

Evaluation of Polyhydroxybutyrate Extracted from Recombinant *E. coli* DH5 α Harboring the *phbCAB* Operon

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Abstract–Poly- β -hydroxybutyrate (PHB) is a microorganism-produced member of polyhydroxyalkanoate (PHA) polymer family which is considered as a potential alternative to traditional petroleum-based materials in agriculture and tissue engineering. Generally, natural bacteria generate PHB with lower efficiency than recombinant ones. This study evaluated the quality of PHB powder obtained from recombinant *E. coli* strain harboring *phbCAB* operon of *Alcaligenes eutrophus*. Assessment results proved that the PHB products possessed almost equivalent infrared (IR) absorption spectrum to Stigma's standard PHB powder and heavy element content remained at acceptable level. Glass was the most suitable material for the scaffold casting mould, surpassed other tested materials like teflon, silicon, inox. Optimal physical traits (scaffold thickness, tensile modulus, elongation at break, ultimate tensile strength, water vapor transmission rate) were achieved with 0.75 – 1% PHB casting solution and 9cm diameter Petri dish as the mould. The above results are highly useful for the production of PHB scaffold used in tissue engineering in further studies.

Keyword: PHB, *E. coli* DH5 α , *phbCAB*, scaffold, glass, mould.

I. INTRODUCTION

Biodegradable plastics are catching an increasing interest from the public – especially poly- β -hydroxybutyrate (PHB) which attracts much attention from various researchers. PHB is a polyester with similar physical characteristic to traditional synthetic (thermoplasticity, elasticity, heat resistance) while possessing distinctive advantage of biodegradability without generating toxic products^[1]. This polymer is widely applied in health service, agriculture and industry^[2]. Furthermore, *in vitro* experiment results proved that PHB expresses excellent biocompatibility on various growth medium with many kinds of cell types including fibroblast, mesenchymal stem cell (MSC), osteoblast, myelocyte, chondrocyte, epithelial cell, smooth muscle cell^[3]. Various experiments proved cell adhesion to PHB structure, PHB's role in cell viability and proliferation and its application in tissue repair and regeneration^[4]. It is also possible to modify PHB structure to change its properties for different conditions and requirements. Electrospinning, for example, can be used instead of tradition casting to emulate the extracellular structure for improvement of

plasticity, porosity, surface area, hydrophilicity, rate of degradation and increasing cell viability, proliferation and adhesion^[5,6]. Moreover, it is possible to incorporate various kinds of monomers^[2,7,8] and materials to PHB structure, including other polymer like collagen, gelatin, keratin, PGLA to achieve similar results^[1,4,9,10].

In a previous study, a recombinant *E. coli* strain harboring *Alcaligenes eutrophus phbCAB* operon was successfully created for PHB accumulation in 1.5 litre medium using molasses as sole carbon source^[11]. Obtained PHB powder was planned to be the material for scaffolds using in animal tissue engineering. Therefore, it is necessary to assess the quality and characteristics of the PHB product. These assessment results in regards to product purity, heavy element contamination and important physical traits are presented in this study and will be useful for setting the optimal processing of PHB scaffolds used in further studies of animal tissue engineering.

II. MATERIALS AND METHODS

PHB was extracted from *E. coli* follow the description of XYZ. PHB 1% solution was prepared by mixing 1gr PHB powder with 100ml chloroform solvent in Duran flask. The solution was well mixed by a magnetic stirrer at 67°C and 500rpm, and then was cooled to 27°C at room condition.

Casting PHB scaffold: Poured 10ml of aforementioned PHB solution into an 9cm diameter uncontaminated glass dish (used as casting mould) and left in room condition for 24 hours for the chloroform to fully vaporize, leaving behind a 1mg dried whitish PHB scaffold which was used in later assessments^[10].

Assessment of chemical properties: Roughly grinded the 1 mg PHB scaffold with a small amount of KBr, then compressed the mixture into a thin film by a hydraulic compressor. The compressed film was scanned in Equinox55 – Bruker infrared spectrometer 32 times in 2-3 minutes following the program setting. The spectrograms were compared to identify any abnormal peaks due to PHB denaturation^[9,10].

Measurement of heavy element content: Heavy element content was measured by VARIAN 240F3AAS Agilent atomic absorption system following the standard of QCVN 8-1:2011/BYT.

Measurement of tensile strength: Tensile strength was measured by LLOYD INSTRUMENT 5kN universal test machine following ASTM D882 standard at rate of elongation 20 mm/min, 25 °C and 75% humidity. Each sample had standard size and was measured three times^[9,10]. Sample was analyzed at Plastic – Rubber Technology and Energy Training & Management Center, 156 Nam Kỳ Khởi Nghĩa, Bến Nghé Ward, District 1, HCMC.

Identification of surface structure by JEOL JMS 6360LV scanning electron microscope (SEM). Sample was analyzed at Research and Implementation Center – Hi-Tech Park at 13 block, N2 street, Hi-Tech Park, District 9, HCMC.

Measurement of water vapor transmission rate (WVTR): WVTR was assessed according to ASTM E96 standard. Assessment was carried out at Plastic – Rubber Technology and Energy Training & Management Center, 156 Nam Kỳ Khởi Nghĩa, Bến Nghé Ward, District 1, HCMC.

III. RESULTS AND DISCUSSION

Assessment of PHB quality by infra-red spectroscopy

Absorption peaks of all samples were virtually similar, measurement errors remained at acceptable level. There was no abnormal peak in the IR spectrum of PHB products, and also no peak in chloroform IR spectrum (3019 cm⁻¹; 1215 cm⁻¹, 760 cm⁻¹, 670 cm⁻¹ and 500 cm⁻¹) – probably chloroform contamination didn't occurred in PHB products. IR spectrum of PHB film were different from PHB powder due to the higher absorption of the film and different amount of sample used when compressing.

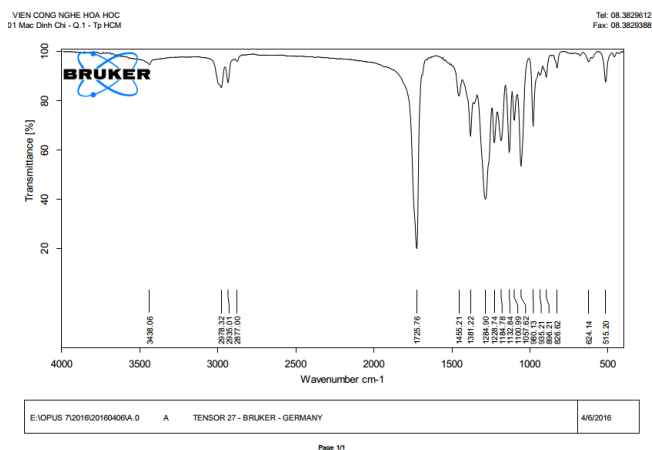


Figure 1. IR spectrogram of standard Sigma PHB

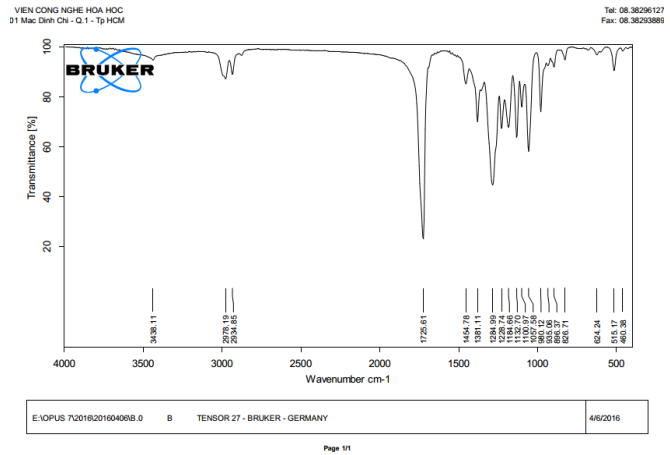


Figure 2. IR spectrogram of PHB products from recombinant *E. coli*

Measurement of heavy element contamination

Table 1 presents heavy element content of PHB powder, which all stayed in the acceptable level according to Ministry of Healthcare's regulation (QCVN 8-1:2011/BYT).

Table 1. Heavy element content of PHB powder

	Element	Content (ppm)	Highest content permitted (ppm)
1	Mercury (Hg)	< 0,06	< 0,06
2	Antimony (Sb)	< 0,02	< 0,05
3	Asenium (As)	< 0,02	< 0,5
4	Cadmium (Cd)	< 0.03	< 0,1
5	Lead (Pb)	< 0.05	< 0,2
6	Zinc (Zn)	0.974	< 4 (mg)

Selection of casting mould

PHB scaffold was casted at room temperature using the protocol of Güven (2008)^[10] with small modifications. Mould diameter was set at 9cm follow the petri dish size and 10ml 1% PHB solution was used. Each experiment was repeated 3 times. Teflon, inox, glass, silicon were chosen as experimented material for casting mould. Suitable material should be chloroform resistant, have smooth surface even in different temperature, and moderate adhesion so that the scaffold can be easily taken away. Results are presented in Table 2.

Table 2. Assessment of PHB casting mould materials.

Material	Silicon	Teflon	Inox	Glass
Mould surface	Deformed	Not deformed	Not deformed	Not deformed
Scaffold traits	Can't form scaffold	Smooth, homogenous	Smooth, homogenous	Smooth, homogenous
Easiness in taking the scaffold	Easy	Easy	Fairly hard	Fairly easy
Cost	High	High	Moderate	Moderate

Assessment results proved that glass and teflon was the most suitable material^[12]. However, while teflon is expensive, glass is much cheaper and more available – abundant 9mm petri dishes can be utilized as casting mould. It is concluded that glass is the best material for PHB casting mould used in further studies.

Measurement and assessment of physical properties

Respectively poured 1% PHB solutions of different volume (5ml; 7,5ml; 10ml and 12,5ml) into petri dishes. Physical properties of the resulted scaffolds are presented at table 3.

Table 3. Physical properties of PHB scaffolds from PHB solutions of different volume

Solution volume	Scaffold thickness (mm)	Tensile modulus (Mpa)	Elongation at break (%)	Tensile strength (N)	WVTR (g/m ² .24h)
5ml	0.0111 ±0.34	5.4463 ±0.34	9.6821 ±0.54	0.6135 ±0.06	1078.82 ±1,52
7,5ml	0.0116 ±0,56	7.3612 ±0,56	7.0735 ±0.36	0.8933 ±0.27	1140.11 ±1,52
10ml	0.0148 ±0,19	7.5897 ±0.19	7.0051 ±2.26	1.1198 ±0.06	1149.87 ±1,52
12,5ml	0.0150 ±0,66	10.4578 ±0.66	7.4962 ±0,69	1.6038 ±0.32	1151.43 ±1.52

Scaffold thickness and mechanical strength increased with larger solution volume. Higher PHB concentration coupled with decrease WVTR due to reduced porous structure and pore sizes. PHB concentration should be around 0.75-1% to maintain desirable nutrient diffusion and water absorption for sustaining tissue viability^[7]. Solution volume should be 10ml to optimize the forming of scaffold and to keep scaffold thickness at acceptable level.

Identification of surface and pore size

Scaffold formed by 0.5% PHB solution had distanced pores with uneven size (averagely 5,135 µm, some cases reached 8µm) (Figure 1). The 0.75% solution provided averagely 2,923 µm pores with uneven, clustered disposition. Fairly even disposition 0,978 µm pores resulted from 1% solution. The 1.25% solution formed roughly 596nm pores mainly positioned at scaffold slots. The 1.5 % solution created 658.5nm pores with large distance between each. The pores were frequently teared due to the separation of scaffold from mould^[13].

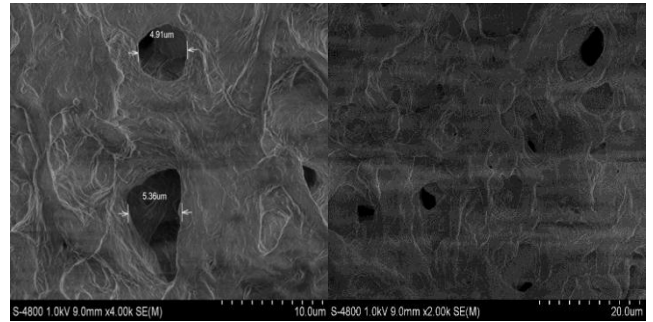


Figure 4. SEM image of PHB scaffold casted from 0.5% solution

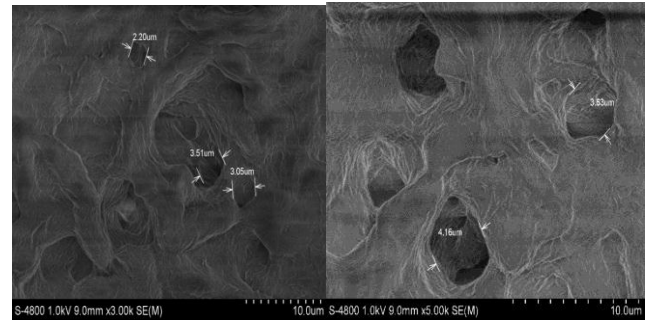


Figure 5. SEM image of PHB scaffold casted from 0.75% solution

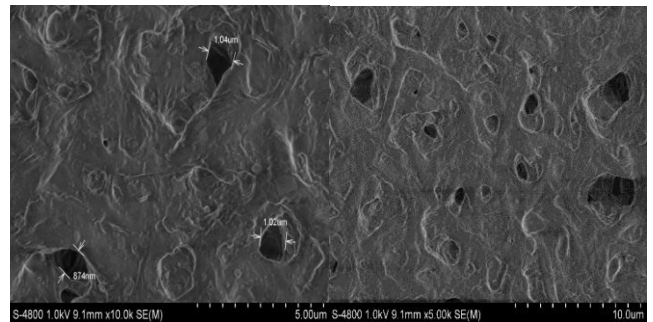


Figure 6. SEM image of PHB scaffold casted from 1% solution

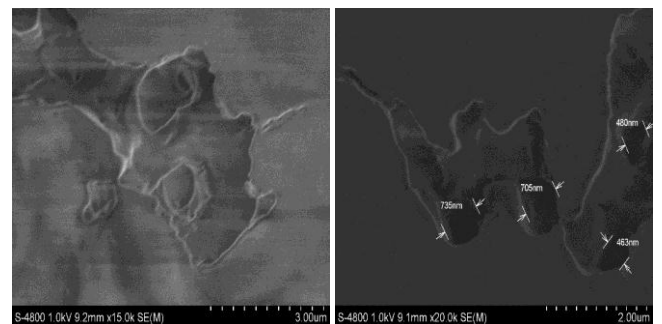


Figure 7. SEM image of PHB scaffold casted from 1.25% solution

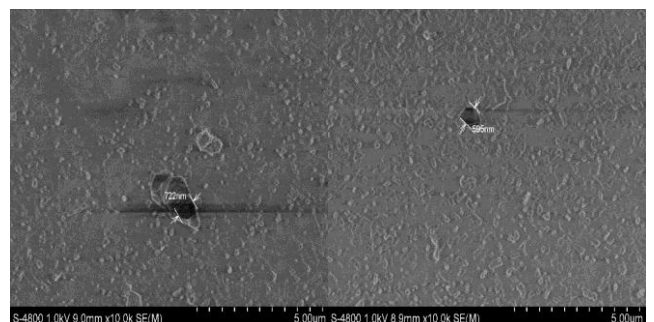


Figure 8. SEM image of PHB scaffold casted from 1.5% solution

Table 4. Porous structure of PHB scaffold casted from solutions with various concentration

PHB concentration	Pore diameter	Pore disposition	General description
0,5%	5,135 ±3µm	Relatively even	Uneven pore size, some reached 8µm diameter.
0.75%	2,923 ±2µm	Clustered	Fairly even pore size
1%	0,978 ±2µm	Fairly even	Fairly even pore size
1,25%	596 ±2nm	On ditches and slots	Highly uneven pore disposition
1,5%	658,5 ±2nm	Uneven	Large distance between pores

SEM images of scaffold surface presented the differences of pore disposition and size of scaffold casted from different solution concentration. Desirable pore sizes occurred in scaffolds casted from 0.5%; 0.75% and 1% solution – although they were still smaller than optimal ones used in tissue engineering (60 - 100µm). Only 1% solution resulted in fairly even pore disposition. Aforementioned pore size guaranteed high cell adhesion, however it also lessens the cell's ability to migrate into the center of the scaffold. The targets of researched scaffold are cells with modest requirements (lipoblast, keratinocyte, fibroblast...)^[8,14] – for example they do not require a support structure; therefore 1% solution is considered to be the most suitable casting material.

IV. CONCLUSION

PHB powder obtained from recombinant *E. coli* harboring *phbCAB* operon possessed equivalent purity and heavy element contamination to SIGMA standard PHB powder. 9mm diameter Germany-made glass dish proved to be the most suitable casting mould. Optimal volume and PHB concentration of casting solution is 9ml and 0.75-1%, respectively. Scaffold properties (thickness, tensile strength, tensile modulus, elongation at break, WWTR) all satisfied the requirements for application in tissue engineering.

ACKNOWLEDGMENT

This research was supported by Ministry of Sciences and Technology, Vietnam, under grant No ĐTĐL2012.G/35 to HDT

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