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# Biotreatment of Industrial Pollutant (Carbon Dioxide) and its Role in Bioplastics Production

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## Abstract

Polyhydroxyalkanoates (PHAs) are a class of biopolymers with characteristics resembling those of petrochemical-based plastics that have gained recent attention and a growing amount of research effort due to their environmental friendliness. Numerous bacteria, including Alcaligenes spp., Pseudomonas spp., Staphylococcus spp., and algae create (PHAs) in the presence of carbon and limiting nutrients like nitrogen, and these biopolymers can successfully replace industrial plastics. Therefore, the goal of this study is to employ carbon dioxide, an industrial pollutant emitted into the environment, as a cheap carbon source to lower production costs and remove pollution at the same time. Only three of the nine bacterial isolates utilized were able to synthesize the polymer in the presence of $CO_2$, and the best isolate was belonging to the genus Alcaligenes after 48 hours of incubation at 30°C and pH 7, which are the optimal conditions for polymer synthesis. Bacterial growth resulted in the production of 5.2gm/l of PHA and 6.2gm/l of biomass under these conditions.

**Keywords:** Alcaligenes, Polyhydroxyalkanoates, $CO_2$, Anaerobic bacteria, Biopolymers.

## المعالجة الحيوية للملوث الصناعي ثاني اوكسيد الكربون ودوره في انتاج البلاستيك الحيوي

**الخلاصة:**

بولي هيدروكسي ألكانوات (PHAs) هي فئة من البوليمرات الحيوية ذات خصائص تشبه تلك الموجودة في المواد البلاستيكية القائمة على البتروكيماويات والتي اكتسبت اهتمامًا مؤخرًا وكمًا متزايدًا من الجهود البحثية بسبب ملاءمتها للبيئة. العديد من البكتيريا، بما في ذلك *Alcaligenes* spp.، *Pseudomonas* spp.، *Staphylococcus* spp.، والطحالب تخلق (PHAs) في وجود الكربون والمواد المغذية المحدودة مثل النيتروجين، ويمكن لهذه البوليمرات الحيوية أن تحل محل المواد البلاستيكية الصناعية بنجاح. ولذلك فإن الهدف من هذه الدراسة هو توظيف ثاني أكسيد الكربون، وهو أحد الملوثات الصناعية المنبعثة في البيئة، كمصدر كاربوني رخيص الثمن لخفض تكاليف الإنتاج وإزالة التلوث في نفس الوقت. ثلاثة عزلات بكتيرية من التسعة المستخدمة في البحث اثبتت قدرتها على تصنيع البوليمر في وجود ثاني أكسيد الكربون، وأفضل عزلة كانت تابعة للجنس *Alcaligenes* بعد حضانة 48 ساعة في 30 درجة مئوية والاس الهيدروجيني 7، وهي الظروف المثلى لتخليق البوليمر. أدى نمو البكتيريا إلى إنتاج 5.2 غم/لتر من PHA و6.2 غم/لتر من الكتلة الحيوية في ظل هذه الظروف.

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## 1. Introduction:

Oil wealth has received wide attention at the international and regional levels, given that it poses a threat to the environment, public health, and biodiversity, as well as because it poses a threat to the environment, public health, and biodiversity due to the misuse of this wealth [1]. It is the responsibility of the state and its institutes to protect the environment, whether through preventing pollution and damage before they occur and removing and reducing their effects, or through their commitment to treat these pollutants, where the competent authorities must take all measures to stop them or impose respect for the legal rules related to the environment that aim to prevent environmental risks or stop violating them [2]. $CO_2$ capture and storage are considered to be one of the promising techniques to reduce $CO_2$ [3]. In recent years, the synthesis and research of polymers that are based on derivatives of carbonic acids have received a significant amount of attention. In the category of biodegradable polyesters, such as polylactides and polyglycolides, polyesters of microbial origin, known as polyhydroxyalkanoates (PHAs), hold a unique and significant position. The primary benefit of using genes from Ralstonia [4]. *Ralstonia eutropha* housing *Pseudomonas* synthase gene, *E. coli* harboring PHA-synthesis genes from Aeromonas and Ralstonia, which make HB-HHx copolymers, *R. eutropha* harboring *Pseudomonas* synthase gene, *R. eutropha* harbor [5]. PHAs are polyesters with a wide variety of chemical structures, ranging from high-crystallinity thermoplastic polymers to thermo labile rubber-like elastomers. PHAs can be divided into three groups: short-chain- length PHAs (PHASCL), medium-chain-length PHAs (PHAMCL), and long-chain-length PHAs. PHAs are used in a variety of applications, including the production of plastics, elastomers (PHALCL). They are insoluble in water and exhibit an amorphous mobile liquid state in vivo but in laboratory setting, they display variety of mechanical and material properties, from rigid and brittle crystals to elastomers and molds, [6]. PHA biosynthetic pathway is initiated by condensation of two molecules of acetyl-coenzyme-A (acetyl-CoA) by β-ketothiolase to acetoacetyl-CoA which is then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase and then be polymerized into PHA by PHA synthase [7]. They have proven to degrade quickly into carbon dioxide, water, methane and biomass due to the enzymatic activities of microbes [8,6]. The synthesis of multi-component PHAs requires certain conditions to be met in terms of the nutrition provided by carbon [9]. As a general rule, PHAs with medium and long chain lengths are synthesized by employing complex carbon substrates. These complex carbon substrates consist of the primary carbon substrate as well as hydrocarbon acid salts with carbon chains of varied lengths (6 to 9 atoms and longer) as co-substrates. It has only been

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occasionally looked into, but the generation of P(3HB) from $CO_2$ through the autotrophic culture of hydrogen-oxidizing bacteria is a potentially useful use [10]. According to [11], the structure, molecular weight, and content of PHAs might vary significantly depending on the type of microbe, the conditions of development, and the method of polymer extraction. It is possible to extract carbon dioxide from fuel gases such as biogas, which typically includes between 50 and 70 percent $CO_2$. Upgrading of the biogas is accomplished through removal from the biogas. In addition, carbon dioxide can be removed from the exhaust of a gas engine using a scrubber. Methane, when burned in a gas engine, produces carbon dioxide along with a number of other trace elements. Methane serves as a fuel in gas engines. This carbon dioxide is discharged into the atmosphere in the form of a hot gas by the internal combustion engine. The industrial sector may find that carbon dioxide is a useful resource. For instance, it may be put to use as a fertilizer in greenhouses in order to stimulate the development of plants. However, in order to avoid adverse effects on the climate, it would be unwise to generate carbon dioxide ($CO_2$) for this objective by burning fossil fuels. It would be considerably better for the environment if the $CO_2$ that is produced during industrial processes could be filtered out of the exhaust fumes and turned into something beneficial [12]. A wide variety of bacterial species store carbon and energy in the form of Poly-D-3-hydroxybytyric acid, also known as P (3HB), which is accumulated in their cells. Some of them are able to produce the copolymer of D-3-hydroxybutyric acid and other hydroxyalkanoic acid, known as polyhydroxyalkanoate (PHA), by deriving their carbon from constrained sources, such as propionic acid and/or medium chain length fatty acids. It is anticipated that these bacterial polyesters will be utilized in the production of biodegradable polymers as a raw material [13; 14; 15; 16; 17). Hydrogen-oxidizing bacteria are microorganisms that are capable of autotrophic growth using a gas mixture consisting of hydrogen, oxygen, and carbon dioxide as a substrate, and they have been the subject of research for quite some time (18; 19; 20). *R. eutropha*, formerly known as *Alcaligenes eutrophus*, is a species of hydrogen-oxidizing bacterium that is commonly utilized in the synthesis of P (3HB), and it is also the species that has received the most research attention. On the other hand, the generation of P (3HB) from $CO_2$ by the autotrophic culture of hydrogen-oxidizing bacteria has seen very little research in the area of practical application. Through the course of our research, we have investigated a viable fermentation system for the generation of PHA from $CO_2$ through the autotrophic cultivation of *Alcaligenes* spp.

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## 2. Material and Methods

### 2.1 Samples collection

A- From areas with high sewage sludge pollution, such Al-Rustomiya in Baghdad, samples of soil, and sewage water sludge were taken. For this experiment, soil samples were taken from the top 10 to 20 cm, air dried, and sieved into fine particles.

B- The samples were then placed in sterilized glass vials and kept at 4 ° C in the refrigerator.

C- To obtain a pure culture for the bacterial isolates, 100ul was inoculated onto nutrient agar plates and incubated at 30°C for 72 hr after serially diluting all soil and sewage water samples.

D- Bacteria were cultivated in autotrophic conditions using $CO_2$ as the carbon source and mineral salt medium [21, 22] deficient in $NH_4Cl$ to identify the hydrogen-oxidizing bacteria and determine the best growth conditions. MSM contents: 1.0gm of $(NH_4)_2SO_4$, 0.5gm of $KH_2PO4$, 0.2gm of $MgSO_4.7H_2O$, 4.0mg of $CaSO_4$, and 15.0gm of agar in 1 liter of distilled water.

E- The media's pH was adjusted to 7.0 by 1M NaOH before autoclaving for 20min at 120°C. After the medium had cooled, 0.1ml of a solution containing trace elements was added, along with the following amounts: (per litter 0.5 n HCl): 5.56 g $FeSO_4.7H_2O$, 3.96 g $MnCl_2.4H_2O$, 5.62 g $CoSO_4.7H_2O$, 0.34 g $CuCl_2.2H_2O$, 0.58 g $ZnSO_4.7H_2O$, 0.60 g $H_3BO_3$, 0.04 g $NiCl_2.6H_2O$ and 0.060 g $Na_2MoO_4.2H_2O$

F- A vacuum pump removed the anaerobic jar's interior air before adding Gas Pak. The Gas Pak System, according to [23], is an airtight container that creates an anaerobic environment by combining water with sodium borohydride and sodium bicarbonate tablets. In this process, carbon dioxide and hydrogen gas are produced.

### 2.2 Sudan black B in solid medium

Sudan black B powder, 0.02 grams dissolved in 100 milliliters of ethyl alcohol to create a 60% concentration of this reagent. Using this reagent, the granules formed inside the bacterial cells, which represent the produced polymer (PHA), are examined and appear bluish black, while the negative result is represented by the absence of color [24].

### 2.3 Microscopic examination of PHA producing isolates

The Sudan black stain was used to test whether microbial intracellular lipids were present [25]. The lipid inclusion granules are visible under a microscope to be blue-black or blue-grey in color,

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whereas the bacterial cytoplasm is stained pale pink due to the presence of safranine counterstaining.

### 2.4 Morphological and biochemical characterization of PHA-producing isolate.

According to factors like size, color, and form, the most notable phenotypic features were observed and recorded as follows: Under the microscope, Gram staining was used to investigate the morphology of the cells. In order to identify the bacteria that were isolated by the biochemical tests, standard microbiological procedures were applied. Arginine broth, Gelatin, Bromocresol purple broth and oxidase test [26].

### 2.5 Optimization of PHA production

Colonies of the bacterial isolate were isolated, and then transferred to test tubes containing 20 milliliters of liquid mineral medium and 40 milliliters of space for growth. Phosphate buffer, consisting of 1.5 grams of $KH_2PO_4$ and 1.5 grams of $K_2HPO4$, was added to the MSM used for cultivation of bacterial culture in order to prevent a fall in the pH of the culture. After (24-48-72-96-120) hours of cultivation at temperatures ranging from (25-30- 37-40) °C, the percentages of dry weight and PHA content in salt medium with pH (5.5-6-6.5-7-8) were determined [27].

In order to accomplish the biomass reading, the samples were collected at regular intervals (every twenty-four hours), as follows: A total of 50 milliliters of bacterial cell culture growth was removed and pelleted at a speed of 5000 revolutions per minute for a period of 25 minutes. After determining the pellet's dry weight, it was next extracted in a series of solutions containing acetone and ethanol. In order to recover the PHA, an equal amount of 6% sodium hypochlorite was used to re-suspend the pellet, and then the mixture was let to incubate at 37 degrees Celsius for ten minutes. After this step, the lipid granules were separated by centrifuging the mixture at a speed of 5000 rpm for half an hour. After obtaining the pellet, it was subjected to a treatment with hot chloroform and then washed with acetone and ethanol.

## 3. Results and Discussion

### 3.1 Primary Screening

Three of the nine bacterial isolates isolated from Al-Rustomiya-Baghdad sewage water and soil samples from numerous sites contaminated with hydrocarbons and landfills were able to grow in anaerobic conditions and positively identified by Sudan black stain (Figure 1).

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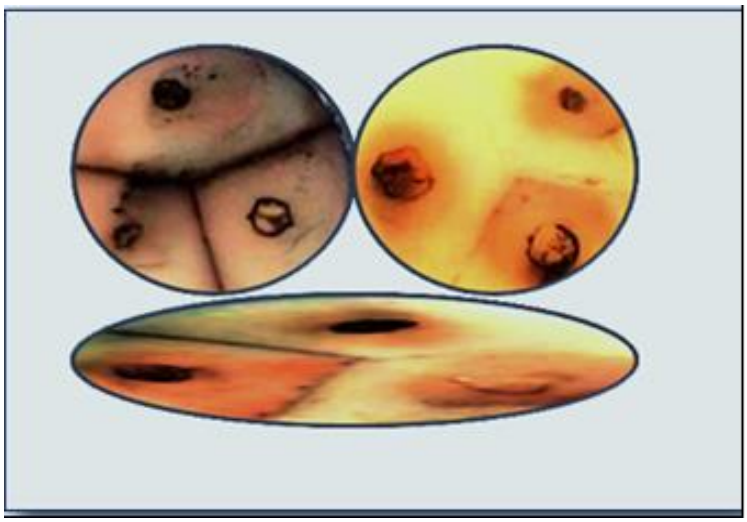

**Fig. (1): Primary screening of Sudan black stained bacterial isolate: producing of PHA with black colour is positive**

According to the research of [28], a PHA-producing bacterial isolate from a rhizospheric soil sample generated a yield of 0.186 gm/l, indicating that they are capable of producing PHA-containing fatty granules.

Figure (2) shows the ability of bacterial isolates to produce bioplastic (PHA) through examination after growing on solid media, bacterial colonies being colored in a bluish black color, as well as the black color of the granules that represent the fatty substance that makes up the bioplastic, as they appear under the microscope after being treated with Sudan Black reagent.

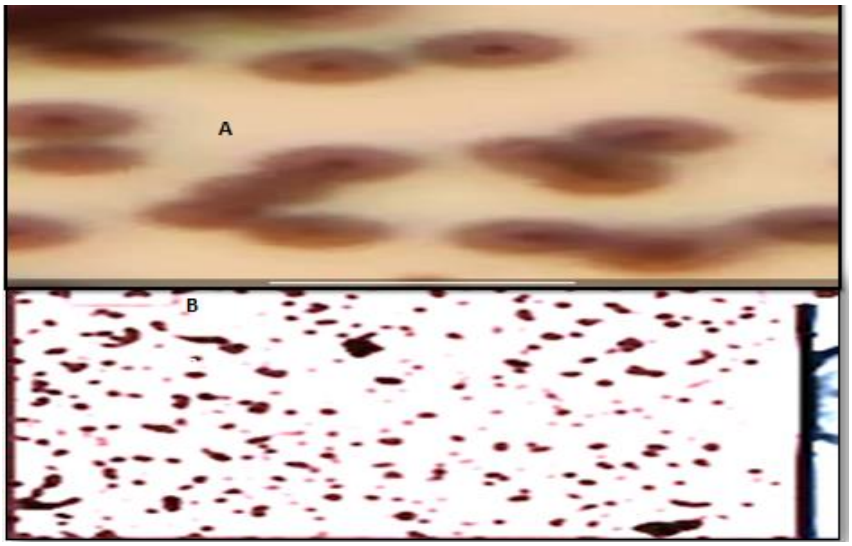


**Fig. (2): Sudan black stained granules in bacterial isolates (A- under contrast phase microscope, B *100)**

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### 3.2 Morphological Characterization of active isolate

The results of morphological and biochemical testing on the most potent Sudan-positive bacteria are displayed in (Table 1).

**Table (1): Tests for identification of active bacterial isolate after culturing on nutrient agar**

| Test | Reaction of isolate |
|---|---|
| **Microscopic appearance** | **Short Bacilli. Gram negative. Motile** |
| **Oxidase production** | **Positive** |
| **Catalase production** | **Positive** |
| **Arginine broth** | **Not hydrolysis** |
| **Gelatin** | **Not liquefied** |
| **Bromocresol purple broth** | **Alkaline** |

According to the results, the isolated bacteria were Gram-negative and capable of anaerobic respiration when exposed to carbon dioxide. Catalase and oxidases were both discovered to be positive. The specification [29] demonstrates that this isolate is an Alcaligenes spp. because of its short, fruity-smelling rods.

### 3.3 Optimization of Culture Conditions

The bacteria that tested positive for Sudan were selected out and cultivated on PHA-production media at various pH levels, temperatures, and incubation times. The optimum condition for dry cell weight (g/l) at pH, temperature ($^0$C), and incubation time (hr), were [7 (5.6), 30$^0$C (5.9), and 48hr (6.2)], respectively, (Figures 2, 4, and 6). Whereas the optimum condition for PHA (g/l) production at pH, temperature ($^0$C), and incubation time (hr), were [7 (4.4), 30$^0$C (5), and 48 hrs (5.2)], respectively, (Figures 3, 5, and 7).

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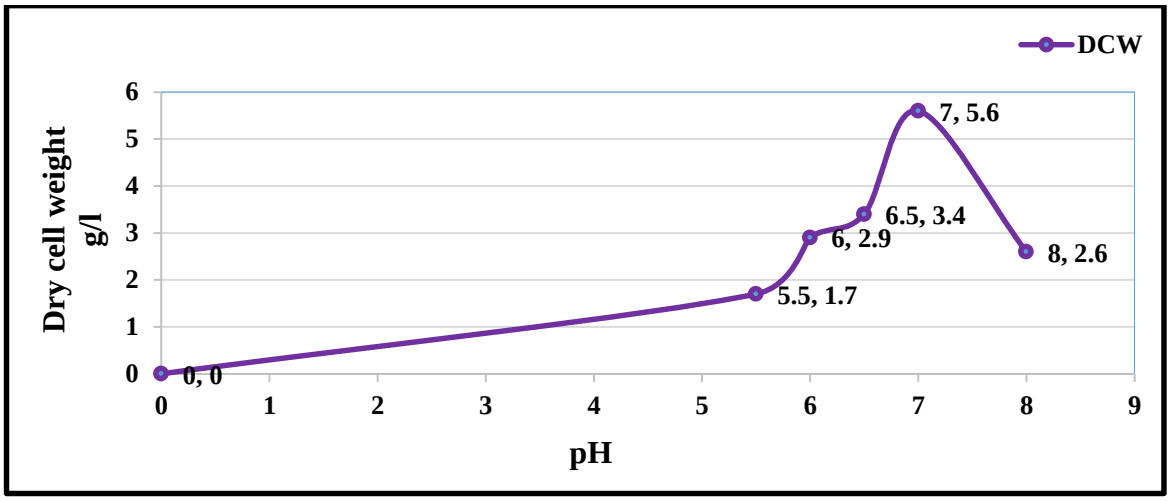


**Fig. (2): DCW of bacterial isolate vs. pH**

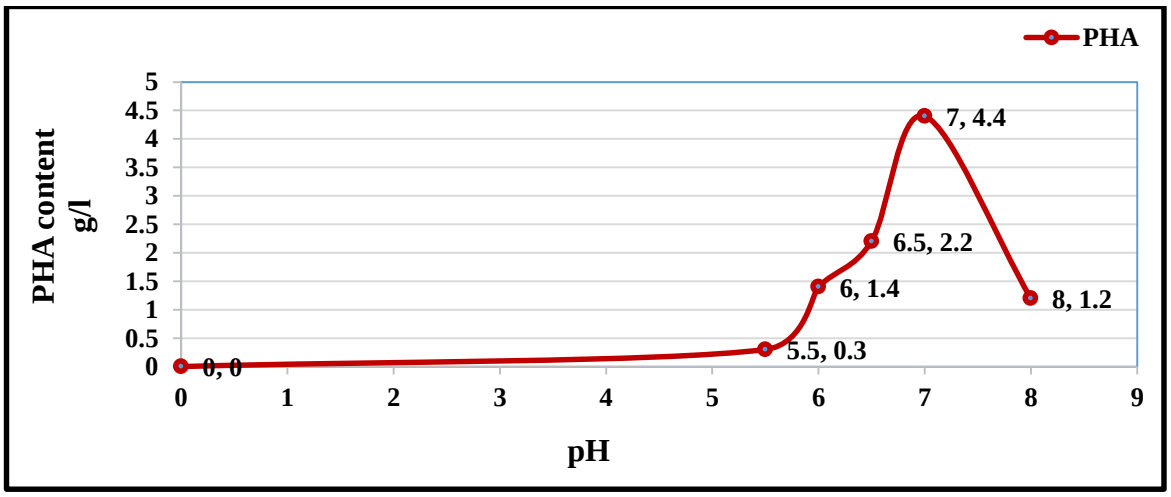


**Fig. (3): PHA concentration produced by bacterial isolates vs. pH**

Degradative enzymes played a significant part in the breakdown of the polymer, allowing polyhydroxy butyrate (PHB) to be consumed at a rate that was almost equal to its synthesis rate, which caused a reduction in PHA output at low and high pH levels above optimal [30]. Small changes in pH have a very clear effect on metabolic processes [30], and it appears that the bioavailability of trace elements through the change in pH directly affects PHA production. [29] reported that the best production of the substance by the bacteria *Alcaligenes eutropicus* was at a pH of 6.8 to 8.0. High pH may affect the effectiveness of enzymes that degrade the polymer, which makes the process of consuming the substance as much as it is formed inside the cells, and therefore its quantity decreases.

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The research resulted in the conclusion that bacteria cannot make PHA at this pH. The best temperature for the growth and production of the polymer was $30^0$C [31], which produced a value of (5.9, 5) gm per liter, respectively, according to the results, which are shown in (Figures 4 and 5).

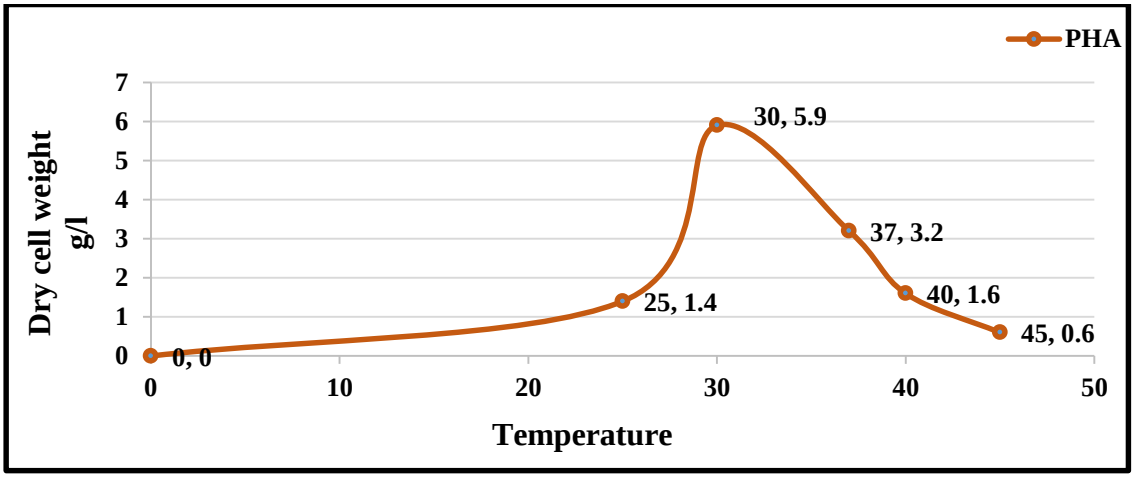


**Fig. (4): DCW of bacterial isolate vs. temperature of incubation**

However, it was able to do so at temperatures between the lowest ($20^0$C) and highest ($45^0$C) tested temperatures, resulting in decreased enzyme activity that affected the microorganism's metabolic activity, resulting in reduced growth and PHA production [32]. It was unable to grow and produce clearly at temperatures higher than $37^0$C and lower than $30^0$C. This response was probably due to the low activity of enzymes involved in biosynthesis of PHA. [32] reported that higher and lower temperatures than 30ºC lead to decrease in cell biomass and PHA synthesis by bacterial isolate.

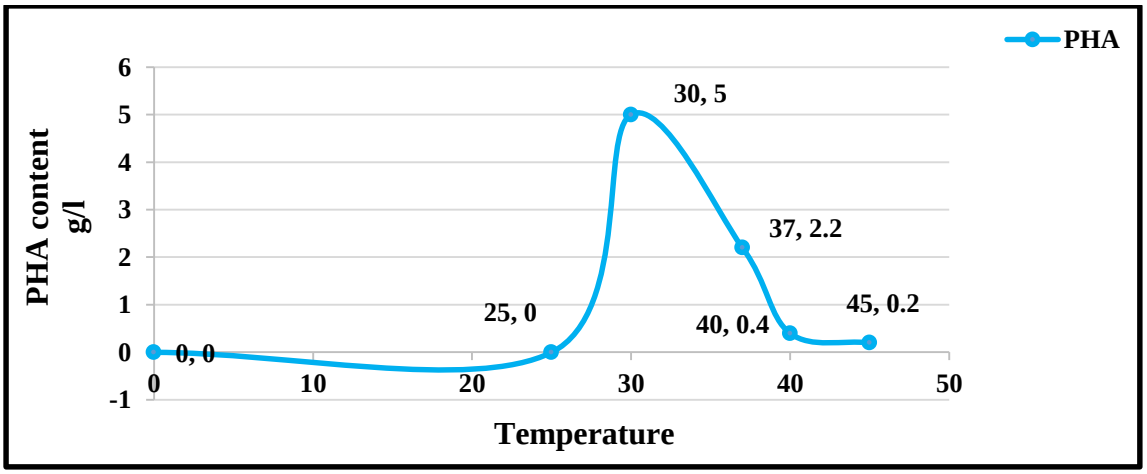


**Fig. (5): PHA concentration produced by bacterial isolate vs. temperature of incubation**

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The effects of different incubation times on the volume of material produced by the bacteria are shown by both (Figures 7 and 8) of these figures.

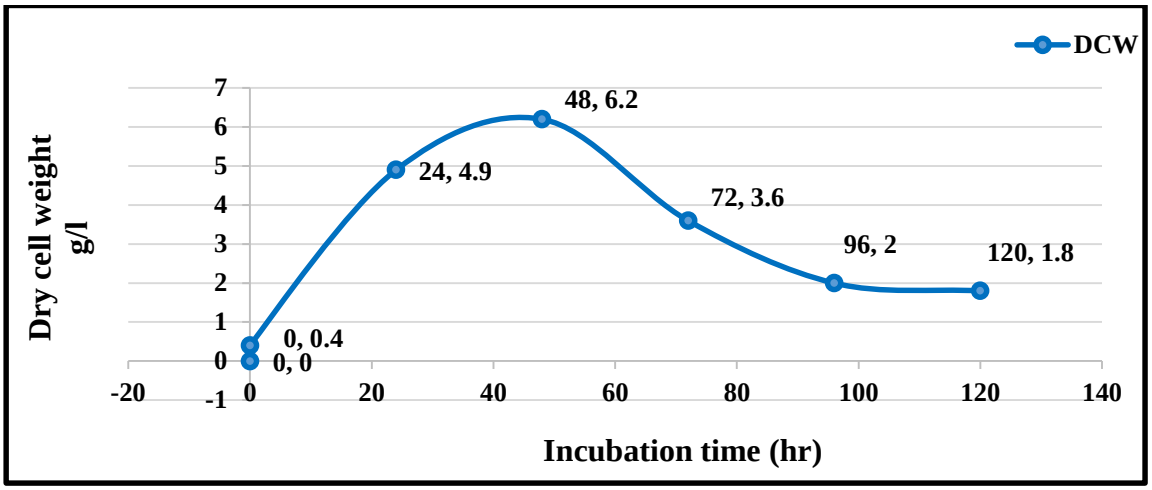


**Fig. (6): Effect of incubation time on growth rate gm/l (DCW) dry cell weight**

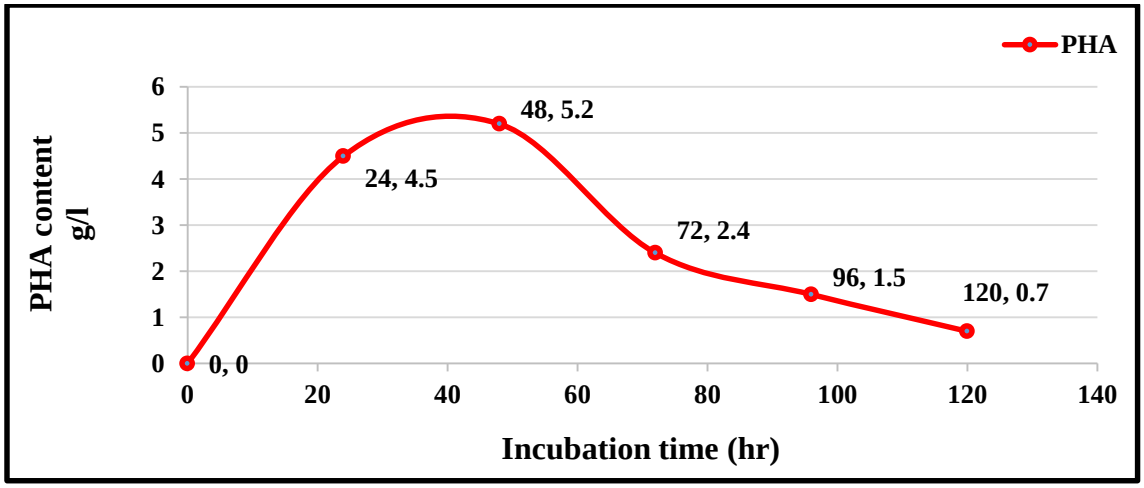


**Fig. (7): Effect of incubation time on PHA concentration**

The bacteria can produce their maximum level of PHA after 48hr of development. With the generation of 5.2gm/l of polymer, the growth rate increased to 6.2gm/l. A PHA-generating bacterial isolate from a rhizospheric soil sample produced a yield of 0.186gm/l, according to the research of [28], showing that they are capable of making fatty granules that contain PHA. At this stage, when it begins to enter an essentially stationary phase, the bacteria *A. latus* produces a biodegradable polymer known as polyhydroxy butyrate. Other research [33] have suggested that the decline in PHA synthesis may be due to bacteria using PHA as a carbon source, which reduces

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the amount of nutrients available in the culture medium. The growth of the bacteria *A. eutropicus* and the production of the substance require an incubation period ranging from 48 to 50 hours. Similar results on several bacterial isolates were supported by [34, 35]. PHA is a polymer with a comparatively high molecular weight. According to [36], A. latus has an unprecedentedly high carbon dioxide level of more than 50% by volume. A number of authors have described PHA production using low-cost substrates from various bacterial species [33, 37]. Purified PHA has opened a new area of study into the potential use of naturally occurring polymers as alternatives for those derived from petroleum because of its physical similarities to manufactured plastics. [38].

A short fermentation duration on an alternative carbohydrate is caused by the accumulation of Poly(3-hydroxybutyrate) (P3HB) from sucrose during cell development, which can account for up to (50-60) weight percent of the dry cell mass [39, 40].

## 4. Technical and Economic Feasibility

In terms of both economic and environmental performance, the process of absorbing environmental chemical pollutants, such as industrial discharges containing carbon dioxide, to produce biomaterials as an alternative to petrochemical materials is a distinct basic purification step. Due to the lower cost of industrial goods, it aids in reducing the usage of basic materials.

## 5. Conclusions

The main goal of this study was to improve production conditions to allow more efficient and cost-effective PHA synthesis. This work demonstrated the importance of modifying environmental conditions and delivering nutrients in a planned manner to enhance polymer production by a locally isolated *A. latus* strain in a laboratory setting. Pollutants such as carbon dioxide and hydrocarbons can be digested by bacteria in soil and sewage sludge, mitigating their negative impact on the environment and human health.

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