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Isolation of Halophilic Bacterial Strains from Mangrove Soil Samples for Polyhydroxyalkanoate Production

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Abstract: More than two hundreds bacterial strains were isolated from mangrove soil samples collected from Quang Ninh province. Among them three of the strains (VK75, VK98 and VK129) were found to accumulate polyhydroxyalkanoate (PHA) in noticeable amounts. Strains VK75, VK98 and VK129 are moderately halophilic, neutrophilic and mesophilic bacteria. The three halophilic bacterial strains were able to synthesize copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from various carbon sources (glucose, sucrose, lactose, glycerol, fructose, maltose and molasses). Glucose and glycerol were found to be most suitable substrates for PHBV synthesis by three selected strains. For experiments performed in shake flasks, strain VK75 reached a maximum cell dry weight (CDW) of 3.3 g/l and PHBV content of 67 wt%, strain VK98 attained a maximum CDW of 4 g/l and PHBV yield of 50% while strain VK129 reached a maximum CDW of 4.3 g/l and PHBV content of 56.8 wt% after 36 h of cultivation. The results obtained in this study are comparable to that of the highest reported so far for other moderately halophilic bacteria.

Keywords: Polyhydroxyalkanoate, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), mangrove, halophilic bacteria.

1. Introduction

In nature, many bacteria can accumulate polyhydroxyalkanoates (PHAs) as carbon and energy storage materials under the condition of excess carbon and limitation of an essential nutrient such as oxygen, nitrogen, and phosphorus. PHAs are biodegradable, biocompatible and thermoplastic materials. The properties of PHAs are similar to those of common petrochemical-based synthetic thermoplastics and can hence potentially replace them. Furthermore, they become completely degraded to carbon dioxide and water under aerobic conditions and to methane and carbon dioxide under anaerobic conditions by many microorganisms in the environment [1, 2].

PHAs have been used in the production of various products, including films, coated paper, compost bags, bone plates, osteosynthetic materials, surgical sutures, vascular grafts, heart valves, and drug delivery systems [3, 4]. One of the major bottlenecks in the commercial application of PHAs is their high price as compared to the conventional petroleum-based plastic materials. A great deal of effort has been devoted to reducing the production cost by isolating and developing more efficient bacterial

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strains, improving fermentation/recovery processes, and utilizing cheap carbon sources [5].

Studies on the production of PHA by halophilic bacteria were recently initiated. Moderate halophiles such as *Halomonas boliviensis*, *Halomonas hydrothermalis*, *Halomonas* TD01 isolated from saline habitats in Bolivia, India and China, respectively, have showed high PHA accumulation [6-8]. *H. boliviensis* could produce PHB from different sugars and agricultural residues. It could accumulate over 80 wt% PHB in fed-batch cultivations. *H. hydrothermalis* was able accumulate 75 wt% PHB from Jatropha biodiesel byproduct. *Halomonas* TD01 could produce PHA under unsterile conditions and the PHB content of 80 wt% was obtained after 56 h of cultivation in fed-batch fermentation. The major advantages of halophilic organisms are the possibility of growing them under unsterile conditions, and also the relative ease of extracting the biopolymer from the cells by hypoosmotic shock of the cells [9].

The objective of the present work is to isolate halophilic bacteria from mangrove soil samples, screening for high PHA producers and testing their ability to utilize of different carbon sources for production of PHA.

2. Materials and methods

2.1. Isolation of bacterial strains

Soil samples from mangrove forests located at Yên Hưng district, Quảng Ninh province were collected and serially dilluted with sterile sea water, and then 100 µl of the dilution were spread on modified MPA medium, containing (g/l): NaCl, 30; meat extract, 5; peptone, 5; granulated agar, 20; and pH adjusted to 7 using 1N NaOH. The plates were incubated at 30 ^{o}C for 48 h. Several hundreds of colonies were isolated by plating them again on fresh solid MPA medium.

2.2. Detection of PHA in bacteria

Bacterial isolates were grown on a solid medium (medium for PHA production - MA) containing (g/l): NaCl, 30; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.09; KCl, 0.5; KBr, 0.06; KH_2PO_4, 0.25, yeast extract, 2; glucose, 20; granulated agar, 20; pH adjusted to 7 using 1N NaOH, and Nile red (Sigma) (dissolved in dimethylsulfoxide) with final concentration of 0.5 µg dye per ml of the medium. Petri dishes were incubated at 30 ^{o}C for 48 h. The agar plates were then exposed to untraviolet light (312 nm) to detect the presence of intracellular PHA granules in the bacteria. The colonies with fluorescent bright orange were chosen for further studies [10].

2.3. Production of PHA by the isolated strains from different carbon sources

The selected bacterial strains were grown in 20 ml of MPA medium in 100 ml flasks at 30 ^{o}C with rotary shaking at 180 rpm for 13 h. Subsequently, 2.5 ml of each culture were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of MA medium with different carbon sources. The cultures were incubated at 30 ^{o}C with rotary shaking at 180 rpm. Samples were withdrawn at 30 h of cultivation for cell dry weight (CDW) and PHA content analysis.

2.4. Effect of temparature, NaCl concentration and pH on the growth of selected bacterial strains

The selected bacterial strains were grown in 20 ml of MPA medium in 100 ml flasks at 30 ^{o}C with rotary shaking at 180 rpm for 13 h. Subsequently, 2.5 ml of each culture were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of MA medium with glucose as carbon source. The cultures were incubated at different temperature, NaCl concentration and pH with rotary shaking at 180 rpm. Samples were withdrawn at 30 h of cultivation for CDW analysis.

2.5. Quantitative analysis

CDW was determined by centrifuging 3 mL of the culture samples at 6 000 rpm for 10 min in a pre-weighed centrifuge tubes, the pellet was washed once with 3 ml distilled water, centrifuged and dried at 105 ^{o}C until constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

Polymer content (wt%) in dried cell mass and its composition were determined by gas chromatography (GC). Approximately 10 mg lyophilized cells were mixed with 1 ml methanolysis solution [contains 15% H_2SO_4, 85% methanol (v/v) and 0.4% benzoic acid (w/v)] and 1 ml analytical chloroform. The methanolysis process was carried out for 3 h at 100 ^{o}C. After cooling down to room temperature, 0.5 ml MiliQ water was added to the mixture and vortexed for 30 seconds. Bottom layer containing methyl ester was transferred to sodium sulphate to remove remaining water, and analyzed by Agilent HP5890-II system (Hewlett Packard CO,USA) equipped with capillary HP-5 collumn (Hewlett Packard CO, USA) and flame ionization detector (FID) [11]. PHBV containing 24% valerate (Sigma) were used as a standard for calibration.

3. Results and discussion

3.1. Isolation and screening of PHA producing bacteria from soil samples

Soil samples were dilluted and spreated on MPA medium. After 48 h of cultivation at 30 ^{o}C, more than 200 of randomly chosen colonies were collected and grown on MPA medium. The isolate strains were then re-cultivated on agar MA medium containing Nile red for 48 h and then exposed to UV light. Three bacterial strains (named VK75, VK98, and VK129) that exhibited a very strong fluorescence were selected for further studies.

The selected bacteria were then cultivated in flasks using MA medium with seven different carbon sources at 30 ^{o}C, rotary shaking at 180 rpm for 30 h. The results of GC analysis shown that three selected bacterial strains were able to syntheze copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV) (figure 1). Among the big familly of PHA, poly(3-hydroxybutyrate) PHB is the most common type of PHA synthesized by microorganisms, and is rigid and brittle. However, PHBV is less crystalline, more elastic and tougher than PHB, and has broader range of applications [3].

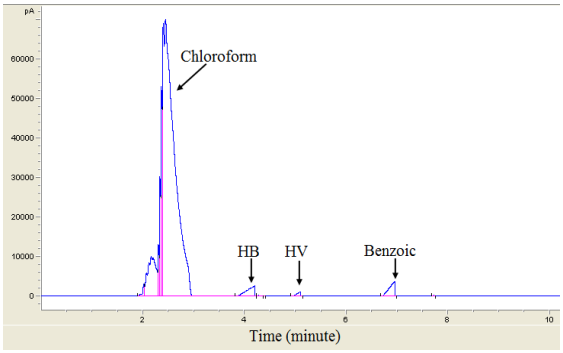


Figure 1. GC analysis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).

As summarized in figure 2, among the seven carbon sources tested: glucose and glycerol were found to be most suitable substrates for both cell growth and PHA accumulation by three selected strains. Maximum CDW of 3.11 g/l, 3.87 g/l, and 3.0 g/l; PHA content of 50.8 wt%, 44,2 wt%, and 50,1 wt% were respectively obtained by three selected strains (VK75, VK98, and VK129) when glucose was used as carbon substrate. Sucrose and molasses were also found to be good for cell growth, but led to lower accumulation of PHBV; while xylose, fructose and maltose served as poor substrates for cell growth and PHA synthesis. For further study, glucose was choseen as carbon substrate for three selected bacterial strains.

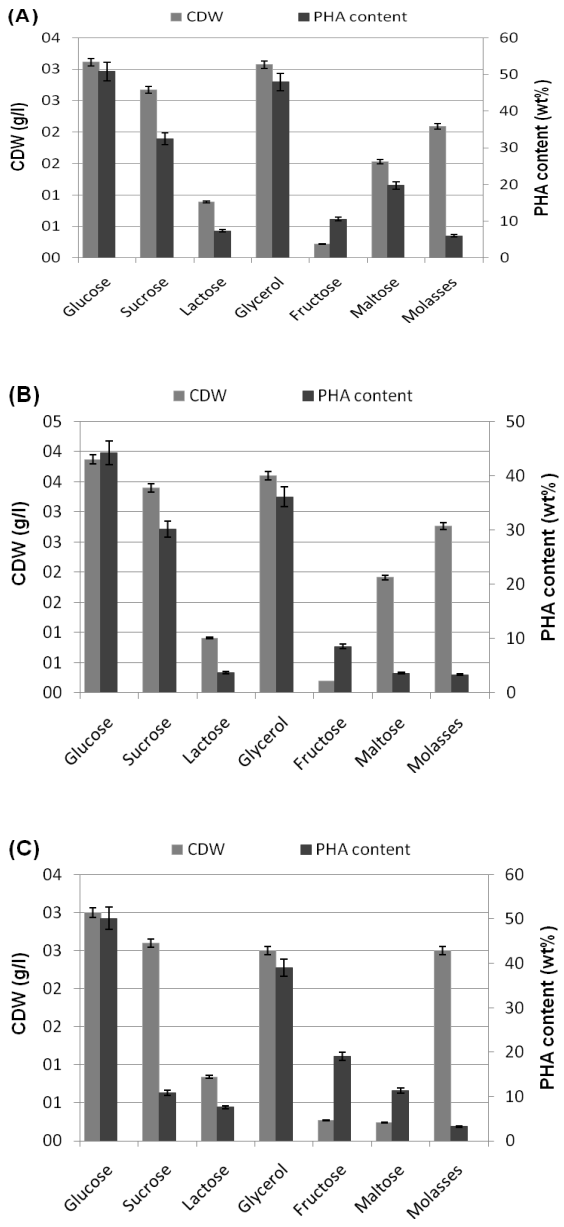


Figure 2. Effect of different carbon sources on CDW and PHBV content of (A) bacterium strain VK75, (B) bacterium strain VK98, and (C) bacterium strain VK129.

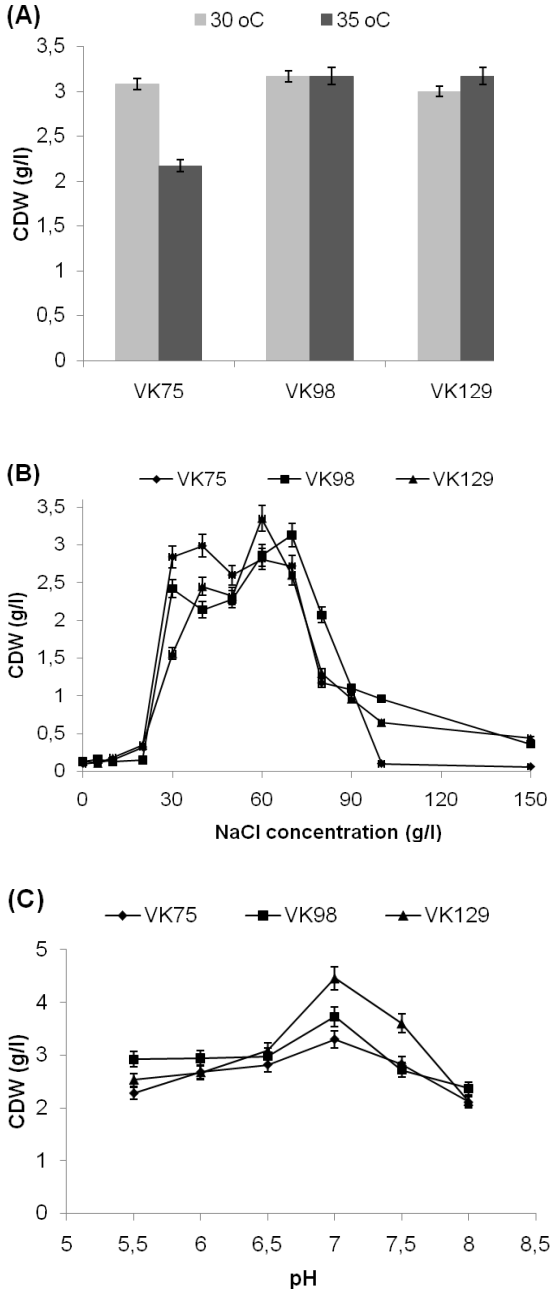
3.2. Effect of temperature, NaCl concentration and pH on cell growth of three selected strains

In order to evaluate the effect of temperature on the growth rate of three selected bacterial strains, the organisms were grown on MPA medium (solid medium) and then incubated at different temperatures for 48 h. The results indicated that the temperature range from 30°C to 35°C found to be optimal condition for the growth of three bacterial strains. In addition, to confirm the optimal temperature, strain VK75, VK98 and VK129 were then cultivated on MA medium (liquid medium) at 30°C and 35°C for 30 h. The results of CDW analysis show in figure 3A. Bacterial strain VK75 grows fastest at 30°C (CDW of 3.08 g/l), in contrast strain VK129 grows fastest at 35°C (CDW of 3.17 g/l), where as changing temperature between 30°C and 35°C seem to be not affect on growth rate of bacterial strain VK98 (constant CDW of about 3.17 g/l).

Figure 3B shows the effect of NaCl concentration on cell growth rate of three bacterial strains. All of three tested strains could not growth on the medium without NaCl. Strain VK75 was able to grow at NaCl concentration of 100 g/l, with optimum of about 30-40 g/l. Strain VK98 and strain VK129 are more halophilic than strain VK75, they could grow at NaCl concentration up to 150 g/l. The optimum NaCl concentration for the growth of strain VK98 and VK129 are 70 g/l and 60 g/l, respectively. According to the classcification of halophilic group based on optimum NaCl concentration for growth [12], three selected bacterial strains can be recognized as moderate halophilic bacteria (optimum growth between 0.5 and 2.0 M NaCl). Previous studies have demonstrated that the cultivation of the halophilic bacteria such as *Halomonas* TD01 [8] and *Yangia* sp. ND218 [13] under unsterile condition was contamination-free when the NaCl concentration in the medium was 60 g/l. Therefore, there is a possibility to design an unsterile process for cell growth and PHA production by three selected bacterial strains.

The effect of pH on cell growth rate of three selected strains was also investigated. Figure 3C indicated that neutral pH (pH 7.0) was found to be optimum pH for growth of three bacterial strains. This optimal pH value for the

growth of three bacterial strains is similar to the pH of collected mangrove soil samples, which was used for this study.

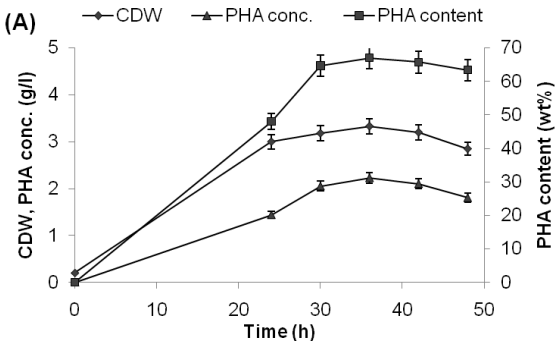


Figue 3. The effect of (A) temperature, (B) NaCl concentration, and (C) pH on growth rate of three selected bacterial strains.

3.3. PHA production by three selected bacterial strains in shake flasks

Three selected bacterial strains were grown in share flasks at optimal temperature, NaCl concentration and pH. Samples were taken at different time intervals for CDW and PHA content and PHA concentration analysis. Monitoring the CDW and PHA accumulated with time during cultivation of three bacterial strains showed that maximum CDW, PHA content and PHA concentration were obtained after 36 h of cultivation. After that CDW and PHA accumulation decreased due to the degradation of bacterial cells and PHA content. Maximum CDW of 4.3 g/l was obtained by strain VK129, followed by strain VK98 (4 g/l) and strain VK75 (3.3 g/l). However, maximum PHA content of 67 wt% was achieved by strain VK75, followed by strain VK129 (56.8 wt%) and strain VK98 (50 wt%) (figure 4A, 4B and 4C).

The CDW and PHA content obtained in this study by three halophilic bacteria are comparable to that of the highest halophilic producers reported so far. They are in same range as reached by *Halomonas boliviensis* (CDW of 4 g/l and PHA content of 62 wt%) [6], lower than those obtained by *Halomonas* TD01 (CDW of 6 g/l and PHA content of 69 wt%) [8].



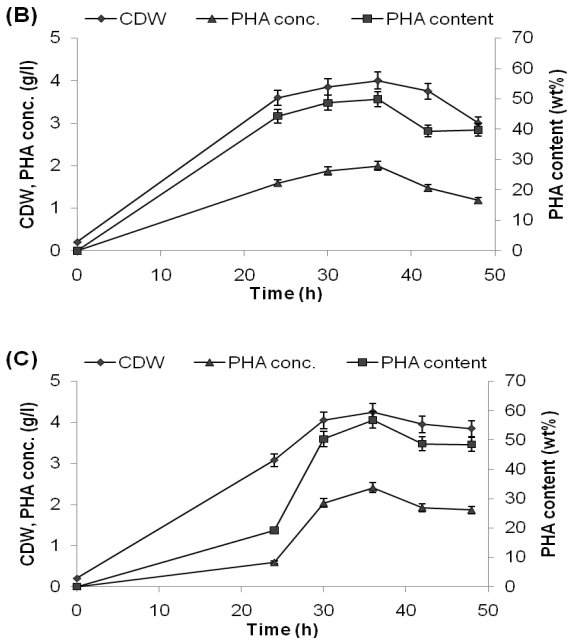


Figure 4. Profile of CDW, PHBV content and PHBV concentration during cultivation of (A) strain VK75, (B) strain VK98, and (C) strain VK129 in shake flasks.

4. Conclusion

Three PHA producing bacterial strains were isolated and selected from mangrove soil samples collected from Quảng Ninh province. They are moderately halophilic, neutrophilic and mesophilic bacteria. Three bacterial strains were able accumulate PHBV from different carbon sources. Maximum CDW of 3.3 g/l, 4 g/l and 4.3 g/l; and PHBV content 67 wt%, 50 wt% and 56.8 wt% were respectively obtained by strain VK75, strain VK98 and strain VK129 after 36 h of cultivation in shake flasks. The results obtained in this study are comparable to that of the highest reported so far for other moderately halophilic bacteria.

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References

[1] A.J. Anderson, E.D. Dawes, Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates, Microbiology Review 54 (1990) 450-472.

[2] S.Y. Lee, Bacterial polyhydroxyalkanoates. Biotechnology and Bioengineering 49 (1996) 1-14.

[3] S. Philip, T. Keshavarz, I Roy, Polyhydroxyalkanoates: biodegradable polymers with a range of applications, Journal of Chemical Technology and Biotechnology 82 (2007) 233-247.

[4] G.G. Chen, Industrial production of PHA. In: Chen GQ (ed) Plastics from Bacteria Natural Functions and Applications. Springer, Heidelberg Dordrecht London New York, (2010) pp 121-132.

[5] J. Choi, S.Y. Lee, Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Applied Microbiology and Biotechnology 51 (1999) 13-21.

[6] J. Quillaguamán, O. Delgado, B. Mattiasson, R. Hatti-Kaul, Poly(β-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1. Enzyme Microbiology and Technology 38 (2006) 148-154.

[7] A. Shrivastav, S.K. Mishra, B. Shethia, I. Pancha, D. Jain, S. Mishra, Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing *Jatropha* biodiesel byproduct. International Journal Biological Macromolecules 47 (2010) 283-287.

[8] D. Tan, Y.S. Xue, G. Aibaidula, G.Q. Chen, Unsterile and continuos production of polyhydroxybutyrate by *Halomonas* TD01, Bioresources Technology 102 (2011) 8130-8136

[9] J. Quillaguamán, H. Guzmán, D. Van-Thuoc, R. Hatti-Kaul, Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. Applied Microbiology and Biotechnology 85 (2010) 1687-1696.

[10] P. Spiekermann, B.H. Rehm, R. Kalscheuer, D. Baumeister, A. Steinbüchel, A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Archives Microbiology 171 (1999) 73-80

[11] L.F. Silva, J.G.C. Gomez, M.S. Oliveira, B.B. Torres, Propionic acid metabolism and poly-3-

hydroxybutyrate-*co*-3- hydroxyvalerate (P3HB-*co*-3HV) production by *Burkholderia* sp. Journal of Biotechnology 76 (2000) 165-174.

[12] D. Gilmour, Halotolerant and halophilic microorganisms. In:Edwards C (ed) Microbiology of Extreme Environments. McGraw Hill Publishing Co, New York (1990) pp 147-177

[13] D.V. Thuoc, Utilization of an unsterile medium for production of polyhydroxyalkanoate (PHA) by Yangia sp. ND218. Journal of Science of HNUE 57 (2012) 104-110.

Phân lập các chủng vi khuẩn ưa mặn sinh tổng hợp polyhydroxyalkanoate từ đất rừng ngập mặn

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Tóm tắt: Trong nghiên cứu này, từ các mẫu đất rừng ngập mặn tỉnh Quảng Ninh chúng tôi đã phân lập được hơn hai trăm chủng vi khuẩn. Trong số này, ba chủng vi khuẩn (VK75, VK98 và VK129) có khả năng tích lũy lượng lớn polyhydroxyalkanoate trong tế bào đã được lựa chọn nghiên cứu. Chủng VK75, VK98 và VK129 là các chủng vi khuẩn ưa mặn trung bình, ưa ấm và ưa pH trung tính. Ba chủng vi khuẩn ưa mặn này có khả năng tổng hợp loại polymer hỗn hợp poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) từ nhiều nguồn các bon khác nhau (glucose, sucrose, lactose, glycerol, fructose, maltose and molasses). Trong bảy nguồn các bon sử dụng thì glucose và glycerol là hai nguồn các bon thuận lợi nhất cho sự sinh trưởng và sinh tổng hợp PHBV. Khi nuôi cấy ba chủng trong bình nón sử dụng glucose là nguồn các bon, hàm lượng tế bào khô (CDW) và lượng PHBV tích lũy đạt cực đại sau 36 giờ nuôi cấy: CDW và PHBV cực đại của chủng VK75 là 3.3 g/l và 67 wt%, của chủng VK98 là 4 g/l và 50 wt%, và của chủng VK129 là 4.3 g/l và 56.8 wt%. Kết quả thu được trong nghiên cứu này tương đương với kết quả của các chủng vi khuẩn ưa mặn trung bình khác đã công bố.

Từ khóa: Polyhydroxyalkanoate, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), rừng ngập mặn, vi khuẩn ưa mặn.