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CONSTRUCTION OF KNOCK-OUT MUTANTS OF Escherichia coli BW25113 FOR IMPROVED POLYHYDROXYALKANOATE PRODUCTION

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By

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Polyhydroxyalkanoate~~s~~ (PHA~~s~~) is linear polyester produced through fermentation of sugar or lipid. Biosynthesis of PHA involves three enzymes which are acetyl-CoA acetyltransferase, acetoacetyl-CoA reductase and PHA synthase. Under growth conditions, PHA is synthesized when excess carbon sources and essential nutrients are limited. *Comamonas* sp. is one of the strains commonly used for PHA production. However, the strain consist of PHA depolymerase gene in its genome which will influence PHA production. Thus, *E. coli* was used as a host for PHA production since its genome is well characterized and no depolymerase gene was reported. In this work, PHA biosynthesis operon of *Comamonas* sp. EB172 was introduced into *Escherichia coli* BW25113 through pGEM'-T vector. The strain was used for further modification to enhance PHA production thorough metabolic engineering approach. Metabolic engineering through one-step single deletion approach was carried out to identify specific gene related to PHA metabolism in *E. coli*. Seven genes *pgi, frdC, fdnG, focA gltA, pta,* and *poxB* were found to be associated with PHA metabolism. In addition, P1 transduction was conducted to introduce multiple knock-outs in order to enhance PHA production from *E. coli*. A deletion of two genes of *E. coli* BW25113 *frdCgltA*::kan/pGEM'-*phaCAB*$_{Co}$ has produced 53 wt.% of PHA compared to the control strain *E. coli* BW25113/pGEM'-*phaCAB*$_{Co}$ which was 46 wt.%, respectively. Finally, a combination of three genes deletion were found to give highest PHA production at 64 wt.% as engineered *E. coli* BW25113 *frdCgltApta*::kan/pGEM'-*phaCAB*$_{Co.}$ PHA profiling of was compared with *Comamonas* sp. EB172 and it showed the engineered strain is about 3-fold higher compared to *Comamonas* sp. EB172 which is only 23 wt.%. Overall, the results indicate that the genes deletion has enhanced PHA production and the genes of *frdC, fdnG, focA* and *gltA* were first to report that improve PHA production in *E. coli*.

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PEMBINAAN MUTAN-MUTAN DARIPADA *Escherichia coli* UNTUK MENINGKATAKN PENGHASILAN POLIHIDROKSIALKANOAT

Oleh

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Polihidroksialkanoat (PHAs) adalah jujukan poliester yang dihasilkan melalui fermentasi gula atau lemak. Biosintesis PHA melibatkan tiga enzim iaitu acetyl-CoA acetyltransferase, acetoacetyl-CoA reductase dan PHA synthase. *Comamonas* sp. adalah salah satu bakteria yang biasa digunakan untuk menghasilkan PHA. Walau bagaimanapun, bakteria ini mempunyai gen degradasi di dalam genom yang akan mempengaruhi penghasilan PHA. Oleh itu, *E. coli* telah digunakan sebagai tempat penghasilan PHA kerana genomnya telah dikaji secara mendalam dan ketiadaan gen degradasi dilaporkan. Dalam hasil kerja ini, PHA biosintesis operon daripada *Comamonas* sp. EB172 telah diperkenalkan ke dalam *Escherichia coli* BW25113 melalui pGEM-T vektor. Bakteria ini telah digunakan untuk di ubahsuai bagi meningkatkan penghasilan PHA melalui teknik kejuruteraan metabolik. Kejuruteraan metabolik telah dijalankan melalui teknik langkah pembuangan satu gen bagi mengenal pasti gen tertentu yang berkaitan dengan metabolisma PHA dalam *E. coli.* Tujuh gene *pgi, frdC, fdnG, focA, gltA, pta* dan *poxB* telah di dapati terlibat dengan aktiviti metabolisma PHA. Di samping itu, P1 transduksi telah dijalankan untuk memperkenalkan pembuangan beberapa gen bagi meningkatkan lagi penghasilan PHA daripada *E. coli.* Pembuangan dua gen dalam *E. coli* BW25113 *frdCgltA*::kan/pGEM'-$phaCAB_{Co}$ telah menghasilkan 53 wt.% PHA jika dibandingkan bakteria kawalan *E. coli* BW25113/pGEM'-$phaCAB_{Co}$ sebanyak 46 wt.%. Akhir sekali, pembuangan tiga gen memberi penghasilan PHA tertinggi sebanyak 64 wt.% sebagai manipulasi bakteria *E. coli* BW25113 *frdCgltA pta*::kan/pGEM'-$phaCAB_{Co.}$ Profil PHA telah dibandingkan antara manipulasi bakteria dan *Comamonas* sp. EB172, dimana manipulasi bakteria menunjukkan peningkatan sebanyak tiga kali ganda berbanding *Comamonas* sp. EB172 hanya sebanyak 23 wt.%. Secara keseluruhan, keputusan ini menunjukkan bahawa pembuangan gen-gen dapat meningkatkan penghasilan PHA dan gen *frdC, fdnG, focA* dan *qltA* adalah pertama di laporkan membantu meningkatan penghasilan PHA di dalam E.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

CoA	Coenzyme A
CDW	Cell dry weight
E. coli	*Escherichia coli*
FRT	flanking repeated site
GC	Gas chromatography
GPC	Gas permeation chromatography
HPLC	High performance liquid chromatography
LB	Luria Bertani
Mn	Number average of molecular weight
Mw	Weight average molecular weight
MSM	Mineral salt medium
mcl	medium chain length
PHA	Polyhydroxyalkanote
PHB	Polyhydroxybutyrate
PCR	Polymerase chain reaction
PDI	Polydiversity index
Rpm	Rotation per minute
RE	Restriction enzyme
scl	short chain length
TEM	Transmission electron microscope

CHAPTER 1

INTRODUCTION

1.1 Background of study

Synthetic plastics are among the greatest inventions of mankind since the 1940 that were developed into a major industry and essential commodity in human's life (Sudesh and Iwata, 2008). Plastics have become valuable materials and successfully utilised in a wide range of applications in domestic, medical and industrial fields in the form of disposable gears, packaging and furniture (Khanna and Srivastava, 2009). Since the past few decades, plastic has been widely used and is expected to be continuously utilised until 2020 (Patel *et al*., 2005). However, the accumulation of petrochemical plastic wastes in the environment is increasing, which also affects the survival of many species.

The natural environment was continuously polluted with plastic accumulation as well as the rapid depletion of natural resources used in their production, which further encouraged many researchers to study other sources and tools as the alternatives to petroleum based polymers (Amache *et al*., 2013). Several solutions were taken to enhance plastic waste management such as source reduction, incineration and recycling. However, most solutions proposed were found to have problems such as the employment of plastics incineration is potentially dangerous and expensive as well as that recycling might be tedious and time consuming (Khanna and Srivastava, 2005).

Biopolymer materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are the potential candidates to replace synthetic plastics (Steinbüchel, 2001). Biopolymers have the properties of biodegradability, eco-friendly manufacturing processes and a wide range of applications in many sectors. Besides, most biopolymers are biocompatible, not harmful to the biological systems with some of them are bacterial origin, which produced as a result of defence mechanism or as storage materials (Marjadi *et al.*, 2010; Sukan *et al.*, 2015).

There are many biodegradable plastics developed such as polyhydroxyalkanoates (PHAs) with promising results due to inherent biodegradability, sustainable and environmental-friendly (Salehizadeh and Van Loosdrecht, 2004). PHAs are completely biodegradable, which makes them useful in medical field for cardiovascular system devices, wound management, urological stents, nano- and microspheres for controlled drug delivery (Castilho *et al.*, 2009; Jain *et al.*, 2010; Keshavarz and Roy, 2010).

However, the production cost for PHA is still far above the price of conventional plastics (Salehizadeh and Van Loosdrecht, 2004). Several strategies were applied to upstream metabolic regulation or downstream fermentation optimisation (Chen, 2009; Nikodinovic-Runic *et al*., 2013). Thus, recombinant microbial strains were developed to make the process economically attractive and to achieve both high substrate conversion rate and close packing of PHAs granules in the host cell (Taguchi *et al.*, 2003; Kahar *et al.*, 2005; Agus *et al.*, 2006; Nikel *et al.*, 2006; Sujatha and Shenbagarathai, 2006).

Escherichia coli (*E. coli*) is an ideal host for synthesising PHA due to proper cultivation and adequate studies (Horng *et al.*, 2010). Moreover, the production of PHA in *E. coli* have several advantages such as fast growth, high cell density, the ability to use inexpensive carbon sources and easy purification (Fidler *et al.*, 1992; Hahn *et al.*, 1995). The vast amount of knowledge on *E. coli* genetics and the metabolic necessary for PHA synthesis is important to improve PHA production through metabolic engineering approach. It is expected that *E. coli* will play a role for determining the mechanism of PHA synthesis and commercialisation of PHA products (Khanna and Srivastava, 2005).

1.2 Problem statement

Materials derived from petroleum-based plastics are made up from synthetic polymers and are not biodegradable. The depletion of natural resources and pollution awareness have lead the government, industry and community to seek an economical replacement from petroleum-based into renewable and environmental friendly sources (Chanprateep, 2010).

Generally, PHA biosynthesis process is initiated by three key enzymes known as PHA synthase (*phaC*), *ß*-ketothiolase (*phaA*) and NADPH-dependent acetoacetyl-CoA reductase (*phaB*) (Rehm and Steinbüchel, 1999). PHAs are degraded by either intracellular or extracellular PHA depolymerases. Intracellular depolymerase is able to hydrolyse endegenous carbon reservoir of native PHA granules, which consists of layers of protein and phospholipid (Knoll *et al*., 2009). This is a significant feature to introduce PHA producing operon into a host that do not have a PHA depolymerase gene.

E. coli is natively lack of PHA degradation capacity and due to the extensive studies on its genome, it is easier to manipulate the genome to get a high production of PHA (Aldor and Keasling, 2003). Accumulation of PHA in *E. coli* can be regulated by metabolic engineering (Wang *et al*., 2009). Two important intermediates in PHA production are acetyl-CoA and NADPH (Leong *et al*., 2014). The acetyl-CoA is one of the monomers essential for PHA biosynthesis. Meanwhile, Acetyl-CoA is the important central intermediate in *E. coli* that is consumed through pyruvate pathway.

There are few studies on metabolic engineered strain for PHA improvement. To date, only Jian *et al.* (2010) created multiple gene deletion in *E. coli* for studying PHA. Previous study showed that the inactivation of single *pta* gene in the *E. coli* can help to improve PHA production since the pathway for acetate production using acetyl Co-A can be disrupted, which leads to the accumulation of acetyl-CoA (Miyake *et al.*, 2000). Hence, this study attempts to remove several genes contributing to PHA production in *E. coli* and to create metabolic engineered *E. coli* from glucose for PHA improvement.

PHA biosynthesis genes related (*phaC*, *phaA* and *phaB*) were isolated from *Comamonas* sp. EB172 (Yee *et al.*, 2012a). The *Comamonas* sp. EB172 was found able to utilise mix organic acids (acetic acid: propionic acid: butyric acid) derived from palm oil mill effluent (POME) to produce 59 wt.% of PHAs (Zakaria *et al*., 2008; Mumtaz *et al.*, 2010). The isolated genes were then cloned in one operon namely *phaCAB*$_{Co}$ operon with promoter from *C. necator* (Yee *et al.*, 2012a). The *phaCAB*$_{Co}$ operon was inserted into commercially available modified cloning vector pGEM' identified as pGEM'-*phaCAB*$_{Co}$. The pGEM'-*phaCAB*$_{Co}$ was transformed into *E. coli* JM109 and achieved 46.4% (w/w) PHA with 1% (w/v) glucose as the carbon source and 1% (w/v) of nitrogen source, respectively (Yee *et al*., 2012b). For the continuity study via metabolic engineering approach, the pGEM'-*phaCAB*$_{Co}$ was isolated and transformed into wild-type *E. coli* BW25113 and Keio mutants provided by KEIO library (National Institute of Genetics, Japan) as a new host for metabolic engineering studies to enhance PHA production. In this study, *E. coli* BW25113 serve as the host strain due to its derivative from *E. coli* K-12, one of the well characterised microorganisms in molecular biology with *E. coli* BW25113 used as Keio collection of single gene deletion. Several genes were screened to improve PHA production by removing organic acid byproducts from glycolysis pathway and increase the concentration of acetyl-CoA. Further genes deletion in *E. coli* BW25113 were carried out to improve PHA production via P1 transduction methodology with their properties compared using control strain, *E. coli* BW25113/ pGEM'-*phaCAB*$_{Co.}$

1.3 Objectives

The objectives of this study are:-

1. To construct an engineered *Escherichia coli* strain for PHA production using one-step single gene deletion approach.
2. To produce higher PHA production from engineered *Escherichia coli* strain through multiple genes deletion using P1 phage transduction technique.

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