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Characterization of Polyhydroxybutyrate Nanoparticles

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Abstract

Preparation of nanoparticles is one of the important ways to increase the biological effectiveness of materials. There are several methods to prepare the polyhydroxybutyrate (PHB) nanoparticles. Here, a new method is used based on exposing PHB to ultrasound waves under variable pH conditions. In the present study, PHB was added to distilled water and pH was adjusted to 4 by HCl (1 N). The suspension was exposed to ultrasound waves at 4500 kh for 25 seconds. Then, pH was readjusted to 10 by NaoH (1N) and the mixture was incubated for 2 h at 21 ^{o}C. Finally, the pH was adjusted to 7 by HCl (1 N) and the mixture was incubated at 21 ^{o}C for 18 h. The characterization of the prepared nanoparticles was achieved by using atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV) spectrophotometer, X-ray powder diffraction (XRD), and scanning electron microscopy (SEM). The results demonstrated the formation of nanoparticles, especially after examinations by SEM and AFM, which showed that the diameter of particles was between 22.9 and 73.95. The present study confirmed that the method of exposing PHB to gradient pH and high levels of ultrasonic waves could produce PHB nanoparticles.

Keywords: Polyhydroxybutyrate (PHB), PHB nanoparticles, pH gradient method, Ultrasonic waves.

توصيف مادة البوليهايدروكسيبيوتاريت النانووية

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الخلاصه

يعد تحضير الجسيمات النانوية أحد الطرق المهمة لزيادة الفعالية البيولوجية للمواد. هناك عدة طرق لتحضير مادة البوليهايدروكسيبيوتاريت النانووية (PHB). هنا تم استخدام طريقة جديدة تعتمد على تعريض PHB للموجات فوق الصوتية في ظل ظروف الأس الهيدروجيني المتغيرة. في الدراسة الحالية تمت إضافة PHB إلى الماء المقطر وتعديل الأس الهيدروجيني إلى 4 بواسطة HCl 1 N). تعرض المعلق للموجات فوق الصوتية عند 4500 ك.س لمدة 25 ثانية. ثم أعيد تعديل الأس الهيدروجيني إلى 10 بواسطة NaoH 1N) وخلطة لمدة ساعتين عند 21 درجة مئوية. أخيرًا ، تم تعديل الرقم الهيدروجيني للخليط إلى 7 بواسطة HCl 1 N) واحتضانه عند 21 درجة مئوية لمدة 18 ساعة. تم توصيف الجسيمات النانوية المحضرة بواسطة المجهر الماسح الذري (AFM) ، وجهاز مطياف الأشعة تحت الحمراء (FTIR) ، مقياس الطيف الضوئي فوق البنفسجي (UV) ، جهاز الأشعة السينية (XRD) المجهر الإلكتروني (SEM). عزا توصيف الجسيمات

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المحضرة الموضحة في الاختبارات أعلاه الجسيمات على أنها جسيمات نانوية خاصة بعد فحصها بواسطة SEM و AFM والتي أظهرت قطر الجسيمات بين 22.9 إلى 73.95. أكدت الدراسة الحالية أن طريقة تعريض PHB لتدرج الأس الهيدروجيني والمستوى العالي من الموجات فوق الصوتية تنتج جسيمات PHB النانوية.

Introduction

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate polymer belonging to the polyesters class that has the area of interest as bio-derived and biodegradable plastics. The poly-3-hydroxybutyrate (P3HB) form of PHB is probably the most common type of polyhydroxyalkanoate, but other polymers of this class are produced by a variety of organisms. These include poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO), and their copolymers [1]. PHA is one of many biopolymers of linear-chain ester groups that are produced as a carbon source and energy reserve in bacteria during physiologically stressful conditions . They exhibit good physiochemical properties that are exploited for many biomedical applications. PHB is the first to be found under the category of four carbon chained homopolymers. It is universally known as an alternative for plastics. PHB is being used as an implantation material, biofuel, etc. [1].

PHB is a material which also generates interest by having different approaches for synthesis. There are three routes of obtaining PHB, the first being by means of polymerization synthesis [2]. Nanoparticles (NPs) are commonly used as antimicrobial agents that affect microbial growth in different ways, especially biofilm formation [3] and occasionally by stimulating the innate immune response to pathogens *in vivo* [4]. Nanoparticles are ultrafine particles that are ranging between 1 and 100 nm in size. The chemical nature of nanoparticles may be metallic or polymeric. Polymeric nanoparticles may be synthetic or natural and exhibit nano-sized colloidal structures [5]. Based on the preparation method of nanoparticles, drugs can be loaded into or onto the surface. In nano capsules, the drug is entrapped by the polymeric surface surrounding the molecule. Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Nanoparticles (NPs) are methods used to detect

In the present study, we used a new method of NPs synthesis that is based on exposing the PHB to ultrasound under different pH values.

Materials and methods

Materials

PHB was purchased from Sigma-Aldrich, USA. PHB used in the present study was derived from microbial fermentation.

Synthesis of PHB Nanoparticles-

One gram of PHB was added to 50 ml of distilled water and pH was adjusted to 4 by HCl (1 N). The mixture was placed in an ultrasonic path at 4500 kh for 25 seconds. The pH was readjusted to 10 by NaoH (1N). The mixture was mixed by a magnetic stirrer for 2 hrs. at 21 ^{o}C. The mixture was incubated at 21 ^{o}C for 18 hrs. and then the pH was readjusted to 7 by HCl (1 N). The characterization of the prepared nanoparticles was achieved by using atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV) spectrophotometer, X-ray powder diffraction (XRD), and scanning electron microscopy (SEM) [7].

Characterization of PHB Nanoparticles

Different standard methods were followed to judge whether the prepared particles of PHB were PHB nanoparticles.

UV-Vis spectral analysis
PHB nanoparticles synthesis was confirmed by measuring the wavelength of the prepared mixture by UV-Vis spectrum at a resolution of 1 nm in 2 ml quartz cuvette with 1 cm path length. Scanning range for the samples was 190-1100 nm at a scan speed of 500 nm/min. A blank reference was used for the correction of the spectrophotometer. The UV-Vis absorption spectra of all the samples were recorded and numerical data were plotted [5].

Atomic force microscopy (AFM) analysis
Atomic force microscopy was used to analyze the prepared PHB nanoparticles. A thin film of the prepared PHB nanoparticles was deposited on a silica glass plate by dropping few drops of the mixture of PHB nanoparticles on the plate and allowing them to dry at room temperature in the dark. The deposited film glass plate was then scanned with the AFM [8].

Scanning electron microscope (SEM)
The main features of morphology and diameter of nanoparticles were characterized using SEM. The dried sample of PHB nanoparticles solution was sonicated with distilled water; few drops of the prepared sample were placed on a glass slide and allowed to dry. After that, a thin layer of platinum was coated to make the samples conductive [9].

X- ray diffraction
In this technique, a glass slide was prepared that contains PHB nanoparticles at a concentration of 20 mg/ml (several drops) to form a thin layer of PHB suspension with a thickness of 0.5 mm. After that, it was examined using an X- ray diffraction device by exposing to K and Cu rays over the model to be measured, at different angles (10^o to 80^o), with a wavelength of 1.5406 Å, a voltage of 40 kilovolts, and an amount of 2^o /min [7].

Fourier-transform infrared spectroscopy (FTIR)
FTIR is a technique which is used to obtain infrared spectra of absorption, emission, and photoconductivity of solid, liquid, and gas. It was used to detect different functional groups in PHB. FTIR spectra are recorded between 4000 and 400 cm−1. For FTIR analysis, the polymer was dissolved in chloroform and layered on a NaCl crystal. After the evaporation of chloroform, the polymer film was subjected to FTIR. The spectrum of PHB showed peaks at 1724 cm−1 and 1279 cm−1, which correspond to specific rotations around carbon atoms. The peak at 1724 cm−1 corresponds to C–O stretch of the ester group present in the molecular chain of a highly ordered structure. The adsorption band at 1279 cm−1 corresponds to ester bonding [12].

Results
PHB nanoparticles preparation
UV-Vis spectral analysis
The PHB nanoparticles were examined with visible and UV spectrophotometers. The results showed that three peaks were raised. The absorbance values of the peaks were 2.967 and 0.595 at the wavelengths of 283 and 311.00, respectively (Figure 1).

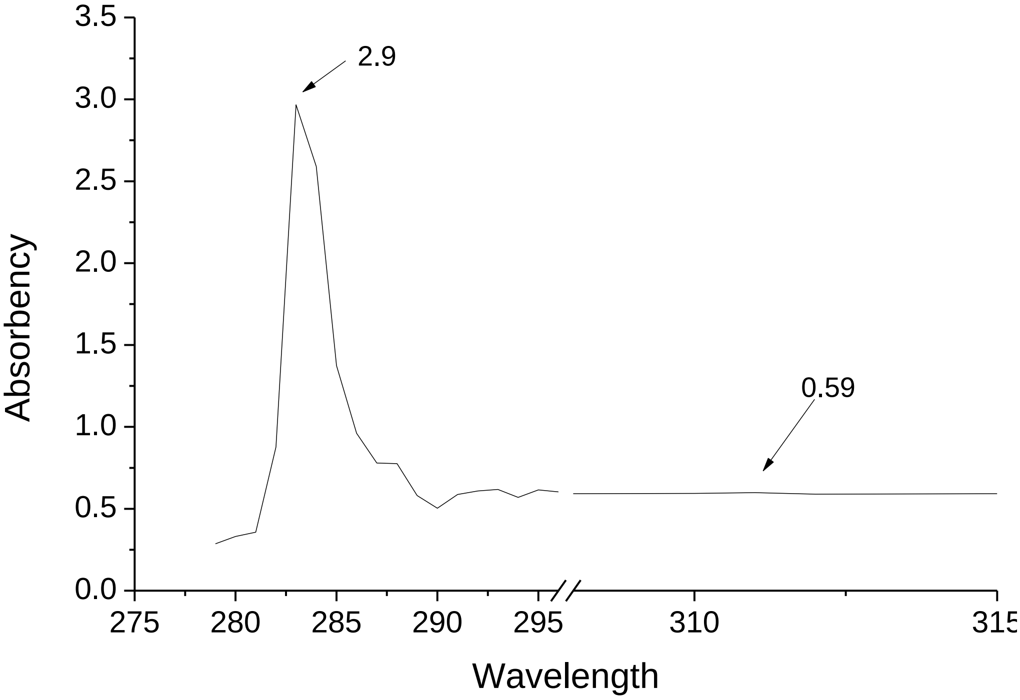
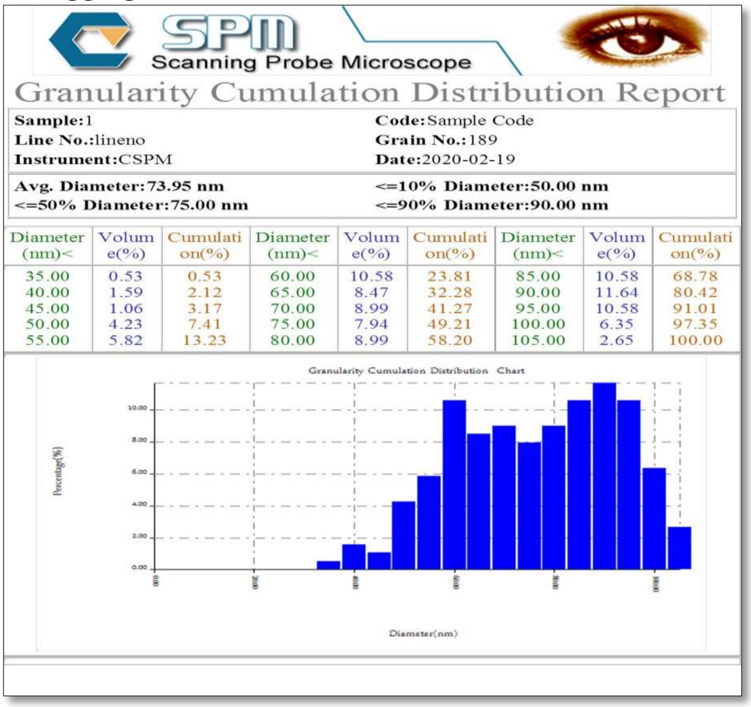


Figure 1-Absorbency of PHB nanoparticles under different wavelengths. The range of wavelength was 0000 to 0000 nm.

Atomic force microscopy (AFM) analysis

In the present study, PHB nanoparticles that were prepared from PHB were examined under AFM apparatus to confirm the presence of PHB nanoparticles. The results demonstrated the existence of a number of nanoparticles with different diameters, with an average of 79 nanometers (Figures 2 and 3). In all examined samples, the nanoparticles were found to be spherical-shaped and aggregated, with smooth surfaces.



SPM Scanning Probe Microscope

Granularity Cumulation Distribution Report

Sample:1 **Code:**Sample Code
Line No.:lineno **Grain No.:**189
Instrument:CSPM **Date:**2020-02-19

Avg. Diameter:73.95 nm **<=10% Diameter:50.00 nm**
<=50% Diameter:75.00 nm **<=90% Diameter:90.00 nm**

Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)
35.00	0.53	0.53	60.00	10.58	23.81	85.00	10.58	68.78
40.00	1.59	2.12	65.00	8.47	32.28	90.00	11.64	80.42
45.00	1.06	3.17	70.00	8.99	41.27	95.00	10.58	91.01
50.00	4.23	7.41	75.00	7.94	49.21	100.00	6.35	97.35
55.00	5.82	13.23	80.00	8.99	58.20	105.00	2.65	100.00

Figure 2-Atomic force microscopy (AFM) analysis showing the diameters of PHB nanoparticles.

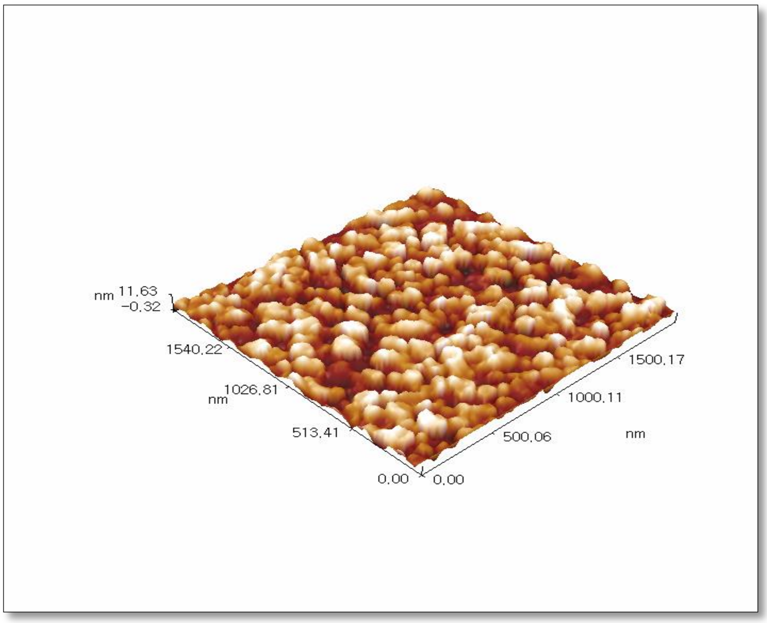


Figure 3-Scanned picture under AFM apparatus showing the diameters of the prepared PHB nanoparticles.

Scanning electron microscopy

The PHB nanoparticles prepared in the present study were analyzed by SEM to determine their diameter and shape. The results showed clearly that the range of diameters of the PHB nanoparticles was from 15.5 to 34.8 nm, with an average of 22.9 nm. The data of size of particles were collected by using the Digimizer software. The present study showed that the prepared PBH typically formed nanoparticles with a very small size. These results confirmed that the method used in preparing PHB nanoparticles was very efficient (Figure 4a and b).

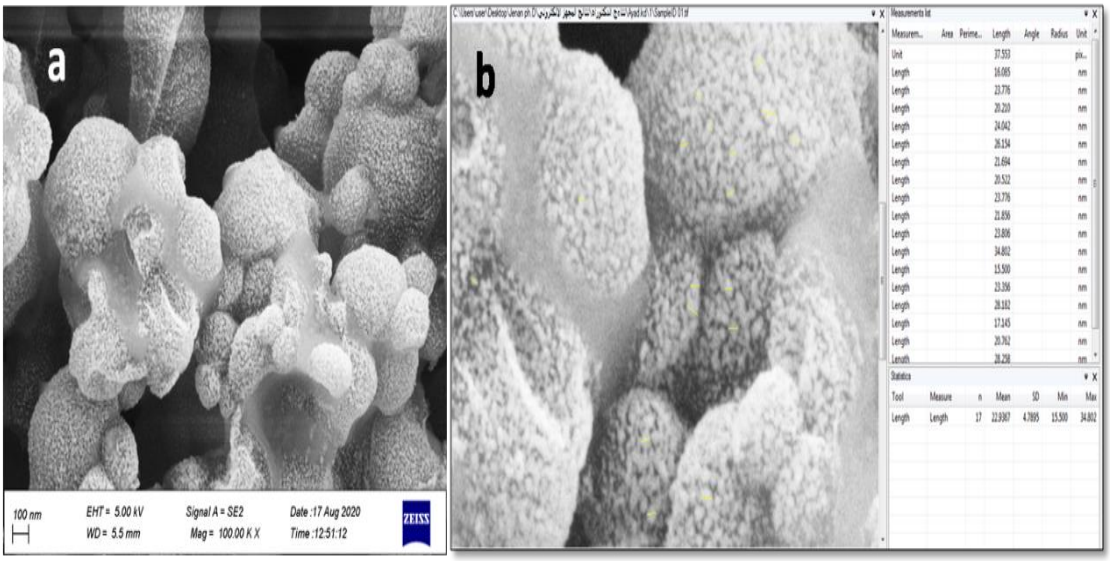


Figure 4-a and **b.** PHB nanoparticles picture taken by scanning electron microscope. The diameters of particles ranged from 34.8 to 15.5 nm with an average of 22.9 nm. The data were analyzed by Digimizer software.

X- Ray diffraction

The prepared PHB nanoparticles were examined by XRD and the results showed different peaks, but there were only four main peaks at 13.4, 17.2, 32 and 45.8 Å. The largest peak was seen at 45.8 Å (Figure 5).

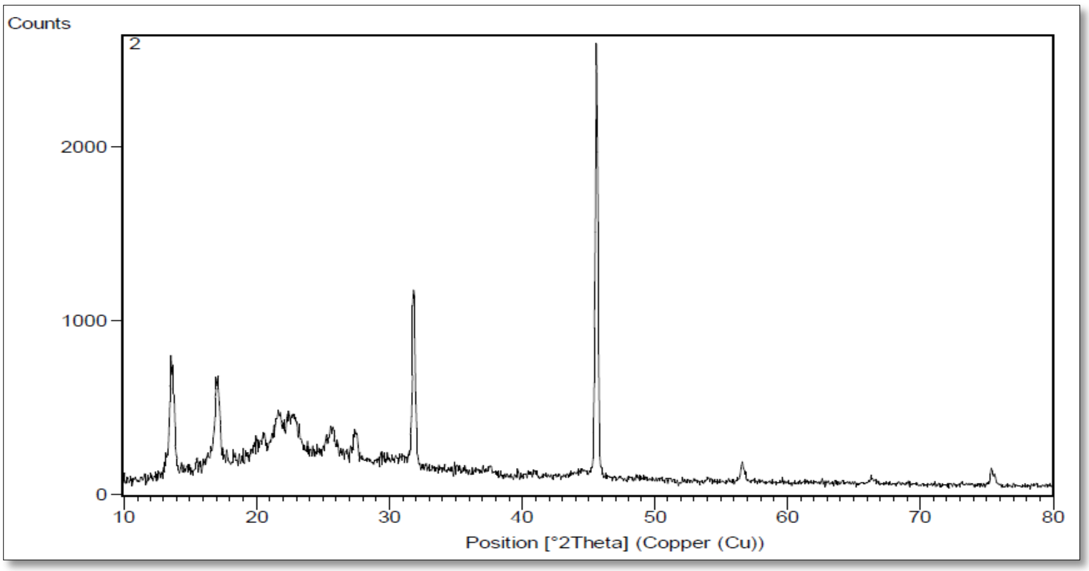


Figure 5-The XRD patterns of PHB nanoparticles. The scale shows four main peaks.

Fourier-transform infrared spectroscopy

The prepared PHB nanoparticles were subjected to FTIR analysis, as shown in Figure 6. From the spectrum obtained, it is inferred that the bands at 3458.17 cm^{-1} and 3440.77 cm^{-1} correspond to OH (Hydroxyl) group, whereas the band at 1728.1 cm^{-1} represents C=O (Carbonyl) and COO (ester) groups. The band at 1371 cm^{-1} represents COH bond, whereas the band at 1288 cm^{-1} corresponds to CH. The results also showed asymmetrical stretching and bending vibration in the CH3 group stretch of bands ranging 1058.85 -1288.36 cm^{-1} , which showed C-O bonding. The analyzed results are consistent with the reports of Arun *et al*. (2009) and Padermshoke *et al*. (2004), confirming that the isolated compound was PHB.

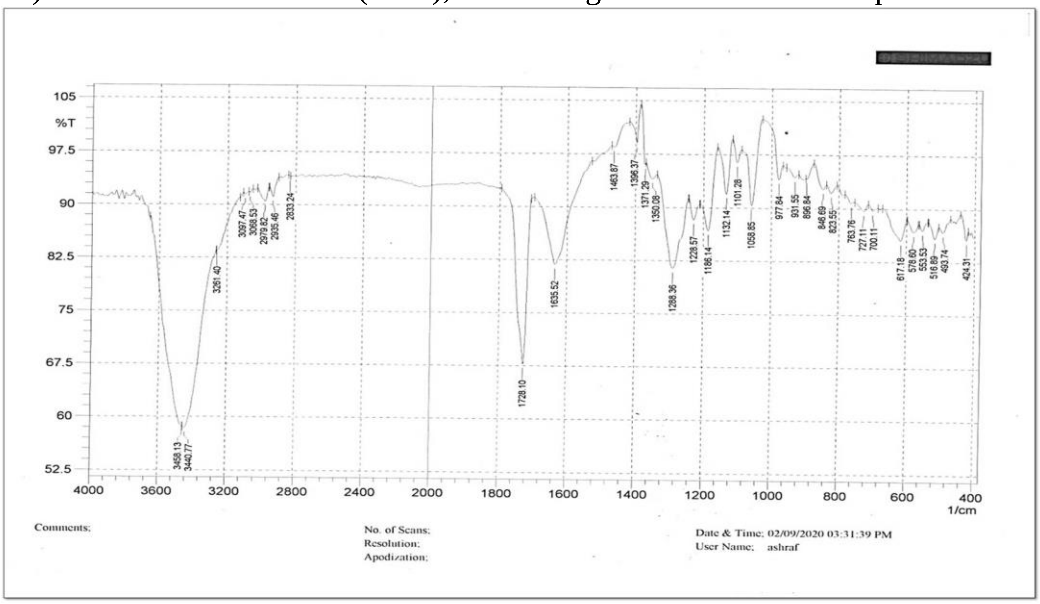


Figure 6-Fourier transform infrared spectroscopy spectral analysis of PHB nanoparticles in the 400–4000 cm^{-1} wave number region.

Discussion

There are several methods that were used by many investigators to prepare PHB nanoparticles [13, 14]. In the present study, a different method was used for the preparation; the method was dependent on exposing the PHB suspension to ultrasound at different pH values. Different tests (UV-Vis spectra analysis, AFM, SEM, XRD and FTIR) were performed to check the presence of PHB nanoparticles as an indicator of success of the method used in the present study.

The results of previous studies, which used UV-Vis spectra analysis to check the presence of PHB nanoparticles, showed that the peaks of PHB absorbency for PHB prepared from *Azohydromonas australica* DSM 1124 were found at 235 nm [15]. A similar result was found by Alarfaj *et al*. (2015) [16]. These results are similar to, but not the same as, those found in the present study in terms of the peak of absorbency, because the particles used in the present study are nanoparticles.

The size of PHB nanoparticles varies according to several factors, such as the method of preparation, source of PHB, and environment of PHB nanoparticles preparation. Senthilkumar *et al.* (2018) found that PHB nanoparticles prepared by the nanoprecipitation method with different solvent systems, i.e. Chloroform: DMSO (CD), Chloroform: Water (CW), Ethylacetate: DMSO (ED), and Ethylacetate: Water (EW), had different sizes that ranged 110 – 300 nm after examination by AFM [17]. Pachiyappan *et al.* (2019) prepared PHB nanoparticles by following the nanoprecipitation method with different solvents and surfactant (Tween 80) concentrations. They found that the diameters of the nanoparticles, as examined by AFM, ranged from 60 to 300 nm [18].

SAM is one of the most important and accurate methods to determine the sizes and shapes of nanoparticles obtained through practical experiments. Most of previous studies that dealt with preparing nanoparticles relied on SAM to determine their success in obtaining nanoparticles, since, by using this method, the nanoparticles can be seen and their pictures can be acquired [15-18].

Previous studies also showed the results of the utilization of XRD to detect PHB nanoparticles. Liau *et al*. (2014) showed very small peaks at 2θ of 2.195, 2.275, and 2.325∘ with 40.20 , 38.78 , and 38.00 ̊A interlayer spacing values, respectively. This increment of d-spacing indicates that polymer chains are intercalated into the clay layers to form intercalated type nanocomposites [19]. Mottin *et al*. (2016) examined the prepared PHB nanoparticles using an XRD apparatus and their results are slightly different from those of the present study because the nanoparticles they prepared are nanofiber particles [20]. The results of other previous studies were in agreement with those of the present study [21, 22].

When the prepared PHB nanoparticles were examined by FTIR, different peaks were seen that match those observed in other studies. Those prepared and purified PHB nanoparticles from several sources that according to the several reasons such as the source of PHB [17].

The present study showed that the PHB particles prepared by the selected method are typically nanoparticles. This leads to conclude that the method used in the present study is useful to prepare PHB nanoparticles.

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