

Isolation and characterization of novel *Rhodobacter* spp. with the sodium removal ability from mangrove forest sediment in Southeast Vietnam

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Abstract

Salt contamination is one of the major problems of agricultural land. It is important to find new bacteria with sodium removal ability, which can be used to reduce salts from soils. This study aims to isolate and characterize the novel *Rhodobacter* spp. with the sodium removal ability from mangrove forest sediment in Southeast Vietnam. After screening of morphological characteristics of the cell size, shape, and presence of protrusions, seven strains were identified. The results also showed that these strains were able to reduce NaCl from Glutamate-malate medium (GM). The concentration of NaCl decreased from 28.57% to 36.42% for the treatment with 25 ppm NaCl after 14 days of incubation. However, in the 35ppm NaCl concentration was absorb proficiency of decrease by approximately 5% compare with the 25 ppm NaCl concentration. The gene sequences of the 16S rDNA and puMf gene regions of the isolated strains shared high similarities with genus *Rhodobacter*, which were identified as *Rhodobacter sphaeroides* and *Rhodobacter johrii*. Our results showed a high diversity in the isolated strains belonging to the *Rhodobacter* species with sodium absorption ability. This study suggests the possibility of using isolated *Rhodobacter* strains to remove salinity from soils.

Keywords: Collection, Mangrove forest, Salinity, Reduce, *Rhodobacter* spp.

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Introduction

Purple phototrophic bacteria (PPB) are photosynthetic bacteria that are commonly employed in wastewater treatments due to their absorption capability of heavy metals (HMs), such as Hg, As, Cd, Pb, Cr and Cu (Watanabe et al., 1998, 2003; Xu

and Jia, 2011; Sasaki et al., 2012). PPB are also utilized to produce biofuel, secondary compounds and nutrients. The study in Thailand indicated that the two purple nonsulfur bacteria (PNSB) strains *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24 isolated from shrimp pond sediment have the ability to removal various HMs



(maximum of 39%) from contaminated shrimp pond water, and with removal ability of the NW16 strain was even higher in wastewater with the presence of 3% NaCl (Panwichian et al., 2011). The *Rhodobacter* species was also reported with the removal capability of radionuclides such as Co, Sr and U at a rate of 58%, 82% and 95%, respectively (Sasaki et al., 2012; Sasaki and Takeno, 2014).

In addition, the photosynthetic bacteria were reported with the ability of denitrification of aging biogas slurry from livestock farm and chemical oxygen demand (COD) from municipal wastewater (Anpi et al., 2017; Stefania et al., 2017). The reported that the *Rhodospseudomonas* species was a promising candidate for denitrification of aging biogas slurry and swine sewage wastewater (Anpi et al., 2017; Hongyi et al., 2016) and that the highest removal efficiency of NH₃-N reached 99.75% in aging biogas slurry. In municipal wastewater, the removal efficiency of NH₃-N, that of NH₄ and COD reached 95%, 70% and 69%, respectively (Anpi et al., 2017; Stefania et al., 2017). Other strains of photosynthetic bacteria were reported with a lower removal capability of NH₃ and NH₄ (Ahmadldi et al., 2015). In another study, PPB was able to simultaneously remove 88% of phosphorous from primary settled domestic wastewater (Tim et al., 2014, 2016). Photosynthetic bacteria were combined with vegetables to remove total phosphorous, total nitrogen, COD, ammonia, nitrate, and nitrite from shrimp wastewater (Luo et al., 2012).

Another function of photosynthetic bacteria is removal of dyes. Some strains were utilized in the treatment of color contaminants in textile industries. The removal of color is attributed to the azoreductase enzyme, in photosynthetic bacteria species, such as *Rhodospseudomonas palustris* (AS1.2352), *R. blasticus*, *R. adriaticus*, *R. capsulatus* and *Rhodovulum strictum*. These species contain the azoreductase compound which has ability of decolorization of color or photosynthetic bacterial biofilms (Elizabeth et al., 2003). The *Rhodobacter capsulatus* and *R. sphaeroides* species have been widely investigated as model organisms for anoxygenic photosynthesis, nitrogen and carbon fixation and chemotaxis (Lang et al., 2012). *R. capsulatus* strain is a model organism of the photosynthetic bacteria group, which is representative for the study of a gene transfer (Wu and Bauer, 2008; Masepohl and Hallenbeck, 2010). Recently, the microbial desalination cell (MDC) has

been applied as a new method to reduce the salinity in water and soils (Xiaoxin et al., 2009; Luo et al., 2011; Ahmed et al., 2014). This method is promising because water desalination can be accomplished without electrical energy input or high water pressure by using a source of organic matter as the fuel to desalinate water (Xiaoxin et al., 2009). The used a microbial desalination cell as a reverse osmosis pre-treatment method to remove salinity from water. For a 30–35g NaCl/L treatment, their results demonstrated substantial approximately 43–67% desalination, a sodium removal efficiency >90% and an increase in desalination a sodium concentration of 98 - 99% (Maha et al., 2010; Ahmed et al., 2014; Younggy and Logan, 2011; Kim and Logan, 2011; Luo et al., 2011). The performance of the microbial desalination cell can be improved by using an electro dialysis stack and concentrating cells.

However, the information and database on microbial desalination remain limited. Only a limited number of species for biodesalination have been reported, such as two bacterial, *R.sphaeroides* SSI and *Rhodovulum* sp. and two cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (Kei et al., 2017; Jaime et al., 2014). Thus screening, selecting and collecting microbial communities for research and application of salinity reduction is a very urgent request for agriculture. This study, which focused on finding photosynthetic bacteria, has the potential to remove sodium from mangrove forests. The results of this study are promising for the application of microbial desalination in agriculture by seawater intrusion, which is causing rising seawater levels, due to global climate change.

Material and Methods

Isolation of photosynthetic bacteria

Sediment and water samples were collected from the mangrove forest in the Ca Mau Biosphere Reserve of the United Nations Educational, Scientific and Cultural Organization (UNESCO), Vietnam. Photosynthetic bacteria were isolated by transferring a 1 ml or 1 g sample into a 5 ml tube containing the GM medium or modified GM medium (added sodium at a concentration of 35 ppm) (Kei et al., 2017). All samples were grown under light (*ca.* 3000 lux) for approximately 7 days. After incubation, we chose the samples with a red, red-brown or dark red collar and then performed the streaked method onto



GM agar, selected a single colony. The morphological characteristics reveal Gram-negative bacteria with varied shapes, such as spherical, rod, arc and spiral shapes. The diameters of the cells range from approximately 0.3–1.5 μm (Imhoff, 1992).

Screening of *Rhodobacter* spp. with sodium removal ability

The count method was employed to measure the biomasses of the bacterial cells in the culture process by absorption spectrometry nephelometry at $\text{OD}_{660\text{nm}}$. The cells were washed 3 times in the 60% sucrose solution and were taken as the control group to calculate the absorption spectra (Feng, 2016). The culture was incubated in a screw cap test tube containing 18 ml of GM broth in a 15×150 mm tube with the addition of 10% (v/v) of stock bacteria solution. The samples were incubated in room temperature with a light intensity of 3,000 lux for 2 days and with NaCl concentration of 35ppm in the medium. The growth rate of photosynthetic bacteria was observed at $\text{OD}_{660\text{nm}}$. The sodium concentration was measured with a spectrophotometer and ATAGO equipment. The strains selected for the screening had growth rates exceeding 0.5. Bacteria strains with a resistant ability at 35 ppm NaCl in broth GM were collected. The culture broth was centrifuged at $10,000 \times g$ for 20 min in 4°C to isolate the supernatant, and then the sodium concentration was measured.

Identify *Rhodobacter* spp.

Bacterial cells were grown under anaerobic conditions at a light intensity (*ca.* 3000 lux) in broth GM at room temperature. The cells were collected by centrifuge in the growth phase after 4 days and then extracted using isolated genomics DNA with QIAGEN kit. Amplification and analysis of 16S rDNA sequences was carried out with eubacterial universal primer 27F and 1492R and special primer pufM 557F and 750R of the photosynthetic bacteria group (Lane, 1991; Laurie et al., 2001). All of the polymerase chain reaction (PCR) products were purified by using a QIAGEN kit before sequencing.

Primers and PCR amplification.

To identify the photosynthesis bacteria, the 16S rDNA gene and specific pufM gene were amplified by using the 27F forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R reverse primer (5'-GGTTACCTTGCTATGACTT-

3'), and the specific pufM primer 557F (5'-CGCACCTGGACTGGAC-3') and 750R (5'-CCCATGGTCCAGCCAGAA-3'), respectively (Laurie et al., 2001). The PCR amplification was prepared in a volume of 25 μL containing 1 μL of DNA, 9.5 μL of Master mix 1X (Bioline), 1 μL of per primer, 1 μL of DNA template (*ca.* 100 ng) and nuclease-free water. For the amplification of the 27F and 1492R primers, the conditions were in the cycled first step at 94°C for 4 min; repeated in the second step with 30 cycles at 94°C for 20 seconds, 54°C for 15 seconds and 72°C for 45 seconds; a continuous extension step was performed at 72°C for 10 min and a final step at 4°C . For the pufM 557F and 750R primers, the amplification was cycled at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. An extension step was performed at 72°C for 10 min, and a final step at 4°C .

Sequences and phylogenetic analysis.

The sequences of the 16S rDNA region and pufM gene have been popular for gene identification of bacteria taxonomy. In addition, the pufM gene is specific to identify the photosynthetic bacteria. They were compared with reference sequences downloaded from the National Center for Biotechnology Information (NCBI) database and aligned by using Clustal X (Thompson et al., 1997). The phylogenetic tree was constructed from the evolutionary distance data (Kimura, 1980) using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap method based on 1,000 random repeated was employed for the sequence analysis (Felsenstein, 1985). Furthermore, in the phylogenetic trees were determined by contributions from group members of the genus *Rhodobacter* spp. with separation of other *Bacillus* genera.

Results and Discussion

Isolation of *Rhodobacter* spp.

Rhodobacter were isolated from 78 sediment and water samples collected from mangrove forests in the Ca Mau biosphere reserve of UNESCO, Vietnam. In total, 48 strains were isolated and selected from GM agar medium after purification by restreaking on the plate. All strains were identified as Gram-negative, oval or rod - shaped cell under a microscope and the seven strains with the highest sodium removal ability were selected (Table 1). Our results showed that isolated the strains had the same morphological



characteristics as the *Rhodobacter* species (Imhoff et al., 1984).

Table-1: Morphology cell and Gram of isolated bacteria

| The strains | Morphology cell | Gram |
|-------------|-------------------|----------|
| CM4.2 | rods shaped | Negative |
| CM8 | rods shaped | Negative |
| CM15 | rods shaped | Negative |
| CM37 | rods shaped | Negative |
| CM53.2 | rods shaped | Negative |
| CM58.1 | rods shaped | Negative |
| CM63.3 | Ovals/rods shaped | Negative |

Screening of *Rhodobacter* spp. with sodium removal ability

The 48 strains were able to grow in GM broth for 14 days; all strains had been grown well. In GM with 25 ppm NaCl, only 7 strains (CM53.2, CM58.1, CM8, CM37, CM4.2, CM63.3 and CM15) survived and grew (Fig. 1a) after incubation. They were selected as strains with sodium removal ability in this study.

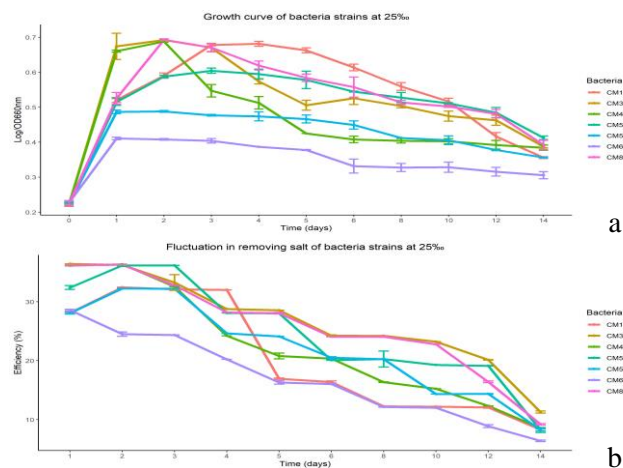


Figure-1: Growth and sodium removal by seven strains (CM53.2, CM58.1, CM8, CM37, CM4.2, CM63.3 and CM15) at 25 ppm NaCl treatment.

The removal rate showed significant differences between the 25 ppm and 35 ppm NaCl treatments. In most strains, the removal efficiencies for the 35 ppm NaCl treatment were lower than the removal efficiency in the 25 ppm treatment with a decrease from 1% to 5%, depending on the growth of the strains (Fig. 1b, Fig. 2a,b). Our results indicated that sodium concentration affected the salinity removal capability during the first few days. The results of

this study show agreement with the report indicated that *Rhodobacter* was able to remove sodium with a removal efficiency of 39.3% in a 3% NaCl treatment (Kei et al., 2017). In another report, they found that both *Rhodobacter* strains (NW16 and KSM24 strains) can remove a maximum of 31 % of Na from water that contained 3% NaCl (Panwichian et al., 2011; Saijai et al., 2010). Our study confirmed the promising sodium removal ability of some *Rhodobacter* species in desalination from Vietnam.

The sodium removal was dependent on the pathway of photosynthetic metabolism and respiration through the chloride and sodium pump. The light energy directs is affects the halorhodopsin protein of the chloride pump onto the membrane cell in a study of *Halobacterium halobium* strain (Dohrmann and Müller, 1999). In the desalination phase, the light will makes chloride pump of opening and Cl⁻ import into the cell, which will then change the membrane potential (V_m) inside and outside the cell. The sodium pump will open and draw Na⁺ into the cell by *Cyanobacteria* species, which means that sodium as removed from the environment (Dimroth, 1990; Jaime et al., 2014; Minas et al., 2015).

The pathways of the metabolic bacteria are based on their life cycle. Thus, the salinization processing also depends on the growth phase and nutrients in the experiments. Sodium ion is necessary for metabolism bacteria pathways, such as nutrient uptake (e.g., Na⁺/HCO₃²⁻ transport), cell division (e.g., in heterotrophic cyanobacteria), nitrate assimilation, nitrogen fixation and photosynthesis (Apte and Thomas, 1983; Maeso et al., 1987; Espie et al., 1988). With the species *R.sphaeroides* SSI, the rate is higher with Na removal after 2 days of cultivation under static light conditions. In another case, the Na concentration was reduced after cultivation for 8 days under aerobic dark conditions with *Rhodovulum* sp. (Kei et al., 2017). The cell growth characteristics of *R. sphaeroides* O.U.001 showed the start of exponential growth phases in 24 h and the stationary phase in 80 h (Nitai and Debabrata, 2009).

From the 1st day to the 4th day, the strains depended on the lag phase, log phase and stationary phase. They had an exponential increase in the number of living bacterial cells and cell division. Therefore, sodium ion has increased the absorption of sodium into the cells. Especially, the receptive capacities of the cell of each strain is completely different and limited. In our experiments, the results show that the salt removal potential decreases with the passage of time,



which was suitable in the growth phase of the *Rhodobacter* species in sodium concentration medium (Fig. 1b and Fig. 2b).

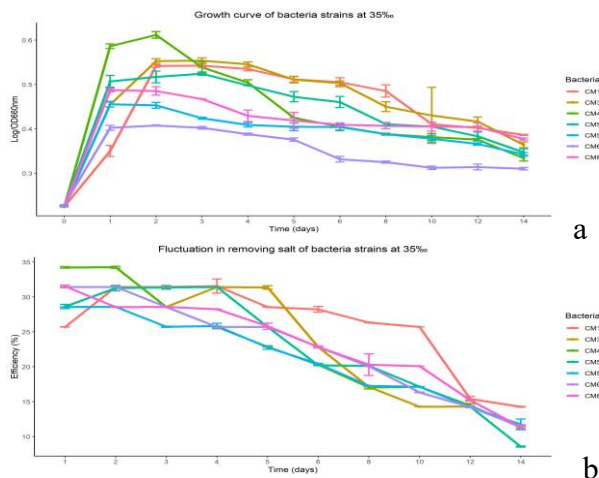


Figure-2: Growth and sodium removal by seven strains (CM53.2, CM58.1, CM8, CM37, CM4.2, CM63.3 and CM15) at 35 ppm NaCl treatment.

Identify *Rhodobacter* spp. belongs to the 16S rDNA and puMf sequences gene

The phylogenetic tree of 16S rDNA gene sequences showed that the seven isolated strains from this study located in clusters within the genus of *Rhodobacter* (Fig. 3). Our sequences were aligned with the gene database of known bacteria using the BLAST tool. They are similar and are considered to represent a single species. In this case, these strains CM37, CM63.3 and CM4.2 were closely related to *Rhodobacter johrii*, and four strains belong to *R. sphaeroides* (Fig. 3).

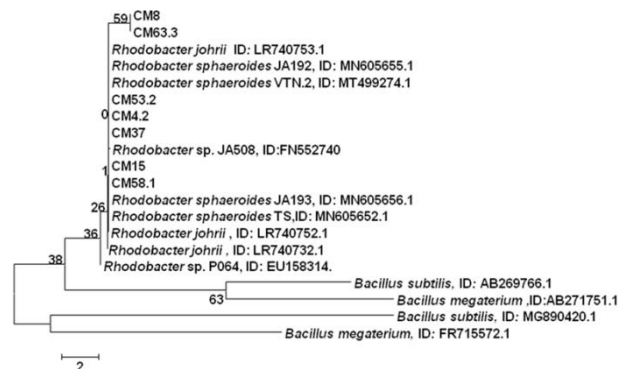


Figure-3: Phylogenetic tree for seven strains of Vietnam constructed by the neighbor-joining method based on 27F and 1492R primers.

However, the phylogenetic tree of the *pufM* gene sequences showed that three of seven strains (CM58.1, CM37 and CM15) were closely related to *Rhodobacter johrii* and four other strains have high similarity with *R. sphaeroides* (Fig. 4). Our results suggested that seven of our strains belong to *Rhodobacter* genus and are closely related *R. johrii* and *R. sphaeroides*.

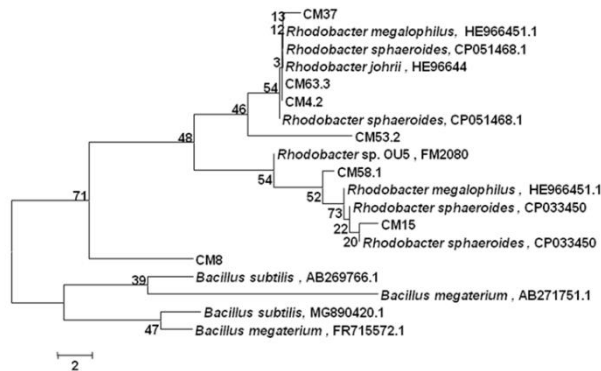


Figure-4: Phylogenetic tree for seven strains of Vietnam constructed by the neighbor-joining method based on puMf 557F and puMf 570R primers.

Our strains, namely, CM37, CM8.1 and CM15, shared 98.28% similarity to *R. johrii* (Girija et al., 2009). The *puMf* sequences gene of these three trains were submitted to the GenBank database with accession numbers MT645162, MT645163 and MT645164. On the other hand, the 16S rRNA gene was accepted as a new species with a similarity of 97% (Stackebrandt and Goebel, 1994). In yeast species, when nucleotide shown a difference of 1% or more in the D1/D2 domain, it indicates a difference in species (Kurtzman and Robnett, 1998). The *puMf* gene of the *R. johrii* strain in the database description was verified (Girija et al., 2009).

Conclusion

In this study, seven strains of photosynthesis bacteria belonging to *Rhodobacter* genus were isolated from the mangrove forests in the Ca Mau Biosphere Reserve in Southeast Vietnam. The bacteria are Gram-negative with oval- or rod-shape cells and were morphologically identified as *Rhodobacter* spp. The results of 16S rDNA and *puMf* gene sequences indicated that they were closely related to *R. sphaeroides* and *R. johrii*. The results also confirmed that all seven strains have ability to remove sodium

from GM medium with removal efficiency from 28.57% to 36.42% in the 25 ppm NaCl treatment. The removal efficiency decreased in the higher NaCl treatment at 35 ppm. Our results suggested that all strains have the potential for desalination and could be utilized for biodesalination in agricultural environments.

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Contribution of Authors

Duy ND: Conceived the idea, designed research methodology, literature review, data analysis and interpretation, manuscript final writing and reading

Dao DTH: Designed research methodology, sampling and data collection

Dung NH: Designed research methodology, literature review, and interpretation, manuscript writing

Nhung VTT, Vu PA, Loan LQ & Diep HT: Sampling and data collection

Luu PT: Literature review, data analysis and manuscript writing

Khanh HQ: Literature review and interpretation, manuscript final reading