ORIGINAL ARTICLE

# Effect of co-substrate on production of poly-β-hydroxybutyrate (PHB) and copolymer PHBV from newly identified mutant *Rhodobacter sphaeroides* U7 cultivated under aerobic-dark condition

**Kemarajt Kemavongse[1], Poonsuk Prasertsan[2], Apichat Upaichit[3] and Pawadee Methacanon[4]**

## Abstract

**Kemavongse, K., Prasertsan, P., Upaichit, A. and Methacanon, P.**
**Effect of co-substrate on production of poly-β-hydroxybutyrate (PHB) and copolymer PHBV from newly identified mutant *Rhodobacter sphaeroides* U7 cultivated under aerobic-dark condition**
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**Photosynthetic bacterial mutant strain U7 was identified using both classical and molecular (16S rDNA) techniques to be *Rhodobacter sphaeroides*. The glutamate-acetate (GA) medium containing sodium acetate and sodium glutamate as carbon and nitrogen sources was used for production of poly-β-hydroxy-butyrate (PHB) from *R. sphaeroides* U7 cultivated under aerobic-dark condition (200 rpm) at 37°C. Effect of auxiliary carbon sources (propionate and valerate) and concentrations (molar ratio of 40/0, 40/20, 40/40 and**

**[1]M.Sc. (Biotechnology), [2]Ph.D. (Biotechnology), Assoc. Prof., [3]Dr. Techn (Technical Chemistry), Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand. [4]Ph.D. (Chemistry), National Metal and Materials Technology Center, National Science and Technology Development Agency, Ministry of Science, Technology and Environment, Pathum Thani, 12120 Thailand.**
**Corresponding e-mail: poonsuk918@yahoo.com**
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**40/80) on copolymer production were studied. Both combinations of acetate with valerate and acetate with propionate were found to induce the accumulation of poly-β-hydroxybutyrate-co-β-hydroxyvalerate (PHBV) within the cell. Acetate with propionate in the molar ratio of 40/40 gave the highest poly-β-hydroxyalkanoates (PHA) content (77.68%), followed by acetate with valerate at the same molar ratio (77.42%). Although their polymer contents were similar, the presence of 40 mM valerate gave more than 4 times higher hydroxyvalerate (HV) fraction (84.77%) than in the presence of 40 mM propionate (19.12% HV fraction).**

**Key words :** identification, co-substrate, PHB, PHBV, *Rhodobacter sphaeroides*

## บทคัดย่อ

**เขมรัฐ เขมวงศ์[1] พูนสุข ประเสริฐสรรพ์[2] อภิชาติ อู๋ไพจิตร[3] และภาวดี เมธะคานนท์[4]**

**ผลของแหล่งคาร์บอนร่วมต่อการผลิตพอลิบีต้าไฮดรอกซีบิวทีเรท และโคพอลิเมอร์ชนิด PHBV จาก *Rhodobacter sphaeroides* U7 ภายใต้สภาวะมีอากาศ-ไร้แสง**

**ว. สงขลานครินทร์ วทท. 2550 29(4) : 1101-1113**

**จำแนกแบคทีเรียสังเคราะห์แสงสายพันธุ์กลาย U7 ด้วยวิธีแบบดั้งเดิมและเทคนิคทางโมเลกุล สามารถจำแนกเป็นเชื้อ *Rhodobacter sphaeroides* เมื่อเลี้ยงเชื้อดังกล่าวในอาหารเลี้ยงเชื้อกลูตาเมท-อะซิเตทภายใต้สภาวะที่มีอากาศ-ไม่ให้แสง บนเครื่องเขย่า (200 รอบ/นาที) ที่อุณหภูมิ 37 องศาเซลเซียส พบว่าเชื้อสามารถสะสมโฮโมพอลิเมอร์ชนิดพอลิเบต้าไฮดรอกซีบิวทีเรท (poly-β-hydroxybutyrate; PHB) จากการศึกษาผลของแหล่งคาร์บอนร่วม (กรดโพรพิโอนิกและกรดวาเลอริก) และความเข้มข้น (อัตราส่วนโมลระหว่างโซเดียมอะซิเตทต่อกรดโพรพิโอนิกหรือกรดวาเลอริกเป็น 40/0 40/20 40/40 และ 40/80) ต่อการผลิตโคพอลิเมอร์ พบว่าการใช้แหล่งคาร์บอนร่วมกันมีผลในการชักนำให้มีการสะสมโคพอลิเมอร์ชนิดพอลิ-เบต้า-ไฮดรอกซีบิวทีเรท-โค-เบต้า-ไฮดรอกซีวาเลอเรท (poly-β-hydroxybutyrate-co-β-hydroxyvalerate; PHBV) ภายในเซลล์ การใช้อะซิเตทร่วมกับกรดโพรพิโอนิกด้วยอัตราส่วนโมล 40/40 ให้ปริมาณ PHA ภายในเซลล์สูงสุด (77.68%) รองลงมาคือ การใช้อะซิเตทร่วมกับกรดวาเลอริกที่อัตราส่วนโมลเดียวกัน (77.42%) อย่างไรก็ตาม แม้ว่าปริมาณพอลิเมอร์ใกล้เคียงกัน แต่การใช้กรดวาเลอริกความเข้มข้น 40 มิลลิโมลาร์ ให้ค่าส่วนที่เป็นไฮดรอกซีวาเลอเรท (HV unit fraction) สูงกว่าถึง 4 เท่า (84.77%) เมื่อใช้กรดโพรพิโอนิกที่ความเข้มข้นเดียวกัน (19.12%)**

**[1]ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112**
**[2]ศูนย์เทคโนโลยีโลหะและวัสดุแห่งชาติ ตำบลคลองหนึ่ง อำเภอคลองหลวง จังหวัดปทุมธานี 12120**

The exponential growth of human population has led to the accumulation of huge amount of non-degradable plastic materials. Discovery of new environmentally friendly materials will help solving the global environment and solid waste management problem (Luengo *et al.*, 2003).

Polyhydroxyalkanoates (PHA) are polyesters of hydroxyalkanoates (HA) and consist of β-hydroxyacyl as monomer. PHA are synthesized by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cell under the condition of limiting nutrients such as nitrogen and phosphorus but in the presence of excess carbon source (Lee *et al*., 1995). Nevertheless, it was reported that nitrogen-stressed condition was omitted for good PHA production (Makhopadhyay *et al.*, 2005). Copolymer, such as poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV), is less brittle and less crystalline than its homopolymer, poly-β-hydroxybutyrate (PHB), and making it more suitable for commercial applications such as bone tissue engineering materials and biodegradable drug carriers (Chen and Wu, 2005).

One of the most important factors influencing PHA production is carbon source as different

intermediate would be generated along the PHA production pathway. Acetate or butyrate is oxidized to acetyl-Co A, then β-hydroxybutyrate (HB) to produce PHB while propionate is oxidized to propionyl-CoA, then β-hydroxyvalerate (HV) to produce PHV. Therefore, HB and HV are C4 and C5 monomers of the copolymer PHBV, respectively (Steinbuchel and Schlegel 1991). They can be obtained from switching between these two carbon sources (Iadevaia and Mantzaris, 2007) and incorporated into the growing chain by the PHA synthase, encoded by *phaC* gene. The higher prevalence of HV repeating units over HB repeating units was needed for production of copolymer PHBV (Berlanga *et al.*, 2006)

The purple non-sulfur bacteria (*Rhodospirillaceae*) especially *Rhodobacter sphaeroides* are the most studied producer of homopolymer or copolymer of PHA under aerobic and anaerobic conditions. The UV mutant strain of *Rhodobacter sphaeroides* ES16 was found to accumulate PHB (Madmarn 2002). The strain was further studied on the identification and effect of co-substrate on the production of PHB and its copolymer PHBV.

## Materials and Methods

### Microorganism

The halotolerant photosynthetic bacterial strain U7 was the UV mutant of *Rhodobacter sphaeroides* ES16 (Madmarn 2002). The culture was maintained in glutamate-malate (GM) medium containing 3% NaCl (Tangprasitiparp *et al.*, 2007) using stab technique, kept at about 4°C and subcultured every 2 months.

### Cultivation medium

Glutamate-acetate (GA) medium contained (g/l): L-glutamic acid 3.8, $CH_3COONa$ 3.26, yeast extract 2.0, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, $(NH_4)_2SO_4$ 0.2, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.053, $MnSO_4.5H_2O$ 0.0012, and NaCl 30, vitamin (mg/l): nicotinic acid 1.0, thiamine 1.0, biotin 0.01, pH was adjusted to 7.0 (Sangkharak *et al.*, 2005). This media was used as basal medium for production of intracellular polymer.

### Dry cell weight

Culture sample (10 ml) was centrifuged (15,000 xg, 15 min, 4°C) and the cell pellet was washed with deionized water, recovered by centrifugation again, and dried (105°C, 24 h) to constant weight (Jung *et al.*, 2000).

### Determination of PHB

#### The qualitative analysis of PHB

A sodium dodecyl sulfate solution (1% w/v sodium dodecyl sulfate, 10 ml, pH 10) was added to the biomass pellet. The mixture was incubated on an orbital shaker (60 min, 200 rpm, 37°C). The solids were recovered by centrifugation (15 min, 15,000 x g) and washed with 1 ml commercial sodium hypochlorite diluted to 5 ml. The pellet was centrifuged (15 min, 15,000 x g), washed with deionized water (5 ml), and centrifuged again. The final pellet was dried (105°C, 24 h) to constant weight in preweighed aluminum dishes. The PHB yield coefficient relative to biomass was calculated as the mass of PHB obtained per unit dry cell weight (Grothe and Chisti, 2000). Measurements were performed in triplicate.

#### The quantitative analysis of PHBV

Cells were harvested, lyophilized and subsequently treated with chloroform/methanol/sulfuric acid (1ml/0.85ml/0.15ml) at 100°C for 140 min to convert fatty acids to their corresponding methyl esters. The samples were analyzed by gas chromatograph and mass spectroscopy (GC-MS). After cooling to room temperature, 1.5 ml deionized water was added and the mixture was vortexed. The layers were allowed to separate, and the organic layer (bottom) containing the methyl esters was removed and dried over anhydrous sodium sulfate. The β-hydroxymethylesters were assayed. The compositions of the methyl ester monomers were confirmed by GC-MS using an HP 5890 GC with an HP 5972 mass selective detector. Separations were made with an HP-5 column (30 mm × 0.25 mm × 25 mm). The injector and detector temperature for the GC-MS were 230°C and 240°C, respectively, and an oven temperature program (hold at 100°C for 2 min, then increased to 230°C at 10°C/min) was used to separate the methyl esters

(modified from Ganzeveld *et al.*, 1999).

**Determination of volatile fatty acid (VFA)**

**Volatile fatty acid determination**

Volatile fatty acid was determined by GC in a AutoSystem XL gas chromatograph (Perkin Ekmer Co, USA) equipped with a capillary column OPTIMA 5 (0.1 mm × 0.1μm × 10m), after acidification of sample with 0.2 N HCl. Acetic acid, propionic acid and valeric acid (Sigma) were used as external standard (modified from Silva *et al.*, 2000)

**Identification of the mutant strain U7**

**Classification on the group of photosynthetic bacteria**

The 24 h culture of halotolerant photosynthetic bacteria mutant U7 was inoculated on sulfide medium and thiosulfate medium (Watanabe *et al.*, 1981) under anaerobic- light (3,000 lux) condition. No growth performance on sulfide medium and thiosulfate medium could divide to the family of *Rhodospirillaceae*; purple non-sulfur bacteria (Staley *et al.*, 1989).

**Determination of physiological and nutritional characteristics**

The strain was characterized by morphology, substrates photoassimilation, vitamin requirement and Gram staining. Cell morphology was observed by using thin-section electron microscopy and transmission electron microscopy (TEM). The motility was observed by stabbing the culture into semi-solid medium and inoculated into broth in order to determine slime formation. Nutritional requirement was used for determination of Photolithotroph (Photoautotroph) or Photoheterotroph, Chemolithotroph or Chemoheterotroph using succinate medium (Staley *et al.*, 1989). For photoassimilation experiments, simple media (Watanabe *et al.*, 1981) was used to activate growth. The medium contained 0.5% each of nitrogen source (ammonium sulfate) and various carbon sources; benzoate, citrate, malate, lactate, glucose, glycerol, fructose, acetate, sorbitol, glutamate, propionate, tartrate and succinate.

**Absorption spectra and electron microscopy**

The absorption spectra of cell suspensions in 4 M sucrose were recorded against a blank (4 M sucrose) for determination of bacteriochlorophyll (Heising *et al.*, 1996) using a Hitachi U-2000 scanning spectrophotometer. The approximate maximum absorption of major peaks was determined. A transmission electron microscope (TEM, JEM-2010, JEOL), set at 0.3 nm point-to-point resolution and 50,000 x magnification, was used to examine the isolate. Slide preparation was used for general microscopy and Gram staining.

**Study of 16S rDNA technique**

The 16S rRNA gene was amplified using two primers; UFUL (5'-GCCTAACACATGCAA GTCGA-3') and 536R (5'-GTATTACCG CGGCT GCTGG-3') and sequenced using Automate DNA Sequencer (3100-Avant Genetic Analyzer, ABI). DNA sequences from 16S rDNA were compared with other sequences in Gene Bank (http://www.ncbi.nlm.nih.gov). The 16S rDNA was amplified and sequenced by Central Instrument Facility (CIF) and MU - OU: CRC at Mahidol University.

**Effect of the co-substrate carbon source and concentration for production of copolymer poly-β-hydroxybutyrate-co-β-hydroxyvalerate; PHBV**

Starter culture was prepared by cultivating the halotolerant photosynthetic bacterial strain U7 in GA medium containing 40 mM acetate as carbon source, pH was adjusted to 7 (by sterile 3 N NaOH) and incubated at 37°C on rotary shaker (200 rpm) for 24 hrs. The culture adjusted to OD660 at 0.5 was used as starter culture.

The starter culture (10%) was added into GA medium (pH 7) to study on the effect of auxiliary carbon source, propionic acid and valeric acid at 20, 40 and 80 mM. Cultivation was conducted under aerobic-dark condition at 200 rpm at 37°C for 96 hrs and samples (10 ml) were taken for measurement of growth, pH and production of PHB and PHBV. Copolymer production was determined by GC-MS.

## Results and Discussion

### Identification of the mutant strain U7

The halotolerant photosynthetic bacterial mutant strain U7 was identified based on morphology, pigment (bacteriochlorophyll) and photoassimilation substrates. The mutant strain U7 exhibited the ability to utilize organic substance for carbon source and received energy from light. Therefore, the strain could be considered as photoheterotroph. No growth in sulfide and thiosulfate medium could classify the mutant strain U7 to the family of *Rhodospirillaceae* or purple non-sulfur bacteria. The mutant strain was Gram-negative, ovoid-shaped motile bacterium with a length of 0.8-1.0 μm and a width of 0.3-0.5 μm (Figure 1). The cells divided symmetrically by binary fission. The bacteria formed smooth, round and pink to dark red colonies with a diameter of 2-4 mm on agar media and brownish-red cell suspension in liquid media under phototrophic conditions including red cell suspension under aeration without light. The strain produced photosynthetic pigments when grew under anoxygenic phototrophic conditions. Absorption spectra of cell suspension in 4 M sucrose had three major peaks at 588, 800 and 877 nm indicating that bacteriochlorophyll *a* was present. There were no absorption peaks for bacteriochlorophyll *b* and *c* which are nornallly the characteristics of purple non-sulfur bacteria. The maxima between 450 and 550 nm indicated the presence of carotenoids of the spheroidene series (Heising *et al.*, 1996). Transmission electron micrograph of this strain illustrated the presence of PHA as the inclusion body (Figure 2).

The photoassimilation experiments, simple media were used to support the growth. This strain appeared in oval to spherical shape and could be classified to genus *Rhodobacter, Rhodopseudomonas* and *Rhodomicrobium*. The cell multiplied by binary fission which could be classified to genus *Rhodobacter*. Genus *Rhodobacter* consisted of 5 species namely; *Rhodobacter capsulatus, R. sphaeroides, R. sulfidophilus, R. adriaticus* and *R. veldkampii*. Determination of electron donor or

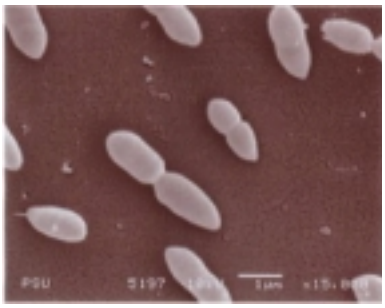

**Figure 1. Scanning electron micrograph (SEM) (x15,000) of the halotolerant photosynthetic bacterial mutant strain U7.**

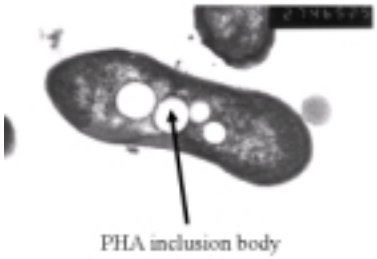


**Figure 2. Transmission electron micrograph of the halotolerant photosynthetic bacterial mutant strain U7 indicated PHA as the inclusion body.**

utilization of each carbon source under anaerobic-light condition (photoassimilation) could identify the strain to species level. All species of *Rhodobacter* are able to utilize acetate, propionate, lactate, malate, succinate, glucose and glutamate but could not utilize benzoate. *R. capsulatus, R. sphaeroides* and *R. sulfidophilus* can utilize fructose, mannitol and sorbitol while citrate could be used by *R. capsulatus* and *R. sphaeroides* and glycerol by *R. sphaeroides* and *R. sulfidophilus*. The use of tartrate could identify the strain to *R. sphaeroides*. Both biotin and thiamine were absolutely required as growth factors whereas NaCl was not required. Results of photoassimilation were given in Table 1 and strain U7 was identified as *Rhodobacter sphaeroides*.

Result of homology analysis of the sequenced 16S rDNA from 364 base pairs were blasted with the blast program on the web site of

**Table 1. Morphological and biochemical characteristics of the photosynthetic bacterial mutant strain U7.**

| Characteristics | Results | | | | |
|---|---|---|---|---|---|
| 1. Growth form | Photoheterotroph, Chemoheterotroph | | | | |
| 2. Growth on sulfide and thiosulfate | - | | | | |
| 3. Bacteriochlorophyll | *a* | | | | |
| 4. Gram staining | negative | | | | |
| 5. Cell shape | ovoid | | | | |
| 6. Cell size (μm) | 0.3-0.5 x 0.8-1.0 | | | | |
| 7. Motility | + | | | | |
| 8. Gelatin degradation | - | | | | |
| 9. Slime formation | + | | | | |
| 10. Visible colour of cell suspension | An-L : brownish red, Ae-D : red | | | | |
| 11. Vitamin requirement | biotin, thiamine | | | | |
| 12. Carbon source for electron donor | | | | | |
| Acetate | ++ | ++ | ++ | ++ | ++ |
| Propionate | ++ | ++ | ++ | ++ | ++ |
| Butyrate | ++ | ++ | ++ | — | ++ |
| Lactate | ++ | ++ | ++ | ++ | ++ |
| Malate | ++ | ++ | ++ | ++ | ++ |
| Succinate | ++ | ++ | ++ | ++ | ++ |
| Tartrate | ++ | — | — | — | — |
| Citrate | + | + | — | — | — |
| Glutamate | ++ | ++ | ++ | | ++ |
| Benzoate | — | — | — | — | — |
| Glucose | ++ | ++ | ++ | o | ++ |
| Fructose | ++ | ++ | + | — | — |
| Mannitol | ++ | + | + | — | — |
| Sorbitol | ++ | + | + | o | — |
| Glycerol | ++ | — | ++ | ++ | — |
| 13. Identified as | *Rhodobacter sphaeroides* | *Rhodobacter capsulatus* | *Rhodobacter sulfidophilus* | *Rhodobacter adriaticus* | *Rhodobacter veldkampii* |
| 14. References | This study | | Staley *el al.*, 1989 | | |

**Note + represent positive result, - represent negative result, An-L represent Anaerobic-Light condition, Ae-D represent Aerobic-Dark condition. ++ represent well growth, + represent a little growth. — represent no growth, o represent not determined.**

Gen-Bank: http://www.ncbi.nlm.nih.gov (Figure 3) and this confirmed the above results indicated high similarity with 99 % identity to *Rhodobacter sphaeroides* (gi/56541583/dbj/AB196355.1). In addition, phylogenetic analysis of the mutant strain U7 was based on the 16S rDNA gene sequence and also grouped the mutant strain U7 with other selected photosynthetic bacteria members in alpha subdivision of the division Proteobacteria generated from an alignment of approximate 400 bp of 16S rDNA obtained from GenBank database (Figure 4). The mutant strain U7 was identified to be *Rhodobacter sphaeroides* which is in agreement with the classical identification as mentioned above.

Identification procedure in this experiment was the same as Hiraishi and Ueda (1995) who isolated and characterized some purple nonsulfur bacteria from colored blooms in tidal and seawater pools and identified as *Rhodovulum stricum*.

| | | | | |
|---|---|---|---|---|
| 1 | GACTTAGCGG | CGGCACGGGT | GAGTAACGCG | TGGGAACGTG |
| 41 | CCCTTTGCTT | CGGAATAGCC | CCGGGAAACT | GGGAGTAATA |
| 81 | CCGAATGTGC | CCTTTGGGGG | AAAGATTTAT | CGGCAAAGGA |
| 121 | TCGGCCCGCG | TTGGATTAGG | TAGTTGGTGG | GGTAATGGCC |
| 161 | TACCAAGCCG | ACGATCCATA | GCTGGTTTGA | GAGGATGATC |
| 201 | AGCCACACTG | GGACTGAGAC | ACGGCCCAGA | CTCCTACGGG |
| 241 | AGGCAGCAGT | GGGGAATCTT | AGACAATGGG | CGCAAGCCTG |
| 281 | ATCTAGCCAT | GCCGCGTGAT | CGATGAAGGC | CTTAGGGTTG |
| 321 | TAAAGATCTT | TCAGGTGGGA | AGATAATGAC | GGTACCACCA |
| 361 | GAAG | | | |

**Figure 3. 16S rDNA sequence of mutant strain U7 by using UFUL and 536R as orward and reverse primer for PCR.**

Similarly, the purple non-sulfur bacterium *Rhodopseudomonas palustris* was isolated from an alkaline lake sample in Turkey and identified by Donmez *et al.* (1999). *Rhodopseudomonas* spp. was found in mature microbial mats used for metal sequestration and organic pollutant degradation experiments (Mehrabi *et al.*, 2001). Moreover *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* could utilize taurine (Novak *et al.*, 2004). The purple non-sulfur bacteria strain PS9 isolated from an anaerobic swine waste lagoon was identified as *Rhodobacter* sp. (Do *et al.*, 2003).

**Effect of the co-substrate carbon source and concentration for production of copolymer poly-β-hydroxybutyrate-co-β-hydroxyvalerate; PHBV**

The volatile fatty acids were used as co-substrate or auxiliary carbon source for cellular growth and copolymer production from *R. sphaeroides* U7 cultivated in GA medium with 40 mM acetate as the main carbon source. The concentrations of acetate and propionic acid or valeric acid were the molar ratio of 40/0, 40/20, 40/40 and 40/80.

The initial concentrations of propionic acid (0-80 mM) had influence on cell growth and PHBV accumulation for this mutant strain. Growth of *R. sphaeroides* U7 appeared as diauxic growth with the first lag phase in 24 h and slightly increased until 72 h when they started to increase again (Figure 5). PHA accumulation increased as growth increased, therefore, it was growth-associated substance. The maximum PHA concentration of 2.37 g/l was significantly different ($p<0.05$) from the values of 1.81, 1.88 and 1.63 g/l obtained from the molar ratio of acetate to propionic acid of 40/0, 40/20, 40/40 and 40/80, respectively after 60 h cultivation. In addition, the PHA contents in the cells were 82.29, 70.57, 77.68 and 72.37%, respectively (Table 2). The composition of PHA monomers analyzed by GC-MS indicated that the HB fractions were in the range of 80-100% whereas the HV fractions were present only in much smaller amount (0-20%). This strongly illustrated that *R.sphaeroides* U7 in culture medium supplemented with propionic acid as co-substrate gave higher mol percentage of HB unit fraction than HV unit fraction. This was due to the fact that β-oxidation of propionic acid converts propionyl-CoA to acetyl-CoA that induced to form β-hydroxy-butyryl-CoA as occurred in mutant strain of


**Figure 4. Phylogenetic analysis of the mutant strain U7 and selected photosynthetic bacteria in alpha subdivision of the division Proteobacteria generated from an alignment of 364 bp of 16S rDNA obtained from GenBank database.**

*Burkhoderia* sp. (Silva *et al.*, 2000). β-hydroxy-butyryl-CoA was the monomer for polymerization. Propionic acid was one of efficient co-substrate to use for PHBV production from *Alcaligenes eutrophus* and *Pseudomonas oleovorans* (Kocer *et al.*, 2003). For the mutant strain U7, propionic acid could provide the HV fraction upto about 20% with its concentrations had no significant effect.

The residual substrate concentration in the GA medium measured by GC-FID indicated the assimilation of both acetate and propionic acid for growth and PHA production (Figure 6). Acetic acid concentration decreased continuously with the residual about 10 mM at 24 h and all disappeared at 96 h. Similar profile was obtained for propionic acid but with the residual of 20 mM from the initial concentration of 80 mM. The initial growth within the first 24 h of *R. sphaeroides* U7 seemed to be supported by the smaller molecule of acetate rather than propionic acid which was consumed simultaneously thereafter. The concentration of volatile fatty acid is generally much higher within the cell than outside. A fundamental principle is that different concentrations of a given solute will

**Table 2. Cell yield, PHA and copolymer PHBV contents obtained from *Rhodobacter sphaeroides* U7 after 60 h under aerobic-dark cultivation in glutamate-acetate (GA) medium containing propionic acid as co-substrate.**

| Molar ration of substrate (mM/mM) | DCW (g/l) | PHA (g/l) | PHA content (%) | Copolymer PHBV | |
|---|---|---|---|---|---|
| | | | | HB fraction (mol %) | HV fraction (mol %) |
| Acetate/Propionic acid | | | | | |
| 40/0 | 2.88[a] | 2.37[a] | 82.29[a] | 100 | 0 |
| 40/20 | 2.56[b] | 1.81[b] | 70.57[b] | 80.38 | 19.62 |
| 40/40 | 2.42c | 1.88[b] | 77.68c | 80.88 | 19.12 |
| 40/80 | 2.40c | 1.63c | 72.37[d] | 79.25 | 20.75 |
| Acetate/Valeric acid | | | | | |
| 40/0 | 2.88[a] | 2.37[a] | 82.29[a] | 100 | 0 |
| 40/20 | 2.80[a] | 1.51[b] | 54.04[b] | 20.51 | 79.49 |
| 40/40 | 2.49[b] | 1.93[c] | 77.42[c] | 15.23 | 84.77 |
| 40/80 | 1.33[c] | 0.71[d] | 53.38[d] | 22.83 | 77.17 |

**a,b,c,d, The different character showed the mean difference that is significant at the 0.05 level.**

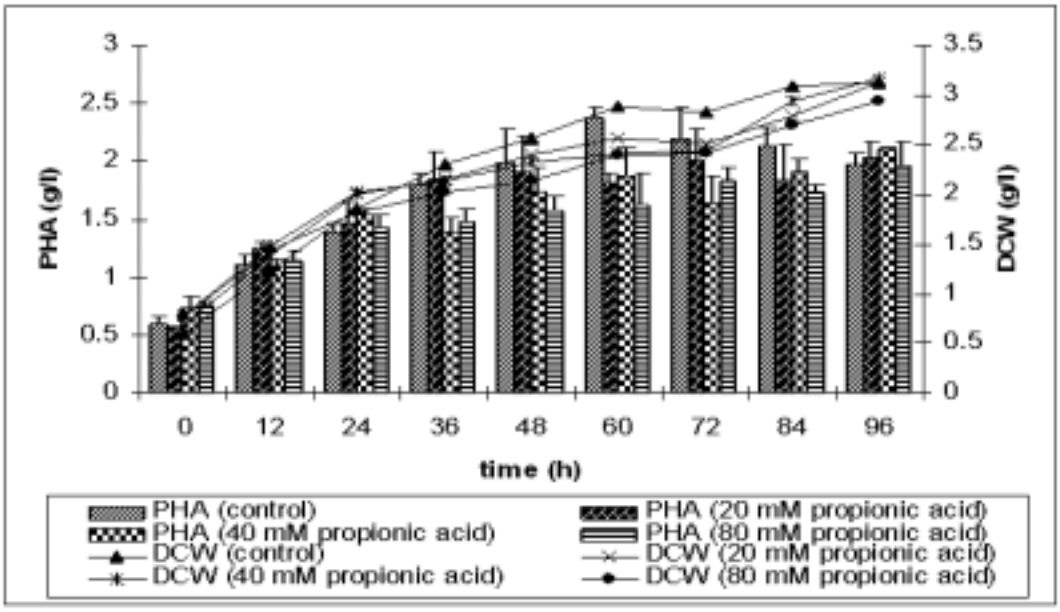


**Figure 5. Effect of propionic acid concentrations on growth and PHA accumulation in *R. sphaeroides* U7 during anaerobic-light cultivation at 37°C in the medium containing acetate as a carbon source.**

tend to equilibrate across the boundary due to diffusion. The volatile fatty acid was transported across cell membrane by facilitated diffusion via transport proteins, such as uniporters and channel proteins, along a concentration gradient from an area of higher concentration to lower concentration. Facilitated diffusion is powered by the potential energy of a concentration gradient and

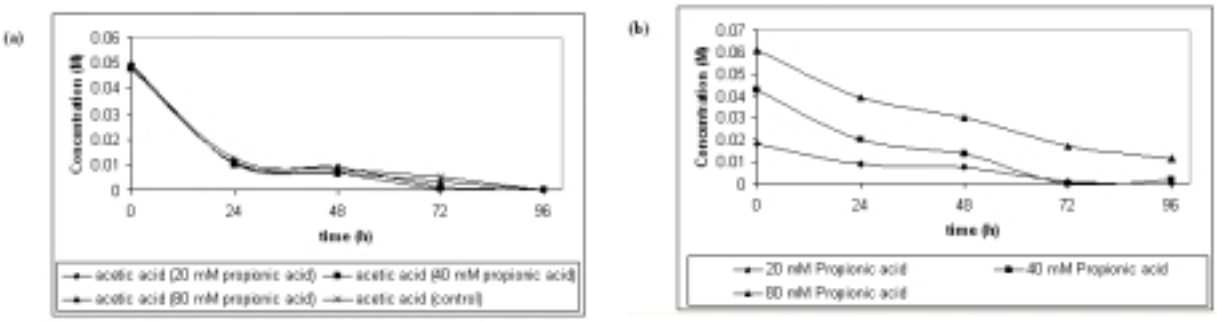


**Figure 6. The residual concentration of acetic acid (a) and propionic acid (b) during growth and PHA accumulation of *R. sphaeroides* U7 cultivated under anaerobic-light condition at 37°C in the medium containing acetate as a carbon source.**

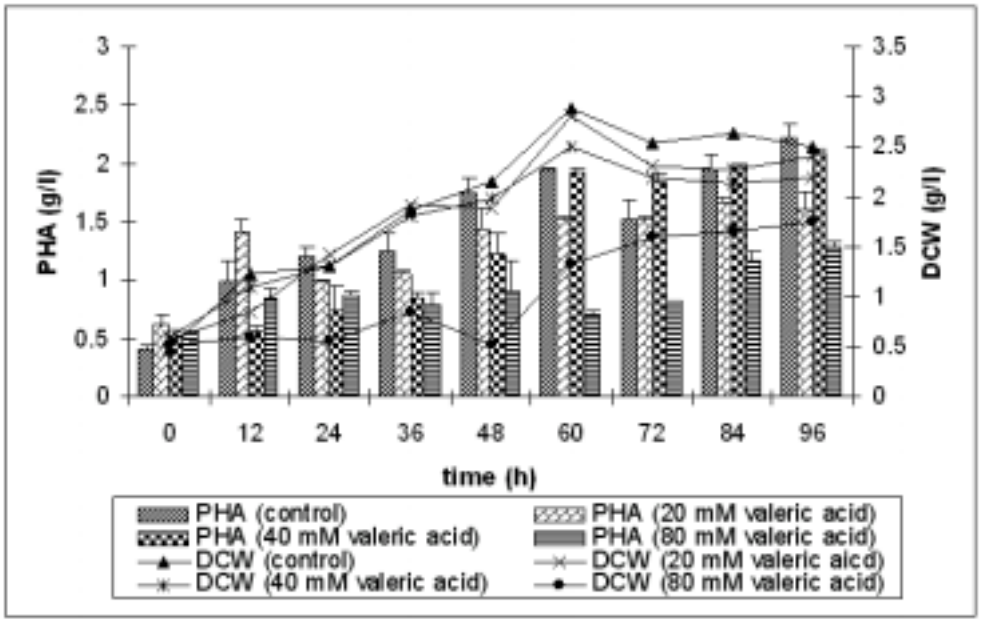


**Figure 7. Effect of valeric acid concentrations on growth and PHA accumulation in *R. sphaeroides* U7 during anaerobic-light cultivation at 37°C in the medium containing acetate as a carbon source.**

does not require the expenditure of metabolic energy (http://www.microbiologytext.com. Cited 26 Nov 2006).

Valeric acid was used as a co-substrate (0-80 mM) to convert to a HV monomer. It had a great influence on cell growth and PHBV accumulation of *R. sphaeroides* U7 (Figure 7). The highest growth (2.88 g/l), PHA concentration (2.37 g/l), and PHA content (82.29%) obtained at the molar ratio of 40/0 at 60 h cultivation were significantly ($p<0.05$) higher than those obtained from the molar ratio of 40/20 (2.80, 1.51 g/l and 54.04%, respectively) and 40/40 (2.49, 1.93 g/l and 77.42%, respectively). At the molar ratio of 40/80, lag phase was very long (48 hrs) as affected by high concentration of valeric acid. The GA medium supplemented with valeric acid gave the mol percentages of HV unit fraction (79.49, 84.77and 77.17) higher than those

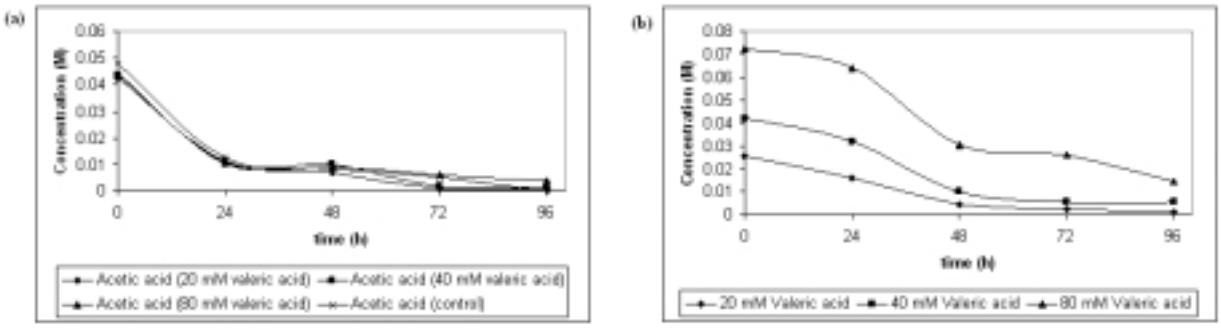


**Figure 8. The residual concentration of acetic acid (a) and valeric acid (b) during growth and PHA accumulation of *R. sphaeroides* U7 under anaerobic-light cultivation at 37°C in the medium containing acetate as a carbon source.**

of HB unit fraction (20.51, 15.23 and 22.83) from the molar ratio of 40/20, 40/40 and 40/80, respectively (Table 3). The above results indicated the increase of HV fraction from 0 to 84.77 mole % when 40 mM valeric acid was used as co-substrate. This was similar to the results from *Alcaligenes latus* in which the mol percentage of HV fraction increased from 15 to 38 % (Ramsay *et al.*, 1990). The HV fraction of this halotolerant mutant of *Rhodobacter sphaeroides* was nearly 6 times higher than that of photosynthetic bacterium *Rhodopseudomonas palustris* SP5212 (14.36 mol %) with the addition of valerate (0.1% w/v) as co-substrate (Mukhopadhyay *et al.*, 2005). Using 1% acetate with 2% fructose as carbon sources, *Rhodobacter sphaeroides* 14F produced PHA with the highest PHA content about 60% but gave less than 3% of hydroxyvalerate in repeating units of polymer (Lorrungruang *et al.*, 2006).

In the case of using acetate with valeric acid as co-substrate, very similar profiles of substrates assimilation was obtained and only difference in the residual valeric acid concentration (65 mM at 24 h and 20 mM at 96 h cultivation) from the initial concentration of 80 mM valeric acid. Valeric acid could be converted to valeryl-CoA, to L-(+)-β-hydroxyvaleryl-CoA, then to the important intermediate 3-ketovaleryl-CoA before partly metabolized to D-(-)-β-hydroxyvaleryl-CoA which is PHBV monomer. This intermediate is used as a substrate for the PHA synthase reaction or PHA polymerase reaction, resulting in the synthesis of the D-(-)-β-hydroxyvaleryl-CoA units (Yamane *et al.*, 1996).

## Conclusion

The halotolerant photosynthetic bacterial mutant strain U7 was identified as *Rhodobacter sphaeroides* using both classical and molecular techniques. For production of copolymer PHBV in the medium with acetate (40 mM) as a carbon source, valeric acid was a better co-substrate as it gave higher HV unit fraction than propionic acid which in turn gave higher HB unit fraction. The optimal molar ratio of acetate to valeric acid for copolymer production was 40/40 which gave the highest poly-β-hydroxyalkanoates (PHA) content of 77.42% in the cells. Repeating unit of the co-polymer contained almost 85% hydroxyvalerate (HV) fraction.

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