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Polyhydroxyalkanoates production by a bacterium isolated from mangrove soil samples collected from Quang Ninh province

Vi khuẩn sinh tổng hợp polyhydroxyalkanoates phân lập từ đất rừng ngập mặn tỉnh Quảng Ninh

Research article

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A PHA producing bacterium (strain QN271) was selected from mangrove soil samples collected from Quang Ninh province by using the Nile red dying technique. PHA accumulation in the selected bacterium strain was confirmed by transmission electron microscope. With the exception of maltose or sucrose, the bacterium strain was found to be able to synthesize PHA from various carbon sources (glucose, xylose, fructose, glycerol, and glucose plus propionate). The strain accumulated poly(3-hydroxybutyrate) from glucose, fructose, xylose, and glycerol whereas poly(3-hydroxybutyrate-co-3-hydroxyvalarate) was produced when a combination of glucose and propionate was included in the culture medium. Fructose was found to be most suitable substrate for PHA synthesis by strain QN271. PHA content of 63.3% and CDW of 6 g/L were obtained after 32 hrs of cultivation in fructose medium.

Chủng vi khuẩn có khả năng sinh tổng hợp PHA đã được phân lập từ đất rừng ngập mặn tỉnh Quảng Ninh nhờ kỹ thuật nhuộm với Nile red. Ảnh quan sát dưới kính hiển vi điện tử dẫn truyền chứng tỏ rằng chủng vi khuẩn này có khả năng tích lũy lượng lớn PHA trong tế bào. Chủng vi khuẩn tuyển chọn có khả năng sinh tổng hợp PHA từ nhiều nguồn các bon khác nhau như glucose, xylose, fructose, glucerol, glucose và propionate nhưng không có khả năng tổng hợp PHA từ maltose hoặc saccharose. Chủng vi khuẩn tuyển chọn tổng hợp poly (3-hydroxybutyrate) từ các nguồn các-bon như glucose, xylose, fructose, hay glycerol, trong khi đó poly (3-hydroxybutyrate-co-3-hydroxyvalarate) sẽ được tổng hợp khi phối hợp sử dụng hai nguồn các-bon (glucose và propionate). Fructose là nguồn các-bon tốt nhất cho chủng QN271 sinh tổng hợp PHA, khi nuôi cấy trong môi trường có fructose chủng vi khuẩn này có thể tạo ra lượng sinh khối là 6 g/L trong đó có chứa 63.3% PHA sau 32 giờ.

Keywords: biopolymer, mangrove, poly(3-hydroxybutyrate), polyhydroxyalkanoate

1. Introduction

Mankind has become highly dependent on fossil resources for their need of energy, chemicals and materials. However, the fossil resources will sooner or later come to an end and also that they are found only in some regions of the world. That has led to a global interest in finding alternative sources that are renewable and easily accessible. The other problem that motivates a shift from fossil resources is the negative environmental impact of the processes and products in terms of greenhouse gas emissions, global warming and climate change. Fossil energy and plastics are among the most environmentally damaging products used in enormous amounts (Braunegg *et al*., 1998). The current global consumption of plastics is more than 250 million tonnes with annual increase in consumption of approximately 5%. In order to overcome the problem of pollution caused by non-degradable plastics, there is considerable interest in the development of biodegradable polymers such as polyhydroxyalkanoate (PHA), polylactic acid (PLA) (Lee, 1996; Salehizadeh and Van Loosdrecht, 2004).

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Polyhydroxyalkanoates (PHAs) are polyester of hydroxyalkanoates, accumulated intracellularly as carbon and energy storage materials in numerous microorganisms, usually when growing under the limitation of a nutrient and in the presence of excess carbon (Anderson and Dawes, 1990; Valappil *et al*., 2007). After extraction from the cells, PHAs possess the common features of non-toxic, biocompatible, biodegradable and recyclable thermoplastics. The main applications of PHAs include replacing petrochemical polymers currently in use for packaging and coating, as well as disposable plastic items. PHAs are also widely employed as bone plates, osteosynthetic materials, surgical sutures, vascular grafts and heart valves (Philip *et al*., 2007).

Mangrove forests are specialized ecosystems situated at the inter-phase between land and sea of the tropical and subtropical areas. Mangrove forests occupy a total estimated area of 152,000 km^2 and distributed in 123 countries and territories (Spalding *et al*., 2010). Microbes play an important role in governing the biogeochemical cycles of any ecosystem. Many different microorganisms including bacteria, fungi, protozoa and algae have been found in mangrove ecosystems. Among these microbes, the bacterial population is many-fold greater than the other. Because of its diversity, bacterial activity is responsible for most of the mineral cycle and the carbon flux in the mangrove ecosystems, and act as a carbon sink (Holguin *et al*., 2001).

The present study reports the isolation of a PHA accumulating bacterium from soil samples collected from mangrove forests located at Yen Hung district, Quang Ninh province. The ability to produce PHA from different carbon sources by the isolate strain was also investigated.

2. Materials and methods

2.1 Isolation of bacterial strains

Soil samples from mangrove forests located at Yen Hung district, Quang Ninh province were collected and serially dilluted with sterile sea water, and then 100 µL of the dilution were spread on solid HM medium (Quillaguamán *et al*. 2004), containing (g/L): NaCl, 30; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.09; KCl, 0.5; NaBr, 0.06; peptone, 5; yeast extract, 10; glucose, 1; and granulated agar, 20; and pH was adjusted to 7 using 2N NaOH solution. The plates were incubated at 35^{o}C for 30h. Several hundreds of colonies were isolated by plating them again on fresh agar medium.

2.2 Detection of PHA in bacteria

Bacterial isolates were grown on a modified solid HM medium (HM-1) containing (g/L): NaCl, 30; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.09; KCl, 0.5; NaBr, 0.06; KH_2PO_4, 0.25, yeast extract, 2; glucose, 20; granulated agar, 20; pH adjusted to 7 using 2N 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 35^{o}C for 2 days. The agar plates were then exposed to ultraviolet 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 (Spiekermann *et al*., 1999).

2.3 Transmission electron microscopy (TEM) observation

PHA containing cells were fixed and observed under TEM following a protocol reported previously (Quillaguamán *et al*. 2006).

2.4 Production of PHA by the isolated strain

The selected bacterial strain was grown in 20 mL of HM medium in 100 mL flasks at 32^{o}C with rotary shaking at 180 rpm for 13h. Subsequently, 2.5 mL of each culture were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of HM-1 medium with different carbon sources. The cultures were incubated at 32^{o}C with rotary shaking at 180 rpm. Samples were withdrawn at 30h of cultivation for cell dry weight (CDW) and PHA content analysis.

2.5 Quantitative analysis

CDW was determined by centrifuging 3 mL of the culture samples at 4000 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.

PHA content analysis was performed using a gas-chromatographic method (Huijberts *et al*., 1994). Sample volume of 2 µL was injected into the gas chromatography column (VARIAN, Factor Four Capillary Column, CP8907). The injection temperature was 250^{o}C, the detector temperature was 240^{o}C, and the column temperature was 60^{o}C for the first 5 minutes and then increased at 3^{o}C/min to 120^{o}C. PHB and PHBV containing 12% valerate (Sigma) were used as a standard for calibration.

3. Results and discussion

3.1 Isolation and detection of PHA producing bacteria from soil samples

Several different bacteria were isolated from soil samples collected from mangrove forests located at Yen Hung district, Quang Ninh province. Three hundred randomly chosen colonies were collected and grown on agar HM medium. The isolate strains were then re-cultivated on agar HM-1 medium containing Nile red for 2 days and then exposed to UV light. The dye produced orange fluorescence on binding to PHA granules or other lipid storage compound in the cell (Figure 1). About fifty of fluorescent bacteria were observed, among them one bacterial strain that exhibited a very strong fluorescence was selected for further studies, named as QN271 (where QN means Quang Ninh).

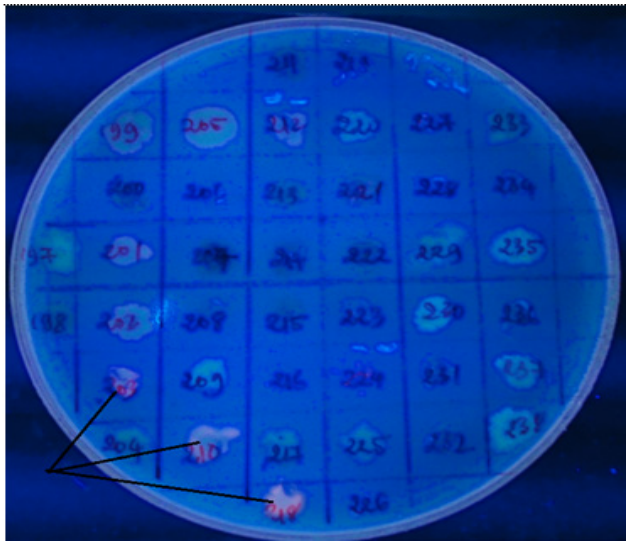

Figure 1. Fluorescent Nile red staining of strains from mangrove soil samples. The dye produces orange fluorescence on binding to polymer granules in the cell

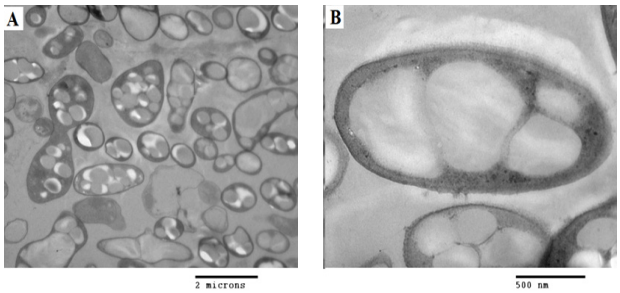

Figure 2. Transmission electron micrographs of strain QN271 grown on HM-1 medium with glucose for 48 h

To confirm the formation of PHA granules in the selected bacterial strain, cells from the 48h cultures grown on HM-1 medium (without Nile red) were observed on a transmission electron microscope. Most of the cells of selected strain showed the presence of PHA granules in the cytoplasm (Figure 2A, 2B). As shown in the figure, PHA exists as gray granules, with about 1 to 10 granules per cell and with maximum diameter of 0.5 μm.

3.2 PHA production from different carbon sources by the selected strain QN271

One of the most important factors affecting PHA production is the carbon sources. In this concern, the effect of different carbon sources on PHA production by the selected strain QN271 was investigated. As showed in Table 1 PHA was accumulated by strain QN271 when glucose, xylose, fructose, glycerol, and glucose plus propionate were used as sole carbon source. However, the remaining two carbon sources (maltose and sucrose) were not induced the synthesis of PHA by strain QN271. Fructose was found to be most suitable for growth and PHA accumulation. The maximum PHA content of 60.5% and CDW of 5.5 g/L were attained when fructose was used as carbon source. Gas chromatography analysis of the PHA indicated that 3-hydroxybutyrate (3HB) is the main component of the polymer, 3-hydroxyvalerate was only found in the polymer when glucose and propionate were supplied. Propionate has been used as a precursor for 3HV synthesis by many bacterial strains (Chen *et al*., 1991; Reddy *et al*., 2009). In this study the presence of propionate in the culture broth was also induced the synthesis of 3HV by the selected strain QN271.

Table 1. Effect of different carbon sources on cell growth and PHA accumulation by the selected strain QN271

Carbon source	CDW (g/L)	PHA content (% CDW)	PHA conc. (g/L)	PHA productivity (g/L/h)	Monomer composition (mol%)	
					3HB	3HV
Glucose	5.0	47.6	2.39	0.08	100	0
Maltose	3.4	0	0	0	0	0
Xylose	4.3	28.9	1.25	0.04	100	0
Sucrose	3.4	0	0	0	0	0
Fructose	5.5	60.5	3.33	0.11	100	0
Glycerol	4.6	36.5	1.67	0.06	100	0
Glucose + propionate	4.7	31	1.46	0.05	95	5

3.2 PHA polymer production by the selected strain QN271 in fructose medium

The effect of incubation time on cell growth and PHA accumulation by strain QN271 when grown in HM-1 medium containing fructose as carbon source was investigated. As showed in the Figure 3, strain QN271 grew rapidly and reached stationary phase within 32 h. The CDW, PHA content, and PHA concentration increased until hour 32th of cultivation and were 6 g/L, 63.3%, and 3.82 g/L, respectively (Figure 3). The PHA content of 63.3% and CDW of 6 g/L reached in flask experiments by strain QN271 were lower than those reached by *Azotobacter vinelandii* (74% and 10 g/L) (Page, 1992), *Cupriavidus necator* (54% and 9.4 g/L) (Doi *et al*., 1988) and a recombinant *E. coli* strain (80.8 wt% and 8.9 g/L) (Lee *et al*., 1994). These bacteria attained among the highest productions of PHA, and are recognized for their potential utilization at industrial scales.

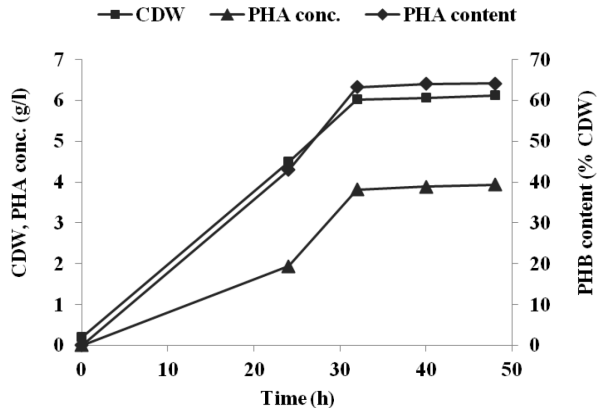

Figure 3. CDW, PHA content and PHA concentration of strain QN271 as a function of incubation time

4. Conclusion

A PHA producing bacterium strain QN271 was isolated from soil samples collected from Quang Ninh mangrove. The bacterium strain was able to accumulate high PHA (PHB or PHBV) from different carbon sources. Maximum PHA content of 63.3% and CDW of 6 g/L were obtained by strain QN271, the results obtained here are comparable to that of the highest reported so far for other microorganisms. Further work on improving PHA production by strain QN271 is being investigated.

5. Acknowledgements

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