

PRODUCTION OF POLYHYDROXYBUTERATE (PHB) FROM INDUSTRIAL WASTE

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ABSTRACT

Biodegradable polymers are of reasonable medical and agricultural applications nowadays. Samples from various natural environments in South Riyadh area, Saudi Arabia, were screened for microorganisms that are capable of producing poly (3-hydroxybutyrate-co-4- hydroxybutyrate). A total of 50 isolates were collected and two out of those were identified as possible PHA producers based on Nile red staining methods. The isolate NG-2 obtained in this study was cultivated in MSM containing date palm syrup as inexpensive carbon source for economic production. Moreover, PHA was extracted from the NG-2 isolate and analyzed by IR followed by electron microscope to confirm the capability of producing PHB. Efforts have been paid to determine the optimal condition of the culture growth and its biodegradation activity. It was found that despite of the type of the polymer-containing media used, the optimum incubation temperature for the tested microorganism was 37°C, while pH value of 7.2 resulted in the best growth pattern in date palm syrup at concentration of 8 % containing media.

Our major goal is to develop biodegradable drug carriers as effective drug delivery systems that release particular nutrient or drug at a particular site. This biotechnology of biological delivery has a unique physiochemical and mechanical properties help in field of pharmaceutical and nutraceutical targeting. This sort of nanotechnology will appear in subsequent works.

Keywords: marine bacteria _ Polyhydroxyalkanoate _ Nile red _ PHA producer _

INTRODUCTION

Plastics has a lot of applications in our life and have demand increase each year. They are utilized in almost every manufacturing industry, ranging from automobiles to medicine. According to its chemical structure plastics has importance in industry, it can be manipulated in a wide range of shapes. The elasticity and strength of plastic make it easily be molded into different applicable shapes ranging from fibers to thin films (**Madison and Huisman, 1999**).

Most of plastics and synthetic polymers are produced from petrochemicals and because of its hazards on human health and its damage effects on environment, there are growing studies concerning the use of biodegradable polymers as an ecologically useful alternative. The biopolymer are produced from renewable resources and do not depletion of the resources.

Among various biodegradable polymer materials, plastics are replaced by the polyhydroxy alkanoates (PHAs) because it has the same properties of petrochemical plastics. Moreover, it can be completely degradable and easily be used in different applications (**Page, 1992**). They are accumulates in numerous microorganisms under unbalanced growth

conditions as a mechanism of storage excess carbon and energy (Ojumu *et al.*, 2004 and Mahishi *et al.*, 2003).

Many bacterial strains either Gram-negative or Gram-positive accumulate PHB and can grow on different types of growth media with different carbon and nitrogen sources. Among of these strains are *Ralstonia eutropha*, which represent the most promising microorganism used now for production of commercial PHB (Lee, 1996).

For commercialization of PHAs, tremendous efforts have been devoted to reduce the production cost by the development of better bacterial strains and more efficient fermentation recovery processes (Choi & Lee, 1998 and Ahn *et al.*, 2000).

From past decade, PHAs has attention from different scientific studies as metabolism, biochemistry, physiology and molecular genetics. Now the genetic studies cleared that the polyhydroxy alkonlate (PHAs) are produced from different variety of microorganisms and can be produced in a large scale production by using gene transformation.

PHA biopolymer has many advantages, they are biodegradable, insoluble in water, non-toxic, biocompatible, thermoplastic. These advantages make them unique substances suitable for different applications such as packaging industry, medicine, pharmacy, agriculture, food industry, and the production of paints (Christopher and Anthony 2012).

The quantity of the storage biopolymer inside bacterial microorganisms is affected by different environmental and physiological conditions. Incubation temperature, incubation time, hydrogen ion concentration, and the nutrients may enhance or stimulate PHAs production (Gulab *et al.*, 2011).

The present study aimed to investigate the bacterial strains isolated from one of Saudi industrial area with the ability to accumulate PHAs as a unique storage material.

MATERIALS AND METHODS

Isolation, screening and selection of PHA producing bacteria

Bacterial strains were isolated from industrial waste water samples, industrial area, South Riyadh. The isolation procedure of all isolates was performed according to the method of Quillaguamán *et al.* (2004) as follows: 100 μ L of water sample was spread on solid HM medium, containing (g/L): 30 NaCl; 0.25 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.09 CaCl_2 ; 0.5 KCl; 0.06 NaBr; 5 peptone; 10 yeast extract; 1 glucose; and 20 agar. The pH was adjusted to 7 and the plates were incubated at 35°C for 30h. The colonies produced were isolated by plating them again on fresh agar medium.

Bacterial isolates were grown again on a modified solid HM containing (g/L): NaCl, 30; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; CaCl_2 , 0.09; KCl, 0.5; NaBr, 0.06; KH_2PO_4 , 0.25, yeast extract, 2; granulated agar, 20 and pH 7. The previous media supplemented with adding of 5% date palm syrup (Khalas) as inexpensive carbon source.

Nile red (Sigma) with final concentration of 0.5 μ g dye per mL of the medium was added. Plates were then exposed to ultraviolet light (300 nm) to monitor the accumulation of the PHA. The lighted plates were recorded as positive result.

Factors affecting PHAs production

More investigation was carried out on the NG-II isolate to study the culture conditions that affects production of PHA. Therefore, three different media as YT media

containing (g/l) 16g trypton, 10g yeast extract and 10g NaCl, the pH was adjusted to 7.0 (**Hanahan, 1985**), SG media containing 25% NaCl (w/v), 2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v), 0.2% KCl (w/v), 3% trisodium citrate (w/v), 1% yeast extract (w/v), and 0.75% Casmino acids (w/v), the pH was adjusted to 7.0, and LB media containing (g/l) 10 g NaCl, 10 g trypton and 5g yeast extract were used with different cultivation conditions (Lee, 1996, Ramsay *et al.*, 1990 and Kim *et al.*, 1994). Moreover, incubation temperature, pH, various carbon sources were also tested, with respect to their PHA accumulation, in flasks that incubated on a shaker (200 rpm).

Extraction of the PHA

Recovery by chloroform extraction

Solvent extraction is the most extensively adopted method to recover PHA from the cell biomass. In this study, PHA granuals were extracted by using methods described by **Santhanam and Sasidharan (2010)** as follows: The PHA was directly extracted using chloroform. First, the bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The lipids were removed from the cell pellet-using methanol (40 times the volume of cell pellets) and the cells were incubated at 95°C for 1 h. before filtered to remove the methanol completely and the sediment granules were incubated in an oven at 65°C till dry. Chloroform was added to the dried granules and was incubated at 95°C for 10 min and after cooling, the mixture was gently mixed overnight. The solution was then filtered to get the debris. Finally, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried.

Recovery by Sodium hypochlorite

According to the method of **Lillo and Valera, (1990)**, 5 ml of sodium hybochlorite (12 %) was added to about 0.2 g of lyophilized cell biomass, and the mixture was leaved at 40°C for 1 h. The PHA granules were collected by centrifugation (2000 xg), and the pellet was then washed by water then by ethanol and acetone, and the final pellet was dissolved in chloroform, while the insoluble residue was discarded. The chloroform was evaporated at room temperature and the PHA granules were collected and weighed.

Recovery by NaOH

PHA was extracted from bacterial cells by using the method of (Choi and Lee, 1998) as follows, cells were treatment in 0.1 M sodium hydroxide (NaOH) and incubated for 1 hour at 30°C. Every 15 minutes, each tube was vortex for approximately 3 minutes. PHA was separated from the aqueous fraction containing cell debris by centrifugation. Then, the pellet was washed using 20% ethanol for 3 hours and rinse with distilled water. After centrifugation, the polymers were then kept and dry in freeze-dryer.

Infra Red (IR) spectroscopic analysis of PHA

The extracted PHA was analyzed for identification of its functional groups through FTIR analysis. The solid samples (PHA) were dissolved in chloroform (CHCl_3) and deposited as a film on a KBr disk. The PHA samples analyzed using Fourier Transform Infra Red Spectroscopy, (Model: FTIR-4100 JASCO). The peaks were observed from 4000-400 cm^{-1} (**Ravenelle and Marchessault, 2002**).

RESULTS AND DISCUSSION

This study has been designed to isolate PHA accumulating bacteria from ecosystem resources and select the more efficient strains with the optimal condition of growth.

Screening of culture collection for PHAs producers

The bacterial isolates were screened for estimation of the presence of PHAs by using Nile- red assay. The results indicated that two bacterial isolates are of strong fluorescence when stained with Nile-red, therefore they recorded as PHA positives.

Zuriani et al. (2013) used Nile red fluorescence as a rapid quantitative measurement of accumulated polyhydroxyalkanoate (PHA) by microorganisms. Likewise, Landazuri and Maldonado, (2008) reported that various microorganisms isolated from the soil were screened for their PHA production and confirmed by the orange fluorescence emitted after staining with Nile blue dye under UV transilluminator. On the other hands, **Juan et al. (1998)** used the method of Sudan Black B stain and he considered black stained isolate positives for PHA production and the poorly stained a negative result.

Extraction of PHAs from bacterial cells

To extract the PHAs, various extraction methods either chloroform, sodium hypochlorite or sodium hydroxide were performed. The results presented in Fig. (1) indicated that the extraction of PHA using chloroform gave 6 mg/ml with recovery percentage 30% and sodium hypochlorite extraction give 10mg/ml with recovery 50% but the extraction with NaOH made the highest value represent 14 mg/ml with recovery equal 70%. Also, the chloroform extraction method is time consuming and yielded low quantity than the other methods of extraction. On the other hand, the use of NaOH gave the best results than the chloroform and sodium hypochlorite. It gives the highest value of recovery of PHA than the two other methods of extraction. Similar results were observed by **Kunasundari and Sudesh (2011)** they extracted PHA with solvents such as chloroform and 1,2-dichloroethane and reported that this method of extraction has some disadvantage as consumption of large volume of toxic and volatile solvents and it is high capital and operation cost. On the other hand, **Xuan Jiang (2010)** stated that treated biomass with 0.20 N NaOH at $22 \pm 1^\circ\text{C}$, PHA increased purity to 89.3%.

FTIR spectroscopic analysis

The FTIR spectroscopic analysis gave further insights into the chemical structure without a previous hydrolysis of the polymer and reflects the existence of monomeric units. As shown in Fig. (2), the results of NG-II isolate indicate the absorption bands appeared in the spectrum are associated with the side chains from the ester $\text{C}=\text{O}$ stretching vibration at 1740 cm^{-1} , the CH_3 -deformation peak at 1286 cm^{-1} and the ester $\text{C}-\text{O}-\text{C}$ at 1056 cm^{-1} which clearly put the extracted polymer sample obtained from isolate in the class of Polyhydroxybutyric acid. The results reported by **Hong et al. (1999)** indicated that a band at between 1728 cm^{-1} and 1744 cm^{-1} is characteristic for PHA. Our results agree with results reported by **Jarute et al. 2004)** and **Kansiz et al., (2007)** who clearly indicated that this spectrum is associated with the presence of PHB in pure cultures at about 1730 cm^{-1} .

Optimization of the cultivation conditions

Effect of different fermentation media on Cell growth

To achieve enough biomass from NG-II isolate required as inoculum for PHB production, various growth media (YT, LB and SG), and incubation times (24, 48 and 72 hr)

at 30°C were studied. As shown in Fig. (3) After incubation time for 72 hours, YT medium yielded the highest cell biomass to be used in the further studies. Our results agree with results of **Khanafari et al. (2006)** he reported that poly- β hydroxyl-butyrate production was increased in presence of meat extract up to 75% of the cell dry weight after 48h. At same time, the addition of nitrogenous source had a positive effect on poly- β hydroxyl-butyrate production and presence of meat extract made 4.43 g/L of PHB yields.

The effect of hydrogen ion concentration (pH)

In this experiment, the optimized mineral salt medium was prepared as previously described and the medium was initially adjusted at different pH values ranged from 6.0 to 8.5 using either NaOH (1N) or HCl (1N). Under the optimized growth conditions (incubation at 37°C for 48 hour in HM medium), the effect of pH on the growth rate of NG-II isolate was examined. As shown in Fig. (4), the results cleared that the highest growth rate (O.D 600 =0.85) was observed at pH 7.2, a significant decrease in PHB accumulation was observed when the culture grew at lower or higher pH values. Our results seemed to be the same results of **Sreya et al. (2012)** who reported an optimum growth of isolate VK-12 of genus *Burkholderia* pH 7.0, temperature 30 °C and incubation time at 48h.

Effect of different carbon sources on the production of PHB

Under the optimized growth conditions, different carbon sources were tested. The data presented in Fig. (5) showed that the highest PHB yield was recorded with glucose (0.5 mg/ml), Sucrose (4 mg/ml), peptone (2 mg/ml), yeast extract (1 mg/ml), and starch (1.5 mg/ml), However, the highest PHB yield among all the tested carbon sources was observed with sucrose (4 mg/ml).

PHB production from date palm syrup as inexpensive Carbon source

The effect of different concentrations of date palm syrup (1-30% v/v) as inexpensive carbon source was studied. In HM medium with incubation time for 48 h at 37°C, different date palm syrup concentrations ranged from 1 to 30% (v/v) were tested. The data revealed that the highest amount of PHB (4.5 mg/ml) produced by the NG-II isolate was achieved with date palm syrup (Khalas) concentration of 8% as illustrated in Fig (6). Our results closely agree with the results reported by **Omar et al. (2001)** who found that optimal growth and PHB accumulation in *Bacillus megaterium* occurred with 5% (w/v) date syrup.

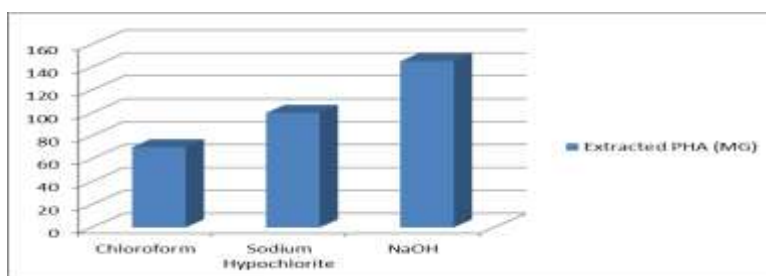


Fig. (1): PHA yields extracted with different extraction methods.

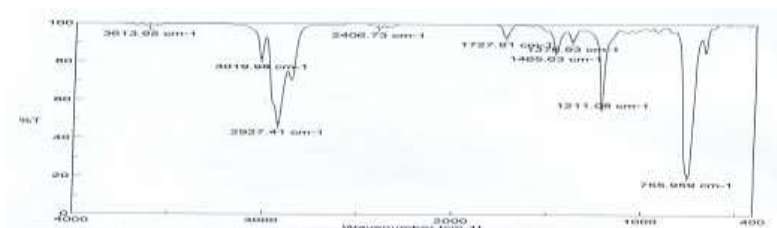


Fig 2: IR spectrum of extracted biopolymer

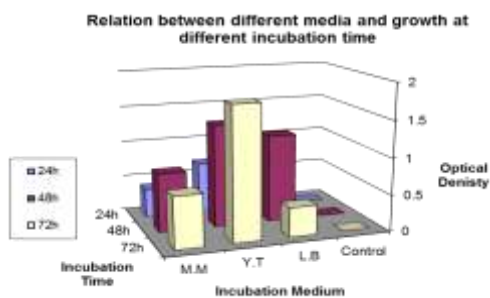


Fig 3: Effect of different nutrient media and different incubation times.

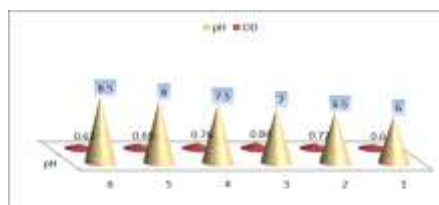


Fig 4: The effect of different pH values on bacterial growth.

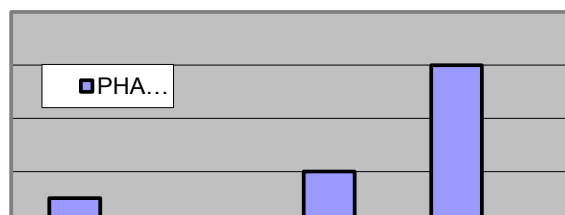


Fig 5: The effect of different carbon sources on PHA content.

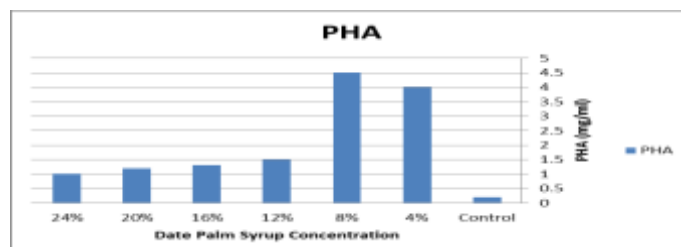


Fig 6: Effect of different Date Palm syrup concentrations

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إنتاج البوليمر البيولوجي هيدروكسي بيوترات (PHB) من مخلفات الصناعة

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أجريت في هذا البحث العديد من التجارب الحيوية المعملية وشبه التصنيعية وذلك بهدف إنتاج بوليمر حيوي (Biopolymer) ذو خاصية تحليله حيوية (Biodegradable) من بعض الأنواع البكتيرية المعزولة من مياه الصرف الصناعي وذلك لدراسة إمكانية استخدامه كبديل حيوي وغير ملوث للبيئة في صناعة البلاستيك وكمكون أساسي في بعض الصناعات الغذائية والطبية وصناعة الدواء. لتحقيق هذا الهدف تم البحث أولاً عن وجود أو عدم وجود هذه البوليمرات الحيوية وخاصة مجموعة البولي هيدروكسي الكانواتس (PHAs) موضع الدراسة بين العديد من السلالات البكتيرية التي تم عزلها من مواقع بيئية عديدة من مناطق الصرف الصناعي ومن مناطق الصرف الصحي المنزلي وباستخدام طريقة الرصد الحيوي بواسطة صبغة النيل الحمراء (Nile-red) والتتبع الايجابي والسلبي لها تحت الاشعة فوق البنفسجية. أثبتت النتائج ايجابية عزلتين لهما القدرة على تخزين البوليمرات الحيوية في خلاياها بدرجات متفاوتة وقد وجد أن عزلة واحدة (NG-II) ذات قدرة أعلى لتخزين هذه البوليمرات. وتم في هذا البحث أيضاً استخلاص وفصل وتنقية البوليمر من العزلة البكتيرية بطرق عدة مثل الكلوروفورم وهيبوكلوريت الصوديوم وثبت أن من أفضلها طريقة الاستخلاص الثالثة بواسطة هيدروكسيد الصوديوم.

تم دراسة انسب العوامل اللازمة لنمو هذه العزلة البكتيرية مثل درجة الحرارة المثلى و فترة التحضين وانسب مصدر كربوني وكذا الرقم الهيدروجيني. ولقد أثبتت النتائج أن افضل درجة حرارة هي 37 درجة مئوية عند اس هيدروجيني 7.2 وعند فترة تحضين 48 ساعة وباستخدام مصدر كربوني رخيص هو عصير التمر بنسبة اضافة 8% الى الوسط الغذائي.

الدراسات مستمرة لاستغلال هذا المركب العديد في مجال الصيدلة و التغذية و فقا لتكنولوجيا النانو.