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Isolation of *Bacillus cereus* C042 capable of producing polyhydroxybutyrate by using native rice bran waste materials from rice bran oil production as carbon source

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Abstract

A replacement of polyhydroxyalkanoates (PHAs) for existing petroleum-based plastics is limited due to their high production cost. Thus, increased production of PHAs on cheap substrates is an important challenge to support the commercialization. Normally, the pre-treatment of cheap substrates is required which results in increased costs and time. In this study, native rice bran waste materials from rice bran oil production, using the cold press method which generates various fatty acids, were used as the main carbon source for poly(3-hydroxybutyrate) (P(3HB)) production. Fifty soil samples obtained from various locations in Thailand, including industrial sites, refuse sites, garden and community areas, were screened for PHAs-producing microorganisms. Each soil sample was diluted in distilled water and the soil suspension was poured on MSA agar supplemented with 3% (w/v) native rice bran waste materials. The microorganisms that grew on the medium were further screened for PHAs production by using Sudan Black B staining. The production of P(3HB) was confirmed by using Gas Chromatography-Mass Spectrometry (GC-MS) by comparing to standard P(3HB). The P(3HB) content was measured by using GC and percentage of P(3HB) content per cell dry weight was calculated. It was found that 55 of 336 isolates (16.4%) which grew were positive for PHAs. The isolate designated C042 showed the highest potential for PHAs production and was used for further experiments. Biopolymer was extracted from C042 dried cells by using chloroform. From GC-MS analysis, it was found that biopolymer was P(3HB) by comparing the mass spectrum to standard P(3HB). The isolate C042 was gram positive bacilli with spore forming abilities and was further identified as *Bacillus cereus* by using 16S RNA sequencing method. The percentage of P(3HB) production per cell dry weight of *Bacillus cereus* C042 was 11.6 (w/w) when the cells were grown on MSA agar supplemented with 21% (w/v) native rice bran waste materials.

Keywords**:** *polyhydroxyalkanoates (PHAs), biopolymer, native rice bran waste materials*

1. Introduction

The use of plastics has grown rapidly over the past few decades, with about 150 million tons of plastic materials being consumed yearly worldwide. At least half of this amount is used in short-term applications such as packaging materials. The petroleum-derived synthetic plastics can persist in the environment for an average of 100 years, and this accumulation of plastics in the environment has become a worldwide problem. Moreover, methods for the adequate disposal of plastics are problematic. In landfills, the degradation rates of plastics are extremely slow. Incineration is expensive and may generate toxic by-products (Castilho, Mitchell, & Freire, 2009). Recycling can be done but the sorting of the wide variety of discarded plastics is a very time-consuming process (Khanna & Srivastava, 2005). To decrease the environmental impact of plastics, they need to replace conventional petroleum derived plastics with biodegradable ones.

Polyhydroxyalkanoates (PHAs) are a group of biodegradable polymers consisting of homopolymers such as polyhydroxybutyrate or poly (3-hydroxybutyrate) (P(3HB)) and copolymers such as poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)). PHAs are produced by microbes and have received increased interest in recent years, as they have been considered to be good substitutes for petroleum-derived synthetic plastics (Singh, Patel, & Kalia, 2009). It is important to mention, PHAs have the advantage of being biocompatible (ie., characterized by the absence of any toxic compound generated during polymer degradation, as well as by the shape and surface porosity of the material.) Therefore, PHAs are a potential candidate for use in medical applications, such as in wound management (suture, skin substitutes),

vascular system devices (heart values, vascular grafts), orthopaedy (scaffolds for cartilage engineering, screws, bone graft substitutes), micro- and nano- spheres for controlled drug delivery, urological stents and others (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 and food packaging. P(3HB) is biocompatible plastic used in the medical devices, medicine, pharmacy, veterinary sciences, since P(3HB) is a normal blood constituent and is found in the cell envelope of eukaryotes (Wiggam et al., 1997). But commercial production of PHAs is limited by the high cost of production compared to synthetic plastics (Ngozi & Adeniyi, 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 waste and by-products as material for the cultivation of microbes (Choi & Lee, 1999). A number of inexpensive and renewable carbon substrates, such as molasses, whey, wheat bran, rice bran, starch and starchy wastewaters, and waste frying oil, are widely studied. The end result being a much reduced cost of the polymer which can compete with petroleum-derived synthetic plastics (Sangkharak & Prasertsan, 2012; Gamal et al., 2013; Morgan-Sagastume et al., 2014; Queirós, Rossetti, & Serafim, 2014). Production of PHAs from inexpensive carbon sources by solid-state fermentation or by mix culture are also proposed, with the aim of reducing the cost and/or increasing the yields (Castilho, Mitchell, & Freire, 2009; Queirós, Rossetti, & Serafim, 2014). Unfortunately, productivity and PHAs content are usually lower for bacteria grown in crude, inexpensive substrates (Choi & Lee, 1999). For application of agro-industrial residues for PHAs production, pre-treatment was done by hydrolysis of agro-industrial by-products or waste such as potato starch, wheat bran, extruded rice bran and extruded corn starch (Huang, Duan, & Hoang, 2006). The hydrolysis of agro-industrial residues are normally performed by amylase, alcalase and lipase enzymatic reactions for starch, proteins and lipids, respectively (Shamala, Vijayendra, & Joshi, 2012) which are costly and time consuming. Therefore, the development of efficient processes based on crude carbon sources, such as native agro-industrial by-products and wastes, remains a challenge to be pursued (Castilho, Mitchell, & Freire, 2009).

In this study, attempts were done to screen a bacterium that can use raw or native rice bran waste materials obtained from rice bran oil cold press-production as the main carbon source without any pretreatment and can still produce P(3HB).

2. Objectives

This study aimed to isolate a newly identified bacterium capable of producing P(3HB) from soil samples in Thailand by using native rice bran waste materials from rice bran oil cold press-production as the main carbon source.

3. Materials and methods

3.1 Isolation procedure

Fifty soil samples were collected from various locations in Thailand included industrial sites, refuse sites, garden and community areas. Each sample was diluted in sterilized distilled water and plated on minimal salt agar (MSA) supplemented with 3% 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%. Sterilization of MSA+ native 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. Representative colonies were obtained and purified by repeated streaking on MSA+ 3% native rice bran waste materials. Each clonal colony was individually picked based on distinct morphological characteristics.

3.2 Screening method for PHAs accumulation in bacteria

The pure isolates were grown on MSA+3% (w/v) native rice bran waste materials plates and incubated at 30 ^{o}C for 48 hours. Each isolate was smeared on a glass slide and air-dried. Then, the dried slides were heat-fixed and stained with Sudan Black solution using a modified

method that was previously described (Ngozi & Adeniyi, 2012). Briefly, heat fixed slides were stained with 0.3% Sudan black B in 95% ethanol (w/v) for 10 minutes and rinsed off with slowly running water, counterstained with 0.5% aqueous safranine for 5 seconds and rinsed off with slowly running water. The slides were air dried and viewed under oil immersion lenses. The PHAs granules appeared as blue-black granules inside pink cells, referred to as positive cells, and only pink for those cells that contained no granules and were referred to as negative.

3.3 Extraction and analysis of P(3HB)

The C042 isolate was cultured on MSA+ 3% native rice bran waste materials at 30 °C for 48 hours. Then, the cells were harvested and washed once with distilled water by centrifugation at 5,000 rpm for 30 min and the pellet was dried at 105 °C in a hot air oven (Memmert U50) to obtain a constant weight. Twenty milligrams of dried cells were added 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 80 °C for 3.5 hours with mixing at 30 minutes intervals. After that, the tube of mixture was placed on ice and let it cool down. Then, 2 mL of distilled water was added, mixed vigorously by vortex for 5 minutes and let the tube stand overnight. The chloroform layer was transferred into a vial and used for analysis of P(3HB) by Gas Chromatography-Mass Spectrometry (GC-MS). The authentic P(3HB) was run in parallel. The analysis of P(3HB) was performed at Scientific Equipment Center, Prince of Songkla University by Gas Chromatography-Mass Spectrometry (Perkin Elmer Clarus 500 GC / MS 17) with capillary column (MS), 30 m x 0.25 mm coated with DB-5, 0.25 μm film thickness: column oven temperature of 80 °C increasing to 150 °C at the rate of 10 °C /min., injection port temperature 230 °C, constant pressure of carrier gas (helium), flow rate 1 mL/min. One μL of sample was injected into the GC-MS for analysis of P(3HB) and compared to P(3HB) standard (Fluka).

3.4 Identification of isolated P(3HB) producing bacterium

The positive P(3HB) producing bacterial isolate C042 was identified by using gram staining, spore staining and 16S rRNA sequencing. The 16S rRNA sequencing was kindly performed by Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, Mahidol University, Bangkok, Thailand.

3.5 Extraction and determination of P(3HB)

After incubation, *Bacillus cereus* C042 cells on MSA+ 3,6,9,12,15,18,21 and 24% native rice bran waste materials were harvested, washed twice with minimal salt broth and followed with distilled water by centrifugation at 2,500 rpm for 10 minutes and lyophilized. P(3HB) from dried cells was extracted by incubating 50 mg dried cell with chloroform and methanol as described above, except heated at 100 °C for 4 hours with mixing after the first 30 minutes. Following these steps, the mixture was allowed to cool down at room temperature overnight before adding distilled water. The chloroform layer was used for determination of P(3HB) 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 250 °C (isothermal), column oven temperature of 100 °C increasing to 250 °C at the rate of 5 °C /min., detector (FID) temperature 250 °C, split ratio 50:1, constant pressure of carrier gas (helium) at flow rate 1 mL/min. One μL of chloroform sample was injected into GC instrument by auto-loading system.

3.6 Calculation of % P(3HB) per cell dry weight

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.

3.7 Extraction and analysis of raw rice bran waste components

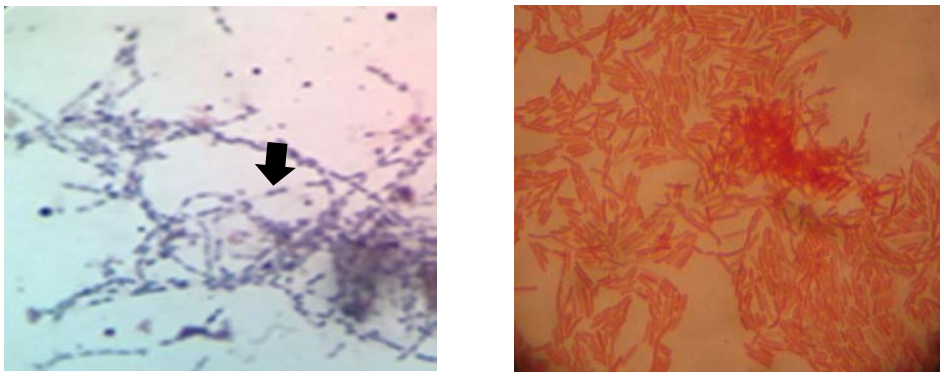
Native rice bran waste material was extracted by using three organic solvents; methanol, chloroform and hexane. Briefly, 1 g of native rice bran waste materials in 16x125 mm screw cab tube was added with 5 mL of methanol, chloroform or hexane, each tube was mixed vigorously, and placed in a sonicator-water bath at 30^o C for 45 min. The organic solvent was then filtered through 0.45 micron nylon filter membrane and readied for GC-MS analysis. The composition analysis of native rice bran waste materials was

performed at Rangsit University–Science and Technology Equipment Research Center (RSU-STREC), Rangsit University by using Gas Chromatography-Mass Spectrometry (Agilent Technologies 7890 GC) with capillary column (MS), 30 m x 0.25 mm coated with DB-5, 0.25 μm film thickness: column oven temperature of 80 °C increasing to 150 °C at the rate of 10 °C/min., injection port temperature 230 °C, constant pressure of carrier gas (helium), flow rate 1 mL/min. One μL of sample was injected into the GC-MS for analysis of various compounds compared to standard mass spectrum.

4. Results

4.1 Screening of PHAs producing microbes from soil samples

From the total number of 50 soil samples obtained from various places in Thailand, there were 336 isolates that could grow on MSA+3% native rice bran waste materials after incubation at 30 ^{o}C for 48 hours. The isolates were screened for PHAs production by using Sudan Black B staining. It was found that there were 55 isolates from these 336 isolates (16.4%) that showed positive cells for PHAs granules which appeared as blue-black granules inside the cells as shown in figure 1 (A). Among them, the isolate named C042, that was isolated from a refuse site at Amphur Pimai, Nakornrajasrima Province, showed the highest percentage of positive cells as shown in figure 1 (A). If the cells, such as *E. coli*, lack PHAs granules, the cells will be stained with safranin and show pink color as seen in figure 1 (B).



(A) (B)

Figure 1 Sudan Black staining-photomicrograph of isolate *Bacillus cereus* C042, showing the PHAs granules in form of blue black granules inside the bacterial cells (A) compare to negative cells of *Escherichia coli* (B) (1000X Magnification)

4.2 Identification of isolate C042 by 16S rRNA sequencing

The isolate C042 is a gram positive bacilli with spore forming ability. By using16S rRNA sequencing, the strain C042 was identified as *Bacillus cereus* and was designated with the name *Bacillus cereus* C042.

4.3 Analysis of P(3HB) produced by *Bacillus cereus* C042

P(3HB) produced by *Bacillus cereus* C042 was identified by using GC-MS. GC chromatograms of an extract obtained from *Bacillus cereus* C042 and standard P(3HB) are represented in Figure 2. We determined that the mass spectrum of the polymer extract at retention time 14.30 from *Bacillus cereus* C042 corresponded to standard P(3HB) (Figure 3).

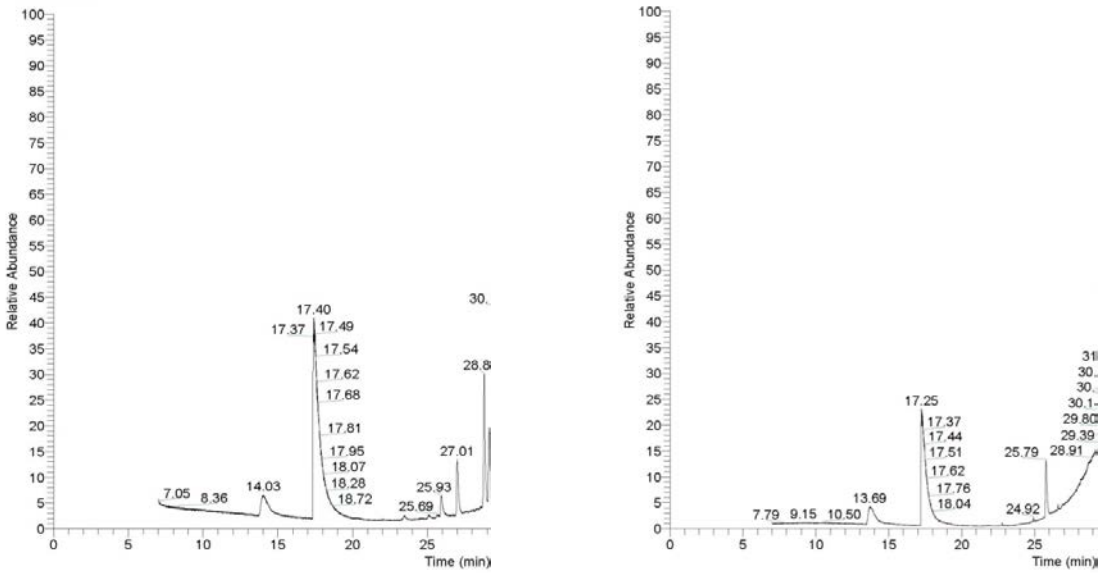
4.4 P(3HB) production by *Bacillus cereus* C042 using native rice bran waste materials from rice bran oil production by cold press as the main carbon source

Bacillus cereus C042 was cultured on MSA supplemented with native rice bran waste materials from rice bran oil production by cold

press method at these concentrations; 3, 6, 9, 12, 15, 18, 21 and 24% (w/v). After 48 hours incubation period at 30 ^{o}C, the cells were harvested, lyophilized and extracted for polymer using the 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 determined that the percentage of P(3HB) content per cell dry weight of the cells was 1.8, 2.6, 4.8, 5.6, 8.0, 9.6, 11.6 and 10.97, respectively. The example of GC chromatogram was illustrated in Figure 4.

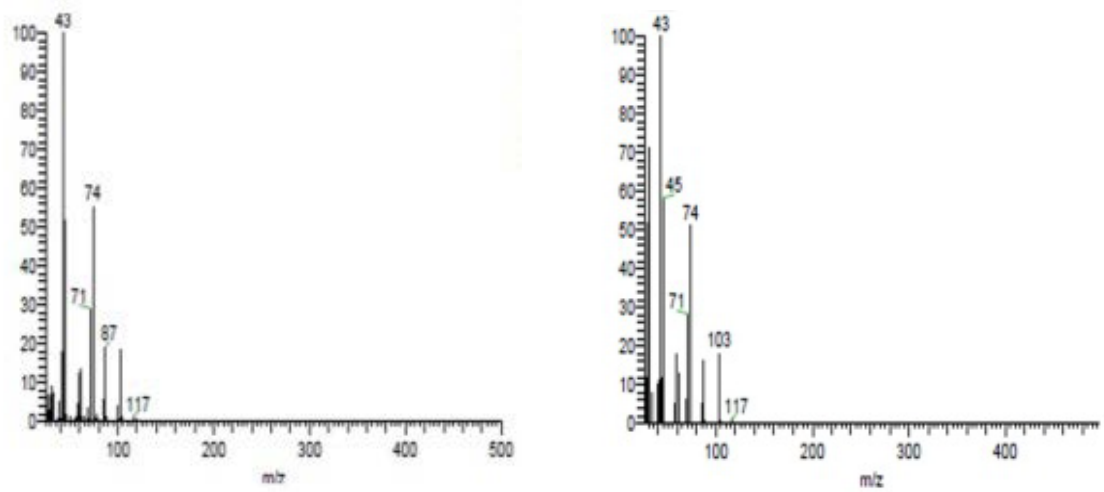
4.5 Analysis of native rice bran waste materials composition by using GC-MS

By using GC-MS, seven fatty acids were found in native rice bran waste materials extracts: palmitic acid, linoleic acid, elaidic acid, adipic acid, mesitoic acid, myristic acid and oleic acid as shown in Table 1.



(A) (B)

Figure 2 GC chromatogram of the chloroform extract obtained from *Bacillus cereus* C042 at the peak of 14.03 (A) and standard P(3HB) at the peak of 13.69 (B). (Benzoic acid internal standard peak at 17.40 and 17.25, respectively)



(A) (B)

Figure 3 Mass spectrum of the chloroform extract obtained from *Bacillus cereus* C042 at the peak of 14.03 (A) compared to standard P(3HB) at the peak of 13.69 (B)

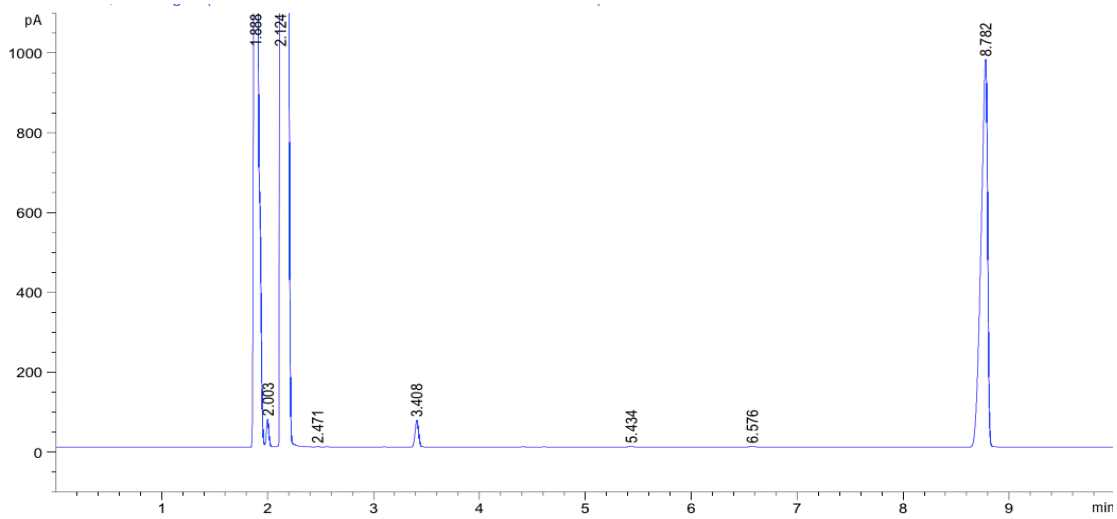


Figure 4 GC chromatogram of the extract obtained from *Bacillus cereus* C042 cultured on MSA+ 21% of native rice bran waste materials from rice bran oil production by cold press method, peak at 1.8-2.2, 3.4 and 8.7 are chloroform, P(3HB) and benzoic acid internal standard, respectively.

Table 1 The components in native rice bran waste materials extracted with various organic solvents and analyzed by GC-MS

Organic solvent	Retention time (min)	Components
Methanol	45.212	Palmitic acid
Methanol	50.446	Linoleic acid
Methanol	50.665	Elaidic acid
Methanol	58.915	Adipic acid
Chloroform	27.305	Mesitoic acid
Chloroform	40.073	Myristic acid
Chloroform	45.218	Palmitic acid
Chloroform	50.443	Linoleic acid
Chloroform	51.498	Stearic acid
Hexane	40.041	Myristic acid
Hexane	45.210	Palmitic acid
Hexane	50.443	Linoleic acid
Hexane	52.736	Oleic acid

5. Discussion

Recent studies have focused on the use of renewable carbon sources for the production of PHAs that can reduce the production costs of PHAs biopolymer (Chaudhry, Jamil, Ali, Ayaz, & Hasnain, 2010), but most of cheap carbon materials cannot be used as native materials, because they require pre-treatment such as enzymatic hydrolysis before using as carbon source for PHAs production (Saranya, Vijayendra, & Shamala, 2012). Thus, we explored the newly isolated bacterium that could utilize native rice bran waste materials obtained from rice bran oil production by using cold press method as its main carbon source for bacterial P(3HB) production without requiring any pre-treatment process. From GC-MS analysis, extracts of the rice bran waste were determined to be composed of various fatty acids: palmitic acid, linoleic acid, elaidic acid, adipic acid, mesitoic acid, myristic acid and oleic acid as reported earlier (Saranya, Vijayendra, & Shamala, 2012). 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) depending on fatty acid type and concentration (Jiun-Yee, Yifen, Mohd-Razip, & Kumar, 2010). In this study, we isolated a bacterium capable of utilizing native rice bran waste as the carbon source. The bacterium, *Bacillus cereus* C042 was isolated from the soil obtained from a refuse site at Amphur Pimai, Nakornrajasrima Province that was considered as a rich site for organic carbon. The biopolymer in chloroform extracted from its dried cells was preliminarily identified to be P(3HB) by using GC-MS. The peaks of produced biopolymer and standard showed the difference of retention time to be not more than +/-5% from standard P(3HB peak (Figure 2) and also showed the same mass spectrum as standard P(3HB) (Figure 3).

Recently, it has been revealed that gram negative bacteria such as *Ralstonia eutropha*, *Alcaligenes latus* and recombinant *E. coli* are among those which have been exploited for industrial scale P(3HB) production. Their outer membrane contains lipopolysaccharides (LPS) that are highly toxic to human beings making the purification of PHAs more complicated (Singh, Patel, & Kalia, 2009). Gram positive bacteria such as *Bacillus*, lack lipopolysaccharide and are better sources of PHAs used for biomedical applications (Valappil, Rai, Bucke, & Roy, 2008). *Bacillus* accumulates P(3HB) with varied production range of P(3HB) per cell dry weight when grown on different substrates or wastes such as *Bacillus cereus* SPV (41.9%) (Valappil et al., 2007), *Bacillus cereus* CFR06 (46.0%) (Halami, 2008) and *Bacillus sp.* NA10 (66.6%) (Bhuwal, Singh, Aggarwal, Goyal, & Yadav, 2014). In this study, *Bacillus cereus* C042 was a newly isolated bacterium that used native rice bran waste as main carbon source, but the percentage of P(3HB) per cell dry weight was found to be 11.6% when grown on MSA+21% native rice bran waste. This suggests that the optimization study for higher yield and percentage of P(3HB) per cell dry weight should be performed.

Though wide ranges of 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 (Khanna & Srivastava, 2005). As reported earlier, some *Bacillus* such as *Bacillus megaterium* have a high possibility that they can accumulate different types of PHAs copolymers (Valappil, Rai, Bucke, & Roy, 2008). *Bacillus subtilis* contains expression of self-lysing genes on completion of PHAs biosynthetic process for easy and timely recovery and it can use agro-industrial materials as feed. These characteristics enable *Bacillus* to be a strong contender in the future as an industrial PHAs producer (Singh, Patel, & Kalia, 2009). 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, Vijayendra, & Joshi, 2012). For sustained commercialization, it is essential to explore economic substrates for bacterial growth and copolymer production (Anil Kumar et al., 2007). There have been reports on supplementation of organic acid such as propionic acid, levulinic acid and etc., to the bacterial media which can result in increased copolymer production (Jain, 2001), however this may increase the cost of copolymer production. Conversely, other studies on copolymer production by using hydrolyzed agro-industrial wastes or by-products have also been successful (Shamala, Vijayendra, & Joshi, 2012). Therefore, we suggest that the studies of the utilization of cheap carbon sources without any supplementations or processes for P(3HB) and novel PHAs copolymers production are still required.

6. Conclusion

The newly identified bacterium, *Bacillus cereus* C042 capable of utilizing native rice bran waste materials as its main carbon source for P(3HB) production was isolated from soil sample obtained from a refuse site in Thailand. From GC-MS analysis of chloroform extract, one of the polymers was P(3HB). The P(3HB) content per cell dry weight when the cells were cultured on MSA supplemented with 21% (w/v) native rice bran waste material was 11.6%. It seems reasonable to carry out further investigations on other PHAs copolymers produced by this strain and the optimized conditions necessary to increase P(3HB) and perhaps other polymer yields.

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