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# Research Article
# Isolation and Identification of Cadmium-Reducing Bacteria from Contaminated Coastal Sediment in the Northern Coast of Indramayu, Indonesia

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## Abstract

**Background and Objective:** Cadmium (Cd) is one of the heavy metal pollutants and its accumulation impacts the sustainability of marine organisms. Current research aimed to isolate and identify the cadmium-reducing bacteria from contaminated coastal sediment in Karangsong Port, Indramayu, Indonesia. The isolates were investigated for their potential to reduce cadmium and showed the cadmium reduction drastically up to 50% at 6 hrs treated under different cadmium concentrations of 0, 5, 1 and 1.5 ppm, respectively. **Materials and Methods:** Morphological characteristics were observed in most of the isolates. Out of 8 isolates, two selected strains such as Karangsong Cd 3 and Cd 7 were identified by 16S rRNA sequencing as *Pseudoalteromonas issachenkonii* strain KMM 3549 (Acc. No. NR 025139.1) and *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Acc. No. NR 041787.1), respectively. **Results:** The cadmium resistance profile showed that the selected isolates were resistant to various concentrations of cadmium (Cd). The isolates reduced the concentration of cadmium drastically up to 50% at 6 hrs. The results demonstrated the two bacteria are possible to remove the cadmium from seawater containing cadmium. The gram staining showed bacterial colony morphology were diplobacilli and coccobacillus. **Conclusion:** These results suggested that the Karangsong Cd 3 and Cd 7 could facilitate the new references of future microbial applications for bioremediation efforts.

Key words: *Pseudoalteromonas* sp., contamination, bioremediation, heavy metal, cadmium

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The sustainability of the marine environment has been negatively affected by the rapid growth of industries. The discharge of waste and harmful pollutants from industrial activities has increased in seawater, leading to the endangerment of marine ecosystems and living beings, including humans[1]. Marine pollution has now become a critical problem and it has been receiving significant attention from various scientific and international communities[2,3]. A considerable number of hazardous heavy metals in seawater are often associated with industries, mining and agriculture activities[4]. Cadmium is one of the toxic pollutants to marine organisms and is frequently present as a by-product of zinc refining[5,6]. Due to its solubility and its harmful effects on marine ecosystems, cadmium is considered an environmental hazard. This anthropogenic contaminant is discharged into coastal regions through rivers and groundwater[7].

Studies have shown that cadmium exposure can have detrimental effects on marine organisms' fertilization, growth rate and embryonic development[8,9]. Industrial and agricultural activities are common sources of cadmium bioaccumulation in marine environments, such as the Karangsong Port located in the Northern Coastal area of Indramayu, Indonesia. The area is experiencing rapid industrial growth in manufacturing, long-term waste oil from oil companies and waste from fish auctions. Furthermore, numerous studies have reported high levels of cadmium accumulation in the Karangsong Coast area[10]. However, there have been no experiments to develop a consortium of indigenous bacteria as a bioremediation agent for Cd metal pollution in waters contaminated with industrial waste in Karangsong Port.

Microorganisms have been found to have a high tolerance for heavily polluted areas, with physiological and genetic changes that include the secretion of a soluble enzyme and resistance mechanisms[11-13]. A comprehensive analysis of bioremediation from contaminated coastal sediment could enhance our understanding of the availability of cadmium-reducing bacteria. Therefore, in this study, the objective was to isolate and identify bacteria from the sediments at Karangsong Port that have the potential to reduce cadmium. The cadmium-reducing bacteria were isolated from Karangsong sediments and identified as Karangsong CD3 and CD7. Their corresponding 16S rDNA sequence analysis was aligned for phylogenetic trees with other diverse *Pseudoalteromonas* sp. Furthermore, cadmium metal tests were performed on the isolated bacteria from Karangsong Port sediment.

## MATERIALS AND METHODS

**Study area:** This study was conducted in Karangsong, Indramayu Regency. Laboratory and data analysis were carried out from February to July, 2018 at the Biogeochemistry Laboratory, Bioprocess Laboratory, Fisheries Technology Process Laboratory and Research Center of Aquatic Resources and Environment, Universitas Padjadjaran.

**Sediment collection:** The sediment samples were collected from Karangsong Harbour located in the Northern Coastal Area of Indramayu, West Java, Indonesia: L.site (S 06°56'2"E 107°46'38"). Samples were taken at the Ports approximately 50 cm above sea level around the contaminated with heavy metal cadmium (Cd) immersed area[10].

**Cadmium concentration reduction assay (Cd):** To determine the Cd concentration reduction assay, seawater was filtered with a 0.22 µm filter and transferred into a 500 mL test bottle. Three levels of Cd (0.5, 1 and 1.5 mg/L) were prepared and added to the filter seawater solutions. The source of bacteria amounting to about 10 mL was also added and gently swirled. Then, each test medium was mixed into an 80 mL vial bottle and monitored starting for 0, 12, 24 and 48 hrs, respectively.

**Bacterial isolation:** To isolate the bacteria-reducing cadmium, Karangsong sediment was collected from Karangsong beach using a piston core of 60 cm depth and the sediment was immediately stored in a flask and kept at 4°C. Sediments were dispersed in distilled water with serial dilution and the pore water was collected. Aliquots suspension (1 mL) of $10^{-5}$ dilution were spread onto Petri dishes using NA medium dissolved with 100% natural seawater for the isolation bacteria. The agar plates were incubated at 37°C for 24 hrs. Growing colonies of bacteria obtained from this step were separately streaked on NA medium plates to maintain the inoculum of pure cultures.

**Molecular identification:** The genomic DNA of cadmium-reducing isolates was extracted using a Trisure Bioline Extraction kit (Bioline, United Kingdom) and the 16S rRNA was amplified via Polymerase Chain Reaction (PCR). The universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1542R (5'-AGAAAGGAGGTGATCCAGCC-3') were used. The reaction was performed in 60 µL with Master Mix GoTaq G2 (Promega, Wisconsin, USA). The PCR product with an

amplicon size of 1500 bp was sent to First Base company using Illumina MiSeq, NovaSeq 6000 sequencer. The sequences were analyzed using BioeditTM software and submitted to the gene bank database.

**Phylogenetic analysis:** The MEGA x software V1.0 was used to generate the phylogenetic tree using the neighbor-joining tree method. A bootstrap value of 1,000 replicates was used to estimate the confidence level with values of 70% or higher indicating a reliable clustering.

**Testing of cadmium-resistant isolates:** The cadmium resistance study was carried out to identify the cadmium-resistant isolates. A loop of isolates was inoculated in Nutrient Broth and incubated at 37°C for 18 hrs. The isolates were exposed to 1, 1.5 and 2 mg/L concentration of Cd for 18 hrs. The cell density was measured using a spectrophotometer (Agilent Technologies, California, USA).

## RESULTS AND DISCUSSION

**Analysis of cadmium-reduction bacteria:** The isolates were carried out for their potential to reduce cadmium in nutrient broth having initial cadmium concentrations of 0.5, 1 and 1.5 ppm (Fig. 1a), respectively. It was observed that the isolates reduced the concentration of cadmium drastically up to 50% at 6 hrs (Fig. 1b). However, the isolates had stable growth cultured at different intervals and at different levels of cadmium concentrations. These results suggested that some bacteria is possible to remove the cadmium from seawater containing cadmium. It was reported that the bonding of heavy metals in a solution occurs with the exchange process of the ions on the cell wall where they are replaced by heavy metal ions[14]. Presumably, several factors play an essential role in the reduction and absorption processes of heavy metals, comprising phenotype factors, biomass factors and medium factors.

**Morphology and molecular identification of bacterial strains:** The morphological characteristics of Karangsong Cd colonies were irregular, round and spindle as shown in Table 1. Bacterial strains isolated from Karangsong Port were mainly off-white and yellowish as examined by the light microscopy (Table 1 and Fig. 2). The gram staining observation showed that bacterial colonies were diplobacilli and coccobacillus (Table S1). The genomic DNA isolation and 16S rDNA PCR were performed for the cadmium-reducing isolates and the samples were analyzed for sequencing. The universal primer of 16S rDNA was designated and subjected to PCR amplification and showed a clear target band of about 1500 bp (Fig. 3). Results of the BLAST analysis showed that the Cd 3 isolate was 77.28% similarity index with that *Pseudoalteromonas issachenkonii* strain KMM 3549 (Accession number NR 025139.1). The Cd 7 bacterial isolate was identical to *Pseudoalteromonas spiralis* strain Te-2-2 (Accession number NR 114801.1) with an identity value of 95.88%. The result showed morphological and 16S rDNA sequence analysis, selected isolates were found identical to *Pseudoalteromonas* sp.

The isolation of novel isolates from Karangsong Port showed 77 and 97% identity values observed by using BLAST search (Table S2). Two bacterial species were

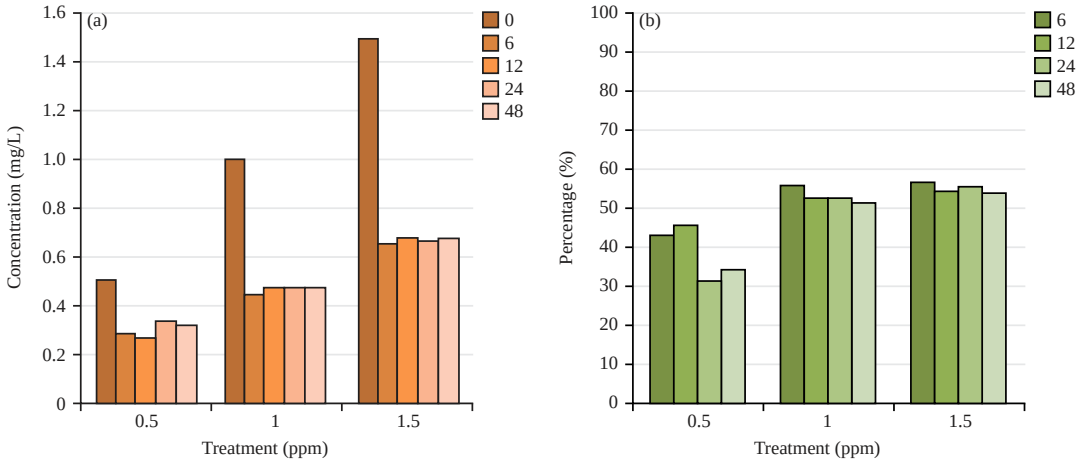


Fig. 1(a-b): Detection of cadmium reduction, (a) Cadmium reduction concentration of bacteria from Karangsong Port and (b) Cadmium degradation capacity (%)
Bacteria reduction test was carried out for its potential to reduce cadmium with different cadmium concentrations

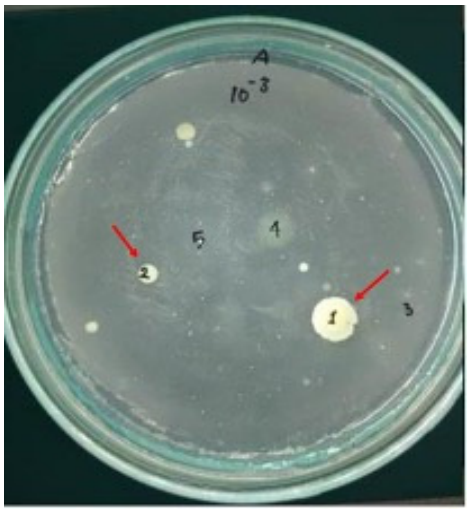


Fig. 2: Characterization of the Cd strains and morphological observation of Cd bacterial colonies

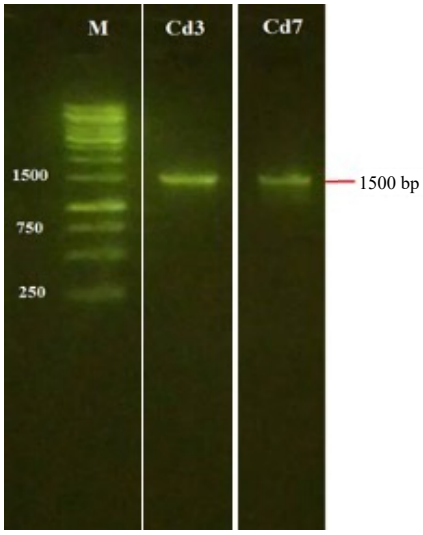


Fig. 3: Agarose DNA gel electrophoresis of PCR products, the size of PCR products are indicated on the right

Table 1: Colony morphology of isolated bacteria

| | Colony features | | | |
|---|---|---|---|---|
| Isolate codes | Size | Elevation | Shape | Color |
| A1 | Large | Plateau | Irregular | Yellowish white |
| A2 | Moderate | Flat | Round | Yellowish white |
| A3 | Small | Flat | Spindle | Transparent white |
| A4 | Moderate | Flat | Round | Transparent white |
| A5 | Small | Convex | Spindle | Yellowish white |

confirmed identical of which homology level shows at least 97%[15]. The selected two isolates were identified by 16S rDNA gene sequencing (Fig. 3). Chosen isolates were identified as closely related to *Pseudoalteromonas issachenkonii* strain KMM 3549 and *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Fig. 4). The bacteria species of *Pseudoalteromonas issachenkonii* strain KMM 3549 is classified as part of the genus *Pseudoalteromonas* bacteria, the order Alteromonadales, the Gammaproteobacteria class and the proteobacteria phylum. This species has both heterotrophic and aerobic

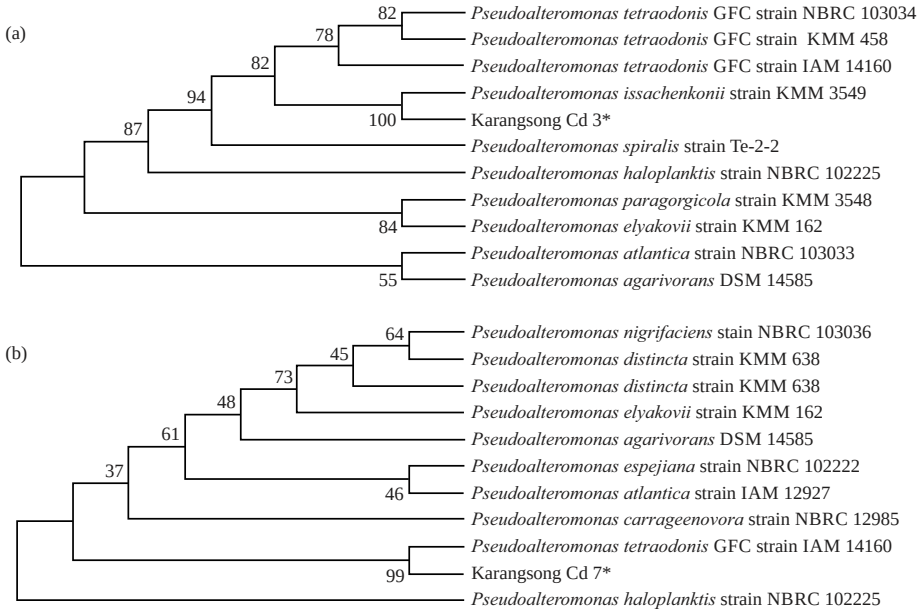


Fig. 4(a-b): Phylogenetic tree analysis of isolates bacteria in red solid stars from Karangsong Port sediments, (a) Phylogeny tree of Cd 3 and (b) Phylogeny tree of Cd 7

Phylogenetic trees were constructed based on the neighbor-joining method with 16S rRNA sequence and 1000x bootstrap. Isolates of bacteria Cd 3 and Cd 7 were marked with solid stars in red color

properties[16] and it is one of the proteobacteria groups that are abundant and widely distributed in the marine environment[17]. This species grows at temperatures ranging from 4-37°C with pH level of 6.0-10.0. The optimum growth is at the temperatures between 28-30°C with an optimum pH of 7.5-8.0. Species cannot grow at temperatures over 40°C. This species is a gram-negative bacterium, spindle transparent colony form, with rod-shaped cells, non-endospore, 0.7-0.9 µm in diameter and 1-1.2 µm in length.

**Phylogenetic analysis:** The evolutionary relationship distances were constructed using the neighbour-joining tree method and statistically tested using the bootstrap method with 1,000 replications (Fig. 4). The phylogenetic tree that Cd 3 sequence is closely related to *Pseudoalteromonas issachenkonii* strain KMM 3549 (Fig. 4a). The phylogeny tree also shows a kinship relationship between the two high sequences with a bootstrap value of 100% and an identity of 77%. Furthermore, Cd 7 sequence is related to *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Fig. 4b). Kinship relationship is also found in the phylogeny tree showing two high sequences with a bootstrap value of 99% and an identity of 97% indicating that, from 1000 reconstructed phylogeny trees, Cd 7 sequence is 99% related to *Pseudoalteromonas tetraodonis* GFC strain IAM 14160.

The species *Pseudoalteromonas issachenkonii* strain KMM 2549 (Accession Number NR 025139.1) was first isolated from the brown algal thallus (*Fucus evanescens*) collected in Kraternaya Bay, Kurile Islands in the Pacific Ocean[18]. Based on the isolation results obtained during the study Lewaru *et al.*[19], the Cd 3 bacterial isolate has morphological characteristics in the form of bacilli (rods), with the colony in the form of spindles, transparent white and possesses gram-negative properties. These morphological characteristics are in accordance with the characteristics of *Pseudoalteromonas issachenkonii* strain KMM 2549 (Accession Number NR 025139.1).

The bacterial species *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 belongs to the genus *Pseudoalteromonas* bacteria, the order Alteromonadales, the Gammaproteobacteria class and the Proteobacteria phylum. This species grows at temperatures ranging from 4-35°C with pH levels 5.5-9.5. The optimum growth is at a temperature between 25-30°C supported by an optimum pH of 7.5-8.0. Species cannot grow at temperatures over 40°C. These species are gram-negative, aerobic, rod-shaped, non-endospore, with flagellated cells. It was reported that this bacterium was first Isolated from puffer fish mucus (*Fugu poecilonotus*) to show tetrodotoxin prevalent in toxin-carrying species[20,21] in association with biota[22,23]. Pseudoalteromonas was classified

Table 2: Resistant efficiency of isolates from Karangsong Port sediments

| Bacteria code | Optical density (OD) | Bacterial density |
|---|---|---|
| **Initial** | | |
| Control | 0 | 0 |
| Cd 2 | 0.0773 | $6.18\times10^{7}$ |
| Cd 3 | 0.0915 | $7.32\times10^{7}$ |
| Cd 6 | 0.0905 | $7.24\times10^{7}$ |
| Cd 7 | 0.0725 | $5.80\times10^{7}$ |
| **End** | | |
| Control | 0 | 0 |
| Cd 2 | 1.5863 | $1.27\times10^{9}$ |
| Cd 3 | 2.02253 | $1.62\times10^{9}$ |
| Cd 6 | 1.8187 | $1.45\times10^{9}$ |
| Cd 7 | 1.94043 | $1.55\times10^{9}$ |
| **End-initial** | | |
| Control | 0 | 0 |
| Cd 2 | 1.509 | $1.21\times10^{9}$ |
| Cd 3 | 1.931 | $1.54\times10^{9}$ |
| Cd 6 | 1.728 | $1.38\times10^{9}$ |
| Cd 7 | 1.868 | $1.49\times10^{9}$ |

Optical density (OD) value at t = 0 and t = 18

as *Alteromonas tetraodonis* in 1990 but was reclassified in 2001 to the genus Pseudoalteromonas. Based on the isolation results obtained during the study by Lewaru *et al.*[19], the Cd 7 bacterial isolate has rod-shaped morphological characteristics with gram-negative properties and exactly these morphological characteristics are in accordance with the characteristics of *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Accession Number NR 041787.1).

**Test of cadmium-reduction on selected bacterial isolates:** To observe the potential of Karangsong Cd bacterial isolates on growth treated upon cadmium, Karangsong bacterial Cd 2, Cd3, Cd 6 and Cd 7 isolates were chosen for further analysis based on their ability to grow on the LB agar containing Cd. The sensitivity of selected Cd bacterial isolates to cadmium was assessed. The selected bacteria isolates, Cd 2, Cd 3, Cd 6 and Cd 7, exhibited the 1.27×109, 1.62×109, 1.45×109, 1.55×109, respectively. The resistant efficiency of the four selected isolates was higher in Cd 3 and Cd 7 (Table 2).

In this study, some of the isolates of Karangsong Port were found to remove the cadmium from seawater containing cadmium (Table 2). The bacteria were successfully isolated and coded Cd 3 and Cd 7 from Karangsong Port sediments. To the best of our knowledge, this is the first report on the isolation of isolates from Karangsong Port sediments and the identification of their associated bacteria species. Cadmium-reducing mechanisms in bacteria were separated into two phases binding phase and active transport[24,25]. The binding phase depends on the cell metabolism, where absorption occurs through the cell wall or external surface, followed by an active transport, that depends on the cell metabolism[26,27]. In metabolic processes, heavy metals can accumulate in cell membranes (extracellular) and within the cytoplasm (intracellular)[28-30]. Current study results showed two novel isolates from Karangsong Port sediments implies the potential basis of using indigenous cadmium (Cd) reducing bacteria for bioremediation application (Table 2). It should be interesting to inspect the efficiency of bioremediation agents by using these novel indigenized bacteria for removing Cd toxic pollution in contaminated waters from different polluted areas.

## CONCLUSION

Two novel bacteria were isolated from Karangsong Port sediment that showed reduced cadmium (Cd). The Cd 3 and Cd 7 are closely related to *Pseudoalteromonas issachenkonii* strain KMM 3549 (Acc. No. NR 025139.1) and *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Acc. NR 041787.1), respectively. These results suggested that novel bacterial isolates could facilitate the necessary approaches to be applied to aquatic ecosystems that are known to be heavily polluted by metal cadmium (Cd). Further investigation on the genomic sequencing of Cd 3 and Cd 7 might facilitate the important gene for future bioremediation targets.

## SIGNIFICANCE STATEMENT

The research aimed to isolate and identify the cadmium-reducing bacteria from contaminated coastal sediment in Karangsong Port, Indramayu, Indonesia. The result elucidates the ability of the new microbial *Pseudoalteromonas issachenkonii* strain KMM 3549 (Acc. No. NR 025139.1) and *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 (Acc. No. NR 041787.1), respectively has the potential to be used for reducing the cadmium concentration in the environment. Therefore, the Karangsong Cd 3 and Cd 7 can facilitate the application for future bioremediation.

## ACKNOWLEDGMENT

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## SUPPLEMENTARY MATERIALS

Table S1: Gram stain result (Microscope Magnification 100×)

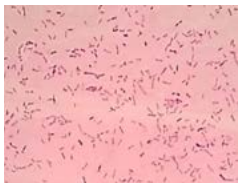
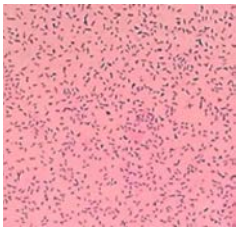
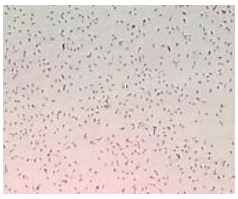
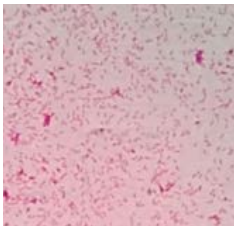
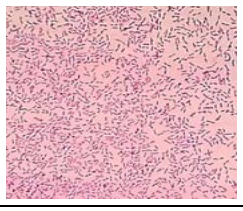
| Isolate code | Figure | Gram type |
|---|---|---|
| A1 |  | Negative |
| A2 |  | Negative |
| A3 |  | Negative |
| A4 |  | Negative |
| A5 |  | Negative |
| A6 |  | Negative |

Table S1: Continue

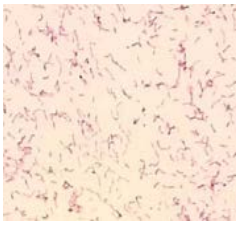
| Isolate code | Figure | Gram type |
|---|---|---|
| A7 |  | Negative |
| A8 |  | Negative |

Table S2: Results of 16S gene isolate identification using the BLAST program

| Isolate code | Species | Query cover (%) | E-value | Identity (%) | Accession number |
|---|---|---|---|---|---|
| Cd 3 | *Pseudoalteromonas issachenkonii* strain KMM 3549 16S ribosomal RNA, partial sequence | 85 | 0 | 77 | NR 025139.1 |
| Cd 7 | *Pseudoalteromonas tetraodonis* GFC strain IAM 14160 16S ribosomal RNA, partial sequence | 89 | 0 | 97 | NR 041787.1 |