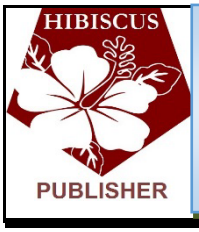
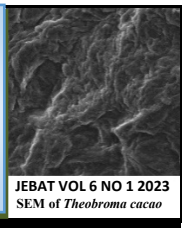
JEBAT, 2023, Vol 6, No 1, 8-11
https://doi.org/10.54987/jebat.v6i1.797



JOURNAL OF ENVIRONMENTAL BIOREMEDIATION AND TOXICOLOGY
Website: http://journal.hibiscuspublisher.com/index.php/JEBAT/index



Isolation, Identification and Characterization of Denitrifying Phototrophic Bacteria from Marine and Aquaculture Wastewater

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HISTORY

Received: 12th March 2023
Received in revised form: 29th June 2023
Accepted: 28th July 2023

KEYWORDS

Denitrification
Denitrifying bacteria
Bacteriochlorophyll
Carotenoids
Nitrogen

ABSTRACT

Denitrifying bacteria occur primarily in the soil and play an important role in the nitrogen cycle and wastewater treatment. This study investigated the possibility of isolating denitrifying phototrophic bacteria from marine and aquaculture wastewater. The denitrifying ability of isolates was examined through their utilization of inorganic nitrogen and nitrate reductase test. The photosynthetic capability of the isolates was determined by detecting the photosynthetic pigments (bacteriochlorophyll and carotenoid). Molecular characterization of the isolates was carried out by the amplification of 16S rRNA gene. Forty-five different isolates were obtained, and photosynthetic pigments were detected in 12 (8 from marine and 4 from aquaculture). Four of the isolates were found to grow on both inorganic nitrate and nitrite as the sole carbon source. Molecular characterization has shown that the isolates are denitrifying bacteria and the relationship between isolates and other denitrifying bacteria has been established by the construction of the phylogenetic tree. Hence denitrification and denitrifying bacteria can occur in both marine and aquaculture wastewaters.

INTRODUCTION

Denitrification is an important part of the world biogeochemical cycle. It provides an avenue for returning inorganic nitrogen from the soil to the atmosphere. It can reduce the amount of nitrogen fertilizer in agroecosystems to an average of 20-30% [1]. It is also capable of degrading some pollutants such as phenol [2], toluene and 1,3-dimethylbenzene [3], nitrilotriacetic acid [4] and carbon tetrachloride [5]. Denitrification can be autotrophic or heterotrophic. Autotrophic denitrification relies on inorganic carbon sources like carbon dioxide or bicarbonate. The bacterial metabolic chain in autotrophic denitrification involves hydrogen gas or sulphur as an electron donor.

The advantages of this process over heterotrophic denitrification includes the use of carbon dioxide as a carbon source which is beneficial in wastewater that contains a low amount of organic matter. It eliminates the harmful effect of some organic carbon. It also lowers the production of biomass and sludge, hence reduces clogging [6]. While autotrophic denitrification uses inorganic carbon sources, heterotrophic denitrification utilizes organic carbon sources which can be soluble or insoluble. The process is characterized by a high rate of denitrification depending on the type and concentration of the carbon source and the C: N ratio [7]. Other factors such as types of microorganism and environmental conditions also influence the rate of denitrification.

Denitrifying organisms are widely distributed in the environment. Their activities are significant in *in situ* bioremediation of groundwater contaminated with nitrogen. Their application in bioremediation of aquifers is easier because they are soluble in water and easily added in comparison with oxygen [5]. This study exploits the isolation, identification and characterization of denitrifying phototrophic bacteria from marine environments and aquaculture wastewater.

MATERIALS AND METHODS

Isolation of Photosynthetic Bacteria

Sampling Site and Sample Collection

For the isolation of bacteria, wastewater samples from an aquaculture shrimp farm located at latitude 5°34'17.73"N and longitude 102°48'26.18"E of Terengganu, Malaysia and marine environment also located in Terengganu, were collected, stored in 4-litre containers and transported in ice to the lab.

Growth Media

Three different growth media were used for phototrophic bacterial isolation. purple non-sulphur bacteria enrichment medium (PNSBEM), [8], G5 medium [9] and the Basal medium. The growth ingredients were weighed appropriately and the required amount of distilled was added as described in Table 1. The medium was carefully mixed, and the pH adjusted before autoclaving at 121°C for 15 minutes. For the solid medium, 15 g (1.5 w/v) of agar was added to each media, autoclaved and left to cool before pouring onto the plate. The compositions of these media are described in **Table 1**.

Table 1. Composition of the Growth Media.

PNSBEM	
Nutrients	**Concentration (g/l)**
NH_4Cl	1.0
$NAHPO_4$	0.5
$MgCl_2$	0.2
Yeast Extract	2.0
Sodium lactate	6 mL OF 80%
pH	7.2
Basal Medium	
Nutrients	**Concentration (g/l)**
K_2HPO_4	0.33
$MgSO_4.7H_2O$	0.33
NaCl	0.33
NH_4Cl	0.5
$CaCl_2.2H_2O$	0.5
Sodium Succinate	1.0
Yeast Extract	1.0
pH	7
G5	
Nutrients	**Concentration (g/l)**
Peptone	5.0
Yeast Extract	5.0
L-glutamic acid	4.0
Malic acid	3.5
KH_2PO_4	0.12
K_2HPO_4	0.18
pH	7

Inoculum Preparation

The samples were inoculated into three different broth media (PNSBEM, G5 and Basal medium) in universal bottles using 10% (v/v) ratio. Subsequently, it was incubated under facultative anaerobic condition with light intensity between 2000-3000 lux at ambient temperature. The facultative anaerobic condition was achieved by filling the 50 mL centrifuge tube with the medium and sample before incubating without shaking. The samples were left for several days until the medium completely turned red in colour due to the production of photosynthetic pigments.

The bacteria were subsequently collected by serial dilution and spread plating and later purified by repeated streaking on the agar plate. Anaerobic condition was found to be necessary for the enrichment of aquaculture samples before subsequent sub-culturing under facultative anaerobic condition. Under aseptic technique, nitrogen gas was sparged onto the medium to provide an anaerobic condition [10].

Serial Dilution and Streak Plating Technique

Because the cultures were very concentrated due to high turbidity and red coloration, they were first serially diluted before spread plating. The series dilution was performed by the preparation of four different tubes, each containing 9 mL of distilled water (diluent). In the process, 1 mL of the mixed bacterial culture was transferred into tube A after mixing. Subsequently, after mixing tube A, 1 mL was transferred into tube B. A similar pattern of transfer was followed for tubes C and D making the dilution in range of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for tube A, B, C and D respectively. One millilitre of each dilution was then spread on each respective agar plate and incubated at ambient temperature with light intensity between 2000-3000 lux. Individual colonies from each plate were streaked on a fresh agar plate of either, PNSBE, G5 or Basal medium depending on the respective agar plate used during spread plating. The colonies were purified by repeated streaking. The isolation technique utilized in this study was based on the procedure described in [11].

Screening and Selection of Denitrifying Photosynthetic Bacteria

The isolated bacteria were screened for photosynthetic and denitrifying abilities via the following experimental activities.

Determination of Photosynthetic Pigments

In order to determine bacteriochlorophyll and carotenoids pigments, a single colony of pure culture was inoculated into a broth and incubated at 37°C with a shaking speed of 150 rpm. Thereafter, 10% (v/v) was transferred into a fresh medium and incubated at ambient temperature under light without shaking. The whole cell spectrum was measured in the range from 300 to 1100 nm using the DR 6000 spectrophotometer [12].

Utilization of Inorganic Nitrogen Sources

Three different basal media comprising different nitrogen sources ($NaNO_2$, $NaNO_3$ and NH_4Cl) were prepared in form of agar and broth. All the isolated bacteria were streaked onto each of the different nitrogen sources. Those that showed positive (visible) growth on the agar were later inoculated into their respective broth and nitrogen consumption levels were monitored by measuring the amount NH_4-N, NO_3^- and NO_2^-.

Nitrate Reductase Test

The test was performed to assess the ability of the isolates to reduce nitrate to nitrite or beyond the nitrite stage (that is to either ammonium or nitrogen gas). It was carried out according to the protocol outlined by [13].

Molecular Identification of the Isolate

Genomic DNA from the bacterium was extracted using the promega wizard genomic DNA purification kit following the manufacturer's protocol. The concentration and purity of genomic DNA were determined using agarose gel electrophoresis and Nano drop. The agarose gel was prepared by dissolving 5 g of agarose powder into 50 mL of TAE buffer bringing the concentration to 1% (w/v) and incubated in microwave for 1 min. The solution was allowed to cool down to room temperature before 0.1 µL of ethidium bromide (EtBr) was added and the solution was poured onto the gel tray and appropriate comb was placed to create a well for loading the sample. After solidification, the gel was transferred into the electrophoresis tank and the TAE buffer was added until it was completely covered. In the first well, 5 µL of the gen ruler ladder was loaded as a marker. The second well was loaded with 5 µl of DNA sample mixed with 2 µL of loading dye. The electrophoresis was run for 45 min at 80 volts. The DNA band was later observed using the UV trans illuminator [14].

The 16S rRNA gene was amplified under the following PCR condition: 3 min at 95ºC, 30 cycles of 1 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and 10 min at 72ºC for initial denaturalization, denaturalization, annealing, extension and final extension respectively [12]. The universal primers used for the amplification were: forward primer 14-F (5" AGAGTTTGATCCTGGCTCAG3") and reverse primer 1492-R (5" GGTTACCTTGTTACGACTT3"). The PCR products were sent to 1st Base Sdn Bhd for purification and sequencing. BioEdit v 7.2.5 software and online BLAST program at (http://blast.ncbi.nlm.nih.gov/blast.cgi) were used for sequence analysis. Phylogenetic tree was constructed using neighbor joining method with MEGA 5.2 software and sequence was submitted to NCBI.

RESULTS AND DISCUSSION

Isolation of Phototrophic Bacteria

Phototrophic bacteria are found in nearly all aquatic environments. They can be easily recognized by their ability to form a "bloom". Their diversity in aquatic environments is likely due to their versatile metabolic activities. They can photoassimilate a variety of organic compounds, hence their presence depends on the extent to which the aquatic environment is polluted with organic matter. A total of 45 different isolates were obtained from aquaculture wastewater and the marine environment. However, photosynthetic pigments were only detected in 12 isolates, 8 from marine environments and 4 from aquaculture wastewater. The growth potential of the twelve isolates using different growth medium on agar plates is presented in **Table 2**.

Table 2. Isolated bacteria, isolation source and growth on different media.

Isolates	Source	Growth media		
		Basal	G5	PNSBEM
PSC	aquaculture	+	+	+
PSE	aquaculture	+	+	-
PSF	aquaculture	+	+	+
PSK	aquaculture	+	+	+
PSA	marine	+	+	+
PSB	marine	-	+	+
PSD	marine	+	+	+
PSH	marine	+	+	+
PSJ	marine	+	-	+
PSS	marine	-	+	+
PST1	marine	+	+	+
PST2	marine	+	+	+

Determination of Photosynthetic Pigments

The analysis of photosynthetic pigment was the first criterion used for screening photosynthetic bacteria in this study. Photosynthetic pigments are present in all photosynthetic organisms as a light-harvesting centre. However, these pigments differ in different organisms. While plants, green algae and cyanobacteria possess chlorophyll used for the absorption of light during photosynthesis, phototrophic bacteria use bacteriochlorophyll (s) instead for the same purpose.

It was observed that one of the main distinguishing features of most of the isolates was the reddish colour because of the presence of photosynthetic pigments (bacteriochlorophyll and carotenoids). The pigments were observed in 12 isolates. bacteriochrophyll *a* with absorption peaks between 800 and 865 nm and carotenoid related to the spheroidene series with peaks ranging between 376-589 nm were also observed (see **Fig. 1** and **Table 3**). Both absorption peaks correspond to the ones reported by (15). Absorption peaks around these ranges are typical characteristics of purple non-sulphur bacteria [12].

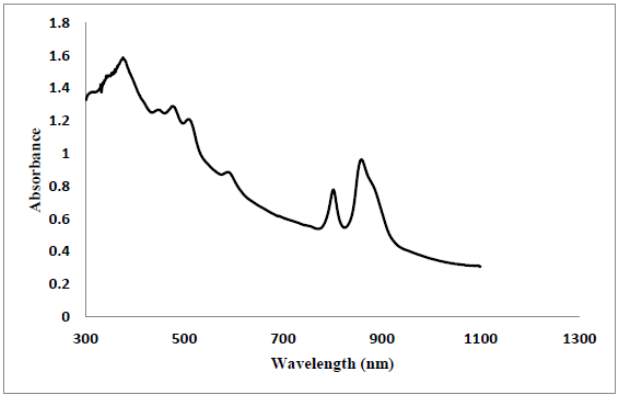

Fig. 1. Absorption spectrum showing bactrochlorophyll and carotenoides peak of PSA.

Table 3. Bacteriochlorophyll *a* and carotenoids peaks of the isolates.

isolate	bacteriochlorophyll *a* (nm)	carotenoids (nm)
PSC	855-804	470-486
PSE	851-808	466-513
PSF	865-803	472-489
PSK	856-804	450-489
PSA	856-805	474-518
PSB	851-803	456-507
PSD	854-805	465-486
PSH	862-802	479-511
PSJ	876-805	468-521
PSS	858-795	481-501
PST1	864-805	437-475
PST2	868-804	439-500

Utilization of Inorganic Nitrogen Compounds

To determine the utilization of nitrates and nitrites, the isolates were screened by culturing them on $NaNO_3$ and $NaNO_2$ as sole sources of nitrogen [16]. They were streaked onto the agar plate of the basal medium containing either $NaNO_3$ or $NaNO_2$ as the only source of nitrogen, then incubated as described earlier. Four isolates designated as PSC, PSD, PST1 and PST2 were found to grow in $NaNO_3$ and $NaNO_2$ as the only carbon source. These isolates were then grow in a broth culture containing $NaNO_3$ as the only source of nitrogen. The results showed reduction in nitrate, bubbles formation and an increased in the pH. These are preliminary features indicating denitrification [17].

Nitrate Reductase Test

The purpose of the nitrate reductase test is to determine the denitrifying ability of the bacteria. It reveals the ability of some bacteria to convert nitrate to nitrite, ammonia or nitrogen gas. The result from this test showed that, the isolate "PSD" can reduce nitrate into NH_4^+ or N_2, which indicate the reductase enzyme's activity. This result is in accordance with [18], which shows that photosynthetic bacteria possess the ability to reduce NO_3^- to NH_4^+ and subsequently used it for growth. The ability of PSD in denitrification was earlier reported in [19].

Table 4. Nitrate reductase test of the isolates.

Sample	Solution A And B	Addition of Zinc	Possible end Products
PSC	+		Nitrite
PSD	-	-	NH_4 or N_2
PST1	+		Nitrite
PST2	+		Nitrite
Control	-	+	Nitrate

Negative (-) = no red coloration, positive (+) red coloration

Molecular Identification of the Isolates

Three isolated strains were identified by amplification of the 16S rRNA genes. The sequences obtained were deposited in NCBI (https://www.ncbi.nlm.nih.gov/) with the following accession numbers: KP776627, KP412477, and KP065811. The accession number KP412477 represents *Rhodobacter sphaeroides* AZ101 with 1354 bp, KP776627 represents *Rhodabacter sphaeroides* ADZ 101 with 1367bp and KP065811 represents *pseudomonas stutzeri* AZ101 with 1433bp. Photosynthetic pigments were not detected in KP065811 but its ability to utilize inorganic nitrogen was very high.

The phylogenic tree constructed described the evolutionary relationship between the isolates and other denitrifying bacteria. Evolutionary analyses were conducted with MEGA X software [20] while the evolutionary history was inferred using the neighbor-joining method [21]. The evolutionary distances were computed using the maximum composite likelihood method [22] and are in the units of the number of base substitutions per site. The optimal tree with the sum of branch length = 2.77781940 is shown in **Fig 2.** The analysis involved 16 nucleotide sequences and all ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1465 positions in the final dataset.

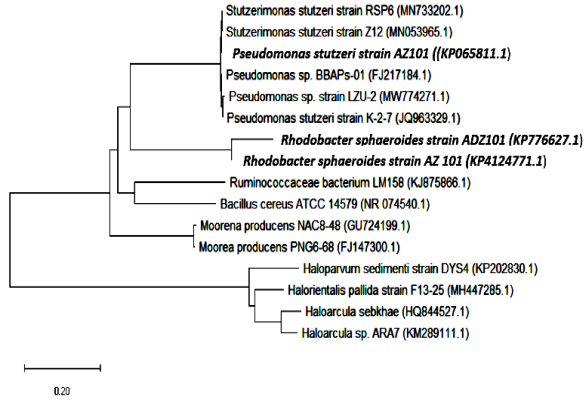

Fig. 2. phylogenetic tree describing the evolutionary relationship between the isolates and other organisms.

CONCLUSION

The results indicated that phototropic bacteria can be obtained from both marine environments and aquaculture wastewater, indicating the possibility of the denitrification process in such environment and the ubiquitous nature of denitrifying bacteria.

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