VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 233-241

Bacteria Isolated from the Sediment of a Bioelectrochemical System Installed in a Simulated Aquaculture Pond Operated with Brackish Water

Tran Thi Hien, Vu Thuy Linh, Pham The Hai*

VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam

Received 02 June 2016
Revised 02 August 2016; Accepted 09 Septeber 2016

Abstract: The brackish-water-adaptive electrochemical bacterial consortia in the sediment bioelectrochemical system (SBES) installed in a model tank simulating an aquaculture pond operated with brackish water were successfully enriched after 15 days. Total bacteria counts in the inoculum, the sediment of the SBES anode and the sediment of the control tank varied from 3.9 x 10^5 to 2.71 x 10^6 cfu g^{-1}. Halophilic bacteria such as *Vibrio* sp., *Pseudomonas* sp. were found dominant in the anode of the SBES and might play a key role in the electron transfer process as well as in the performance under the saline conditions of the corresponding SBES. The composition and the diversity of the anode bacterial community enriched in the SBES were significantly different from that of the control not having electrodes but only slightly different from that of the inoculum.

Keywords: Brackish-water-adaptive electrochemical bacteria, sediment bioelectrochemical systems (SBESs), brackish aquaculture.

1. Introduction

The aquaculture sector, which has contributed greatly to exportation, has been considered as one of the key economic sectors in Vietnam. To respond to the increase of the per-capita consumption demand of aquatic products, aquaculture farmers, who want to increase aquaculture production, have applied more intensive practices to their aquaculture ponds and utilized large amounts of nutrient-rich feed. Thus, the uneaten nutrient-rich feed, dead phytoplankton, fish excreta and other metabolic wastes have caused many negative consequences such as pathogenic bacteria food source, harmful gas, sediment deterioration, poor water quality effecting to aquatic animals health [1, 2]. Aquatic animal epidemics are the direct threats to aquaculture production and it can cause severe damages to aquaculture farmers. Besides, contaminated water from ponds released into the environment can create serious problems [3].

In fact, there are many ways to overcome environmental pollutions such as handling pathogens in the environment; selecting and controlling good, disease-free breeds; good feed management; changing the water and aeration frequently; using nanomaterials (e.g. silver

*Corresponding author. Tel.: 84-913318978
Email: phamthehai@vnu.edu.vn

nanoparticle solution). In the world as well as in Vietnam, there have been many studies on measures to reduce water pollution in aquaculture ponds, such as the artificial aeration systems and constructed wetlands have been investigated [1, 4-7]. Other biological treatment or physicochemical treatment have been applied such as probiotics supply or land reclaimation liming. Although these solutions are effective, each of them has advantages and disadvantages. More importantly, cost-effective solutions that are able to reclaim the water quality of aquaculture ponds in a sustainable manner are currently demanded.

The SBES - a new technology researched and developed recently shows many potentials for on-site reclamation of the water quality of aquaculture ponds with simple operation as well as low cost [1, 8]. Research on this system has only been done with freshwater aquaculture ponds [1, 9-10] as the research objects while in fact there are a lot of brackish-water aquaculture ponds in Vietnam. Therefore, we carry out an initial study to develop a bioeletrochemical system for *in situ* reclamation of the water quality of brackish aquaculture ponds.

Electrochemically active bacteria are the microorganisms which have the ability that can transfer electrons outside the cell. This kind of microorganisms is able to directly transfer electrons to a chemical or material that can function as the immediate electron acceptor. By studying the microbial consortia in the anode of an SBES, the diversity and the composition of the microbes in relation to the performance of the system can be understood [11, 12]. Furthermore, the electrochemically active bacteria enriched in a brackish water SBES may promisingly have many new exciting characteristics, because they are both electrochemically active and able to operate in a high-salinity environment. Based on that, the correlation between the microbial community and the capacity of electricity generation as well as the treatment efficiency of the system can also be assessed. Thus, in this study, we isolated and investigated bacteria of the SBES anode and the control tank (without the SBES) as well as their possible roles in the performance of the systems.

2. Materials and methods

2.1. The model aquaculture tank set up with the SBES and the control

Two brackish aquariums were constructed from two rectangular parallelepiped glass tanks which had dimensions of 30 cm × 20 cm × 25 cm; the volume ratio was approximate1:169 to an actual water column in a real aquaculture pond. One tank was used for experiments with a bioelectrochemical system installed and the other tank served as the control. The total projected surface area of the anode was 600 cm^2 and that of the cathode was 105 cm^2, and each graphite cloth had dimensions of 15 cm × 7 cm × 0.9 cm. The graphite cloth of the anode was installed horizontally at the experimental tank bottom. The graphite particle layer was spread onto this graphite cloth and covering the entire tank bottom. The sediment was collected from existing aquaculture ponds and filled in the experimental tank up to a height of 3 cm from bottom. The cathode was positioned horizontally in the oxic water at a nearest distance of 10 cm from anode top edge. Cathode electrode floats on aquarium water surface that mean the cathode was contacted with both the aquarium water and the air. The anode and the cathode were connected with copper wire through an external load of 10 Ω to make the external circuit. The remaining volume of the tank was filled with artificial brackish water thus simulating a real aquaculture pond (Fig.1).

SBES was operated in batch mode during the experiment process at room temperature (22 ± 3°C). The pond had an area of 1000 m^2, a depth of 1.3 m and a hypothesized stocking density of 100 shrimp/m^2, along with a shrimp feeding rate of 5 kg/1000 m^2 (for 100,000 shrimp in total) per day for 30-day-old shrimp

[13]. The feed left over was provided similar to actual condition: We estimated that 50% of feed was uneaten, equivalent to about 0.153 g organic food supplied per day for each aquarium without shrimp.

Control experiment: the other aquarium was also operated to evaluate the performance of the sediment without the presence of bio-electrode system. Control set up had no electrode system in it and was filled with the same amount of sediment and aquaculture water as used in SBES.

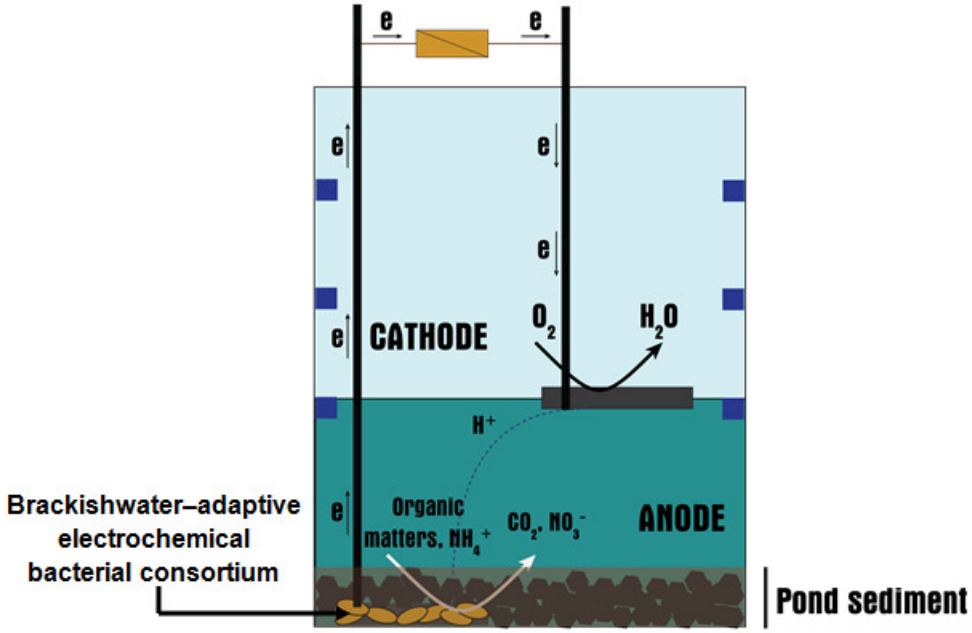


Figure 1. The model aquaculture tank setup with the SBES.

2.2. Sediment sampling

The microbial source which was used for the enrichment of the SBES was a mixture of sediment mud samples collected from three different brackish-water shrimp ponds in different locations of at Bàng La lagoon, Ấp Bắc road and the shrimp lagoon of Đồ Sơn aquaculture enterprise, Ngọc Xuyên ward, Đồ Sơn district, Hải Phòng city.

All collected microbial samples were mixed together. This mixture was used for inoculation into anode of SBES and control tank (without SBES). After successfully enriching the microbial community in the SBES, we collected microorganisms from the sediments of the SBES and the control tank in sterile Falcon tubes (20 to 25 g) and stored them at 4°C along with the inoculum.

2.3. Analysis of samples

Viable count for enumeration of cells by dilution method

1 g sediment sample mixed with 9 ml of sterilized saline and shaken well, which resulted in a 10-fold dilution. This suspension was then further diluted to different levels (10^2-fold, 10^{-3}-fold and 10^{-4}-fold, etc.). Next, 0.1 ml fluid from each diluent was removed, transferred to a Petri dish containing 1.5% NaCl LB agar. A separate plate for each sample was used. Each sample was spread using sterile bent glass rod over the plate and incubated at 37°C overnight and then observed. The number of colonies on a plate were counted and calculated.

Cultivation and isolation of bacteria

Separate colonies were observed in terms of morphology (shape, size and color) and then these single colonies were picked up and transferred to another agar dish containing 1.5% NaCl LB medium to purify the isolates by the streaking method.

Gram-staining observations of the bacteria

The cells of all the purified isolates were Gram stained and observed under microscopy after incubating at 37°C for 24 h. Gram staining was done following standard procedures with *E. coli* and *Bacillus subtilis* as controls. A light microscope (Carl-Zeiss, Germany) was used for observation, and images of cells were photographed with a Canon G10 camera (Japan).

Analysis of 16S rDNA for identification of bacteria

The PCR-amplified 16S rDNA fragments of single strains (~1400 bp), after checked by electrophoresis on 1% agarose gel, were sequenced by FirstBase (Singapore). The sequencing data were then analyzed by Chromas software version 2.4. The refined sequence of each fragment was compared with 16S rDNA sequences of similar species which were published in the database of GenBank sequences by BLAST Search tool.

3. Results

3.1. Culture-based microbial community analysis

The quantities of aerobic bacteria in the the sediments of the simulated brackish water shrimp ponds before and after the microbial enrichment with and without the SBES are shown in Table 1. Each number is the average count of viable colonies that grew on 1.5% NaCl agar plates for each sample. The cell density of the SBES sediment (near the anode), was 2.71 x 10^6 cfu g^{-1}, equivalent to that of the inoculum (2.63 x 10^6 cfu g^{-1}) and an order higher than that of the control tank sediment (3.9 x 10^5 cfu g^{-1}). Each community had about 20 to 21 isolates; the types and the presence frequencies of the isolates were significantly varied among the communities (Fig. 2, Fig. 3).

The bacteria in the three communities were isolated and identified. There seemed to be 4 or 5 strains dominating in each community and they are different among the communities.

Based on investigating the morphology of colonies and cells of the isolates from the microbial communities (Fig. 3), we found that the similarity between the compositions of the communities was relatively low. Most of their cells were rod shaped and Gram - negative. Three isolates of the inoculum community (I4, I5 and I15) were similar with three isolates of the SBES anode community (T10, T4 and T1 respectively). Other three isolates of the inoculum community (I1, I18 and I21) were similar with three isolates of control community (Đ3, Đ20 and Đ5 respectively). There were obviously differences between the SBES anode community and the control community. Only one strain T11 from the former was found to be similar to Đ10 of the latter. They only account for low proportions in the communities. This fact illustrates a significant difference in community composition of a sediment bacterial community with and without an electrode system installed.

Strikingly, two I4 and I5 strains isolated from the inoculum community with very low presence frequencies (both I4 and I5 account for 2.28%) appear to resemble two strains with relatively higher presence frequencies in the communities enriched in SBES: T10 (24%) and T4 (50%), respectively (Fig. 3). These bacteria probably adapted better to the anodic conditions of the SBES and outgrew the others.

Table 1. Bacteria quantity

Community (cfu/g)	Quantity of bacteria
Inoculum	2.63 x 10^6
Anode of the SBES	2.71 x 10^6
Control aquarium sediment	3.9 x 10^5

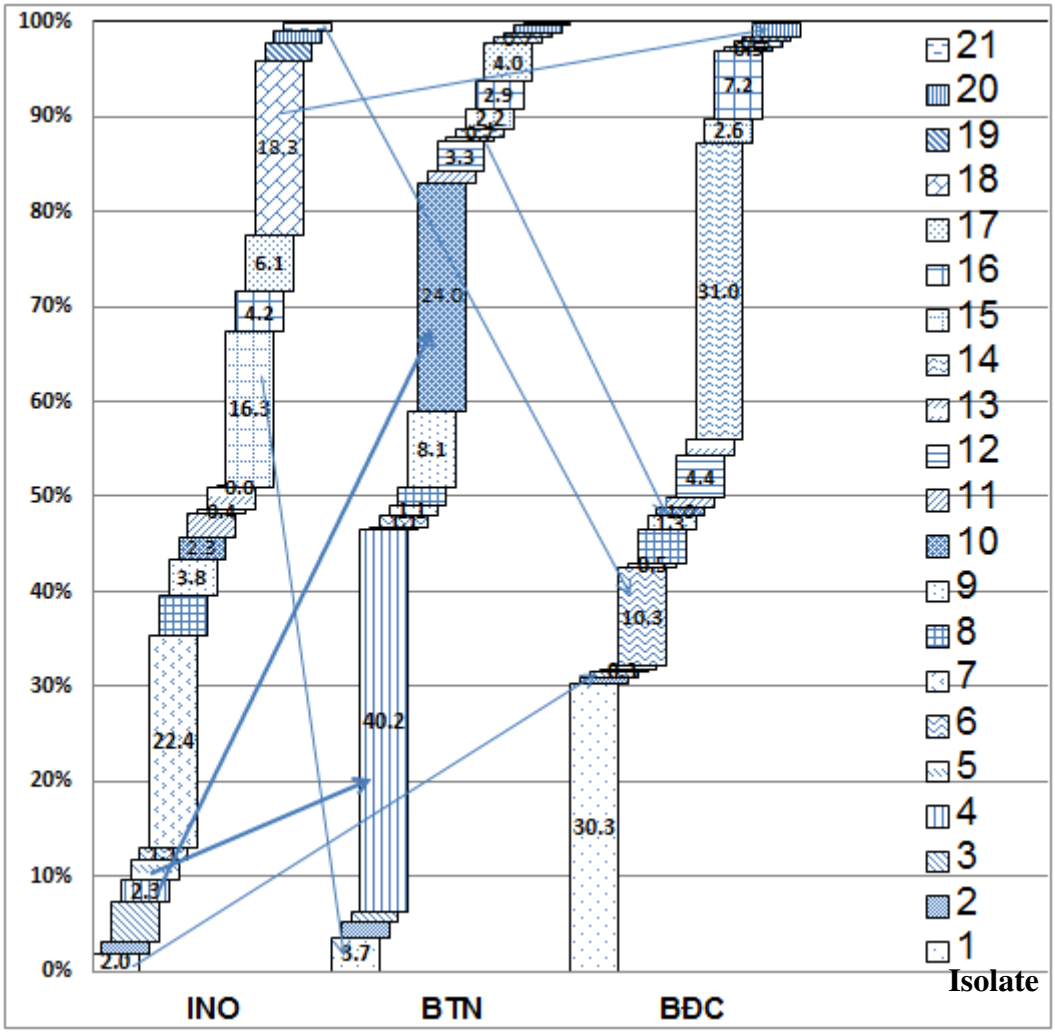


Figure 2. The correlation between the bacterial isolates of three investigated communities.

Note: INO: inoculum community; BTN: the SBES anode bacterial community; BĐC: bacterial community from the sediment of the control tank after 15 days of enrichment. The same patterns do not indicate that the corresponding isolates are the same. Each arrow indicates two strains that appeared similar in terms of colony and cell morphology.

3.2. Identification of dominant isolates

In order to assess the role of the dominant isolates in the communities from the anode of the SBES and the control aquarium, we conducted analyses of their 16S rDNA sequences. Especially, we focused on T4, T10 – two dominant strains of the SBES anode community, along with I4, I5 strains of the inoculum community and Đ1, Đ14 - two dominant strains of the control community (Fig.3). They were identified at genus or species level (Table 2). Accordingly, I4 and T10 are highly possible to be members of the genus *Pseudomonas,* while I5 and T4 could be *Vibrio* sp. Đ1 and Đ14 could be phylogenetically related to *Photobacterium halotolerans* and *Microbulbifer pacificus*, respectively.

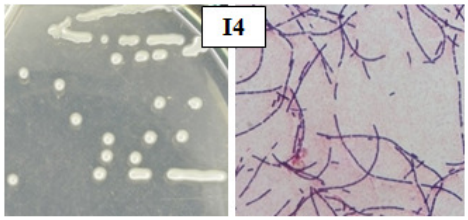
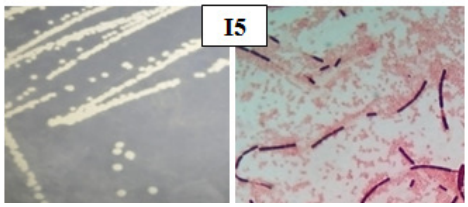
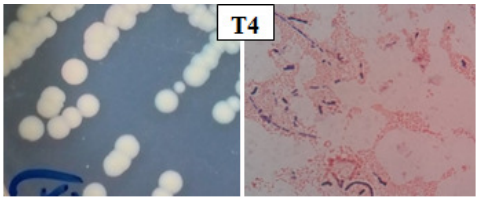
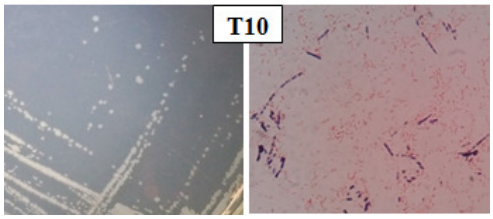
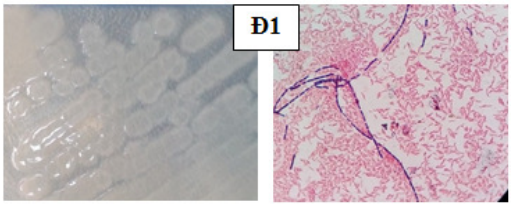
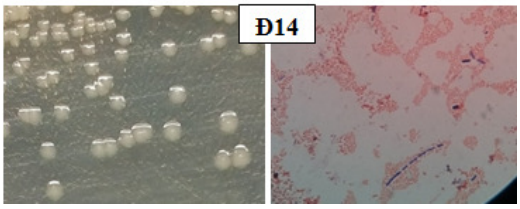
	Colonies: round shape, raised margin, wrinkled center, white Presence frequency: 2.28% Cells: rod shape Gram- negative
	Colonies: circular, dry, creamy, dull, flat Presence frequency: 2.28% Cells: globular shape Gram- negative
	Colonies: circular, dry, creamy, dull, flat Presence frequency: 40.2% Cells: globular shape Gram- negative
	Colonies: round shape, raised margin, wrinkled center, white Presence frequency: 24% Cells: rod shape Gram- negative
	Colonies: mucoid, flat, glistening, dull white Presence frequency: 30.26% Cells: rod shape Gram- negative
	Colonies: cicular, pulvinate, glistening white Presence frequency: 31.03% Cells: rod shape Gram- negative

Figure 3. Colonies and cells of the dominant isolates.

Note: T4, T10 – two dominant isolates of the SBES anode community, along with I4, I5 isolates of the inoculum community and Đ1, Đ14 - two dominant isolates of the control community.

Table 2. Sequence analyses of the 16S rDNA fragments from predominant isolated (using DNA sequence data on NCBI)

Name of strains	Species	Similarity coefficient
I4	*Pseudomonas mendocina*	99%
	Pseudomonas pseudoalcaligenes	99%
	Pseudomonas composti	99%
	Pseudomonas citronellolis	99%
	Pseudomonas oleovorans	99%
	Pseudomonas nitroreducens	99%
I5	*Vibrio parahaemolyticus*	99%
	Vibrio diabolicus	99%
	Vibrio alginolyticus	99%
	Vibrio azureus	99%
T4	*Vibrio parahaemolyticus*	100%
	Vibrio alginolyticus	100%
	Vibrio neocaledonicus	100%
T10	*Pseudomonas xanthomarina*	99%
Đ1	*Photobacterium halotolerans*	99%
Đ14	*Microbulbifer pacificus*	99%

Note: The species name indicates the proposed taxonomic identification of the corresponding isolates based on observation of their colonies and cell morphology and analysis of their 16S rDNA sequences. The percentage of similarity between the 16S rDNA sequence of each isolate and the proposed species was shown correspondingly in the last column.

4. Discussion

The results of 16S rDNA sequence analyses could enable a phylogenetic identification to the genus level. While the morphological characteristics of I4 and I5 strains resembled those of T10 and T4 strains, respectively, their phylogenetic identification results was also similar. The I4 and T10 strains were therefore determined as *Pseudomonas* sp. belonging to *Pseudomonas* genus. This *Pseudomonas* sp. strain accounted for 2.28% of isolates in the inoculum but reached 24% in the anode community of the SBES, probably as a result of the enrichment. In other word, in the anode of the SBES, the bacterial community was dominant by *Pseudomonas* bacteria. The presence of *Pseudomonas* sp. in microbial electrochemical systems (MESs) was also mentioned in previous studies [3]. The researchers at the University of Ghent (Belgium) also discovered a number of bacteria in the anode of MFC, including *Pseudomonas* sp. which could generate mediators for transferring electrons to electrodes [3]. Our study was conducted with the purpose of enriching the brackish-water-adaptive electrochemical bacteria in the anode of SBES, but we observed the presence of *Pseudomonas* sp. However, as shown in Table 2, it is interesting that the *Pseudomonas* sp. in our SBES is most closely related to marine or halo-tolerant/philic pseudomonads such as *P. mendocina* or *P. xanthomarina*. Hence, it can be predicted that this *Pseudomonas* sp. is a halophile and had an important role in the brackish-water-adaptive and electricity-generating bacterial community of the SBES.

The inoculum was taken from the sediment sludge of brackish water aquaculture ponds having relatively good water quality and later it was placed into our system with simulating polluted water; this probably explains why *Vibrio* sp. was present at a ratio of 50% in the anode of the SBES while at only 2.28% in the inoculum. Almost all *Vibrio* species are facultative anaerobes and they often cause diseases in aquatic animals, especially saltwater

fish, shrimp because most of *Vibrio* bacteria live in marine environments. It is questioned whether *Vibrio* species may play some roles in the electrochemical function of the SBES, but if they do, this phenomenon has not been ever reported.

The presence of two predominant isolates in the control aquarium, Đ1 and Đ14, which were defined to be *Photobacterium halotolerans* and *Microbulbifer pacificus,* respectively, is reasonable and consistent because they are derived from the places having high saline concentration. *Photobacterium halotolerant* bacterium is a novel species isolated from a saline lake located in Mallorca, Spain [14] and *Microbulbifer pacificus* is a novel species isolated from a marine sponge sample from the Pacific Ocean [15].

5. Conclusion

In this study, from the brackish-water-adaptive electrochemical bacterial consortia successfully enriched in the SBES, halophilic bacteria such as *Vibrio* sp. and *Pseudomonas* sp. were found dominant and might play a key role in the electron transfer process as well as in the performance under saline conditions of the corresponding SBES.

The composition and the diversity of the anode bacterial community enriched in the SBES was significantly different from that of the control not having electrodes but only slightly different from that of the inoculum.

Acknowledgements

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.04-2015.23. (Nghiên cứu này được tài trợ bởi Quỹ Phát triển khoa học và công nghệ Quốc gia (NAFOSTED) trong đề tài mã số 106-NN.04-2015.23). The authors thank Prof. Bùi Quang Tề for his helpful advices during the study.

References

[1] Sajana, T.K., Ghangrekar, M.M., and Mitra, A. (2013a), "Application of sediment microbial fuel cell for *in situ* reclamation of aquaculture pond water quality", Aquacultural Engineering, 57, pp. 101 -107.

[2] Soraphat Panakorn, Novozymes, Thailand AQUA Culture Asia Pacific Magazine, 7, March/April 2011.

[3] Sumino, T., Isaka, K., Ikuta, H., Saiki, Y., Yokot, T. (2006), "Nitrogen removal from wastewater using simultaneous nitrate reduction and anaerobic ammonium oxidation in single reactor", Bioscience and Bioengineering, 102, pp. 346–351.

[4] Nguyễn Quang Hưng, Nguyễn Vũ Thanh Sơn, Nguyễn Vũ Anh Tú (2015), "Tổng quan các phương pháp xử lý có khả năng áp dụng để xử lý nước thải nuôi trồng thủy sản tại tỉnh Quảng Trị", Tạp chí Khoa học ĐHQGHN: Khoa học Tự nhiên và Công nghệ, 31 , tr. 39-47.

[5] Lê Anh Tuấn (2007), "Xử lý nước thải ao nuôi cá nước ngọt bằng đấtngập nước kiến tạo", Hội thảo Quản lý và xử lý ao nuôi thủy sản, Sở NN và PTNT An Giang.

[6] Boyd, C. E., Ahmad, T. (1987), Evaluation of Aerators for Channel Catfish Farming, Bulletin 584. Alabama Agricultural Experiment Station, Auburn University, Alabama.

[7] Lin, Y.F., Jing, S.R., Lee, D.Y., Wang, T.W., (2002), "Nutrient removal from aquaculture wastewater using a constructed wetlands system", Aquaculture, 209, pp. 169–184.

[8] Reimers, C.E., Tender, L.M., Fertig, S., Wang, W. (2001), "Harvesting energy from the marine sediment–water interface", Environmental Science and Technology, 35, pp. 192–195.

[9] Sajana, T.K., Ghangrekar, M.M., and Mitra, A. (2013b), "Effect of pH and distance between electrodes on the performance of a sediment microbial fuelcell", Water Science and Technology, 68, pp. 537-543.

[10] Sajana, T.K., Ghangrekar, M.M., and Mitra, A. (2014), "Effect of presence of cellulose in the freshwater sediment on the performance of sediment microbial fuel cell", Bioresource Technology, 155, pp. 84-90.

[11] G Muyzer E. C. de Waal, AG Uitterlinden (1993), "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysisof polymerase chain reaction-amplified genes coding for 16S RNA", Applied and Environmental Microbiology, 59, pp. 695-700.

[12] Xu, S. and H. Liu (2011), "New exoelectrogen *Citrobacter* sp. SX-1 isolated from a microbial fuel cell", Journal Apply Microbiology, 111(5), pp. 1108-1115.

[13] Bùi Quang Tề (2010), Công nghệ nuôi tôm chân trắng thương phẩm theo quy phạm thực hành nuôi trồng thuỷ sản tốt (GAP), Viện nghiên cứu nuôi trồng thủy sản I, Tài liệu lưu hành nội bộ.

[14] Rivas R, García-Fraile P, Mateos P. F., Martínez-Molina E., Velázquez E. (2006), "*Photobacterium halotolerans sp.* nov., isolated from Lake Martel in Spain", International Journal of Systematic and Evolutionary Microbiology, 56, pp. 1067-1071.

[15] Jeong S. H. 1, Yang S. H., Jin H. M., Kim J. M., Kwon K. K., Jeon C. O. (2013), "*Microbulbifer gwangyangensis* sp. nov. and *Microbulbifer pacificus* sp.nov., isolated from marine environments", International Journal of Systematic and Evolutionary Microbiology, 63, pp.1335-1341.

Vi khuẩn phân lập từ hệ thống sinh điện hóa với điện cực ở đáy đặt trong ao nuôi thủy sản mô phỏng vận hành với nước lợ

Trần Thị Hiền, Vũ Thùy Linh, Phạm Thế Hải

Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam

Tóm tắt: Quần xã vi sinh vật điện hóa trong hệ thống sinh điện hóa đặt trong một bể mô phỏng ao nuôi thủy sản vận hành trong điều kiện nước lợ được làm giàu thành công sau 15 ngày. Tổng số vi khuẩn đếm được từ quần xã nguồn cấy, quần xã ở đáy cực âm của hệ thống SBES và quần xã ở đáy bể đối chứng biến đổi từ 3.9 x 10^5 to 2.71 x 10^6 cfu g^{-1}. Vi khuẩn ưa mặn như *Vibrio* sp., *Pseudomonas* sp. được tìm thấy ưu thế ở quần xã điện cực đáy và có thể đóng vai trò quan trọng trong quá trình truyền điện tử cũng như trong sự vận hành dưới điều kiện mặn của hệ thống SBES tương ứng. Thành phần và tính đa dạng của quần xã vi khuẩn ở đáy dưới điện cực âm đã được làm giàu trong hệ thống SBES khác biệt đáng kể so với quần xã đối chứng (không có điện cực) nhưng không khác biệt nhiều so với quần xã nguồn cấy.

Từ khóa: Vi khuẩn sinh điện hóa thích nghi nước lợ, *Vibrio* sp., *Pseudomonas* sp., hệ thống sinh điện hóa với điện cực ở đáy (SBES), nuôi trồng thủy sản nước lợ.