally adapted in marine environment and they utilize salt for their growth. Their highest growth level was observed at 2% concentrated salt medium so according to their salt requirement they are slight halophiles grow optimally at 0.2–0.85 mol /L (2–5%) NaCl (Reddy *et al.*, 2011). According to the experimental result of MacLeod and Onofrey, 1957evident showed that the requirement for Na+ at more than trace levels may be a distinctive property of the marine bacteria.

In antibiotic sensitivity of the isolated bacteria against five different antibiotics was checked in our present study. The result showed that isolated A was resistant against four antibiotics including cefuroxime, erythromycin, doxycycline, carbenicillin and intermediate resistance to tetracycline so it was multidrug resistant. On the other hand isolated B was resistant against antibiotics including cefuroxime, tetracycline, doxycycline, carbenicillin and intermediate resistance to erythromycin so it was also multidrug resistant. De Oliveira *et al.,* 2010 isolated multidrug resistant marine bacteria from seawater in southeastern Brazil. In their study, ten antibiotics were used and all the isolates exhibited resistance against cefuroxime, erythromycin, doxycycline and tetracycline (De Oliveira *et al.,* 2010). It was notable that multidrug resistant enterococci was also isolated from coastal bathing waters (Arvanitidou *et al.*, 2001). So, it can be said that our results had similarity with previous findings.

Antimicrobial activity is a very important criterion for bacterial selection as marine bacterial species showed potential antagonists effect against harmful bacteria. Marine derived antibiotics are more efficient at fighting microbial infections than the terrestrial bacteria have not developed any resistance against them (Donia and Humann, 2003). All the known microbes, have long been recognized as prolific producer of useful bioactive metabolites with broad spectrum of activities, which used as potential antibacterial agents (Atta and Ahmad, 2009). According to Narayana *et al.*, 2007 reported that *Streptomyces* sp. were highly susceptible which has been showed various degrees of antibacterial activity against Gram-positive *Staphylococcus aureus* (*S. aureus*) as well as gram-negative bacteria *E. coli* (Narayanna *et al.,* 2007). In their study showed that isolated strain from marine water were able to inhibit the growth of *S. aureus, E. coli* and *Aeromonas hydrophylla* by producing zones of inhibition 19 ,16, and 15 mm diameter respectively at moderate dose. In our present investigation, growth inhibition activity of isolated bacteria were evaluated at doses of 50, 100, 150 and 200 µl/disc against two pathogenic bacteria. In case of isolate A, *S. aureus and E. coli* were susceptible at dose of 200 µl/disc with 17 mm and 16 mm zones of inhibition, respectively. Again, in case of isolate B, susceptible inhibition zone of 18 mm and 16mm was found against *S. aureus and E. coli* at dose of 200 µl/disc respectively. Our result had resemblance with previous findings. From the result it was clear that isolated strains can produce antimicrobial product which can restrain the growth of pathogenic bacteria.
The adaptability of marine bacterial strain A and B in fresh water medium is also investigated. And the result indicates that although both of the strains are halophiles they can be adapted in fresh water media as their growth rate has increased when strain A and B were sub cultured in fresh LB media.

Conclusion

In this present study, the investigation was done to isolate salt tolerant bacterial strain A and strain B from the marine water. The morphological, biochemical and physiological characteristics have been extensively studied in this research project. Isolated strains were identified as halophilic and have strong adaptation ability in different laboratory conditions, more specifically in normal environmental conditions. Our findings also reveal that isolated strain was potential antimicrobial agent which can inhibit the growth of pathogenic bacteria.

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