BIOCONVERSION OF GLYCEROL TO POLYHYDROXYALKANOATE BY HALOPHILIC BACTERIA ISOLATED FROM MANGROVE SOIL SAMPLES

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Received: 26 March 2015; Accepted for publication: 17 July 2015

ABSTRACT

Polyhydroxyalkanoate (PHA) is a group of biodegradable polymer, synthesized by many microorganisms. PHA provide a good fully degradable alternative to petrochemical plastics. One of the major bottlenecks in the commercial application of PHA is their high price as compared to the conventional petroleum-based plastic materials. A potential solution for low-cost PHA production is to utilize glycerol (byproduct of biodiesel production) as carbon source. In this study, we isolated seventeen bacteria strains, which were able to synthesize PHA from glycerol. Among them, three strains named VK32, VK56 and VK72 were chosen for further study. They are moderately halophilic and neutrophilic bacteria, able to metabolize different nitrogen sources. Highest PHA content of 61 wt% was obtained by strain VK75 after 30 h cultivation in flask using KNO_3 as nitrogen source. Maximum cell dry weight (CDW) of 6 g/l was obtained by strain VK75 after 30 h cultivation in bioreactor, however, the PHA content was low (19 wt%). Further studies are being caried out to optimize conditions for both cell growth and PHA accumulation by the three selected strains.

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

1. INTRODUCTION

Polyhydroxyalkanoates (PHA), a group of biodegradable polymers of biological origin, have attracted considerable industrial interest. PHA are accumulated intracellularly as reserves of carbon and energy by a wide variety of bacteria, usually when grown under limitation of a nutrient such as O, N, P, S and in the presence of excess carbon. So far, about 150 different constituents of PHA have been isolated from more than 300 microorganisms. PHA are biodegradable, biocompatible, thermolastics or elastomeric materials. PHA have been proposed as a solution to overcome environmental problem caused by non-biodegradable plastics such as petroleum based plastics [1 - 4]. The major bottleneck for extensive use of PHA is its high

production cost. In this regard, research is focusing on the finding of new PHA producers that can produce high PHA yield and productivity from cheap raw material [5].

Biodiesel is a promising alternative and renewable fuel. Biodiesel production will generate about 10 % (w/w) glycerol as the main byproduct. Due to the increasing demand and production of biodiesel in the world, an excess of glycerol is now available. The effective utilization of crude glycerol will contribute to the viability of biodiesel. One of the many promising applications for the use of glycerol is its bioconversion to value-added products such as PHA through microbial fermentation. However, the presence of high content of NaCl in crude glycerol (3 – 7 % NaCl) will inhibit cell growth and PHA production of normal microorganisms (non-halophiles) [6]. For that reason, searches for halophilic bacterial strains that can convert glycerol to PHA in the presence of NaCl are required.

The objective of the present work is to isolate halophilic bacteria from mangrove soil samples and screen the bacterial isolates for their ability to utilize glycerol as carbon source for the production of PHA.

2. MATERIALS AND METHODS

2.1. Isolation of PHA producing 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. 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; glycerol, 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 [7].

2.2. Production of PHA by the selected strains in flasks

The selected PHA producing 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. 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.

2.3. Effect of NaCl concentration, pH and differrent nitrogen sources on the growth of three PHA producing bacterial strains

Three selected PHA producing 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 NaCl concentration, pH and nitrogen sources. The cultures were incubated at 30 ^{o}C

with rotary shaking at 180 rpm. Samples were withdrawn at 30 h of cultivation for CDW analysis.

2.4. Production of PHA by strain VK75 in bioreactor

Bacterial strain VK75 was grown in 50 ml of MPA medium in 250 ml flasks at 30 ^{o}C with rotary shaking at 180 rpm for 13 h. The medium was then used to inoculate 2.7 L of MA medium in a 10 L bioreactor. Batch fermentation was performed at 30 ^{o}C, pH 7.0. The initial air inflow rate of 0.5 L/min was increased up to 2 L/min during the fermentation. The agitation speed was initially set at 200 rpm and increased to 400 rpm during the fermentation to maintain the dissolved oxygen concentration above 20 %. After first 12 hours of cultivation, the samples were taken every 6 h for CDW and PHA analysis.

2.5. Quantitative analysis

CDW was determined by centrifuging 3 mL of the culture samples at 6000 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) [8]. Approximately 10 mg lyophilized cells were mixed with 1 ml methanolysis solution (contains 15 % H_2SO_4 and 85 % methanol, v/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). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) containing 12 % valerate (Sigma) was used as a standard for calibration.

3. RESULTS AND DISCUSSION

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

Soil samples from mangrove forest located at Yen Hung district, Quang Ninh province were collected, dilluted and spread on MPA medium. After 48 h of cultivation at 30 ^{o}C, more than 200 of randomly colonies were selected and grown on MPA medium. The isolated strains were then re-cultivated on agar MA medium containing Nile red for 48 h and then exposed to UV light. Seventeen bacterial strains (named VK31, VK32, VK38, VK56, VK57, VK58, VK74, VK75, VK84, VK88, VK91, VK94, VK97, VK98, VK123, VK124 and VK129) that exhibited a very strong fluorescence were selected for further studies. To confirm the formation of PHA granules in the selected bacterial strain, cells from the 48 h cultures grown on MA medium (without Nile red) were observed on a light microscope. Most of the cells of selected strain showed the presence of PHA granules in the cytoplasm (Figure 1).

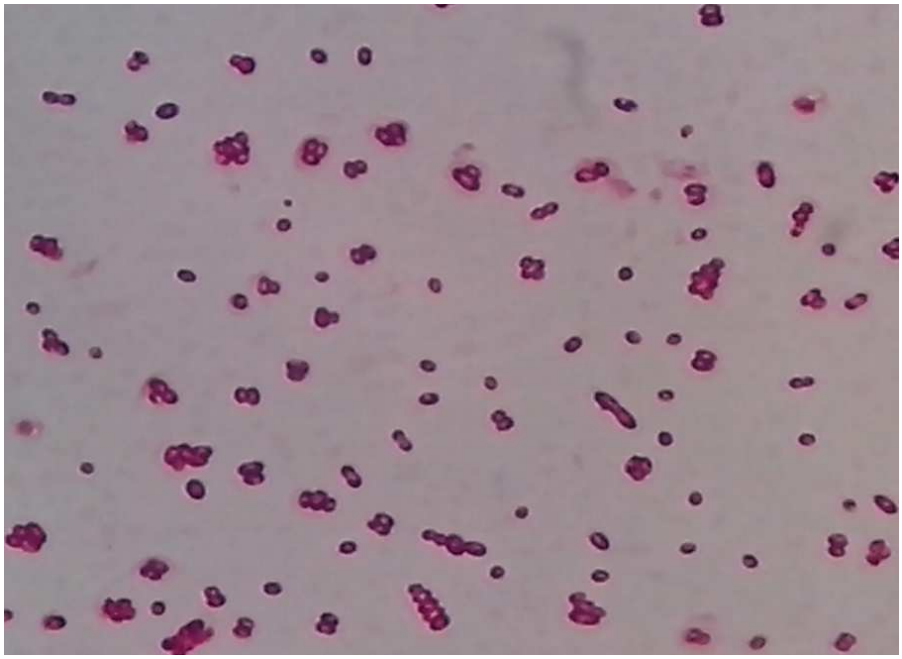

Figure 1. Light micrograph of strain VK75 grown on MA medium using glycerol as carbon source.

3.2. PHA production by selected bacterial strains in flasks

Seventeen selected bacteria were cultivated in flasks using MA medium with glycerol as carbon source at 30 ^{o}C and rotary shaking at 180 rpm, after 30 h of cultivation samples were taken for CDW and PHA analysis. As summarized in Table 1, CDW of between 1.8 and 3.5 g/l were obtained by seventeen selected bacterial strains, maximum CDW of 3.4 and 3.5 g/l were obtained by two strains VK56 and VK84, respectively. The PHA content accumulated by tested strains ranges from 11 to 48 wt%. Among seventeen tested strains, four strains (VK75, VK88, VK98 and VK124) can synthesize polymer with PHA content of more than 40 % of CDW, seven strains have PHA content ranging from 20 to 40 wt% and the rest has PHA content lower than 20 %.

The results of GC analysis showed that the selected bacterial strains were able to synthesize the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Table 1). Normally, there are only few bacteria that can synthesize PHBV from single unrelated carbon sources such as glucose, sucrose or glycerol. Most bacteria synthesize homopolymer poly(3-hydroxybutyrate) (PHB. They can only produce PHBV when HV precursors such as propionate, valerate, and heptanoate are provided to the medium [9]. However, in this study our results showed that all of seventeen selected bacteria strains are able to synthesize PHBV with high 3HV fraction when using glycerol as carbon source. The increase in the 3HV content is correlated with decreased crystallinity and increased elasticity of the copolymer. With high 3HV content, PHBV become less crystalline, more elastic, and has broader range of applications [10, 11].

Among seventeen tested bacterial strains, three strains (VK32 – high HV content, VK56 – high CDW and HV content, and VK75 – high PHA content and HV content) were chosen for further studies.

Table 1. PHA production by seventeen bacterial strains in flasks.

Strains	CDW (g/l)	PHA content (wt%)	PHA conc. (g/l)	PHA composition	
				HB%	HV%
VK31	1.8	11.6	0.2	68.6	31.4
VK32	2.0	21.8	0.4	52.7	47.3
VK38	1.9	27.6	0.5	72.6	27.4
VK56	3.4	32.9	1.1	84.1	15.9
VK57	1.8	25.9	0.5	64.0	36.0
VK58	2.0	13.1	0.3	90.6	9.4
VK74	1.8	15.8	0.3	59.5	40.5
VK75	2.3	45.8	1.0	79.4	20.6
VK84	3.5	35.3	1.2	95.3	4.7
VK88	3.0	44.5	1.3	87.0	13.0
VK91	2.5	13.2	0.3	91.1	8.9
VK94	2.0	11.0	0.2	62.4	37.6
VK97	1.9	10.7	0.2	91.2	8.8
VK98	3.2	47.8	1.5	94.7	5.3
VK123	2.8	48.4	1.4	89.0	11.0
VK124	2.0	31.8	0.6	76.3	23.7
VK129	2.5	39.0	1.0	77.0	23.0

3.3. Effect of NaCl concentration on cell growth of three selected strains

In order to evaluate the effect of NaCl concentration on the growth rate of the three selected bacterial strains, the organisms were grown on MPA medium containing NaCl at different concentrations and then incubated at 30 ^{o}C for 30 h. The results showed that the three selected strains were unable to grow on medium without NaCl. NaCl concentration of between 40 to 50 g/l was found to be optimal condition for the growth of these three strains. Strain VK75 was able to grow at NaCl concentration of 100 g/l, whereas, strain VK32 and strain VK56 can grow at NaCl concentration of up to 150 g/l (Figure 2). According to the classcification of halophilic group based on optimum NaCl concentration for growth [12], the three selected bacterial strains can be classified to the group of moderate halophilic bacteria (optimum growth between 0.5 and 2.0 M NaCl).

3.4. Effect of pH on cell growth of three selected strains

The effect of pH on cell growth of the three selected strains was also investigated. For that, the strains were grown on MPA medium with different pH. After 30 h of cultivation at 30 ^{o}C and 180 rpm, samples were withdrawn for CDW analysis. Figure 3 shows that pH around 7.0 was

found to be optimal pH for the growth of these three bacterial strains. This pH value is similar to the pH of soil samples, which were used for this study.

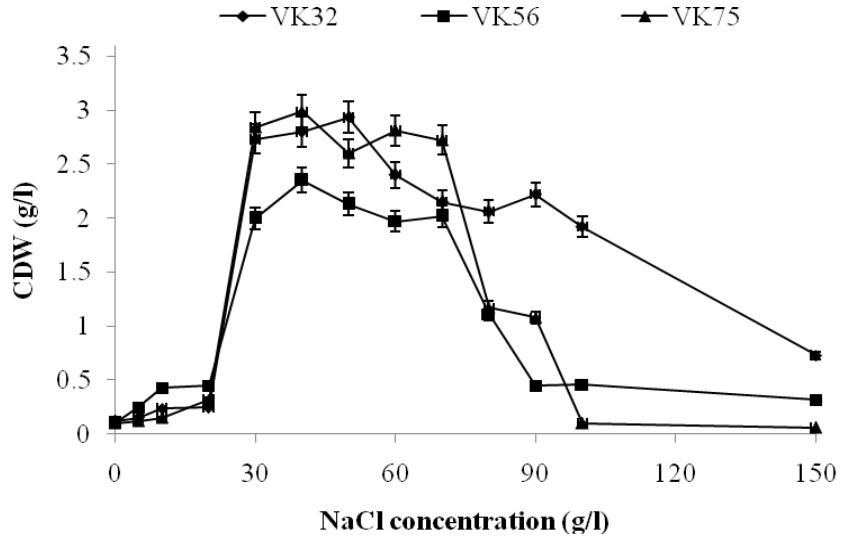

Figure 2. The effect of NaCl concentration on the growth rate of three selected strains

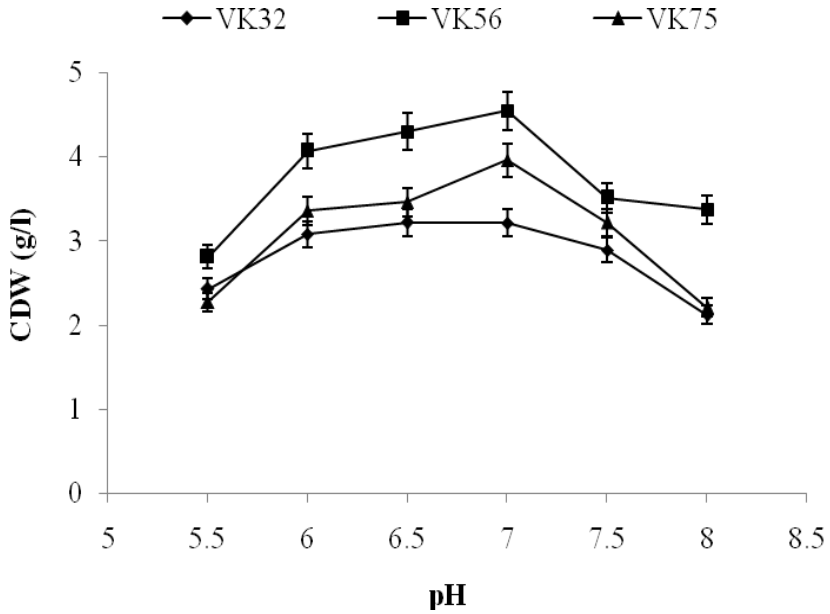

Figure 3. The effect of pH on the growth rate of three selected strains

3.5. Effect of different nitrogen sources on cell growth and PHA production of three selected strains

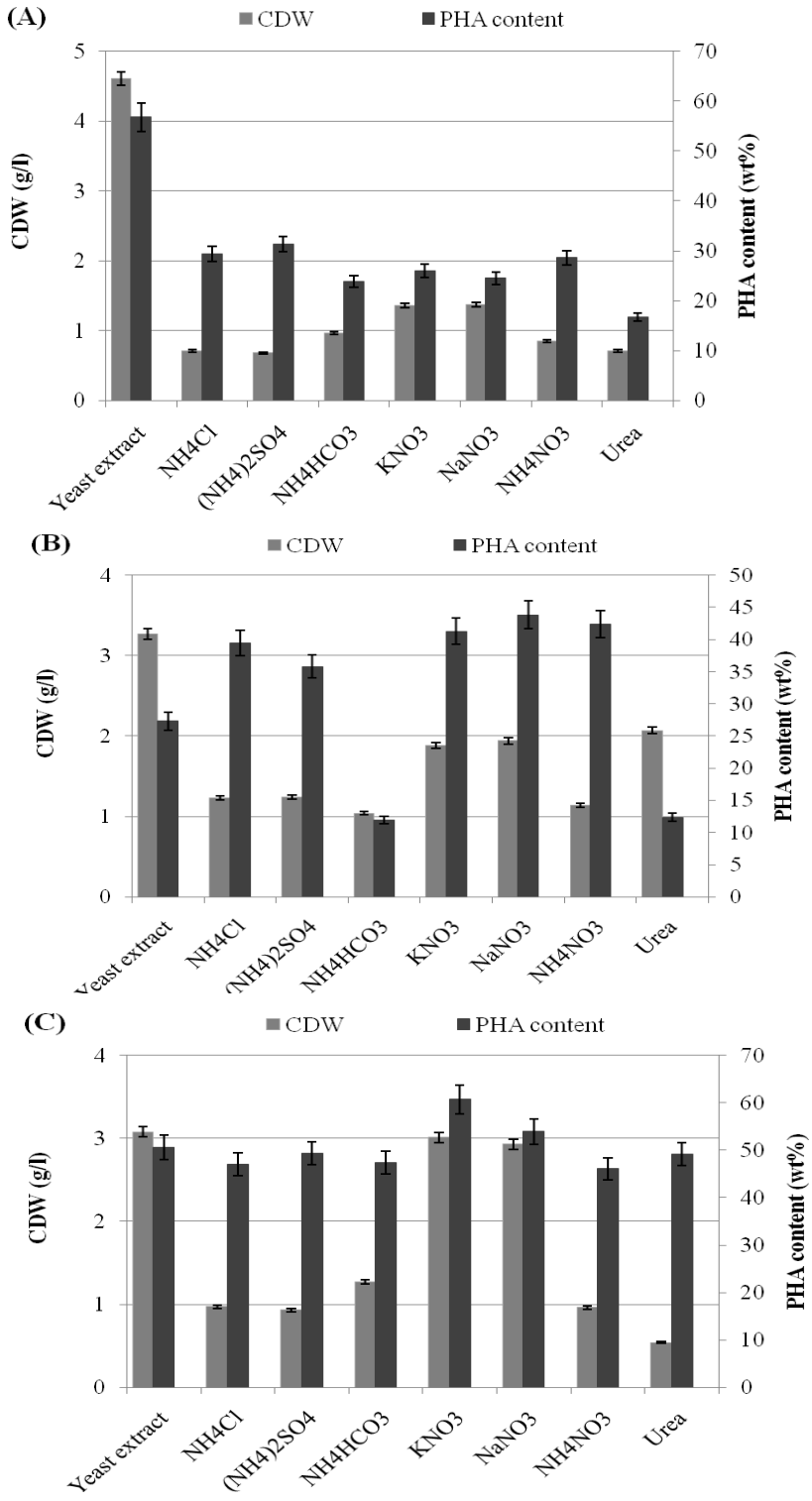

Figure 4. The effect of different nitrogen sources on growth rate and PHA accumulation of three selected strains. (A) strain VK32, (B) strain VK56 and (C) strain VK75 .

The selected bacteria were cultivated in flasks using MA medium with eight different nitrogen sources at 30 ^{o}C, rotary shaking at 180 rpm for 30 h. Figure 4A shows the effect of eight different nitrogen sources on CDW and PHA content of the strain VK32. Yeast extract was found to be a favourable nitrogen souce for cell growth and PHA accumulation by strain VK32. Maximum CDW of 4.6 g/l and PHA content of 57 wt% were obtained by strain VK32 when yeast extract was used as nitrogen souce. Yeast extract was also good for cell growth rate of strain VK56 (CDW of 3.3 g/l), but accumulation of PHA was low (27 wt%). Maximum PHA content of 44 wt% was achieved by strain VK56 when $NaNO_3$ was used as nitrogen source (Figure 4B). All of eight tested nitrogen sources were found to be suitable for PHA accumulation by strain VK75. However, only three of them (yeast extract, KNO_3 and $NaNO_3$) were found to be good for both cell growth rate and PHA accumulation. The highest PHA content of 61 wt% was obtained when KNO_3 was used as nitrogen source. Whereas, maximum CDW of 3.1 was achieved when yeast extract was used (Figure 4C).

Previous studies have demonstrated that limitation of a nitrogen or a phosphorous source is required to achieve the maximum volumetric productivities by PHA producer bacteria. However, the use of a complex nitrogen source such as yeast extract makes it difficult to control the supply of nutrients for achieving high cell density as well as optimal PHA productivity. Replacing yeast extract with another nitrogen source such as KNO_3 or $NaNO_3$ in the medium will thus lead to an increasing the PHA productivity and a drastic reduction in the medium costs [13].

3.6. PHA production by strain VK75 in bioreactor

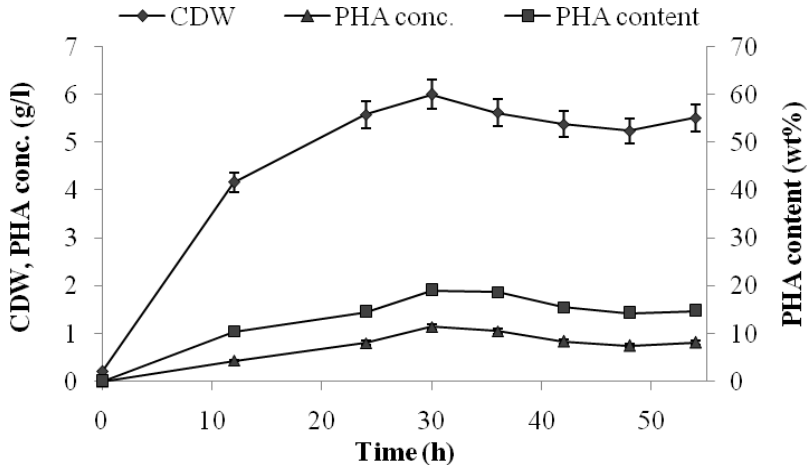

Figure 5. Profile of CDW, PHA content and PHA concentration during cultivation of strain VK75 in bioreactor.

Bacterial strain VK75 was then cultivated in a bioreactor and samples were taken at different times for CDW and PHA analysis. As showed in Figure 5, strain VK75 grew rapidly and reached stationary phase within 30 h. The CDW, PHA content, and PHA concentration increased until 30 h of cultivation and were 6 g/l, 19 wt%, and 1.14 g/l, respectively. The CDW obtained here (6 g/l) is 2 times higher than that obtained in flask experiment (3 g/l). The significant increase in biomass was most likely due to the control of culture conditions such as pH and oxygen during the cultivation process. In contrast, the PHA content obtained in this

experiment (19 wt%) decreased 3 times as compared to that obtained in flask experiment (60 wt%). The optimal conditions for cell growth and PHA accumulation are different with respect to the culture conditions and the amount of nutrient in culture medium. Balance nutrients will be good conditions for cell growth, but imbalance nutrients will be favorable conditions for PHA accumulation [13]. Further studies are being carried out to optimize conditions for both cell growth and PHA accumulation.

4. CONCLUSION

In this study, hundreds bacterial strains were isolated from mangrove soil samples collected from Quảng Ninh province. Among them, seventeen strains were found to accumulate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from glycerol. CDW of 3 g/l and high PHA content of 61 wt% were obtained by strain VK75 after 30 h of cultivation in flask. In bioreactor, CDW was increased to 6 g/l but PHA content was decreased to 19 wt%. Further studies are being carried out to improve both CDW and PHA content.

Acknowledgment. This research work is supported by International Foundation for Science (IFS) (Grant number: F/5021-2).

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TÓM TẮT

CHUYỂN HÓA GLYCEROL THÀNH POLYHYDROXYALKANOATE NHỜ VI KHUẨN ƯA MẶN PHÂN LẬP TỪ RỪNG NGẬP MẶN

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Các polyhydroxyalkanoate (PHA) là một nhóm polymer có khả năng phân hủy sinh học, PHA được tổng hợp bởi rất nhiều các vi sinh vật trong tự nhiên. PHA có nhiều tiềm năng thay thế các loại nhựa có nguồn gốc từ dầu mỏ. Điểm hạn chế ảnh hưởng đến tiềm năng ứng dụng của PHA là giá thành sản xuất cao. Nghiên cứu sử dụng glycerol (phụ phẩm của quy trình sản suất diesel sinh học) sẽ góp phần làm giảm giá thành PHA. Trong nghiên cứu này chúng tôi đã phân lập được 17 chủng vi khuẩn có khả năng chuyển hóa glycerol thành PHA. 3 chủng vi khuẩn (kí hiệu VK32, VK56 và VK75) sinh trưởng phát triển tốt và tổng hợp nhiều PHA đã được lựa chọn để nghiên cứu. Cả 3 chủng vi khuẩn này đều là vi khuẩn ưa mặn trung bình, phát triển tốt ở pH trung tính, có khả năng sử dụng nhiều nguồn ni tơ khác nhau. Chủng VK75 có khả năng sử dụng hiệu quả một số nguồn ni tơ vô cơ như KNO_3, $NaNO_3$. Chủng VK75 có khả năng tích lũy hàm lượng PHA đạt 61 % khối lượng tế bào khô khi nuôi cấy trên môi trường sử dụng KNO_3 làm nguồn ni tơ. Khi nuôi cấy trong nồi lên men, sinh khối của chủng VK75 tăng lên gấp đôi (so với khi nuôi trong bình tam giác) và đạt 6 g/l, tuy nhiên hàm lượng PHA lại giảm 3 lần và chỉ còn 19 %. Nghiên cứu tối ưu điều kiện môi trường nuôi cấy đang được chúng tôi tiến hành để cùng lúc nâng cao sinh khối và hàm lượng PHA tích lũy trong tế bào.

Từ khóa: polyhydroxyalkanoate, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), glycerol, vi khuẩn ưa mặn.