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Isolation and Characterization of a Marine Methanogenic Bacterium from the Biofilm of a Shiphull in Los Angeles Harbor

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Abstract. A marine mesophilic, irregular coccoid methanogen, which shows close resemblance to *Methanococcus* sp., was isolated from the biofilm of shiphulls docked in Los Angeles harbor. Hydrogen plus carbon dioxide or formate served as substrates for methanogenesis in a mineral salt medium. The isolate did not use acetate and methanol as sole source of carbon and energy. The organism had an optimal pH range of 6.8–7.0 and a temperature optimum of 37°C. Elevated levels of sodium chloride were required for optimum growth. Optimum levels of total sulfide and magnesium chloride for growth were 1.0 mM and 10 mM respectively. The isolate used ammonia as nitrogen source. The concentration of 30 mM ammonium chloride supported maximum growth of the isolate.

The atmospheric methane level appears to be increasing [12, 33]. More than 80% of earth's annual output of atmospheric methane is produced biologically in anaerobic environments, mainly from the enteric fermentations of animals and from rice paddy fields, swamps, and marshes [17]. Methanogenesis is a major route in the anaerobic mineralization of organic matter.

Many methanogenic bacteria have been isolated from ruminants, non-ruminants, bovine rumen, and human [3, 4, 25, 26, 27, 30, 35]. Methanogens have been found to thrive in a wide range of habitats, including marine and fresh water sediments, geothermal vents, anaerobic waste digesters, and intestinal tracts of animals [1, 23, 28]. Unidentified methanogens have been isolated from thermal environments, acid peat bog, salt lakes, and human feces [18, 23–25, 29, 39, 40, 42]. Methanogenesis has been detected in termites [33], plankton, fish and goat intestines [28], scarebaeid larvae [16], and the cockroach hind gut [11]. Presence of methanogens has been demonstrated in dental plaques of human and monkey [7, 22], algal mats, volcanic environments, hot springs [37, 38, 41], thermal reactors [19, 34], and rice fields [32]. Thus the presence of methanogens has been demonstrated from a wide variety of ecological niches. However, the possibility of methanogenesis from the marine biofilm of shiphulls was not studied in detail. In this paper we describe the isolation and properties of a pure culture of *Methanococcus* sp. isolated from the biofilm of a shiphull in Los Angeles harbor.

Materials and Methods

Isolation and growth conditions. The anaerobic techniques described by Balch and Wolfe [2] and Daniels et al. [14] were used throughout this study. The medium used for enrichment and pure culture consisted of the following components (mM): KCl (4.1), NH_4Cl (25), $CaCl_2$ (0.61), K_2HPO_4 (1.45), KH_2PO_4 (1.85), NaCl (300), $MgCl_2$ (15), Na_2CO_3 (1.5), resazurin (0.003), Na_2S (1.0), tungstic acid (1 μM), and selenate (1 μM). 10 ml/liter of trace element solution and vitamin mix [14] solution were also added. The pH of the medium was adjusted to 6.8 by the addition of Na_2CO_3 while the medium was bubbled with N_2–CO_2 (80:20 v/v). For nutritional studies various concentrations of NaCl, Na_2S, $MgCl_2$, and NH_4Cl were used. After preparation, media were dispensed into tubes or bottles and made anaerobic as described previously [14]. The tubes or bottles were then autoclaved. The gas phase, except in the carbon source experiment, was H_2–CO_2 (80:20 v/v). In the carbon source experiment, the gas phase was N_2–CO_2 (80:20 v/v).

Marine biofilm samples were collected from different shiphulls (steel hull, fiberglass, and wood), concrete piers, and wooden piers in the Los Angeles harbor. Glass bottles (approximately 20 ml volume) were filled entirely with biofilms and a small amount of sea water. The bottles were sealed tightly with a lid and parafilm and, after transport to the laboratory at 20°–25°C, were kept at 4°C until enrichment cultures were begun 5 days later. A portion of the biofilm sample (1 ml) was added to 27 ml

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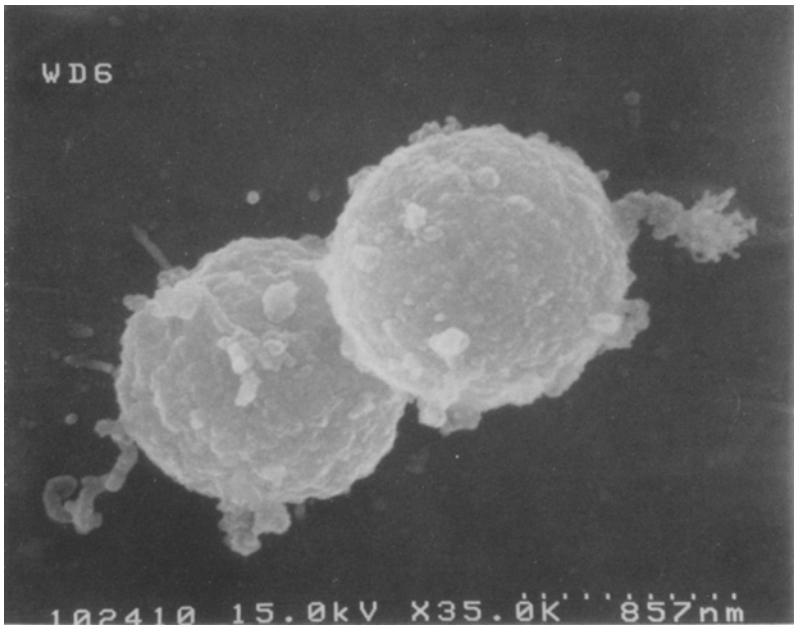

Fig. 1. Scanning electron micrograph of the marine isolate.

anaerobic tube (no. 2048–00150; Bellco Glass, Vineland, New Jersey) containing enrichment medium in 10-ml amounts. The tubes were sealed with lipped black stoppers (aluminum seal rubber stoppers, no. 2048-11800; Bellco) crimped with aluminum seals (no. 224183; Wheaton Scientific). The tubes were incubated at 30°C without shaking. The methane production in the tubes was monitored regularly by use of GC. The cultures showing substantial methane were transferred to the methanogenic medium with H_2–CO_2. After five more transfers, plating was done in an anaerobic glove bag on the H_2–CO_2 methanogenic medium with 2% agar (Becton Dickinson Microbiology System, Cockeysville, Maryland) according to previously described techniques [2, 21, 32]. Isolated colonies were restreaked, and individual colonies were then transferred into liquid medium for further studies. Purity of the broth cultures was checked by microscopic examination and by inoculation into heterotrophic medium under N_2–CO_2 (80 : 20 v/v) atmosphere. Cultures were also grown in the presence of antibiotics (Kanomycin and Streptomycin at 60 μg/ml) to check the purity.

Analyses. Culture turbidity was measured by absorbance at 600 nm with a spectronic 20 spectrophotometer (Bausch and Lomb). Methane was measured by gas chromatography [5].

Scanning electron microscopy (SEM). For SEM, a Hitachi S-4000 SEM was used. Samples were fixed in phosphate buffer containing 2.5% glutaraldehyde for 30 min. The osmolality of the fixing solution was the same as that of the sample and was measured with a Hermann roabling automatic micro-osmometer. After being washed and rinsed in the same buffer, samples were dehydrated in a graded ethanol series and dried at the critical point with liquid CO_2 and a CPD 020 Balzars Union. Samples were sputter coated with gold.

Results

Among the nine different biofilm samples enriched, a significant amount of methane was produced by one biofilm sample from a steel-hulled ship. Methane production was more significant under H_2–CO_2 methanogenic condition than acetoclastic conditions. The H_2–CO_2 methanogenic enrichment culture was transferred five times before a pure culture was isolated. Cells of the isolate were irregular cocci. Cells occurred mainly as doublets and small aggregates. The cells were about 1.0 × 1.5 μm in size (Fig. 1). The purity of the isolate was confirmed by the absence of growth or methanogenesis in basal medium (N_2–CO_2 gas phase) supplemented with the following heterotrophic substrates (%): yeast extract (0.2), peptone (0.2), glucose (0.1), sucrose (0.2), and glycerol (0.1).

In Fig. 2, a typical growth curve of this isolate grown on H_2 + CO_2 or 30 mM each of formate, acetate, and methanol is shown. The isolate grew well on formate and H_2 + CO_2. It did not use acetate or methanol as sole source of carbon and energy. This nutritional character along with the morphology of the isolate led us to believe that this isolate may be a marine *Methanococcus* sp. The methanogenesis of this organism under various substrate conditions is shown in Fig. 3. The culture with H_2 + CO_2 and the culture with 30 mM of formate produced a significant amount of methane (100–125 μmoles of methane/culture tube). The acetate- and methanol-supplemented cultures did not produce any significant amount of methane.

The isolate was examined for its growth and salt requirements. Growth of the isolate was evident between 25° and 45°C with an optimum at 37°C (Fig.

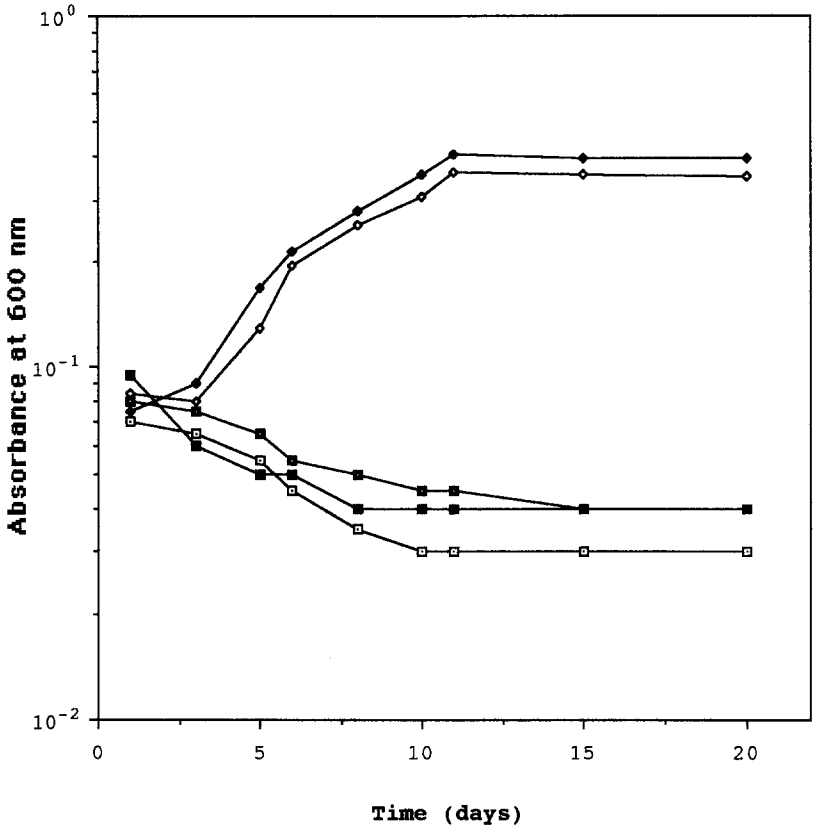

Fig. 2. Comparative growth by the marine isolate on H_2–CO_2, formate (30 mM), acetate (30 mM), and methanol (30 mM). Control culture received no substrate. Data represent mean of two values. ◆, H_2–CO_2; ◇, formate; □, acetate; ■, methanol; ⊡, control.

4). The organism grew in a pH range of 5.8–7.2 with an optimum at pH 7.0 (Fig. 5). The effect of different levels of sulfide on growth was tested and the result is presented in Fig. 6. When no sulfide was present in the medium, no growth was observed. The optimum sulfide concentration for maximum growth was 1.0 mM. Concentrations higher than 1.0 mM of sulfide inhibited the growth of the isolate.

The levels of NaCl required for the optimum growth of the organism were studied. As shown in Fig. 7, the concentration of 300 mM supported good growth. Concentrations higher than 300 mM were found to suppress the activity of the isolate. We also studied the requirements of $MgCl_2$ and ammonium concentration for the optimum growth of the isolate (Fig. 8). The isolate needed $MgCl_2$ for growth. In the absence of $MgCl_2$ no growth was seen. The optimum concentration of 10 mM of $MgCl_2$ supported better growth of the organism. All methanogenic bacteria are known to use ammonia as the sole source of nitrogen for their growth [9]. The concentration of NH_4Cl needed for optimal growth of the organism was determined. Figure 8 shows cell growth with varying concentrations of NH_4Cl in the medium. Optimal growth occurred at >30 mM, and concentrations up to 60 mM had no further effect. The cultures with a concentration of 60 mM of NH_4Cl attained cell densities similar to that obtained with 30 mM of NH_4Cl.

Discussion

The importance of microbial activity in the marine environment as a minor source of atmospheric methane has been established by field measurement studies and by experiments under laboratory conditions. Many marine methanogens have been isolated from marine sediments and deep sea thermal vents. However, to our knowledge, there have been no reports on methanogenesis in the marine biofilms of ship-hulls. The organism described in this study has been isolated from this habitat. On the basis of morphological and nutritional characteristics, the isolate was tentatively identified as a *Methanococcus* sp.

The biofilm samples were collected from ships made of different materials, viz., steel, wood, and fiberglass. It is interesting to note that the biofilm from steel-hulled ship showed significant methanogenesis and the organism was isolated from this biofilm, whereas there were no significant methanogenic activities in the biofilms of ships made of wood and fiberglass. Recently, it has been demonstrated

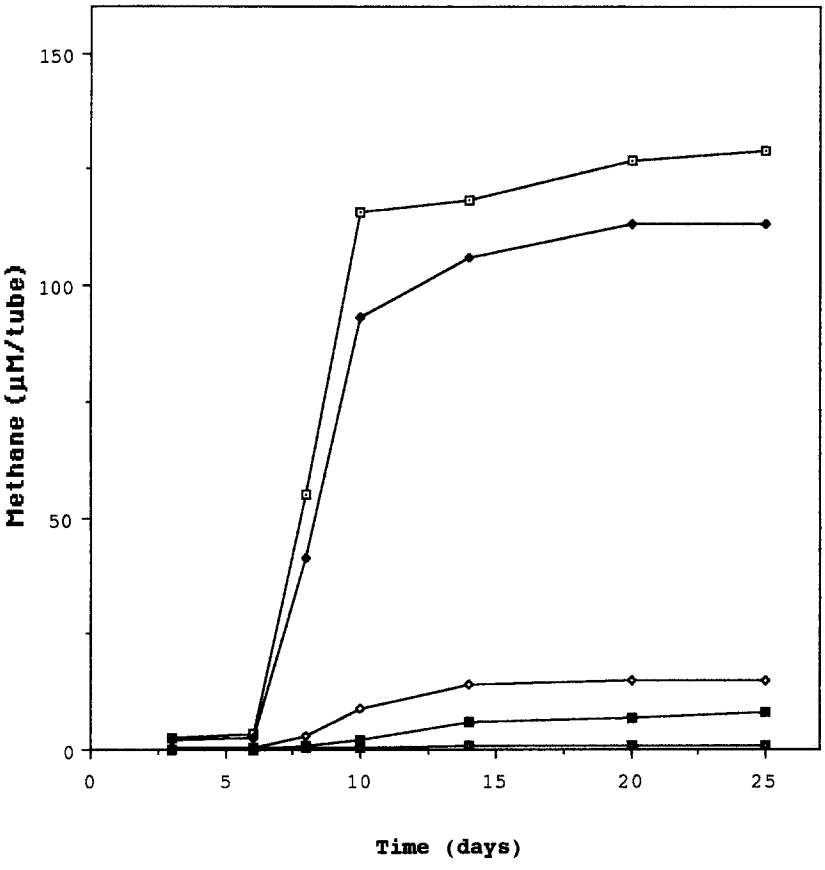

Fig. 3. Comparative methanogenesis by the marine isolate on H_2–CO_2, formate (30 mM), acetate (30 mM), and methanol (30 mM). Control culture received no substrate. Data represent mean of two values. ⊡, H_2–CO_2; ◆, formate; ◇, acetate; ■, methanol; □, control.

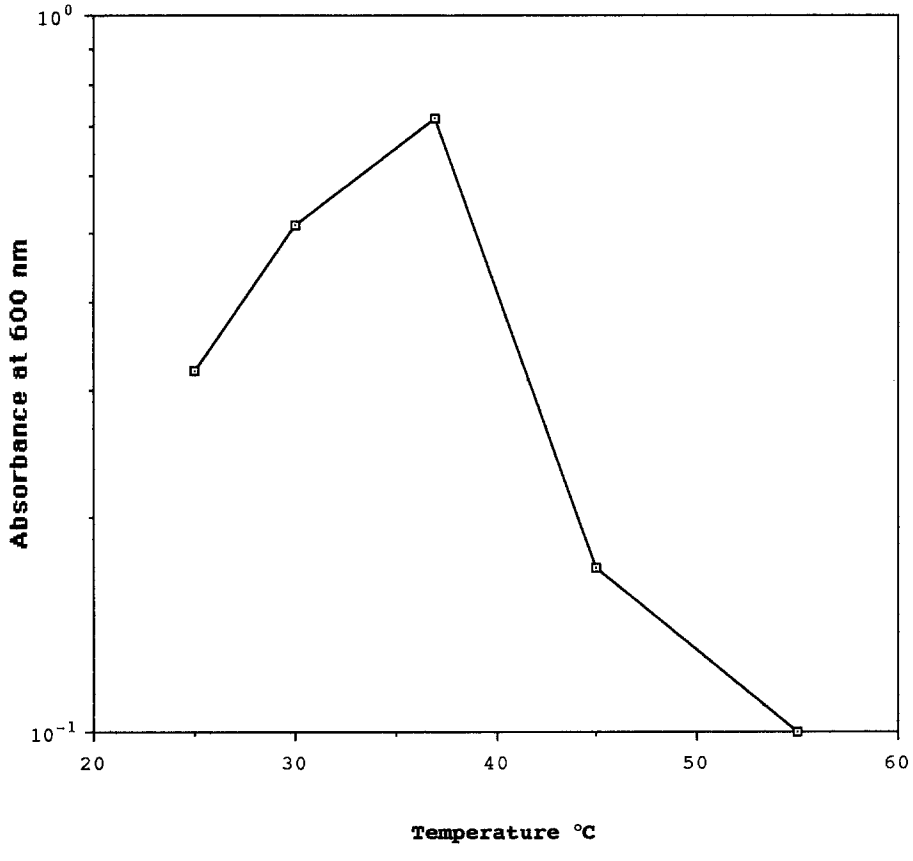

Fig. 4. Effect of temperature on the growth of the marine isolate. Absorbance after 120 h of incubation. Data represent mean of two values. The isolate was grown on H_2–CO_2.

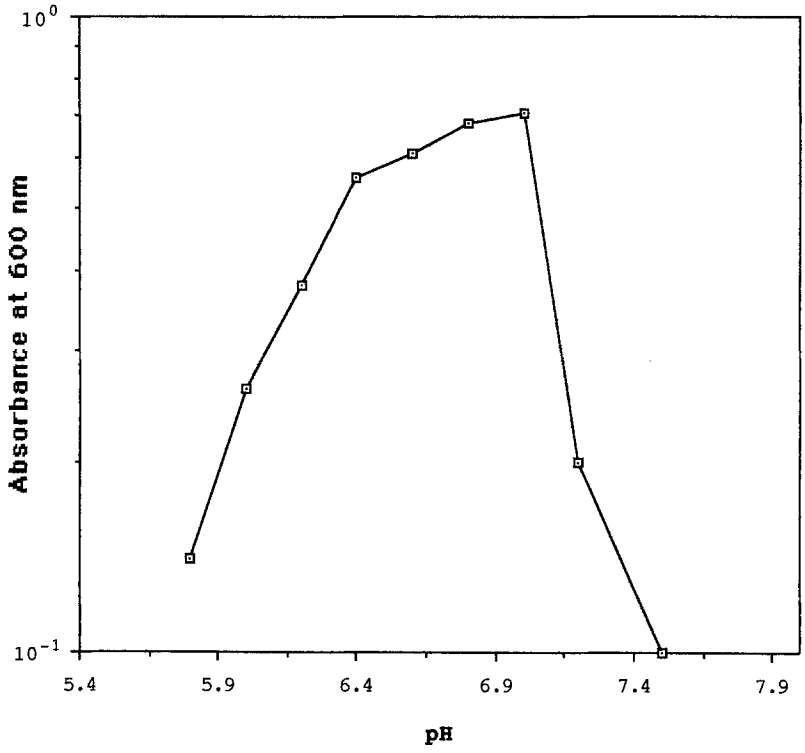

Fig. 5. Effect of pH on the growth of the marine isolate. Absorbance after 120 h of incubation. Data represent mean of two values. The isolate was grown on H_2–CO_2.

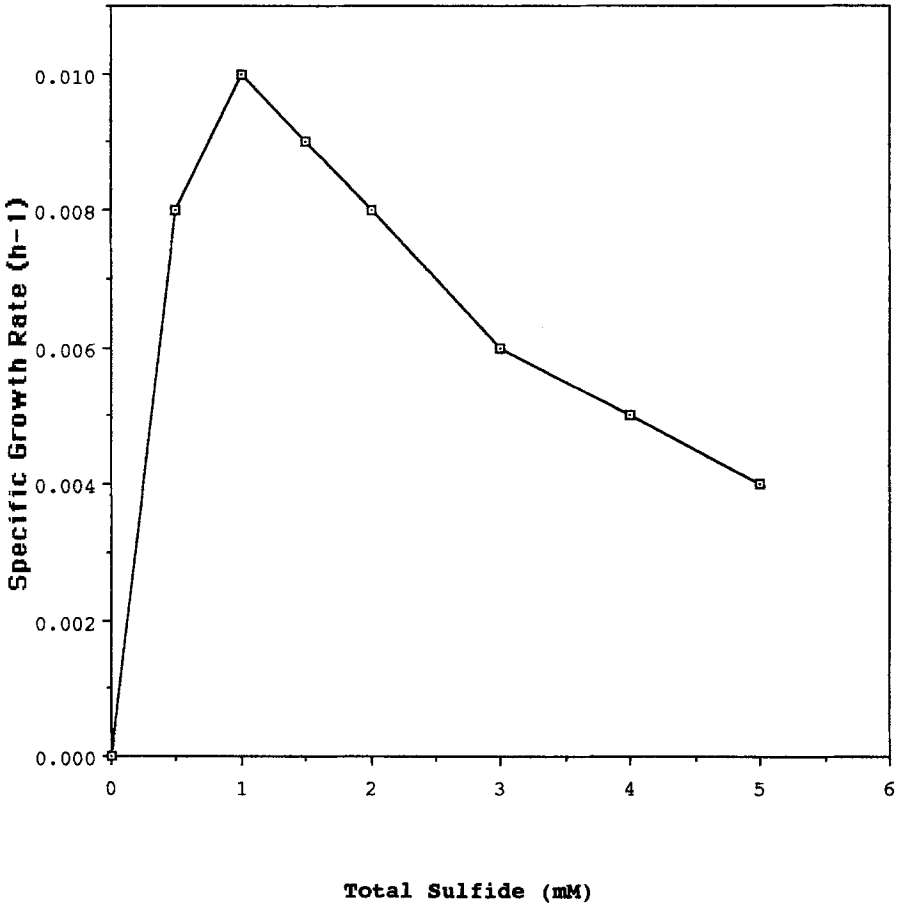

Fig. 6. Effect of sulfide on the growth of the marine isolate. Specific growth rates at the exponential phase of growth were calculated from A_{600} vs. time plots of respective cultures. The isolate was grown on H_2–CO_2.

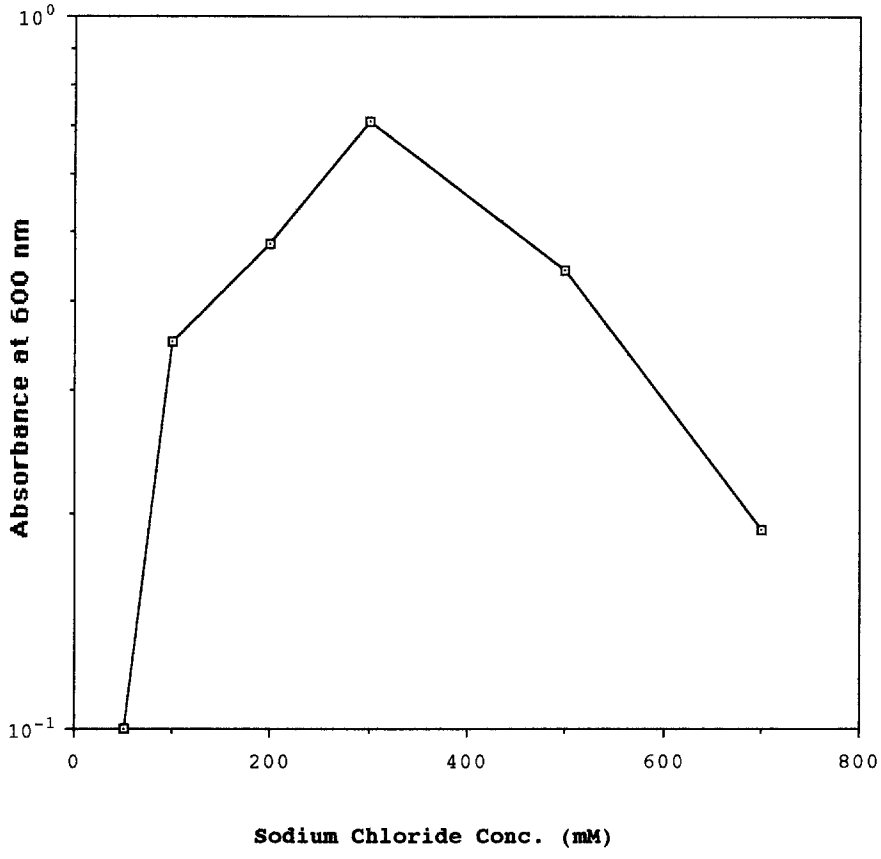

Fig. 7. Effect of NaCl on the growth of the marine isolate. Absorbance after 120 h of incubation. Data represent mean of two values. The isolate was grown on H_2–CO_2.

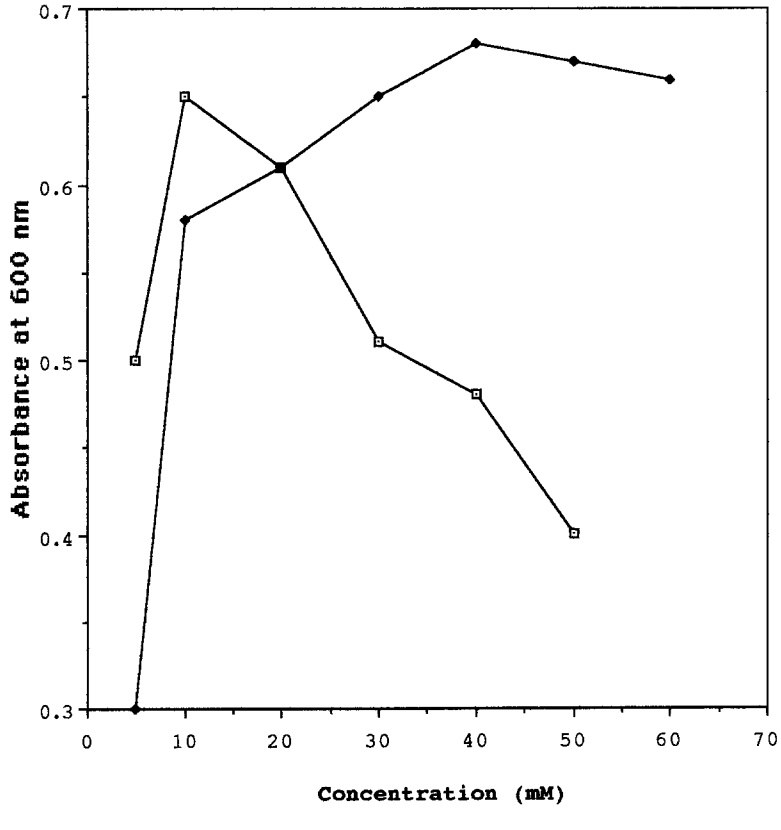

Fig. 8. Effect of $MgCl_2$ and NH_4Cl on the growth of the marine isolate. Absorbance after 120 h of incubation. Data represent mean of two values. The isolate was grown on H_2–CO_2. ⊡, $MgCl_2$; ◆, NH_4Cl.

in the laboratory that both sulfate-reducing bacteria and methanogenic bacteria are capable of using Fe° and other elemental metals [6, 8, 15, 31] as a source of hydrogen for growth and metabolism by use of the mechanism of cathodic depolarization proposed by Van Wolzogen Kuhr and Van der Vlught [36]. In biofilms, the environment is largely anaerobic even with only a thin film established. In this study, we isolated methanogenic bacteria from the biofilm of steel-hulled ship, which suggested that the metal (Fe°)-derived methanogenesis is taking place in that habitat as described earlier by many workers [6, 15]. It is also likely that H_2 is produced in the biofilms by the activities of other heterotrophic bacteria present in the biofilm, and this H_2 is used by the methanogen [10].

The isolate shares many features with mesophilic *Methanococcus* strains. Mesophilic *Methanococcus* present in marine environment are known to grow on H_2–CO_2 and formate [13], as did our isolate. The isolate is sensitive to high concentration of sulfide in the culture medium. The optimum sulfide concentration was 1 mM. A significant level of inhibition was noticed at a concentration of 4 mM of sulfide. Growth of *Methanococcus deltae* and *M. maripaludis* is inhibited at >4 mM of sulfide, while *M. jannaschii* and several *Methanobacterium* species grow well even at 10 mM of sulfide [13, 26, 41]. The NaCl concentration in the natural habitat of the isolate is about 300–500 mM. The growth of the isolate was inhibited by the NaCl level of >300 mM in the medium. The optimum level of NaCl reflects the natural habitat. All the marine methanogens need magnesium for growth [13, 20]. As shown above, this isolate did not grow in the absence of magnesium. Ammonia served as the nitrogen source for the organism. All the optimum concentrations of sulfide, sodium, magnesium, and ammonia obtained in this study showed close resemblance to the *Methanococcus* sp. [13]. The pH and temperature optima of the isolate suggest that this isolate is a mesophilic *Methanococcus* sp.

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