Production of Polyhydroxybutyrate Homopolyester by *Bacillus* sp. 25.1 Bacteria Using Raw Rice Bran Waste from Rice Bran Oil Production as a Nutrient

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Abstract

Plastic has a role in the daily life and also for medical application. Polyhydroxyalkanoates (PHA) are biocompatible plastic and do not cause inflammation or allergy to human tissue so they are suitable for medical applications. However, the PHA production cost is still high, the development of a low-cost PHA production is required. This research aimed to study the production of polyhydroxylbutyrate or P(3HB), one of the PHA polymers by *Bacillus* sp 25.1 using low cost raw rice bran waste from the rice bran oil production via cold press method as main carbon source. The cultivation was performed on minimal salt agar (MSA), pH of medium was adjusted to be 7, temperature for cultivation was at 30 ^{o} C and incubation time was 48 hours. Then, various supplementations and percentage of raw rice bran wastes in medium were studied by comparing the percentage of produced P(3HB) per dry cell weight and P(3HB) yield. The amounts of P(3HB) were determined by using gas chromatography (GC). In preliminary study, it was found that the highest mg% P(3HB) per cell dry weight and P(3HB) yield per plate were 52.54 mg% and 17.13 mg per plate, respectively, when *Bacillus* sp. 25.1 was cultured on MSA+24% raw rice bran waste with citric acid, pH of the medium was 7.0, incubated at 30 ^{o} C for 48 hours. This study showed the new isolated bacteria produced P(3HB) from native rice bran oil waste by using semi-solid cultivation.

Keywords**:** *polyhydroxylbutyrate, P(3HB), semi-solid cultivation, Rice bran waste, Polyhydroxyalkanoates, PHA.*

บทคัดย่อ

พลาสติกมีบทบาทสำคัญในชีวิตประจำวันและด้านการประยุกต์ใช้ทางการแพทย์ Polyhydroxyalkanoates (PHAs) เป็นพลาสติกที่มีความเข้ากันได้กับเนื้อเยื่อมนุษย์และไม่ก่อให้เกิดการอักเสบหรือภูมิแพ้ต่อมนุษย์ ดังนั้นมันจึงเหมาะสมสำหรับการนำมาใช้ทางการแพทย์ อย่างไรก็ตามการผลิต PHA มีต้นทุนสูง การพัฒนาการผลิต PHA ที่มีต้นทุนต่ำจึงมีความจำเป็น งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาการผลิต polyhydroxylbutyrate P(3HB) ซึ่งเป็น PHA ชนิดหนึ่งโดย *Bacillus sp* 25.1 โดยใช้กากรำข้าวเหลือทิ้งจากการบีบเย็นเป็นแหล่งคาร์บอนหลัก การเพาะเลี้ยงเชื้อทำบนอาหารเลี้ยงกึ่งแข็ง (MSA) ค่าความเป็นกรดด่างที่ 7 อุณหภูมิที่ 30 องศาเซลเซียส และเวลาการเพาะเลี้ยงที่ 48 ชั่วโมง จากนั้นทำการศึกษาการใส่สารเสริมต่างๆในอาหารเลี้ยงเชื้อและการเติมกากรำข้าวที่ความเข้มข้นต่างๆ ทำการศึกษาเปรียบเทียบร้อยละของน้ำหนัก P(3HB) ต่อน้ำหนักแห้งของเชื้อและปริมาณ P(3HB)ที่เชื้อสร้างต่อจานอาหารเลี้ยงเชื้อ ผลการวิจัย พบว่าร้อยละของน้ำหนัก P(3HB) ต่อน้ำหนักแห้งของเชื้อและ ปริมาณ P(3HB)ที่เชื้อสร้างต่อจานอาหารเลี้ยงเชื้อที่สูงที่สุด คือ 52.54 มิลลิกรัม% และ 17.13 มิลลิกรัมต่อจานอาหารเลี้ยงเชื้อ เมื่อเลี้ยงเชื้อ *Bacillus sp* 25.1 บน MSA+24% กากรำข้าว ที่มีการเติมกรดซิตริกค่าความเป็นกรดด่างที่ 7 อุณหภูมิเพาะเลี้ยงที่ 30 องศาเซลเซียส เป็นเวลา 48 ชั่วโมง การศึกษานี้จึงแสดงถึงเชื้อแบคทีเรียที่แยกได้ใหม่สามารถผลิต P(3HB) จากกากรำข้าวที่ไม่ผ่านการย่อยโดยใช้อาหารเลี้ยงเชื้อกึ่งแข็งเพาะเลี้ยง

คำสำคัญ: *polyhydroxylbutyrate P(3HB) การเพาะเชื้อแบบอาหารกึ่งแข็ง กากรำข้าว* Polyhydroxyalkanoates PHA.

1. Introduction

The use of petroleum-derived synthetic plastics has grown rapidly results in a serious pollution problem. Accumulation of plastics in the environment has become a worldwide problem (Leda, David & Denise, 2009). An attempt to decrease the environment impacts of plastics is to replace conventional petroleum derived plastics with biodegradable ones. Polyhydroxyalkanoates (PHAs) are a group of biodegradable polymers consist of homopolymer such as polyhydroxybutyrate or poly (3-hydroxybutyrate) (P(3HB)) and copolymer such as poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) as shown in Figure 1. PHAs are produced by several microbes and have been considered to be good substitutes for petroleum-derived synthetic plastics (Mamtesh, Sanjay & Vipin, 2009) especially for medical application (Chen, Wu, Wang & Zheng, 2005). Among PHAs, P(3HB) is commonly produced by various

bacteria. P(3HB) can be used as a biodegradable thermoplastic material for waste management strategies, food packaging and in the medical devices, medicine and pharmacy (Wiggam et al., 1997). But commercial production of PHAs is limited by the high cost of production (Okwuobi & Ogunjobi, 2012). Due to the large impact of the carbon source price on PHAs production cost, one of the important approaches to reduce cost is to use wastes and by-products as material for cultivation of microbes (Choi & Lee, 1999). A number of raw agro-industrial materials as carbon substrates such as wheat bran, rice bran and corn for production of PHAs are widely studied which were targeted to bring down the cost (Huang, Duan & Hoang, 2006; Sangkharak & Prasertsan, 2012; Rawia et al., 2013; Morgan-Sagastume et al., 2014; Queirós, Rossetti & Serafim, 2014). Unfortunately, for application of agro-industrial residues for PHAs production, pre-treatment is required. The hydrolysis of agro-industrial residue normally performed by amylase, alcalase and lipase enzymatic reaction for starch, protein and lipid, respectively (Shamala, Vijayendra & Joshi, 2012) which may result in cost and time consumption. Therefore, the development of efficiency processes based on crude carbon sources, such as native agro-industrial by-products and wastes, remains a challenge to be pursued (Leda et al., 2009).

Figure 1 Chemical structure of (a) 3-hydroxybutyric acid and (b) Poly (3-hydroxybutyric acid) (http://www.google.com/patents/WO2007095709A1?cl=en)

In Thailand, there are a lot of raw rice bran wastes from rice bran oil production using cold press method. From our previous study, *Bacillus cereus* C042 capable of producing P(3HB) by using native rice bran waste materials was isolated but the percentage of P(3HB) was low. (Chobchuenchom, 2016). *Bacillus* sp. 25.1 was earlier screened for P(3HB) production using glucose as sole carbon source showed very high cost of P3(HB) production (Chobchuenchom, Sridori & Tanadchangseang, 2015). Therefore, the attempts were done to study the capability of *Bacillus* sp. 25.1 for P(3HB) production by using raw rice bran waste as substrate.

2. Objectives

This study aimed to study the production of P(3HB) by *Bacillus* sp. 25.1 by using raw rice bran waste from cold press-rice bran oil production as main carbon source.

3. Materials and methods

3.1 Bacterium and culture condition

The bacterium, *Bacillus* sp. 25.1 was isolated from the soil obtained from refuse site at Klong 12, Nakornnayok Province, Thailand. The biopolymer in chloroform extracted from its dried cells was identified to be P(3HB) by comparing GC-MS chromatogram as previously described (Chobchuenchom, Sridori & Tanadchangseang, 2015). For cultivation, 100 μL of starter was spread on minimal salt agar (MSA) supplemented with raw or native rice bran waste materials from rice bran oil production by cold press method using standard spread plate technique. For preparation of MSA + native rice bran waste materials, briefly, 1.0 g ammonium sulphate, 2.0 g potassium dihydrogen phosphate, 0.6 g di-sodium hydrogen phosphate, 0.2 g magnesium sulphate heptahydrate, 0.75 g citric acid, 2.0 g sodium acetate, 0.05 g yeast extract, 15.0 g agar and native rice bran waste materials were mixed in 1 L distilled water to obtain the final concentration (w/v) at 3,6,9,12,15,18,21 and 24 %. In case of minimal salt broth, the medium was prepared as the above, except no agar was added. The pH of MSA was adjusted to be 7.0. Sterilization of MSA+ raw rice bran waste was carried out by autoclaving at 121 ^{o}C for 15 minutes. The plates were incubated at 30 ^{o}C for 48 hours incubation time.

3.2 Starter preparation

Bacillus sp. 25.1 was streaked on nutrient agar (Oxoid) and the cells from single colony were picked up and cultured in nutrient broth at 30 ^{o}C for overnight and used as starter.

3.3 Extraction and determination of P(3HB)

After incubation, *Bacillus* sp. 25.1 cells on MSA+ raw rice bran waste materials were harvested, washed twice with minimal salt broth by centrifugation at 2,500 rpm for 10 minutes and adjusted the volume to 2 mL. After that, 300 µL of cell suspension was dried at 85 ^{o}C for 36 hours and weighed to estimated cell dry weight (Mamtesh, Prasun, Sanjay & Vipn, 2013). P(3HB) from dried cell was extracted by incubating about 50 mg of the dried cell with 2 mL chloroform and 2 mL of 3% H_2SO_4 (v/v) in methanol supplemented with 10 mg/mL benzoic acid as the internal standard and then heated at 100 ^{o} C for 4 hours with mixing in the first 30 minutes. After that, the mixture was cooled down at room temperature for overnight. Then, 2 mL of distilled water was added, mixed vigorously by vortex for 4 minutes and let the tube stand at room temperature overnight. Chloroform layer was used for determination of P(3HB) in milligram by reading from P(3HB) standard curve. For gas chromatography, it was carried out on Gas chromatography instrument (Agilent Technologies 7890A) with capillary column HP5, 25 m x 0.32 mm, 0.52 µm film thickness, injection port temperature of 250 ^{o}C (isothermal), column oven temperature of 100 ^{o}C to 250 ^{o}C at the rate of 5 ^{o}C/min., detector (FID) temperature was 250 ^{o}C, split ratio 50:1, constant pressure of carrier gas (helium) at flow rate 1 mL/min. One µL of chloroform sample was injected to GC instrument by auto-loading system. The calibration curve for quantification of P(3HB) was shown in Figure 2.

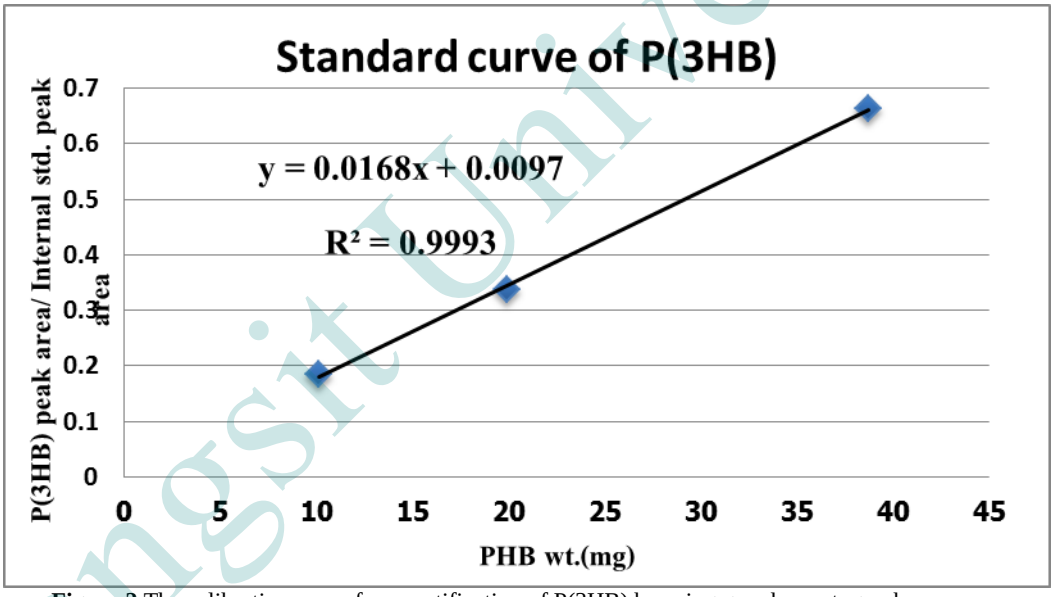

Figure 2 The calibration curve for quantification of P(3HB) by using gas chromatography

3.4 Calculation

Percentage of P(3HB) per cell dry weight (% w/w) was calculated by comparing the amount of P(3HB) in mg by 100 mg of cell dry weight. For P(3HB) yield per plate, it was calculated by multiplying the total amount of cell dry weight per plate by percentage of P(3HB) per cell dry weight (% w/w) then divided by 100.

4. Results

4.1 Cultivation of *Bacillus* sp. 25.1 on MSA and MSB + 3% raw rice bran waste materials

In preliminary study, the cells could grow both on MSA and MSB + 3% raw rice bran waste materials. The cells from MSB could not be harvested easily, because not all raw rice bran waste materials were solubilized in broth. On the other hand, the cells could grow well on MSA+ 3% raw rice bran waste and were easily harvested and prepared in cell suspension. Therefore, the cultivation on MSA + raw rice bran waste was used for further studies.

4.2 P(3HB) production by *Bacillus* sp. 25.1 cultured on MSA + raw rice bran waste materials with and without yeast extract and/or citric acid supplementation

To study the effect of yeast extract and/or citric acid in MSA on P(3HB) production by *Bacillus* sp. 25.1 the various conditions were done as shown in Table 1. It was found that the highest mg% P(3HB) per cell dry weight and P(3HB) yield per plate were 6.59% and 16.53 mg, respectively, when *Bacillus* sp. 25.1 was cultured on MSA+21% raw rice bran waste with citric acid when the pH of the medium was 7.0, and incubated temperature at 30 ^{o}C with the incubation time of 48 hours.

Table 1 P(3HB) production by *Bacillus* sp. 25.1 cultured on MSA (pH7) + 21% raw rice bran waste materials with and without yeast extract and/or citric acid supplementation when incubated for 48 hours at 30 ^{o}C

Yeast extract	Citric acid	mg% P(3HB)	*P(3HB) yield per plate (mg)*
No	No	1.99	6.87
Yes	No	2.02	6.27
No	Yes	6.59	16.53
Yes	Yes	3.89	5.63

4.3 P(3HB) production by *Bacillus* sp. 25.1 cultured on MSA+ raw rice bran waste materials from rice bran oil production by cold press as main carbon source

As *Bacillus sp* 25.1 produced higher P(3HB) when citric acid was added into MSA, therefore MSA with citric acid was used for further experiment. *Bacillus* sp. 25.1 was cultured on MSA(pH 7) supplemented with raw rice bran waste materials from rice bran oil production by cold press method at various concentrations as the following: 3, 6, 9, 12, 15, 18, 21 and 24 % (w/v). After incubation period of 48 hours at 30 ^{o}C, the cells were harvested, dried and extracted for polymer by using chloroform technique. The P(3HB) content was quantitated by using GC and the percentage of P(3HB) content per cell dry weight was calculated. It was found that the percentage of P(3HB) content per cell dry weight of the cells cultured on MSA supplemented with raw rice bran waste materials from rice bran oil production by cold press method at 3, 6, 9, 12, 15, 18, 21 and 24 % (w/v) were 11.02, 5.84, 30.37, 38.41, 38.81, 12.15, 18.24, and 52.54, respectively. The example of GC chromatogram was illustrated in Figure 3. P(3HB) yield per plate was 2.4, 4.13, 4.87, 5.13, 9.07, 8.53, 9.87 and 17.13 mg, respectively.

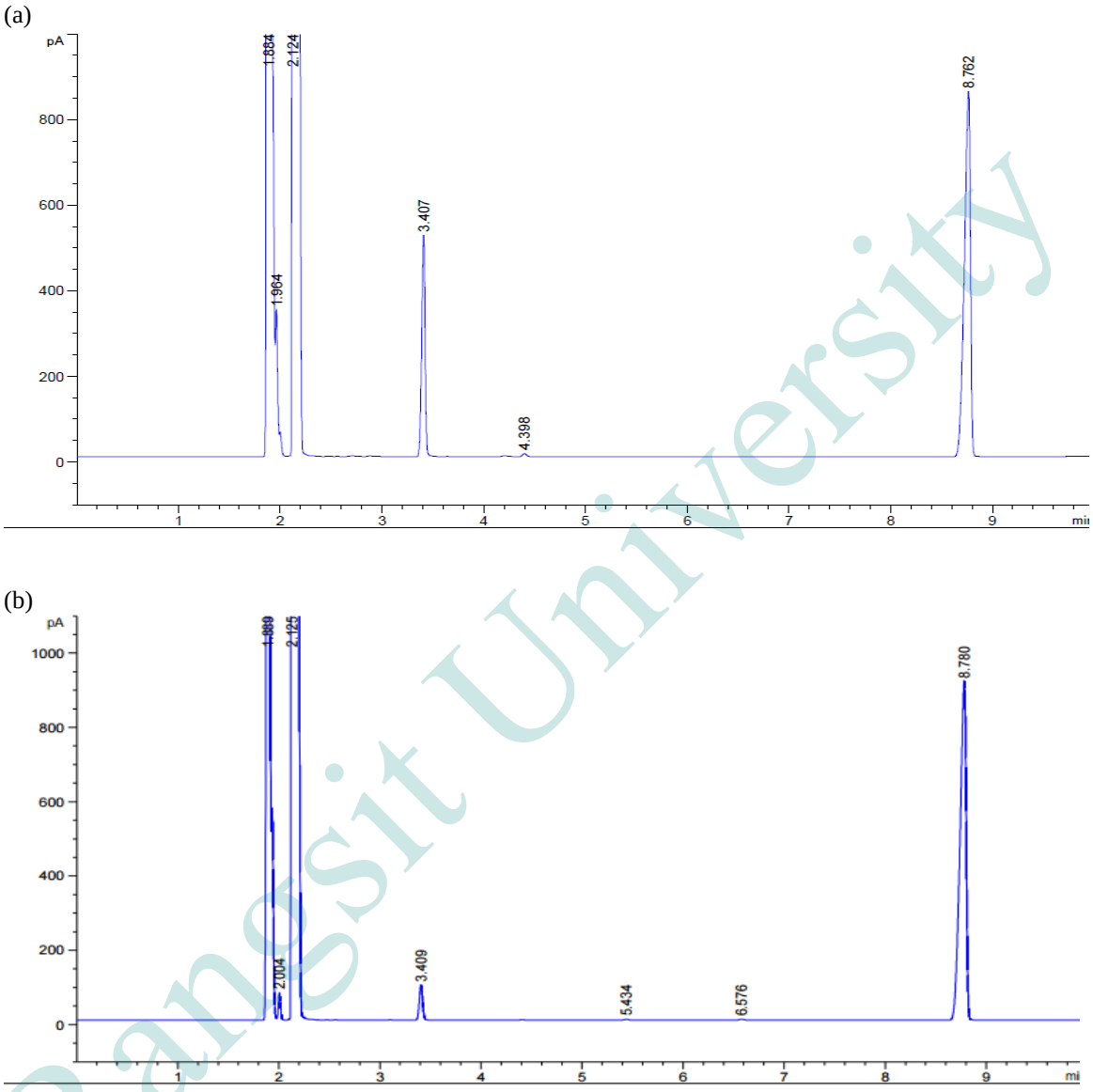

Figure 3 GC chromatogram of the extract obtained from (a) Standard P(3HB) at 19.9 mg and (b) *Bacillus* sp. 25.1 cultured on MSA+ 24 % of raw rice bran waste materials from rice bran oil production by cold press method, peak at 1.6-2.2, 3.4 and 8.7 are chroloform, P(3HB) and benzoic acid internal standard, respectively.

5. Discussion

The use of renewable carbon sources for the production of PHAs that can reduce the production costs of PHAs biopolymer are widely studied (Waqas, Nazia, Iftikhar, Mian & Ayaz, 2010), but most of low cost carbon materials cannot use as native materials. They require pre-treatment such as enzymatic hydrolysis before using as carbon source for PHAs production (Saranaya, Vijayendra & Shamala, 2012). *Bacillus* sp. 25.1 can use native raw rice bran waste materials obtained from rice bran oil production by using cold press method as main carbon source for P(3HB) production without any pre-treatment process. From GC-MS analysis, extracts of the rice bran waste composed of various fatty acids, they were palmitic acid, linoleic acid, elaidic acid, adipic acid, mesitoic acid, myristic acid and oleic acid as reported earlier (Saranaya et al., 2012; Chobchuenchom, 2016). These individual fatty acids can be used as carbon source for P(3HB) production by *Burkholderia* sp. USM (JCM15050) with percentage of P(3HB) per cell dry

weight varied from less than 1 % to 69% (w/w) up to fatty acid type and concentration (Jiun-Yee, Yifen, Mohd-Razip & Kumar, 2010). In this study, *Bacillus* sp. 25.1 capable of utilizing native rice bran waste as carbon source by using semi-solid agar that was suitable for cultivating and harvesting the cells. The highest wt% P(3HB) per cell dry weight and P(3HB) yield per plate were 52.54% and 17.13 mg, respectively, when *Bacillus* sp. 25.1 was cultured on MSA+24% raw rice bran waste with citric acid, pH of the medium was 7.0, incubated at 30 ^{o}C for 48 hours. When compared to percentage of P(3HB) per cell dry weight of other PHAs-producing Bacillus such as *Bacillus cereus* SPV (41.9 %) (Valappil et al., 2007), *Bacillus cereus* CFR06 (46.0%) (Halami, 2008) and *Bacillus* sp. NA10 (66.6%) (Anish, Gulab, Neeraj K, Varsha & Anita, 2014), the percentage of P(3HB) per cell dry weight of *Bacillus* sp. 25.1 was quite high but the yield per plate was not so high. This might result from the low growth of this strain on MSA supplemented with raw rice bran waste. When compare to *Bacillus cereus* C042 from our previous study (Chobchuenchom, 2015), C042 could produce P(3HB) at lower percentage but higher yield because it could grow rapidly on MSA+ rice bran waste without any supplementations (no yeast extract or citric required).

This bacterium is gram positive that lack endotoxin that is a better source of PHAs used for biomedical applications (Valappil, Tai, Bucke & Roy, 2008). Gram negative bacteria such as *Ralstonia eutropha*, and recombinant *E. coli* which have been exploited for industrial scale P(3HB) production and required for endotoxin removal steps. The outer membrane of them contains lipo-polysaccharides (LPS) that are endotoxin and are toxic for human beings. The purification of PHAs is more complicated due to the presence of endotoxin (Mamtesh et al., 2009).

The optimum concentration of raw rice bran waste supplemented in MSA for cultivation seemed to have variation from lot to lot of raw rice bran waste obtained from rice bran oil production factory. Therefore, the carbon content in the raw rice bran waste should be determined and used for calculation of definite suitable C:N ratio of MSA. Unfortunately, the percentage of rice bran waste at 24 % is the highest amount that could be added to MSA for preparation as semi-solid medium.

Though wide range of the microbes can produce P(3HB), this polymer is crystalline and brittle. Due to relatively poor physical properties, extensive efforts are being directed towards the synthesis of copolymers that have better properties. Incorporation of 3- and 5- carbon monomers into a polymer consisting mainly of P(3HB) leads to a decrease in crystallinity and melting point of P(3HB). The copolymers such as poly(hydroxybutyrate-co-hydroxyvalerate) and others have better mechanical properties and are useful for various applications (Shilpi & Ashok, 2005). As reported previously, some *Bacillus* such as *Bacillus megaterium* has high possibility that accumulates different types of PHAs co-polymers (Valappil et al., 2008). It was found that the copolymer production depends on the carbon substrate provided for cultivation and the type of PHAs-synthesis genes present in the microbes (Shamala et al., 2012). For sustained commercialization, it is essential to explore economic substrates for bacterial growth and copolymer production (Anil et al., 2007). There have been reports on supplementation of organic acid such as propionic acid, levulinic acid etc., to the bacterial media can result in increased copolymer production (Jain, 2001). However, this may increase the cost of copolymer production. On the other hand, there have been reports on copolymer production by using hydrolyzed agro-industrial wastes or by-products (Shamala et al., 2012). Therefore, the studies of the utilization of low-cost and renewable carbon sources without any supplementations or processes for P(3HB) and novel PHAs copolymers production are still required.

6. Conclusion

The newly bacterium, *Bacillus* sp. 25.1 capable of utilizing native rice bran waste materials as main carbon source for P(3HB) production was isolated from soil sample obtained from refuse site in Thailand. From GC-MS analysis of chloroform extract, one of the polymers was P(3HB). In this preliminary study, The highest wt% P(3HB) per cell dry weight and P(3HB) yield per plate were 52.54% and 17.13 mg, respectively, when *Bacillus* sp. 25.1 was cultured on MSA+21% raw rice bran waste with citric acid, pH of the medium was 7.0, incubated at 30 ^{o}C for 48 hours. Thus, the optimization and scale up of semi-solid cultivation for P(3HB) production as well as induction for PHAs-copolymer production by this bacterium should be further studied.

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