บทความวิชาการ

การผลิตพอลิไฮดรอกซีอัลคาโนเอตจากจุลินทรีย์

The Production of Polyhydroxyalkanoate by Microorganisms

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บทคัดย่อ

ปริมาณของขยะพลาสติกที่เพิ่มขึ้นในปัจจุบันกลายเป็นปัญหาใหญ่ทางสิ่งแวดล้อม ข้อเสียของพลาสติกสังเคราะห์คือไม่สามารถย่อยสลายได้และยังก่อให้เกิดมลพิษระหว่างกระบวนการผลิตและการย่อยสลายอีกด้วย พอลิไฮดรอกซีอัลคาโนเอต (Polyhydroxylakanoates, PHAs) เป็นสารประกอบพอลิเอสเทอร์ซึ่งผลิตได้จากแบคทีเรียหลายชนิดทั้งแกรมบวกและแกรมลบ กำลังได้รับความสนใจเป็นอย่างยิ่งเนื่องจากเป็นวัสดุที่ย่อยสลายได้ในธรรมชาติโดยอาศัยกระบวนการและเอนไซม์จากจุลินทรีย์และมีคุณสมบัติคล้ายพลาสติกสังเคราะห์ วิธีการทางชีวภาพที่ใช้ในการผลิต PHAs ได้ถูกพัฒนาขึ้นหลากหลายเพื่อให้ได้ PHAs ในปริมาณมาก โดยอาศัยการผลิตของเชื้อจุลินทรีย์ในธรรมชาติและเชื้อที่ผ่านการทำพันธุวิศวกรรม เชื้อที่ได้รับความนิยมใช้ในการผลิตปัจจุบันได้แก่ *Cupriavidus necator Pseudomonas* sp. และ *E. coli* นอกจากนี้การใช้เชื้อจุลินทรีย์สังเคราะห์แสงยังสามารถผลิต PHAs ได้ในปริมาณ 50-88% ต่อน้ำหนักเซลล์แห้ง สำหรับวิธีที่ดีที่สุดในขณะนี้น่าจะเป็นการผลิตจากเชื้อที่ทำพันธุวิศวกรรม ถึงแม้ว่าการผลิตดังกล่าวยังมีปัญหาในเรื่องความคงที่ของยีน การควบคุมปริมาณผลผลิตและราคาของ PHAs ที่ได้

คำสำคัญ : พลาสติกย่อยสลายได้ PHAs PHB พอลิไฮดรอกซีอัลคาโนเอต พอลิไฮดรอกซีบิวทิวเรต

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Abstract

A huge amount of plastic garbage has become a major concern in terms of the environment. The disadvantage of synthetic plastics is that take many decade to be decompose in the nature as well as many toxins produced during the production and degradation process. Polyhydroxyalkanoates (PHAs), polyester compounds naturally accumulated by many Gram-positive and Gram-negative bacteria, are being concerning as an environmental friendly plastics. PHAs completely degraded by an action of various microorganisms and they had similar properties to synthetic plastic. Attempts based on several techniques have been undertaken for mass production of PHAs using natural and genetic-microorganisms. The well known bacteria use for PHAs production such as *Cupriavidus necator Pseudomonas* sp. and *E. coli* in addition to production by photosynthetic bacteria which produced PHAs up to 50-88% of dry cell weight. Although excellent progress has been made in recombinant bacteria, however, the main problems to obtain constant gene expression, control of productivity rate and price of PHAs have still remained.

Keywords : Biodegradable Plastic, PHAs, PHB, Polyhydroxyalkanoate, Polyhydroxybutyrate

1. Introduction

Biodegradable plastics, a substitute for petroleum-derived plastic are being considered as environmental friendly plastics. The best candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have played much attention due to their properties are similar to synthetic plastics. PHAs are a class of natural polyesters, deposited intracellularly in the form of inclusion and may accumulate for up to 90% of the dry cell weight, (DCW) [1, 2]. The simplest and most common member of the PHAs family is polyhydroxybutyrate (PHB). It consists of only one type of monomer, 3-hydroxybutyrate (3-HB). However, about 150 hydroxyalkanoic acids (HAs) rather than 3-HB have been identified as constituents of microbial polyesters as shown in Fig. 1 [3, 4, 5].

PHAs are biopolyester accumulated by a wide range of organisms as a carbon and energy reserves under nutrient restricted condition. To make the production of PHAs feasible for industrial application, research and development to increase the PHAs production with high yield is necessary. This paper reviews various microorganism and substrates to produce significant amounts of PHAs by natural and genetic PHAs-producing bacteria and the advantage and disadvantage of various methods were also reported.

2. The production of PHAs by natural PHAs-producing microorganisms

Many species of bacteria accumulated submicron inclusion bodies composed of PHAs (Table 1). The list of such microorganisms is growing and currently contains more than 300 organisms [6, 7, 8, 9, 10].

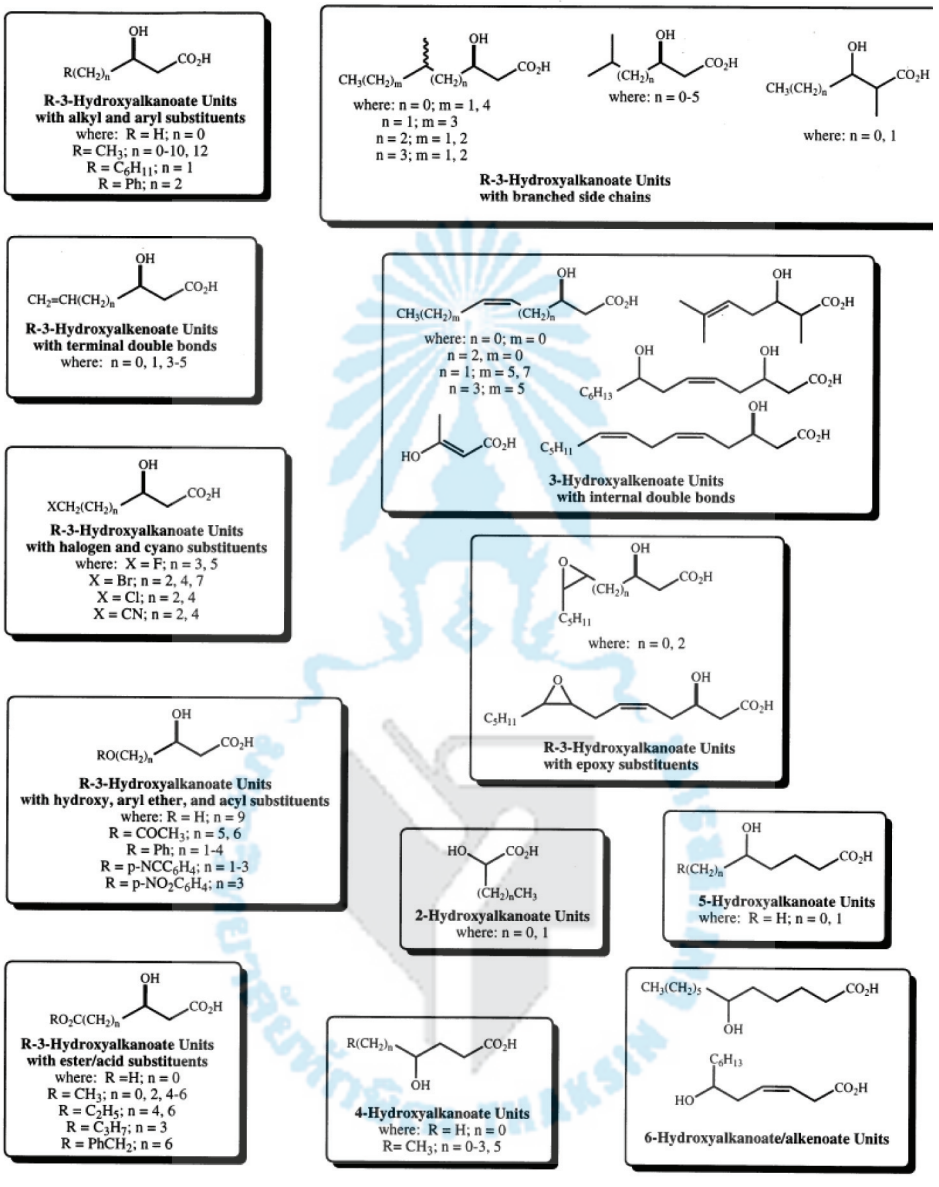

Fig. 1 Examples of monomers that can be incorporated into PHAs polymers.

Source: [3]

Table 1 Poly(3-hydroxybutyrate)-accumulating microorganisms.

Acinetobacter	*Clostridium*	*Micrococcus*	*Rhodopseudomonas*
Actinomycetes	*Derxia*	*Microcoleus*	*Sphaeotilus*
Alcaligenes	*Ectothiorhodospira*	*Microcystis*	*Spirillum*
Aphanothece	*Escherichia*	*Moraxella*	*Spirulina*
Aquaspirillum	*Ferrobacillus*	*Mycoplana*	*Streptomyces*
Azotobacter	*Gamphosphaeria*	*Nitrobacter*	*Syntrophomonas*
Azospirillum	*Haemophilus*	*Nitrococcus*	*Thiobacillus*
Bacillus	*Halobacterium*	*Norcadia*	*Thiocapsa*
Beggiatoa	*Hypromicrobium*	*Oceanospirillum*	*Thiocystis*
Bijerinckia	*Lamprocystis*	*Paracoccus*	*Thiodictyon*
Caulobacter	*Lampropaedia*	*Photobacterium*	*Thiopedia*
Chlorofrexeus	*Leptothrix*	*Pseudomonas*	*Thiosphaera*
Chlorogloea	*Methylobacterium*	*Rhodobacter*	*Vibrio*
Chromobacterium	*Methylosinus*	*Rhodospirillum*	*Xanthobacter*

Among several microorganisms that are known to synthesize PHAs, only a few bacteria have been utilized for the PHAs production. These include *Cupriavidus necator* [1, 11, 12, 13], *Alcaligenes latus* [14], *Azotobacter vinelandii*, several strains of *Methylotrophs* and *Pseudomonas* species [15, 16]. As well as, halotolerant photosynthetic bacteria produce high amount of PHAs (40-90% of DCW) [17, 18, 19, 20, 21, 22]. Aforementioned bacteria have been selected because they can be cultivated efficiently to high cell densities with a high PHAs content (defined as the ratio of PHAs concentration to dry cell concentration expressed as a percentage) in a relatively short period of time, resulting in high PHAs productivity (defined as g PHAs produced l^{-1} h^{-1}) [23].

In summary, PHAs content and its composition are influenced mainly by the strain of microorganism, the type of substrate employed and its concentration and environmental growth conditions. The production of PHAs by different microorganisms using various substrates is summarized in Table 2.

Table 2 Examples of natural bacteria for the biotechnological production of PHAs.

Organism	Substrate ($g\ l^{-1}$)	PHAs % of DCW	References
C. necator	Wheat-based biorefinery	60.0	[24]
C. necator	Corn syrub	31.9	[25]
C. necator	Commercial glycerol (30 $g\ l^{-1}$)	62.0	[26]
	Waste glycerol (30 $g\ l^{-1}$)	38.0	
Pseudomonas putida KT2442	Sodium octanate (10 $g\ l^{-1}$)	66.0	[27]
P. putida KTMQ01	Sodium octanate (10 $g\ l^{-1}$)	86.0	[27]
P. aeruginosa 47T2	Cooking oil	36.0	[28]
Bacillus flexus	Sucrose (20 $g\ l^{-1}$)	45.0	[29]
	Sucrose (20 $g\ l^{-1}$) + castor oil (2 $g\ l^{-1}$)	55.0	
B. cereus SPV	Glucose (20 $g\ l^{-1}$)	38.0	[30]
B. cereus EGU44	Glucose (10 $g\ l^{-1}$)	52.5	[31]
B. cereus EGU3	Glucose (10 $g\ l^{-1}$)	66.6	
B. subtilis EGU163	Glucose (10 $g\ l^{-1}$)	15.5	
B. licheniformis EGU90	Glucose (10 $g\ l^{-1}$)	2.7	
Bacillus sp. EGU91	Glucose (10 $g\ l^{-1}$)	5.3	
Bacillus sp. EGU85	Glucose (10 $g\ l^{-1}$)	30.0	
Caldimonas taiwanensis	Gluconate	14.0	[32]
	Sodium gluconate (15 $g\ l^{-1}$)	70.0	
	Fructose (15 $g\ l^{-1}$)	62.0	
	Maltose (15 $g\ l^{-1}$)	60.0	
	Glycerol (15 $g\ l^{-1}$)	52.0	
	Cassava starch (15 $g\ l^{-1}$) + valerate (0.5 $g\ l^{-1}$)	67.0	
	Corn starch (15 $g\ l^{-1}$) + valerate (0.5 $g\ l^{-1}$)	65.0	
Vibrio sp. (MK4)	Glucose (20 $g\ l^{-1}$)	45.0	[33]
Nostoc muscorum	Glucose (1.6 $g\ l^{-1}$) + Acetate (1.6 $g\ l^{-1}$)	45.6	[34]
Methylobacterium extorquens DSMZ 1340	Methanol (6 $g\ l^{-1}$)	62.3	[35]
Enterobacter aerogenes EGU16	Glucose (10 $g\ l^{-1}$)	7.3	[31]
Proteus mirabilis EGU32	Glucose (10 $g\ l^{-1}$)	33.5	[31]

The advantages of natural PHAs-producing bacteria such as easy to control and operate fermentation system as well as natural strain give high cell densities with high PHAs content. For example, a batch culture of *C. necator* with glycerol concentration control and nitrogen limitation gave a production of PHAs around 38-62% of DCW [26]. *P. putida* KT2442 and KTMQ001 were able to use sodium octanoate (10 g l^{-1}) as the sole carbon source for growth and for the accumulation of PHAs. Approximately 20-25% of the components of the substrate were converted into PHAs. The corresponding yields were 66 and 86% of the DCW for *P. putida* strain KT2442 and KTMQ001, respectively [27]. Thermophilic bacterium namely *Caldimonas taiwanensis* has been studied for PHAs accumulation with soudium gluconate, fructose, maltose and glycerol. In a fully automated batch culture PHAs levels of 70, 62, 60 and 52% of DCW were obtained, respectively [32]. However, high cost of substrate has become the major concern in term of PHAs cost. Even though, many researchers attempted to obtain simple and cheap substrates which can utilize by natural PHAs-producing bacteria, however, external carbon sources still required [24, 25, 26, 28, 29, 32]. *Bacillus flexus*, with castor oil concentration of 2 g l^{-1}, and addition of sucrose (20 g l^{-1}) and complex nitrogen source, accumulated 55% PHAs of DCW [29].

Interesting the production of PHAs by photosynthetic bacteria was also reported in high accumulation [36] compared to aforementioned strains use in an industrial scale. However, only little information has been published. *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* intracellularly produce PHAs content of 45 and 60-80%, respectively under nitrogen-limited source [17, 18, 19, 20, 21, 22]. Some cyanobacteria (e.g. *Aphanothece* sp.) can accumulate PHAs under oxygenic photosynthesis, but their PHAs content is usually not higher than 0.04 -6% of DCW [34].

The natural photosynthetic bacteria are able to produce substantial amount of PHAs and gaseous hydrogen (H_2) under aerobic light culture. This photoproduction was first discovered in 1949 [37]. The wild type *R. sphaeroides* was grown in mineral medium with 7 mM glutamate as the nitrogen source and various substrate e.g. acetate, lactate, pyruvate. With acetate, the wild type produced large amount of PHB up to 70% of DCW but did not produce any H_2, on glucose and fructose PHB production was low because the cell ceased to grow very slowly [36]. The cultivation of *R. sphaeroides* RV on acetate as a sole carbon source under nitrogen-deprived condition gave 40% of DCW [18]. *R. sphaeroides* strain 14F cultivated in a two-stage aerobic dark culture at 37-40 ^{o}C. This strain showed high PHAs production (3.5 g l^{-1} PHAs with 60% PHAs content). Its productivity was 2-3 times higher than other PHAs product from photosynthetic bacteria [38].

There are many advantages of photosynthetic bacteria such as these strains are able to utilize low cost substrate or waste as sole carbon source [17, 39, 40] and high cell density response to high PHAs content. The productions of PHAs from waste materials by *R. spheroides* have been studied from the waste water of sugar refineries and palm oil waste [17, 39]. The production of PHB 60% of DCW was obtained when *R. sphaeroides* IFO12203 was cultivated in palm oil mill effluent (POME) with acetic and propionic acid as carbon sources [17]. The PHAs production from acetic acid by *R. sphaeroides* S and *R. sphaeroides* IL106 gave PHAs content of 38.7 and 66.5% in the cells and 0.30-0.73 g l^{-1} in the medium, respectively [40]. However, all the photosynthetic bacteria as mentioned above were cultured in simple anaerobic light culture along cultivation time, illumination cost was be high, making this culture impractical. Nowadays, the highest

PHAs production was obtained by mutant strains of halotolerant *R. sphaeroides* ES16 designated as N20 which produced PHAs under aerobic-dark condition in glutamate-acetate (GA) medium. The highest PHAs production (8.02 g l^{-1} PHAs with 88% PHAs content) was observed [21]. These results are the highest values ever obtained from photosynthetic bacteria reported so far. Nevertheless, the limitation of photosynthetic bacteria is difficult to recover cell while cultivation in waste as well as high operate cost consume for operation the process under light condition.

2. The production of PHAs by genetic PHAs-producing microorganisms

The most well known of recombinant natural PHAs producer are reported in recombinant of *C. necator* and *Pseudomonas* sp. Recombinant *C. necator* harboring *pha*CAB genes from a plasmid, showed increase in PHB levels from 33-40% of the DCW [41, 42]. Although the increase was insignificant but the recombinant *C. necator* strains could reduce 20% of the cultivation time with the same productivity [43]. This is important for commercial production point of view, since the overall productivity of a PHAs plant would be 20% higher. As well as the PHAs accumulation rates in *C. necator* could conceivably be increased by introducing one or more of plasmid containing the PHAs genes, thus raising the levels of enzymes necessary for catalyzing the final reactions leading to PHAs formation [44].

Alcaligenes latus cells over expressing its own cloned *pha*C gene, exhibited the increased rate of PHB biosynthesis and increase in PHB content [45]. The maximum PHB concentration (3.1-3.7 g l^{-1}) and content of PHB (50.2-65% of DCW) in recombinant *A. latus* increased significantly as compared to the *A. latus* (wild type). In a *Rhizobium meliloti* PHB-mutant strain which lacked of PHB synthesized genes, PHB accumulation was restored to wild type level by the introduction of a plasmid encoded *R. meliloti pha*C gene [46]. In *Paracoccus denitrificans*, an additional *pha*C gene on a plasmid doubles the wild-type PHAs levels in a pentanol-grown parent strain [47]. The *pha*CAB operon from *C. necator* was expressed in *Pseudomonas* strains that normally do not accumulate PHB. PHB accumulation was observed in recombinant *P. aeruginosa, P. putida, P. oleovorans, P. syringe* and *P. fluorescems*, while *P. stutzeri* was unable to synthesize PHB with the *C. necator* genes [48]. *C. necator pha*C-negative (*pha*C^-) strain accumulated about 85% PHB of DCW with gluconate as a carbon source, when transformed with *pha*C gene from *C. violaceum.* However, *P. putida pha*C^- did not accumulate PHAs even when transformed with the *C. violaceum pha*C [27, 49]. *Synechococcus* sp. harboring PHAs biosynthetic genes from *C. necator* accumulated PHB up to 25% of DCW [50].

However the natural producer like *C. necator* has several disadvantages such as the production ability affected by nutrient and environmental limitations as well as this strain grew slowly and was difficult to lyse [1]. On the other hand, *E. coli* is genetic well characterized. Not being a natural PHAs accumulator, PHAs production has to be metabolically engineered in *E. coli* and it does not have any depolymerase activity to degrade accumulated PHAs. The expression of PHAs biosynthetic genes of *C. necator* in *E. coli* for PHAs synthesis opened up the avenues for PHAs production by recombinant organisms [51]. For cost reduction purpose, waste or cheap carbon sources such as molasses, whey etc. have been used for PHAs production [52, 53]. Recent research on the production of PHAs by recombinant bacteria is summarized in Table 3.

Table 3 Examples of recombinant bacteria for the biotechnological production of PHAs.

Organism	Gene containing	Substrates	PHAs % of DCW	References
P. putida KTMQ01	*pha*Z knockout mutant	Sodium octanate (10 g l^{-1})	80.0	[27]
P. putida KTOY06	*fad*BA deletion mutant	Mineral medium + Na-heptanoate (10 g l^{-1})	71.0	[54]
			52.8	
		Luria Bertani medium+ Na-heptanoate (10 g l^{-1})		
P. putida KCTC1639	*pha*J $_{Pseudomonas}$ + *pha*C $_{1Pseudomonas}$	Octanoic acid (5 g l^{-1})	27.0	[55]
Recombinant *E. coli* XL 10-Gold	*pha*CAB $_{C.necator}$	Glucose (10 g l^{-1}) + propionate (1.5-2.0 g l^{-1})	11.0-13.0	[56]
	*pha*CAB $_{Cnecator}$ + *prp*E gene		29.0	62.0
		Glucose (10 g l^{-1}) + propionate (1.0 g l^{-1})		
	*pha*CAB $_{C.necator}$ + *prp*P gene	Glucose (10 g l-1) + propionate (1.0 g l^{-1})		
Recombinant *E. coli* XL-1 Blue	*pha*C $_{C.necator}$	Glucose (1 g l^{-1})	80	[57]
Recombinant *E. coli* ATCC:PTA 1579	*pha*C $_{Streptomyces\ aureofaciens\ NRRL2209}$	Glycerol (10 g l^{-1})	60.0	[58]
		Glucose (10 g l^{-1})	38.0	
		Palm oil (10 g l^{-1})	28.0	
		Molasses (10 g l^{-1})	10.0	
Table 3 (continued).		Sucrose (10 g l^{-1})	10.0	
Aeromonas hydrophila 4AK4	*pha*PCJ $_{A.caviae}$	Lauric acid (8 g l^{-1})	58.6	[59]
A. hydrophila 4AK4	*pha*AB $_{C.necator}$	Dodecanoic acid (8 g l^{-1}) + Propionic acid (4 g l^{-1})	37.2	[60]
A. hydrophila CQ4	*pha*C $_{2P.stutzeri\ 1317}$	Dodecanoic acid (15 g l^{-1}) + Gluconate (15 g l^{-1})	20.9	[61]
Corynebacterium glutamicum	*pha*CAB $_{C.necator}$	MMTG medium (60 g l^{-1} glucose)	52.5	[62]
Recombinant *C. necator* PHB^{-4}	*pha*C $_{2P.stutzeri\ 1317}$	Gluconate (20 g l^{-1})	40.9	[63]
Herbaspirillum seropedicae Z69	*lac*ZY gene	Glucose (0.05 g l^{-1})	36.0	[65]

Not only the integrated of *pha* gene plasmid in *E. coli* or natural PHAs producer but also new transgenic microbial strains is provided which contain PHAs formation gene inserted on the chromosome. The strains are beneficial in PHAs production because no plasmid need to be maintained, no plasmid loss occurs, homogeneous of PHAs product formation and high PHAs productivity (more than 65%) obtained [1, 10].

A novel recombinant *E. coli* strain VG1 (pTU14) was obtained by cloning the *Vitreoscilla globin* gene (vgb) of *Vitreoscilla* into *E. coli.* Introduction of this gene led to decrease not only in the critical oxygen concentration, but also affected the volumetric oxygen transfer coefficient of the recombinant strain. Initially, the KLa was very high and then decreased quickly with growth of VG1 (PTU14) cells. This decrease caused and was unchanged for a very long time when the DO was lower than about 50%, however, a slight increase appeared when DO decreased to about 10%. Thus, high cell density and PHB accumulation at low production cost was obtained at low DO concentration [65].

In addition to the above mentioned microorganisms, PHB production has also been demonstrated in *Thiosphaera pantotropha, Caulobacter crescentus* DSM 4727 [66], *Azotobacter* sp. strain FA8 [67], *Synechococcus* sp. MA19 [68], *Synechococcus* sp. PCC6803 [69], *Rhodopseudomonas palustris* 42OL [70], *Bacillus cereus* UW85 [71], *B. mycoides* RLJ B-017 [72], *Azospirillum brasilense* [73], *Americoccus kaplicenses* [74] and *Rhodobacter sphaeroides* [75, 36, 76].

At above mentioned, recombinant strains have several advantages over other PHAs-producing organisms such as the ability to utilize wide range of carbon sources, high cell density, easy recovery of PHAs and no degradation of PHAs once synthesized [1, 51, 52, 53]. Therefore, the production of PHAs by improved bacterial strains using recombinant *E. coli* has been focus of much attention. For efficient PHAs production using recombinant *E. coli*, it is critical to understand how gene expression, protein synthesis and metabolism are regulated in relation to change in culture conditions. Comprehension of these regulation mechanisms will be able to control culture conditions so that the PHAs production is maximized [41].

3. Conclusion and future direction

Due to the serious environmental problem and oil crisis, the replacing of synthetic plastic with biodegradable plastic becomes a very interesting and important issue. Many researchers reported on the production of PHAs by natural and genetic PHAs-producing microorganism from several substrates. The further research on PHAs production has been explored using bacteria. Moreover, the protein engineering of PHA synthases to alter their specificity properties so as to produce PHAs with desired monomer composition [10]. The potential for the production of PHAs seems to be limited by the availability and costs of the chemicals which can be provided as precursor substrates to the bacteria. Therefore, the chance to obtain cheap PHAs will depend on the successful screening for simple and cheap substrate which can utilize by bacteria to produce PHAs. Furthermore, improvement of PHAs-producing strain using genetic engineering has been focus of much attention. The great advance of the future study should be studied in PHAs production from photosynthesis tbacteria. Due to this strain relies on carbon dioxide and light as well as the great ability to utilized wide range of substrates. However, only little information about PHAs production by photosynthesis bacteria is available. Even so this method appears to be a realistic goal for the future.

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