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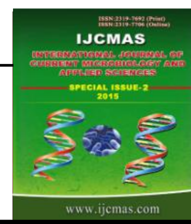

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Original Research Article

Isolation, Biotyping, Biochemical and Physiological Characterization of Marine *Acinetobacter* Isolated from West Coast of India

Ashwini M. Shete[1], Shilpa S. Mujumdar[1,2]*, Smita Bhuyan-Pawar[1,2] and Balu A. Chopade[1]

[1]Department of Microbiology, University, Savitribai Phule Pune University (Formerly: University of Pune), India
[2]Department of Microbiology, Modern College of Arts, Science and Commerce, Shivajinagar, Pune 411 005, India

*Corresponding author

A B S T R A C T

Eleven marine *Acinetobacter* strains were isolated from water and sediment samples collected from west coast of India. Genus *Acinetobacter* was confirmed by chromosomal DNA transformation assay. *Acinetobacter* strains were classified into two species including *A. haemolyticus* (6 strains) and *A. junii* (5 strains) with the help of Bouvet and Grimont system. Marine *Acinetobacter* strains were untypable by API 20 NE system. Zobell marine medium and modified *Acinetobacter* medium were found to be suitable for the isolation of marine *Acinetobacter*. *A. junii* GW2 showed dark yellow pigmentation, *A. junii* GW4 and MW10 showed light orange pigmentation but shades and colony morphology of two strains were different and *A. haemolyticus* MW11 showed dark orange pigmentation. All the strains showed high salt tolerance from 8% to a maximum 20% (w/v). Marine *Acinetobacter* spp. were found to be nutritionally versatile and utilised different carbohydrates and amino acids as sole source of carbon and energy and produced lipase, gelatinase, L-asparginase and phosphatase. Most of the strains had ability to utilise hydrocarbons and organic compounds as sole source of carbon and energy. Marine *Acinetobacter* strains showed high tolerance to metal salts especially to heavy metals like arsenic, lead, platinum, tellurium and selenium however, were highly sensitive to most of the commonly used antibiotics with few exceptions. *A haemolyticus* (6 strains) and *A. junii* (5 strains) contained one plasmid of molecular weight 40 kb. Thus, *Acinetobacter* strains exhibiting peculiar biochemical and physiological characteristics were found to be present on west coast of India. This is a first report of pigment production in the genus *Acinetobacter*.

Keywords

Acinetobacter, west coast of India, Marine

Introduction

The marine expanse is a rich and relatively unexplored source of novel micro-organisms with unusual properties and their metabolites like antimicrobials

biosurfactants and enzymes of commercial interest (Nithya *et al.,* 2011; Subramani and Aalbersberg, 2012; Fu *et al.,* 2014) Gram negative bacteria constitute about 90% of the marine bacterial population (Fenical, 1993). *Acinetobacter* is one of the commonly found Gram negative organism in marine environment (Austin, 1979; Kaneko *et al.,* 1979; Simidu *et al.,* 1980). It is reported as the predominant group of bacteria after *Pseudomonas* in marine environment (Grimes *et al.,* 1984). *Acinetobacter* is a Gram negative, strongly aerobic, encapsulated, pleomorphic, oxidase negative and catalase positive coccobacilli having G+C content 38-47 moles percent (Bergogne-Berezin and Towner, 1996; Tripathi *et al.,* 2014). Members of genus *Acinetobacter* are found ubiquitously in soil, food, sewage. It has been established that *Acinetobacter* is a reservoir of naturally occurring antibiotic and metal resistance plasmids (Chopade *et al.,* 1994a,b; Deshpande and Chopade, 1994; Bhamare *et al.,* 1994 a,b; Naik *et al.,* 1994; Huddedar *et al.,* 2002). It is the only Gram negative bacterium found on human skin (Patil and Chopade, 2001). *Acinetobacter* is an important emerging nosocomial pathogen (Chopade *et al.,* 1994a; Shakibaie *et al.,* 1999: Vahdani *et al.,* 2011). It causes variety of infections like meningitis, septicemia and urinary, genital, respiratory tract and wound infections (Singh *et al.,* 2013).

Acinetobacter is one of the important organisms in marine ecosystem and found to exhibit many interesting properties like hydrocarbon degradation, antibiotic and metal resistance and resistance to different growth inhibitors (Shete *et al.,* 2006; Vahdani *et al.,* 2011; Hou *et al.,* 2013; Tripathi *et al.,* 2014). *Acinetobacter* has been isolated from sea water, sediment, estuaries, deep sea as well as hyper saline areas. It is a member of normal flora of digestive tract of different marine animals like fishes, crabs, prawns etc (Thampuran and Gopakumar, 1993; Chang *et al.,* 1996; Chen, 1995) and also involved in spoilage of preserved stocks of marine food (Shetty *et al.,* 1992; Anggawati *et al.,* 1990; Natarajan *et al.,* 1986). Thus it reveals that *Acinetobacter* plays an important role in marine ecosystem.

Acinetobacter is a versatile organism exhibiting different properties. Along with utilization of different substrates like amino acids, carbohydrates, organic acids and hydrocarbons, it can also produce different enzymes like lipase, protease (Bhuyan, 2012; Luo *et al.,* 2013). *Acinetobacter* isolated from marine animal *M. barbatus* stored at 3°C and - 28°C was suspected to be the putrefactive bacterium as it could produce DNase and gelatinase (Wu and Chen, 1980). *Acinetobacter* spp. capable of degrading hydrocarbons had been reported in many studies (Hanson *et al.,* 1996; Walker *et al.,* 1976; Yamamoto and Harayama, 1996). It exhibits resistance to different heavy metals like mercury (Babich and Stotzky, 1979), lead and other metals (Nieto *et al.,* 1989). *Acinetobacter* isolated from red tides caused by *Mesodinium rubrum* and *Gymnodinium catenatum* in north west coast of Spain showed resistance to different antibiotics and different growth inhibitors (Romalde *et al.,* 1990). *Acinetobacter* isolated as a member of bacterial flora of rotifer was found to be resistant to sodium nifurstyrenate and tetracycline (Tanasomwang and Muroga, 1989).

Until now most of the studies are related to *Acinetobacter* isolated from clinical isolates. Few studies which deal with *Acinetobacter* isolated from marine environment suggest that it shows interesting properties. Howeverdetailed studies from marine *Acinetobacter* are scarce; Therefore the

present study was undertaken to explore some important characteristics of marine *Acinetobacter* from the hitherto unexplored region of the west coast of India.

Materials and Methods

Sampling of marine water and sediment

Location of sampling:

Sampling areas included coastal areas of Mumbai, Thane and Goa. Water and sediment samples were collected from Kalamb beach near Wirar in Thane district and from Dadar Chaupati and India Gate, Mumbai. Samples from Goa were collected from Miramar beach and the off shore sites in the Mandovi estuary.

Marine water and sediment sampling

Approximately 300 ml surface water samples were collected in sterile plastic bottles from all sites. At least 3–4 samples were taken from each site.

At Mandovi estuary, surface water samples as well as water sample from approximately 2 m depth were collected with the help of Niskin sampler. At all the rest sites, only surface water samples were collected.

Sediment samples were collected at beaches at a depth of approximately 1ft whereas in Mandovi estuary, sediment samples were collected from the bottom having approximate depth of 5 m with the help of Grab sampler. Samples from Mumbai were processed after 5–6 hr while Goan samples were processed after 10–12 hr after collection. All samples were stored at 4°C until further use. The Niskin and Grab samplers were provided by National Institute of Oceanography, Goa, India.

Isolation of *Acinetobacter* from marine water and sediment

Different media used for isolation of marine strains include Zobell marine medium (HiMedia Labs Pvt. Ltd, Mumbai, India), cystine lactose electrolyte deficient medium (CLED) (HiMedia), *Acinetobacter* minimal medium (AMM) (Chopade *et al.,* 1985) and modified Acinetobacter medium (mAc) (LaCroix and Cabelli, 1982). All media, except Zobell marine medium, were supplemented with 3.5% (w/v) NaCl. All samples collected from a particular site were pooled together and mixed thoroughly. From the pooled sample, three subsamples were used for further processing. Individual samples were also processed in the same manner. From undiluted and serially diluted (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) subsamples, 100 µl was spread on each medium. All media plates were incubated at 28°C for seven days. After every 24 hr colony morphotypes on each plate were observed.

Enrichment of *Acinetobacter*

Enrichment culture technique was used for isolation of *Acinetobacter.* Enrichment was done using modified *Acinetobacter* broth (mAc). 50 ml medium in 250 ml flask was inoculated with 1ml marine water sample collected from each site and incubated at 28-30°C for 7 days at 250 rpm. For sediment samples, 0.1 gm of sediment was inoculated in 50 ml modified *Acinetobacter* broth and incubated at 28-30°C for 7 days at 250 rpm. 100 µl sample, from the flask inoculated with water and sediment samples, was plated after every 24 hr for 7 days. Sample from each flask was plated on Zobell marine agar, CLED, AMM and mAc.

Chromosomal DNA transformation assay

Chromosomal DNA transformation assay

was done using naturally competent tryptophan auxotrophic mutant, *A. calcoaceticus* BD413 trp E27. 18 hr old culture *A. calcoaceticus* BD413 trp E27, grown on brain heart infusion agar (BHIA) (HiMedia labs. Pvt. Ltd, Mumbai) for three successive transfers for three days at 28°C. A loopful of cell mass was mixed thoroughly with 25µl of purified transforming chromosomal DNA of tentative *Acinetobacter*s with the help of thin, blunt glass rod on BHIA. Chromosomal DNA was purified using method of Juni (1972) and Chen and Kuo (1993). DNA and *A. calcoaceticus* BD413 trp E27 were grown together on BHIA at 28°C. After 18-24 hr, growth from BHIA was transferred on *Acinetobacter* minimal medium (AMM), containing noble agar, without tryptophan, and incubated at 28°C for 7–15 days. Growth of transformants on AMM was visible within 48 to 120 hr. The transformants were confirmed again by transferring the growth to fresh AMM plates. The strains that were able to grow on AMM were confirmed to belong to genus *Acinetobacter*.

Crude genomic DNA preparation

A loopful of 18-24 hr cell paste of tentative *Acinetobacter* strains was suspended in 0.5 ml sterile solution containing 0.05% SDS, 0.15M NaCl, 0.015 M Sodium citrate and mixed thoroughly. This mixture was heated in water bath for 1hr at 60°C. The resulting mixture contained crude transforming DNA which could be used as it is for chromosomal DNA transformation assay.

Purification of genomic DNA

1.5 ml 18 hr old culture of tentative *Acinetobacter* strains, grown in Zobell marine broth, was centrifuged for 10 min at 10,000 rpm. Cell pellet was suspended and lysed in 200 µl lysis buffer containing 40mM Tris-acetate pH 7.8, 20mM sodium acetate, 1mM EDTA and 1% SDS. It was followed by addition of 66 µl 5M NaCl and mixed well. This mixture was centrifuged at 10,000 rpm for 15 min. at 4°C and resulting clear supernatant was transferred to new vial. Equal volume of chloroform was added and the tube was inverted 50 times. The mixture was centrifuged at 10,000 rpm for 15 min and supernatant was transferred to new vial. Chromosomal DNA was precipitated with methanol and dried properly in laminar air flow. Purified DNA was resuspended in T_{10} E_1 buffer (pH 8) (Chen and Kuo, 1993).

Biochemical characterization of marine *Acinetobacter* spp

Biochemical characterization of *Acinetobacter* was done as per the scheme suggested by Bouvet and Grimont (1986, 1987). Growth was recorded daily for 7 days; incubation was done at 28°C unless and otherwise mentioned specifically.

IMViC: All the tests were done as per standard methods described elsewhere (Gerhardt, 1994). In all media, 3.5% (w/v) NaCl was added.

Carbohydrate and amino acid utilization

Carbon source utilization tests were done using M70 medium (Bouvet and Grimont, 1987). M70 was supplemented with 3.5% NaCl. Filter sterilized carbon source solution were added at final concentration of 0.2% (w/v) for carbohydrates and 0.1% (w/v) for other substrates. Andrade's indicator was added to M70 to check acid production while gas production was checked in Durham's tubes.

Haemolysis: Zobell marine agar containing

5% fresh human whole blood was used for checking haemolysis. Plates were observed for haemolysis up to seven days.

Biotyping of marine *Acinetobacter* by API 20NE

API 20NE system (bioMerieux Pvt Ltd, France) was used to classify the *Acinetobacter* strains upto species level. API 20NE kit contained 21 tests, 8 conventional tests and 12 assimilation tests and 21st test was oxidase test. The tests were divided into groups of three and each positive reaction was given value equal to 1, 2 or 4 according to the position in its group, first, second or third, respectively.

The sum of these 3 values (0 for negative reaction) gave the corresponding digit with a value between 0 and 7. Thus a seven digit number was obtained which has to be matched with the API 20NE analytical profile index (BioMerieux, SA 20050 07615E – 03/2000). Necessary instructions given by manufacturers were strictly followed. *A. calcoaceticus* BD413, *A. calcoaceticus* MTCC127, *A. calcoaceticus* MTCC 1271, *A. calcoaceticus* 1425 and *A. lwoffi* MTCC 496 were used as standard control strains.

Physiological characterization of marine *Acinetobacter* species

Growth at different temperature: All *Acinetobacter* strains were streaked on Zobell marine agar and incubated at 28, 37, 41 and 44°C. Plates were observed for seven days. Growth was also checked in Zobell broth at 28, 37, 41 and 44°C.

Growth at different pH: Growth was checked in Zobell marine broth prepared in different buffers in the range of 4-11 at 28^{0}C.

Effect of sodium chloride concentration on growth: Growth at 5,8,10,12,15,20 and 25% (w/v) NaCl was tested on Zobell marine agar supplemented with appropriate amount of NaCl.

Detection of enzyme production by Marine *Acinetobacter* species: 11 Marine *Acinetobacter* strains were checked for production of different enzymes. Following enzymes were checked.

1. **Lipase:** Medium contained (gm/l) peptone 5, yeast extract 3, tributyrin 10 ml, DW 1l, pH 7.5. Cultures were spot inoculated and incubated at 28°C for 15 days. Lipase production was detected by development of clear zone around culture (Smibert and Krieg, 1994).

2. **Amylase:** Medium used contains nutrient agar 100 ml, NaCl 3.5 %, starch 1.0 g. The cultures were spot inoculated on the medium, after incubation for 7 days at 28°C, flooded with 1N iodine solution. Amylase production was detected by development of clear zone around the spot (Smibert and Krieg, 1994).

3. **Gelatinase**: Zobell medium supplemented with 0.04% gelatin was inoculated. After 48 hr, plates were flooded with Frazier reagent (15% w/v $HgCl_2$ in 20 v/v HCl) and observed for the zone of clearance. (Bouvet and Grimont, 1986).

4. **Pectinase:** Medium contained (gm/l) KH_2PO_4 4, NA_2HPO_4 6, pectine 5, $(NH_4)_2SO_4$ 2, yeast extract 1, $MgSO_4$ 2, $FeSO_4$ 0.001, $CaCl_2$ 0.001, NaCl 35, Distilled water 1l. Cultures were spot inoculated, incubated at 28°C for 7 days and the flooded with 1% (w/v) cetrimide for 10 min. Pectinase production was detected by clear zone around the spot (Hankin *et al.,* 1971).

5. **Cellulase:** Medium contained (gm/l) yeast extract 1, K_2HPO_4 0.5, carboxymethyl cellulose 0.5, NaCl 35, Distilled water 1l, pH 7. Cultures were spot inoculated and incubated at 28°C for 7 days. Cellulase production was detected by development of clear zone on flooding the plate with 1% (w/v) cetrimide for 10 min (Hankin and Anagnostakis, 1977).

6. **Xylanase:** Medium contained (gm/l) meat extract 1, yeast extract 1, xylan 2, NaCl 35, Distilled water 1l, pH 7.4. Cultures were spot inoculated and incubated at 28°C for 7 days. Plates were flooded with 0.5% (w/v) congo red solution for 10 min. and periodically with saturated NaCl solution. The isolates showing xylanase activity were detected by colourless zones against red background around the spot (Balkrishnan, 1993).

7. **Phosphatase**: Medium used was Zobell marine medium supplemented with 0.01 % (w/v) sodium salt of phenolphthalein diphosphate. Cultures were spot inoculated and incubated for 2-5 days at 28°C. Few drops of ammonia solution (specific gravity 0.88) were added in the lid of an inverted plate and culture was replaced over it to allow the ammonia fumes to reach the colonies. Cultures producing phosphatase became red in the presence of free phenolphthalein (Smibert and Krieg, 1994).

8. **L-Asparginase**: Zobell marine medium supplemented with 0.2% w/v L - aspargine and phenol red was used. Cultures were inoculated on slants and incubated at 28°C for 48 hr. L-asparginase production was detected by change in colour of medium from yellow to red due to the production of ammonia. (Benny and Kurup, 1991).

9. **Urease:** Christensen urea agar was used which consists of (gm/l) peptone 1, glucose 1, NaCl 35, KH_2PO_4 2, phenol red 0.012, urea (filter sterilised) 20, Distilled water 1l, pH: 6.8 - 6.9. Change in colour of the medium from yellow to pink indicated urease production (Smibert and Krieg, 1994).

10. **Chitinase: C**hitin agar containing (gm/L) K_2HPO_4 0.7, KH_2PO_4 0.3, $MgSO_4.5H_2O$ 0.5, $FeSO_4.7H_2O$ 0.1, $MnCl_2$ 0.1, NaCl 35, Chitin 4.0, Distilled water 1l, pH: 7.5 was used for chitinase production. Cultures were spot inoculated and incubated for 7-10 days at 28°C. Chitinase positive cultures were detected by clear zone of hydrolysis around the spot against opaque background contributed by colloidal chitin (Hsu and Lockwood, 1975).

Utilisation of organic compounds and hydrocarbons

M70 minimal medium was used as a basal medium for testing utilisation of organic compounds and hydrocarbons. Hydrocarbons were used in concentration of O.5% v/v in M70 broth (Bouvet and Grimont, 1986). It was also checked on M70 solid medium supplemented with 1% v/v hydrocarbons. 12 to 18 hr old cultures were inoculated in M70 broth and spot inoculated on plates; incubated at 28°C for seven days and observed every day.

Determination of minimum inhibitory concentration (MIC) of metals

Minimum inhibitory concentration of metal salts was checked by double dilution method employing Zobell marine medium (Dhakephalker and Chopade, 1994). A fresh stock solution of metal salt of concentration 0.1% (w/v) was prepared and appropriate amount was added to Zobell marine medium to get actual concentration. 12 to 18 hr old cultures grown in Zobell broth were spot inoculated on this medium. O.D. of the culture was adjusted to get 10^4 to 10^5cells per spot. Plates were incubated at 28^0C for 24 to 48 hr. Concentration at which growth was totally inhibited within 24-48 hr was considered as minimum inhibitory concentration (MIC). *A. calcoaceticus* BD413 trp E27 and *E. coli* HB101 were used as controls.

Determination of minimum inhibitory concentration (MIC) of antibiotics

MIC of antibiotics was determined by double dilution method using Muller Hinton medium (HiMedia) (Dhakephalker and Chopade, 1994). On this medium, 12 to 18hr old cultures grown in Zobell marine broth were spot inoculated to get 10^4 to 10^5 cells per spot. Plates were incubated at 28°C for 24 to 48 hr. About 35 spots were inoculated per plate. Concentration at which growth was totally inhibited within 24–48 hr was considered as minimum inhibitory concentration (MIC). *A. calcoaceticus* BD413 trp E27 and *E. coli* HB101 were used as controls.

Plasmid profile of marine *Acinetobacter* species

Isolation of Plasmid DNA was attempted by a number of method viz; Kado and Liu (1981), Sambrook *et al.,* (1989) and Birnboim and Doly (1979). Marine *Acinetobacter* strains were grown in Zobell marine broth for 18 hr at 250 rpm at 28°C and used as the starting material. The cells were pelleted by centrifugation at 10,000 rpm for 10 min and used for isolation of plasmid DNA for all above methods. The entire procedure was carried out over ice to prevent damage or shearing of plasmid DNA. The isolated plasmid DNA was stored at -20°C until further use.

Purified plasmid DNA samples were subjected to electrophoresis in agarose gel.

Electrophoresis was done using TAE buffer and 0.8% agarose (Sisco Research Lab) and gel was run at 20 constant volts for 1 hr and then at 50 constant volts for 5-6hr. The gel was stained with ethidium bromide (0.5 µg/ml) for 15–20 min. Excess ethidium bromide was removed by washing with distilled water. Well stained gels were observed and documented using AlphaImager gel documentation system. Size of the plasmid was determined by comparing with the appropriate markers run along with the test plasmid DNA.

Results and Discussion

Isolation of marine *Acinetobacter*

From five sampling sites, total 11 tentative *Acinetobacter* strains were isolated. On the basis of six diagnostic characters, strains were preliminarily identified as belonging to the genus *Acinetobacter*. Gram negative coccobacilli which were non motile, capsulated, catalase positive, oxidase negative and having oxidative or nonoxidative nonfermentative type of metabolism confirmed by oxidation-fermentation test considered as tentative *Acinetobacter*.

Zobell marine medium was found to be the best medium for isolation as 5 tentative *Acinetobacter* strains were isolated from this medium by direct plating the marine water and sediment samples. Enrichment of water sample in modified Acinetobacter medium also found to be useful as 3 tentative *Acinetobacter* strains were recovered by this method. Out of 11 tentative *Acinetobacter* strains, ten were isolated from marine water, whereas one was isolated from sediment. Six tentative *Acinetobacter* strains were recovered from Goa samples whereas five strains were recovered from Mumbai and Thane samples.

Confirmation of genus *Acinetobacter* by Chromosomal DNA transformation assay

Chromosomal transformation assay of the tentative 11 strains was done as described in materials and methods and confirmed that these strains were genuine *Acinetobacter*s. According to Juni (1972), a crude DNA preparation is sufficient for chromosomal DNA transformation. However, it was observed that with crude DNA, transformation rate was low whereas, pure DNA gave good results. It was observed that chromosomal DNA isolated from tentative marine *Acinetobacter* strains required one to two weeks to transform *A. calcoaceticus* BD413 trpE27.

Identification of *Acinetobacter* to species level

Different biochemical tests were carried out for classification of marine *Acinetobacter* strains to species level (Table 1) and the results were compared with the standard results described by Bouvet and Grimont (1986, 1987). Strains GW1, MW3, GW6, MW7, GS8 and MW11 showed two main characters of *A. haemolytics* i.e. haemolysis and gelatin hydrolysis. These strains could also utilize 4 aminobutyrate. Other characters were also matched and it was confirmed that these strains belong to species *A. haemolyticus*. However, being of marine nature, they showed some variations than standard characters i.e. except strains MW7 and MW11, all could grow at 41 and 44^0C and all strains could utilise alanine and phenylalanine as sole source of carbon and energy but could not utilise maleic acid. Also strains GW6, GS8 and MW11 could utilise lactate as sole source of carbon and energy. Strains MW2, GW4, MW5, MW9 and GW10 were classified as *A. junii* since they could grow at 41°C and utilise glucose without acid production. These strains also

showed some variations from standard results like gelatin hydrolysis, except strain MW5 others could not utilise lactate and strains GW4 and MW5 were capable of utilising trans-aconitate as sole source of carbon and energy (Table 1).

Biotyping of marine *Acinetobacter* species with API 20NE system

It was found that API 20NE system was not useful for identification of marine *Acinetobacter* cultures. Among the conventional tests, all *Acinetobacter* strains gave negative results for nitrate reduction, indole production, glucose acidification and arginine hydrolysis. *A. haemolyticus* GW1, MW3 and *A. junii* GW2 only could hydrolyse esculin whereas *A. haemolyticus* GW1, MW3, GW6, MW7, GS8 and *A. junii* GW4, MW5, GW9 could hydrolyse gelatin. Among assimilation tests, except *A. haemolyticus* MW3, all other strains could utilize almost all the substrates as sole source of carbon and energy. Oxidase was not produced by any of the strain. The seven digit number obtained did not exist in the API 20NE catalogue. Thus marine *Acinetobacter* strains were untypable by API 20 NE system. Control strains (mentioned in material and methods) were typable by API 20NE system (Table 2).

Biochemical characteristics of marine *Acinetobacter* species

None of *Acinetobacter* strains produced indole and acetyl methyl carbinol or butanediol from glucose; but all could utilise citrate except *A. junii* GW2. All strains could utilise amino acids histidine, tyrosine, L-glutamic acid, L-aspartic acid, phenyl alanine, and arginine. Strains GW2 and MW3 could not utilise leucine and DL alanine but all remaining strains could utilise both the amino acids. Similarly, except *A. haemolyticus* GW1and MW3 and *A. junii* MW3 and MW5, all other strains utilised ornithin. None of the strains utilised maleic acid and malonate.

Acinetobacter strains utilised a wide spectrum of carbohydrates without production of acid and gas. All the strains could utilise glucose, sucrose and raffinose. Not a single strain could utilise galactose and mellibiose. *A. junii* GW4 and GW10 and *A. haemolyticus* MW3 couldn't utilise xylose, cellobiose, inulin and fructose. Dulcitol could be utilise only by *A. haemolyticus* GW6 and *A. junii* GW9. *A. haemolyticus* GW1 could not utilise arabinose, dulcitol, trehalose, sorbitol and rhamnose. All the strains could utilise 4 amino butyric acid. *A.junii* GW2 and GW4 and *A. haemolyticus* MW3 were unable to utilise phenylacetic acid whereas, *A. haemolyticus* GW1, MW3 and GS8 and *A. junii* GW2 and GW9 could not utilise trans-aconitic acid. Only two strains, *A. junii* MW10 and *A. haemolyticus* MW11 could reduce nitrate (Table 3).

Physiological characteristics of marine *Acinetobacter* species

Physiological characterization of marine *Acinetobacter* included morphology and colony characters on different media. The striking feature was pigmentation shown by marine *Acinetobacter*. *A. junii* GW2 showed dark yellow pigmentation, *A. junii* GW4 & MW10 showed light orange pigmentation but shades and colony morphology of two strain was different and *A. haemolyticus* MW11 exhibited dark orange pigmentation. Growth of all *Acinetobacter* strains was checked in Zobell marine broth and on Zobell marine agar at 37, 41 and 44°C. All *Acinetobacter* strains could grow upto 41°C.

Salt tolerance of marine *Acinetobacter*

strains was found to be quite high. *A. haemolyticus* MW5 and GW9 and *A. junii* MW7 and GS8 could even grow at 20% NaCl concentration. *A. haemolyticus* GW6 could tolerate 15% NaCl concentration whereas, *A. haemolyticus* GW1 and MW3 could grow at 12% NaCl concentration. *A. junii* MW10 and *A. haemolyticus* MW11 could grow up to 10% and *A. haemolyticus* GW2 could grow at 8% NaCl concentration. Both *Acinetobacter* biotypes were able to grow in the pH range of 5–10.

Enzymes produced by marine *Acinetobacter* species

The table 4 indicates that all 11 strains could produce enzymes like L- asparginase and phosphatase and lipase. *A. haemolyticus* GW1, MW3 and MW11 and *A. junii* MW10 were strong lipase producers and *A. haemolyticus* GW6, MW7 and GS8 and *A. junii* (MW5 and GW9) were late producers; they showed clearance zone after 10 to 12 days. Except *A. haemolyticus* GW2 and MW11 and *A. junii* MW10, all other remaining strains could produce enzyme gelatinase. *A. haemolyticus* GW2 and MW11 produced amylase and xylanase and *A. junii* GW4 and *A. haemolyticus* MW11 had capability to produce enzyme urease. None of the strains had the ability to produce chitinase, pectinase or cellulase. It could be concluded that *A. haemolyticus* MW11 had ability to produce maximum number of enzymes like lipase, amylase, xylanase, urease including L-asparginase and phosphatase.

Utilisation of organic compounds and hydrocarbons by marine *Acinetobacter species*

Many marine strains could utilize different organic compounds and hydrocarbons as sole source of carbon and energy (Table 5). *A. haemolyticus* GW6, MW7, GW8, MW11 and *A. junii* GW4, MW5, GW9, MW10 were capable of utilising all the organic compounds and hydrocarbons tested. *A. haemolyticus* GS8 was the only strain which could utilise kerosene. *A. haemolyticus* GW1 and MW3 were unable to utilise most of the organic compounds and hydrocarbons. *A. junii* GW2 could utilise only ethanol 0.1% and 1%, chloroform, propanol, diethylether, acetone, toluene, xylene but could not utilise rest of the compounds.

Metal resistance in marine *Acinetobacter species*

Marine *Acinetobacter* strains showed resistance to many metal salts. The minimum inhibitory concentration of metal salts like lithium chloride, bismuth sulphate, vanedium pentaoxide, sodium molybdate, strontium chloride, lead acetate and potassium dichromate was higher than 1024 µg/ml for marine *Acinetobacter* strains. It was observed that, the strains also showed higher MIC levels for metals like arsenic, platinum, gold and tellurium. All the *Acinetobacter* strains were found to be sensitive to heavy metals like silver and mercury (MIC: 0–32 µg/ml). *A. haemolyticus* GW1, MW3, GW11 and *A. junii* GW2, MW10 were sensitive to cadmium chloride but remaining strains showed moderate resistance (MIC 256 µg/ml). Except *A. junii* GW4 and GW10, all other strains were sensitive to potassium antimony tartarate. It was observed that all the marine *Acinetobacter* strains tested reduced the metal salts, selenium dioxide and potassium tellurite. This was evident from change in colour of the bacterial growth to reddish orange and black respectively when grown in presence of metals (Table 6).

Antibiotic resistance profile of marine *Acinetobacter* species

All the marine *Acinetobacter* strains were sensitive to a number of antibiotics viz., Rifampicin, Streptomycin, Gentamycin, Cefotaxime, Amikacin, Ceftriaxone, Cefuroxime, Ofloxacin, cefalexin and neomycin. *A. haemolyticus* GW6, MW7, GS8 and *A. junii* MW5, GW9 were resistant to antibiotics, Penicillin, Chloramphenicol, Cloxacillin, Nalidixic acid and Norfloxacin (MIC: 320, 1024, 256, 256, 64 µg/ml respectively). *A. junii* GW4 showed resistance to amoxycillin, chloramphenicol, cloxacillin and nalidixic acid (MIC: 512, 128, 32, 32 µg/ml respectively) whereas *A. haemolyticus* GW1 showed resistance to only nalidixic acid (MIC:128µg/ml). MIC levels of chloramphenicol and nalidix acid (MIC: 64–1024 µg/ml) for all strains were high as compared to other antibiotics. Overall marine *Acinetobacter* strains showed lower antibiotic resistance (Table 7).

Plasmid profile of marine *Acinetobacter* species

All eleven *Acinetobacter* strains found to harbor a single plasmid of 40 kb. The method of Kado and Liu (1981) was found to be highly efficient giving reliable and reproducible results.

Acinetobacter plays an important role in marine ecosystem. It has been reported as a free living bacterium in marine water and sediment (Moral *et al.,* 1988; Marquez *et al.,* 1987; Bhuyan, 2012; Fu *et al.,* 2014) as well as attached to different substrates like sea grass, algae or other inorganic material (Wahbeh and Mahasneh, 1984). It is a member of normal flora of gut of marine animals (Ringo *et al.,* 1995, Kantt and Toress, 1993; Maquez *et al.,* 1990). It had been also reported as a causative agent of various infections in marine animals (Hummel *et al.,* 1988). It is also one of the causative agents of spoilage of preserved marine food (Natarajan *et al.,* 1986; Anggawati *et al.,* 1990). Most of these researchers report *Acinetobacter* as 'a group of bacteria alike to *Acinetobacter*' or 'bacteria belonging to *Moraxella* – *Acinetobacter* group'. However, very few reports are devoted solely to *Acinetobacter* isolated from marine environment (Hanson 1996; Amund, 1996). Moreover, there had been no detail characterisation of *Acinetobacter* species found in west coast of India. Therefore, this study aims to find out detail biochemical characteristics and biotyping of marine *Acinetobacter* isolated from Indian west coast.

Chromosomal DNA transformation assay used for confirmation of the genus *Acinetobacter* is a very powerful tool based on the fact that *Acinetobacter* is a naturally competent organism having a unique ability of chromosomal transformation within the genus only (Palmen and Hellingwerf, 1995; Huddedar *et al.,* 2002). In *Acinetobacter* anthranilate synthetase, an enzyme in tryptophan operon, has an unique structure containing two subunits, α and β, and both are required for functional enzyme (Crawford, 1980). No other bacterium has anthranilate synthetase having 2 subunits. The assay was done using tryptophan auxotrophic mutant of *Acinetobacter calcoaciticus* BD413 trp E27. The strains which belong to genus *Acinetobacter* can only transform the mutant and convert it to prototroph which can grow on medium without tryptophan. In this study, 11 strains which were considered as tentative *Acinetobacter* got transformed successfully and therefore can be considered as authentic *Acinetobacter* strains. It has been found that, there is no previous report of marine *Acinetobacter* which can transform *A.*

calcoaciticus BD413 trp E27.

On the basis biochemical tests *Acinetobacter* strains were classified into different species according to Bouvet and Grimont scheme (1986, 1987). However, it was observed that the physiological characteristics shown by marine *Acinetobacter* strains did not match 100% with the standard results. Some characteristics like growth at different temperature had not been considered as deviation from the standard results, as they were inevitable due to change in the environment.

Acinetobacter strains isolated in this study showed some interesting physiological characteristics. High tolerance to sodium chloride is one of the important characteristics of marine bacteria. All the *Acinetobacter* strains isolated during this study could grow at 8% sodium chloride concentration. Some of the *Acinetobacter* strains could tolerate sodium chloride concentration as high as 20% (w/v). High salt tolerance of *Acinetobacter* isolated from clinical samples or soil have not been reported earlier but it appears to be a common feature of marine *Acinetobacter* which has been reported as moderate halophile (Moral *et al.,* 1987; Moral 1988). It had been also isolated from hypersaline saltern (Zuniga, 1991). Non diffusible yellow-orange pigmentation shown by marine *Acinetobacter* was another peculiar feature. Occurrence of bacteria producing non diffusible yellow or orange pigment in marine environment is quiet high (Kaneko *et al.,* 1979; Hauxhurst *et al.,* 1980; Stafsnes et al, 2010). However, pigment production by the members of genus *Acinetobacter* isolated from any environment had not been reported previously.

Members of genus *Acinetobacter* had been reported as nutritionally versatile (Boumann, 1968). This observation is consistent with our results. 90% *Acinetobacter* strains isolated in this study could utilize approximately 90% of the 12 amino acids and 18 carbohydrates tested for utilisation as a sole source of carbon and energy. It could also utilize other substrates like phenyl acetic acid, 4-amino butyric acid and trans aconitic acid as a sole source of carbon and energy. It also has an ability to produce different enzymes like lipase, amylase, urease, xylanase and asparginase. This may be the possible reason for dominance of the *Acinetobacter* in the marine environment and also in gut of marine animals like fishes, shrimps and squids.

Utilisation of organic compounds and hydrocarbons by marine *Acinetobacter* was another an interesting feature. Microbial degradation of petroleum hydrocarbons has been reviewed extensively by Das and Chandran in 2011. Marine *Acinetobacter* has been reported as a leading hydrocarbon degrader (Hanson *et al.,* 1996; Walker *et al.,* 1976; Yamamoto and Harayama, 1996; Kostka *et al.,* 2011; Bhuyan, 2012). Though actual degradation was not tested, utilization of these compounds as sole source of carbon and energy provides an indirect evidence for its degradation. This property is also useful for the study of bioemulsifier production since utilization of hydrocarbon and organic compound is possible only if it is emulsified in the culture medium.

Marine *Acinetobacter* strains were found to be tolerant to high concentrations of metal salts including sodium arsenate and arsenite, potassium tellurium, lead acetate and selenium dioxide. However, all the strains were sensitive to cadmium, silver and mercury. High metal tolerance among marine bacteria is a common phenomenon (Kadri and Salem, 1985; Sabry *et al.,* 1997; Jafarzade *et al.,* 2012).

Table.1 Biotyping of marine *Acinetobacter* by Bouvet and Grimont scheme (1986, 1987)

Name of Test	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GS8	GW9	MW10	MW11
Motility	NM[a]	M	NM	NM	NM	NM	NM	NM	NM	NM	NM
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Growth at different temperatures[b]:											
37^0C	+	+	+	+	+	+	+	+	+	+	+
41°C	+	+	+	+	+	+	+	+	+	+	+
44^0C	+	-	+	+	-	+	-	+	-	+	-
2.1.2.1 Other tests:											
Gelatin hydrolysis	+	-	+	+	+	+	+	+	+	-	-
Haemolysis	+	-	+	-	-	+	+	+	-	-	+
Citrate utilization	+	-	+	+	+	+	+	+	+	+	+
Acid from glucose	-	-	-	-	-	-	-	-	-	-	-
2.1.2.2 Utilisation of[b,c]:											
Histidine	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+	+	-	+
L Aspartic acid	+	+	+	+	+	+	+	+	+	-	+
Maleic acid	-	-	-	-	-	-	-	-	-	-	-
DL Phenyl alanine	+	+	+	+	+	+	+	+	+	+	+
L Arginine	+	+	+	+	+	+	+	+	+	-	+
Leucine	+	-	-	+	+	+	+	+	+	+	+
□ Alanine	+	-	-	+	+	+	+	+	+	-	+
Ornithine	-	-	-	+	-	+	+	+	+	-	-
Malonate	-	-	-	-	-	-	-	-	-	-	-
Lactic acid	-	-	-	-	+	+	-	+	-	-	+
Phenyl acetic acid	+	-	-	-	+	+	+	+	+	+	+
Trans aconitic acid	-	-	-	+	+	+	+	-	-	+	+
4Amino butyric acid	+	+	+	+	+	+	+	+	+	+	+
Ethanol	-	+	-	+	+	+	+	+	+	+	+

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus*
GW2, GW4, MW5, GW9, MW10: *A. junii*,+: Positive; -: Negative
a: Nonmotile, b: Results were observed for 7 days after every 24 hr.
c: M70 minimal medium with 3.5% NaCl was used. +: Positive; -: Negative

Table.2 Biotyping of marine *Acinetobacter* strains with API 20NE system

Name of Test	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GS8	GW9	MW10	MW11
Conventional tests:											
Nitrate reduction	-	-	-	-	-	-	-	-	-	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-
Glucose acidification	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Urease production	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	-	+	+	-	-	-	-	-	-	-
Gelatine hydrolysis	+	-	+	+	+	+	+	+	+	-	-
Galactosidase production	+	-	+	+	-	-	-	+	-	-	+
Assimilation tests:											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	+	-	-	-	+	-	-	-	-	+
Mannose	-	+	-	+	+	+	+	+	-	-	+
Mannitol	+	+	-	-	+	-	+	+	+	+	+
N-acetyl glucosamine	+	+	-	-	+	+	+	+	+	-	+
Maltose	+	+	-	+	+	+	+	+	+	-	+
Gluconate	+	+	-	+	+	+	+	+	+	+	+
Caprate	-	-	-	-	-	+	-	+	-	-	-
Adipate	+	+	-	-	-	+	+	+	+	+	+
Malate	+	+	-	+	+	+	+	+	+	+	+
Citrate	+	+	-	+	-	+	+	+	+	+	+
Phenyl acetate	-	+	-	-	+	+	+	+	+	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
2.1.2.3 API 20NE Number	0474761	0047763	0470000	0072641	0056742	0053773	0056763	0076773	0054763	1044461	1067761

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus*
GW2, GW4, MW5, GW9, MW10: *A. junii*, +: Positive; -: Negative.
Manufacturer's necessary instructions were strictly followed.

Table.3 Biochemical characteristics of marine *Acinetobacter*

Name of Test	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GS8	GW9	MW10	MW11
2.1.2.3.1.1.1.1.1 Carbohydrate											
2.1.2.3.1.1.1.1.2 utilisation[a, b]:											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	ND	ND
Arabinose	-	-	-	-	+	-	+	+	+	-	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	-	+	+	+	+	+	+	+	+
Adonitol	+	+	-	-	+	+	+	+	+	+	+
Xylose	+	+	-	-	+	-	-	-	+	-	-
Dulcitol	-	-	-	-	-	+	-	-	+	-	-
Cellobiose	+	+	-	-	+	+	+	+	+	-	+
Inulin	+	+	-	-	+	+	+	+	+	-	+
Fructose	+	+	-	-	+	+	+	+	+	-	+
Meso-Inositol	+	+	-	-	+	+	+	+	+	+	+
Trehalose	-	+	+	-	+	+	+	+	+	+	+
Sorbitol	-	+	+	-	+	+	+	+	+	+	+
Rhamnose	-	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+
Mellibiose	-	-	-	-	-	-	-	-	-	-	-
2.1.2.3.1.1.1.1.3 Other tests[b]:											
Indol production	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	-	-	-		+	+	-	-	-
VP Test	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	-	-	-	-	-	-	-	-	-	+	+
2.1.2.3.1.1.1.1.4 Growth at different											
2.1.2.3.1.1.1.1.5 NaCl concentrations[b]:											
5 %	+	+	+	+	+	+	+	+	+	+	+
8 %	+	-	+	+	+	+	+	+	+	+	+
10 %	+	-	+	+	+	+	+	+	+	-	-
12 %	+	-	+	-	+	+	+	+	+	-	-
15 %	-	-	-	-	+	+	+	+	+	-	-
20 %	-	-	-	-	+	-	+	+	+	-	-
25 %	-	-	-	-	-	-	-	-	-	-	-

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus* GW2, GW4, MW5, GW9, MW10: *A. junii*, +: Positive; -: Negative

a: Medium used was M70 minimal medium with 3.5% NaCl. b: Results were observed for 7 days after every 24 hr

Table.4 Enzyme production by marine *Acinetobacter* strains

Enzymes	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GS8	GW9	MW10	MW11
Lipase[a]	+++	++	+++	++	+	+	+	+	+	+++	+++
Amylase	-	+	-	-	-	-	-	-	-	-	+
Gelatinase	+++	-	+++	+	++	++	++	++	+	-	-
Pectinase	-	-	-	-	-	-	-	-	-	-	-
Cellulase	-	-	-	-	-	-	-	-	-	-	-
Xylanase	-	+	-	-	-	-	-	-	-	-	+
Phosphatase	+	+	+	+	+	+	+	+	+	+	+
L-Asparginase	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	+	-	-	-	-	-	-	+
Chitinase	-	-	-	-	-	-	-	-	-	-	-

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus*
GW2, GW4, MW5, GW9, MW10: *A. junii*
a: All the strain were late lipase producers.
For all the enzymes results were observed for 10-12 days, after every 24 hr.
+++: Strong Producers
++: Medium Producers
+ : weak producers.
(Ranking done on diameter of zone of clearance)

Table.5 Utilisation of hydrocarbons and organic compounds by marine *Acinetobacter* strains

	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GS8	GW9	MW10	MW11
2.2											
2.3 Hydrocarbons:											
Haxane	-	-	-	-	-	-	-	-	-	-	-
Toluene	-	+	-	+	+	+	+	+	+	+	+
Kerosene	-	-	-	-	-	-	-	+	-	-	-
Xylene	-	+	-	+	+	+	+	+	+	+	+
Organic compounds:											
Ethanol (0.1%)	-	+	-	+	+	+	+	+	+	+	+
Ethanol (1%)	-	+	-	+	+	+	+	+	+	+	+
Ethanol (5%)	-	-	-	+	+	+	+	+	+	+	+
Methanol	-	-	-	+	+	+	+	+	+	+	+
Propanol	-	+	-	+	+	+	+	+	+	+	+
Butanol	+	+	+	+	-	-	+	+	+	+	+
Phenol	+	+	+	+	+	+	+	+	+	+	+
Chloroform	-	+	+	+	+	+	+	+	+	+	+
Diethyl Ether	-	+	-	+	+	+	+	+	+	+	+
Acetone	-	+	-	-	+	+	+	+	+	+	+

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus*
GW2, GW4, MW5, GW9, MW10: *A. junii*, +: Positive; -: Negative
Utilisation of hydrocarbons and organic solvents was checked on solid and in liquid medium.
Medium used was M70 minimal medium

Table.6 Metal resistance showed by marine *Acinetobacter* strains

Metal Salt	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GW8	GW9	MW10	MW11
2.3.2.1.1.1.1 Mercuric ions & organomercuricals:											
Lead acetate	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	1024	1024
Cobolt nitrate	1024	512	512	1024	1024	1024	512	512	1024	512	>1024
Cadmium chloride	02	32	128	256	256	256	256	256	256	32	02
Antimony potassium tartarate	64	32	256	512	128	128	128	128	128	256	64
Silver chloride	32	32	16	16	32	32	32	32	32	16	16
Mercuric chloride	08	16	08	08	32	32	16	32	32	32	00
Anions:											
Sodium arsenate	1024	256	2048	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Sodium arsenite	512	128	1024	1024	1024	1024	1024	1024	1024	1024	512
Potassium tellurite	512	1024	32	2048	128	128	64	128	128	1024	128
Cations:											
Nickel chloride	512	1024	128	>1024	>1024	>1024	>1024	>1024	>1024	>1024	1024
Strontium chloride	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Cupric chloride	1024	1024	1024	>1024	>1024	>1024	>1024	>1024	>1024	1024	1024
Gold chloride	128	256	64	512	256	64	256	128	64	128	64
Platinum chloride	>1024	1024	1024	>1024	>1024	>1024	>1024	>1024	>1024	1024	1024
Potassium dichromate	512	512	512	512	1024	1024	1024	1024	1024	512	1024
Stannous chloride	>1024	1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Palladium chloride	256	256	256	256	256	256	256	256	256	256	256

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus* GW2, GW4, MW5, GW9, MW10: *A. junii*
Controls: *A. calcoaceticus* BD413 trp E27 and *E. coli* HB101.+: Positive; -: Negative
Concentration at which growth was totally inhibited was considered as minimum inhibitory concentration (MIC).

Table.7 Antibiotic resistance profile of marine *Acinetobacter* species

Antibiotics	GW1	GW2	MW3	GW4	MW5	GW6	MW7	GW8	GW9	MW10	MW11
□ lactams											
Penicillin	<5	10	<5	10	320	320	320	320	320	20	<5
Ampicillin	2	4	2	16	8	8	8	8	8	8	2
Ceftriaxone	<1	<1	<1	8	8	8	8	8	8	<1	<1
Cloxacilin	2	2	<1	32	128	256	256	256	256	32	16
Amoxycilin	<1	<1	<1	>512	16	16	16	16	16	<1	<1
Cefuroxime	<1	<1	<1	8	8	8	8	8	8	<1	<1
Cefotaxime	<1	<1	<1	2	16	16	16	16	16	10	<1
Cefazolin	<1	2	<1	4	4	128	4	64	32	64	<1
Cefalexin	<1	<1	<1	16	16	16	16	16	16	32	<1
Aminoglycosides											
Streptomycin	8	16	4	4	32	32	16	32	32	2	4
Kanamycin	2	32	2	4	8	8	8	8	8	2	2
Gentamycin	2	4	2	4	2	2	2	2	2	4	2
Neomycin	<1	8	<1	<1	2	2	2	2	2	<1	2
Ofloxacin	<1	<1	<1	<1	4	4	4	4	4	<1	4
Norfloxacin	4	32	8	8	64	64	64	64	64	4	32
Amaikacin	2	16	2	2	4	4	2	8	4	2	2
3											
4 Others											
5 Chloramphen icol	256	32	256	128	1024	1024	1024	1024	1024	64	64
Nalidixic acid	128	64	64	32	256	256	256	128	256	64	64
Rifampicin	1	1	1	2	1	1	1	1	1	1	1

GW1, MW3, GW6, MW7, GW8, MW11: *A. haemolyticus* GW2, GW4, MW5, GW9, MW10: *A. junii*
Controls: *A. calcoaceticus* BD413 trp E27 and *E. coli* HB101.+: Positive; -: Negative
Concentration at which growth was totally inhibited was considered as minimum inhibitory concentration (MIC).

Marine *Acinetobacter* strains were reported to be resistant to nickel, lead, chromium and arsenate (Nieto *et al.,* 1989) whereas *Acinetobacter* strains isolated from other habitats were highly resistant to cadmium, silver and mercury (Dhakephalker and Chopade, 1994; Deshpande and Chopade, 1994; Bhamare *et al.,* 1994; Shakibaie *et al.,* 1999). Marine water and sediment contain lots of metal salts which are naturally present or enter in the marine environment as a contaminant through dumping of huge amount of industrial and domestic sewage into sea containing salts of lead, arsenic, cadmium, nickel, cobolt, mercury, silver etc (Jafarzade *et al.,* 2012; Chiarelli and Rocherri, 2014) Thus, the constant exposure to the high concentrations of metal salts may be the probable reason for high bacterial metal tolerance. The striking feature in this study was significantly high MIC levels of metal salts as compared to MIC levels of antibiotics. This could be very well explained given the fact that the source of the microorganisms was marine as opposed to a hospital environment.

Plasmid mediated multiple antibiotic resistance is the peculiarity of genus *Acinetobacter* (Chopade *et al.,* 1994 a,b; Shakibaie, 1999; Saranathan *et al.,* 2014). Antibiotic resistance of marine *Acinetobacter* has also been reported by some authors (Romalde *et al.,* 1990; Kelch *et al.,* 1978). In view of this background, it was quite surprising that *Acinetobacter* strains isolated from marine habitat were sensitive to many antibiotics. However, some of the strains were resistant to antibiotics like chloramphenicol, nalidixic acid, penicillin, amoxycillin and norfloxacin.

As mentioned earlier, occurrence of plasmids in the members of genus *Acinetobacter* is a common phenomenon. These plasmids are known to code different characteristics like antibiotic and metal resistance, indole acetic acid production etc. Marine *Acinetobacter* had also been reported to possess plasmids (Di-Cello *et al.,* 1997; Marty and Martin, 1994). In this study we have not directly studied the relation between any character and the plasmid content of the organism. However, high resistance to metals and also to some antibiotics points out the relationship between presence of plasmid and these properties. This relationship could be evaluated in further studies.

From this study, it is clear that marine *Acinetobacter* strains possessed peculiar physiological and biochemical characteristics. Yellow and orange pigment production and ability to grow at high sodium chloride concentration were some of the important physiological characteristics exhibited by marine *Acinetobacter* strains. They also possessed some important biochemical characteristics like enzyme production, resistance to metal salts and ability to utilise hydrocarbons and organic compounds. Overall this study represents a new dimension in the study of marine micro-organisms with special reference to *Acinetobacter* species. Considering the fact that, there is a dearth of ongoing research in this field, the present study will provide a foundation and pave way to further exploration of these marine *Acinetobacter* species.

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