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THE INFLUENCE OF NUTRITIONAL CONDITIONS ON THE ACCUMULATION OF POLY-B-HYDROXYBUTYRATE (PHB) IN *AZOTOBACTER CHROOCOCCUM*

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ABSTRACT

Shaking culture method in conical flasks was used to produce PHB from *Azotobacter chroococcum* using a rotary shaker at 200 rpm at 30°C. The best time for PHB production was after 54 hours and the optimum pH value was 7.4 . The best carbon source for PHB production was glucose syrup at 12.5 % concentration, and the optimum nitrogen source was ammonium nitrate with a concentration of 1.25 g/l . The effect of K_2HPO_4; KH_2PO_4; NaCl; $MgSO_4.7H_2O$; $CaSO_4.2H_2O$; $NaMoO_4.2H_2O$ and Ferric citrate was also investigated and PHB production was 3.03, 3.111, 3.553, 3.747, 3.812, 3.807, 3.81 and 3.81 g/l, respectively.

Key words : PHB, *Azotobacter chroococcm*, batch culture, pH, time course, carbon source, nitrogen source, glucose syrup and ammonium nitrate.

INTRODUCTION

Growth in the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment. Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation. For this reason, there is special interest in producing plastics from materials that can be decomposed **(Suriyamongkol *et al*., 2007).**

In recent years, there is growing interest on the development of biomass-based biorefineries for the production of biofuels, chemicals and biodegradable plastics **(Koutinas *et al*., 2007)**

Poly-b-hydroxybutyric acid (PHB) is a natural, biodegradable polyester, which is accumulated in the form of intracellular granules by a large variety of bacteria. These granules act as energy reserve materials when nutrients such as nitrogen and phosphorous source are available in limiting concentrations in the presence of excess carbon source **(Khanna & Srivastava 2005**) .

PHB are non-toxic, biocompatible, biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, are highly crystalline and insoluble in water. These features make them highly competitive with polypropylene, the petrochemical-derived plastic **(Reddy *et al*., 2003).**

They can be completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage **(Lee, 1996).** The main factor preventing the large-scale production and commercialization of PHB is their high cost of production as compared with that of plastics based on petrochemicals. One of the major factors adding to the cost of PHB is the cost of substrates used for production. Therefore, less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost . Thus, utilization of media containing cheaper carbon and nitrogen sources should be used to reduce the production costs of PHB **(Ahn *et al*., 2001)**.

In PHB production, about 40% of the total production cost is for raw material . Thus, the use of a cheaper carbon source is required in order to reduce the high production cost of PHB. (**Choi and Lee, 1999)**

Toledo *et al*., 1995 reported that, the greatest factor limiting the use of biodegradable plastics (PHB) is its cost of production . In order to find cheaper and better substrate different carbon, nitrogen and mineral sources were tested so as to improve the productivity and at the same time reduce the production cost of PHB. A comparative study was carried out by growing the culture in different carbon sources. It was thought that this would not only help in finding a cheaper carbon source but would also help in accessing the carbon source utilization capacity of the culture. The objective was to find a carbon source, which would be cheaper than fructose and at the same time would give productivity comparable to or greater than that being obtained with fructose. Thus, twelve different carbon sources were selected based on easy availability or cheaper cost.

Pozo et al., 2002 reported that, they strongly suggest that *A. chroococcum* takes up the carbon source from alpechin (a waste has phenolic acids and obtained from a continuous olive processing operation) and stores it after its conversion to bacterial polyester, with higher efficiency in NH4+-medium than in cultures unamended with ammonia. Also they reported that , *A. chroococcum* can form homopolymers (b-hydroxybutyrate) and copolymers (b-hydroxybutyrate-hydroxyvalerate). Since cane molasses contains vitamins and other minor constituents, it can be used as a source of growth factors as can corn steep liquor. The nature of factors contained in molasses and maize extract stimulate the microbial growth, mainly due to action of the betaine and choline contained in molasses and maize extract, respectively. **(Beaulieu *et al*., 1995)**

Glucose syrup and Corn steep liquor (CSL) are by-products produced during the manufacture of starch and other corn products. Glucose syrup contains about 42% total sugars and 0.28% total nitrogen but, corn steep liquor contains 0.5% total sugars and 4.64 % total nitrogen **(Abdel Hafez et al ., 2007)**. Glucose syrup can be produced by hydrolizing starch with either acid or α-amylase enzyme. Glucose syrups were produced from starch using α-amylase at 97°C for 45-90 min liquefaction times, followed by saccharification with amyloglucosidase at 60°C for 18 h .In the present study, *Azotobacter chroococcum* growth was initially carried out in a medium . Thereafter, different carbon and nitrogen sources were tested to analyze their effect on growth and PHB production.

MATERIALS AND METHODS

Microorganism :

A. chroococcum was used in all experiments. The culture was maintained on Modified Ashby's medium **(Abd El-Malek and Ishac, 1968)** slants at 5°C, and sub cultured monthly.

Culture media:

The following media were used throughout this work.

Medium No. 1: Modified Ashby's medium (Abd El-Malek and Ishac, 1968):

10.0 g Mannitol; 10.0 g Sucrose; 0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.20 g NaCl; 0.1 g $CaSO_4$; 5.0 g $CaCO_3$; traces of $MnSO_4.4H_2O$; traces of $FeCl_3.6H_2O$; traces of $NaMoO_4.H_2O$; 1000 ml distilled water and the pH was 7.0.

Medium No. 2: (Pozo *et al*., 2002)

This medium was used for cell growth and production of PHB. The composition of the mineral salts solution was: 0.64 g K_2HPO_4; 0.16 g KH_2PO_4; 0.2 g NaCl; 0.2 g $MgSO_4.7H_2O$; 0.05 g $CaSO_4.2H_2O$; 0.01 g $NaMoO_4.2H_2O$; 0.02 g Ferric citrate; 1000 ml distilled water and the pH was 7.2. This medium was supplemented with 0.12% ammonium acetate and 1% fructose as carbon source. Carbon source and

nitrogen source were sterilized separately at 121°C for 20 min and added to the sterilized medium.

Cultivation system

The seed was prepared in 250 ml conical flasks containing 20 ml of medium No. 2, incubated with a loop of the culture and incubated in a rotary shaker at 200 rpm at 30°C for 48 h. Then the inoculums was transferred into 250 ml conical flasks containing 50 ml of the same medium and incubated in a rotary shaker at 200 rpm at 30°C for 48 h **(Khanna and Srivastava 2006).**

Effect of different carbon sources

glucose, sucrose, mannitol, galactose, fructose, ribose, mannose, dextrose, lactose, starch, can molasses, beet molasses and glucose syrup were added to the mineral salts solution to study the effect of carbon sources .

Effect of different nitrogen sources

Different nitrogen sources investigated were : N_2, NH_4HPO_4, NH_4NO_3, $(NH)_2CO$, NH_4Cl, $(NH_4)_2SO_4$, ammonium oxalate, ammonium acetate, ammonium citrate, $NaNO_3$, $Ni(NO_3)_2$, yeast extract, malt extract, beef extract, peptone and corn steep liquor.

Analytical methods

Biomass determination

Cells from 50 ml were pelleted by centrifugation (5000×*g*, 10 min), washed twice with sterile distilled water, and used for total cell dry weight determination after drying at 100 °C for 24 h (**Khanna and Srivastava 2005).**

Poly-ß-hydroxybutyrate determination

For the quantitative estimation of PHB, cells from 50 ml of culture were collected by centrifugation (5000×*g*, 10 min). 10 ml of hot chloroform was formed in cells at 70 °C 10 min and incubated at 30 °C for 24 h. The resulting solution was collected, allow to the chloroform to evaporated, dried at 100 °C for 24 h and cell dry weight was determination (**Pozo, *et al*., 2002).**

The parameters of polymer production

PHB (%) = gram polymer X 100 / gram biomass dried weight according to **(Lee and Chol 1998)** .

Conversion coefficient (%) = gram polymer X 100 / gram utilized sugar according to (**Wang *et al*., 2007**).

Determination of total carbohydrate

Total carbohydrates were determined as glucose according to the method of **(Dubois *et al*., 1956)**

Determination of reducing sugars

The residual reducing sugars were determined as glucose by **(Nelson, 1944)** method

The physical prosperities determination

The polymer formation was confirmed by infra-red (IR) (MATTISON 5000 FTIR spectrometer) in Mansoura University, Faculity of Science, Chemistry Department, Spectral Analysis Unit. IR spectra of the polymer was measured at a 2 cm^{-1} spectral resolution by using a MATTSON 5000 FTIR spectrometer equipped. An excitation wavelength at 3900 nm was provided and the laser power at the sample position was typically 500 nm . The spectra was obtained with a spectral resolution of 4 cm^{-1}, and 1024.

RESULTS AND DISCUSSION

Effect of time course on PHB production:

The results of the effect of time course on PHB production are presented in Fig. (1). The results showed that the concentration of PHB increased with the increasing the fermentation time. The maximum PHB concentration (0.845 g PHB/L)

was obtained after 54 hours of fermentation and then decline, at this time the cell dry weight was 2.594 g/l and the consumed sugars was 5.379 g/l . The biomass dry weight followed a pattern similar to PHB production. The highest biomass dry weight (3.643 g dried biomass/L culture) was obtained after 72 hours. The pH of culture employed for PHB decreased during fermentation due to the release of production PHB during fermentation of sugars. The lowest value of pH was 6.45. This results are agree with **Toledo *et al*., 1995** reported that, the cultivation time was after 48 hours, and **Kim 2000** was used a rotary shaker for 58 hours. Therefore, this time (54 hours) which proved to be the optimum, was used in the following experiments .

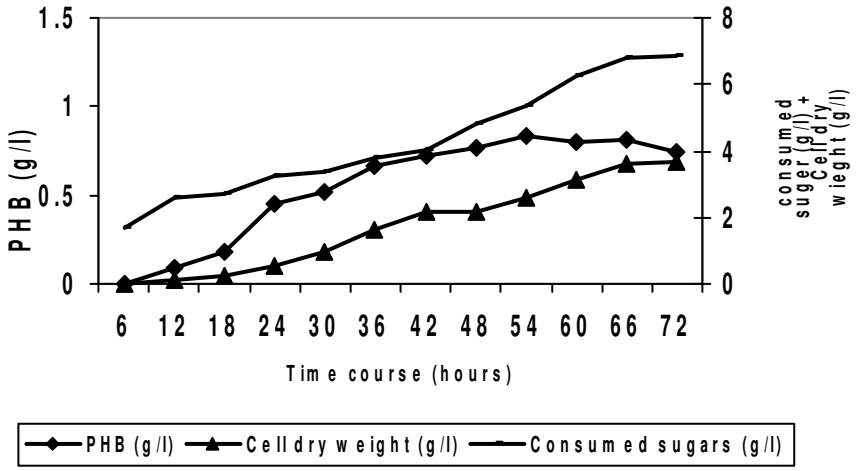

Fig. (1): The effect of time course on PHB production

Effect of the initial culture pH on PHB production:

Since the initial pH of the culture is an important factor that affect the PHB production and the cell growth of *A. chroococcum* , this experiment was undertaken to determine the optimum initial pH . The results on the effect of pH on PHB production are presented in Table (1). The pH range was 6.0 – 8.2, the PHB concentration increased with the increasing of the initial pH from 6.0 to 7.4 and then decreased in the range 7.6-8.2. The maximum amount of PHB was at pH 7.4, in this point, the biomass was (2.878 g /L). The concentration total sugars consumed was 5.512 g /L. PHB % was 36.796 % and conversion coefficient was 19.212 %. This results are in line with **Toledo *et al*., 1995 and Pozo *et al*., 2002** where the pH value was 7.2, but **Kim 2000** was 7.0. Therefore, this pH value (7.4) which proved to be the optimum, was used in the following experiments.

Table (1): The effect of initial pH on PHB production

Initial pH	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)
6.00	4.25	1.954	0.000	0.000	4.948	0.000
6.20	5.35	2.252	0.086	3.818	4.321	1.990
6.40	5.15	2.125	0.144	6.776	5.056	2.848
6.60	5.85	2.005	0.365	18.204	5.209	7.007
6.80	5.85	2.313	0.586	25.335	5.498	10.658
7.00	6.95	2.651	0.758	28.592	5.894	12.860
7.20	6.50	2.584	0.823	31.849	5.480	15.018
7.40	6.45	2.878	1.059	36.796	5.512	19.212
7.60	6.55	2.085	0.969	46.474	5.524	17.541
7.80	6.35	2.829	0.626	22.127	5.654	11.071
8.00	7.45	2.096	0.444	21.183	5.879	7.552
8.20	7.90	2.626	0.102	3.884	5.976	1.706

Effect of different carbon sources on growth and PHB production

The PHB production phase was carried out using glucose, sucrose, Mannitol, Galactose, Fructose, Ribose, Mannose, Dextrose , Lactose, Starch, Can molasses, Beet molasses and Glucose syrup. These carbon sources were added to the mineral salts solution to study the effect of carbon sources . As shown in Table 2, the highest amount of PHB was obtained with glucose syrup being 1.751 g/L, biomass was 3.92 g/L, consumed sugars concentration was 5.608 g/L, PHB % was 44.668 % and PHB conversion coefficient was 31.223%. The other tested carbon sources gave lower PHB concentration. In this sense, the possibility that glucose syrup (a by-product produced during the manufacture of starch) could be utilized as an inexpensive substrate for the industrial application of these microorganism could be of considerable important. Therefore, glucose syrup was the best carbon source for PHB production, replaced for fructose of medium No.2 in the following experiments.

The cell growth and PHA production by *Azotobacter vinelandii* in the 40 g/L glucose medium was as following , cells grew with the consumption of glucose after a 12 h lag period. Only a homopolymer, poly(hydroxybutyrate) (PHB) was produced, and the PHB content increased steadily with fermentation time. After 48 h of incubation, the dry cell mass and PHB mass were 10.2 g/L, 7.6 g/L, respectively **(Cho *et al*., 2001)**.

Pettinari *et al*., 2001 reported that , *Azotobacter* sp. Is an aerobic nitrogen-fixing bacterium that accumulates PHB when cultivated on several carbon sources including sucrose.

Kim, 1992 and Beaulieu *et al*., 1995 were used cane molasses for PHB production . and they used batch cultivation of PHB using different carbon sources has been carried out by *A. chroococcum*. But **Kim 2000** was used starch as carbon source for PHB production by *A. chroococcum*. This results were similar to the results of **Pozo *et al*., 2002**, whose found that, *A. chroococcum* formed homo- and copolymers of polyhydroxyalkanoates (PHAs) up to 80% of the cell dry weight after 48 h of incubation at 100 rev/min and 30°C. Production of PHAs by *A. chroococcum* specially on the cheap substrates is essential if bioplastics are to become competitive products. In an optimized production system, around 2.8 kg of sugar (as glucose equivalents) are needed to produce 1.0 kg of polymer**.**

Table (2): The effect of carbon sources on PHB production

The carbon source	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars (g/l)	Conversion coefficient (%)
Glucose	8.45	4.253	1.255	29.508	5.157	24.335
Sucrose	7.55	3.110	1.051	33.794	5.319	19.759
Mannitol	8.25	4.458	1.124	25.213	6.345	17.714
Galactose	6.70	3.156	1.457	46.166	5.989	24.327
Fructose	7.90	2.711	1.119	41.276	6.741	16.599
Ribose	6.85	3.757	0.128	3.406	7.662	1.670
Mannose	8.05	3.552	0.325	9.149	5.728	5.673
Dextrose	8.40	3.254	1.199	36.846	6.478	18.508
Lactose	6.85	2.755	1.164	42.250	3.625	32.110
Starch	7.50	3.156	1.064	33.713	5.489	19.384
Glucose syrup	6.75	3.92	1.751	44.668	5.608	31.223
Can molasses	7.65	3.656	0.552	15.098	6.024	9.163
Beet molasses	8.75	3.453	0.657	19.026	6.823	9.629

Effect of glucose syrup concentrations on PHB production:

The concentration of sugars in the culture medium had been reveled to be important for PHB synthesis. To investigate the affect of sugar concentration, an

experiment was designed using different amount of glucose syrup which the sugar content varied from 5 to 30 % . Every one was supplemented with the inorganic nutrients of the basal medium. The results on the effect of sugar concentration on PHB production are graphically illustrated in Fig. (2). As shown in this figure , carbon source consumption followed cell growth. The PHB level was increased with the increasing of glucose syrup from 5 to 12.5 % total sugars, and then decline. These results are inharmony with **Koutinas *et al*., 2007 and Khardenavis *et al*., 2007.** At 12.5% sugar concentration PHB reached its maximum being 1.812 g/L, Cell dry weight was 4.387 g/L, consumed total sugars concentration was 7.992 g/L, PHB % was 41.303 % and conversion coefficient was 22.672 %. Therefore, this concentration of total sugars (12.5 g/L) which proved to be the optimum, was used in the following experiments, (12.5 g/L from total sugar equivalent about 30 g/l from glucose syrup) .

This results were higher than **Cho *et al*., 2001** who studies the production of PHB by *Azotobacter* and they fount that the PHB concentration was 0.69 g /L when the medium was supplemented with 20 g glucose. On the other hand **Kim, 2000** was used an inexpensive substrate, starch to produce PHB in fed-batch culture of *Azotobacter* . Fermentation was carried out in a 2.5 liters stirred tank fermentor and the maximum production was 46% PHB.

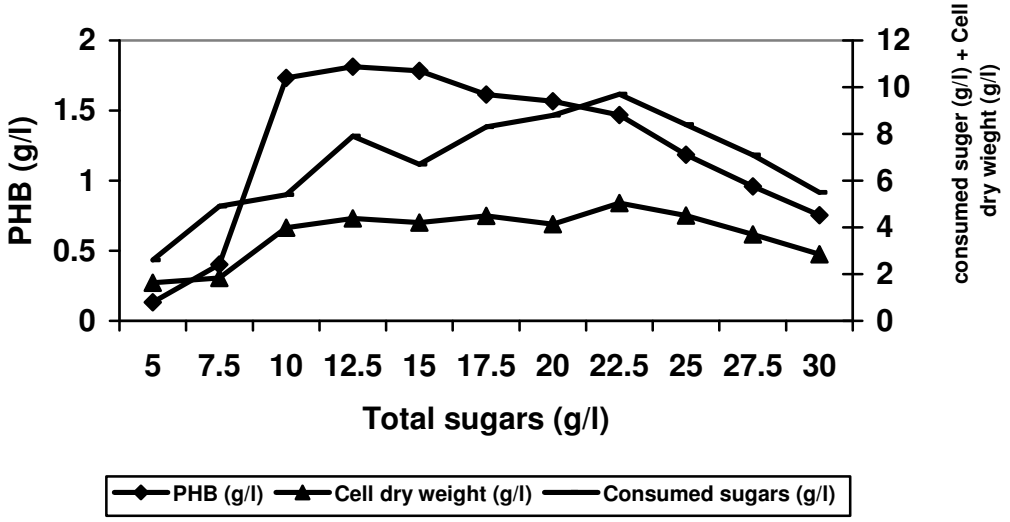

Fig (2): The effect of deferent concentrations of carbon source on PHB production

Effect of different nitrogen sources on growth and PHB production

Different nitrogen sources investigated were : N_2, NH_4HPO_4, NH_4NO_3, $(NH)_2CO$, NH_4Cl, $(NH_4)_2SO_4$, ammonium oxalate, ammonium acetate, ammonium citrate, $NaNO_3$, $Ni(NO_3)_2$, yeast extract, malt extract, beef extract, peptone and corn steep liquor. As shown in Table 3. different salts of ammonium were tested and provided at amounts equivalent to the amount of ammonium acetate taken in the media initially used . Highest growth and PHB production was obtained with NH_4NO_3 as a nitrogen source (6.533 g/l and 2.162 g/l, respectively) followed by $(NH)_2CO$ and NH_4HPO_4 . But PHB production in the case of organic nitrogen sources (peptone, yeast extract, malt extract, beef extract and CSL) was lowest than inorganic sources . Therefore, this nitrogen source (NH_4NO_3) which proved to be the optimum, was used in the following experiments

Khodair, 2003 reported that, it could be recommended to use ammonium sulfate as nitrogen source for highest polymer production .

Khanna and Srivastava 2005, were used relatively cheap corn steep liquor (CSL) in place of expensive yeast extract as a source of vitamins and minerals. The presence of ammonium as a source of nitrogen is an important requirement during the growth phase in order to maximize the concentration of biomass responsible for

accumulation of PHB. This nitrogen source has been studied by **Bitar and Underhill, 1990.**

Table (3): The effect of nitrogen sources on PHB production

The nitrogen source	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)
N_2	5.50	2.893	0.693	23.954	4.662	14.864
NH_4HPO_4	5.80	5.487	2.048	37.324	7.846	26.102
NH_4NO_3	6.15	6.533	2.162	33.093	7.913	27.322
$(NH)_2CO$	6.75	5.187	2.064	39.791	6.424	32.129
NH_4Cl	6.80	3.787	1.566	41.351	5.889	26.591
$(NH_4)_2SO_4$	3.05	3.964	1.449	36.553	6.465	22.412
$NaNO_3$	6.90	2.027	1.187	58.559	4.363	27.206
$Ni(NO_3)_2$	6.25	1.832	0.446	24.344	2.248	19.839
Amm. oxalate	6.25	3.587	1.361	37.942	5.798	23.473
Amm. acetate	6.45	4.255	1.794	42.162	8.011	22.394
Amm. citrate	6.05	3.187	0.587	18.418	7.211	8.140
Yeast extract	4.20	4.232	0.480	11.342	6.469	7.420
Malt extract	4.65	4.667	0.242	5.185	6.985	3.464
Beef extract	4.75	4.253	0.120	2.821	6.344	1.891
Peptone	4.55	4.947	0.053	1.071	6.661	0.795
Corn steep liquor	3.45	4.787	0.093	1.942	7.254	1.282

Effect of NH_4NO_3 concentrations on PHB production

The effects of nitrogen on the cell growth and PHB production were investigated by increasing or reducing nitrogen levels. The results on the effect of NH_4NO_3 on PHB production are graphically in Fig. (3). The PHB concentration was increased with the increasing of NH_4NO_3 then declined. The highest value of PHB concentration 2.663 g/L was obtained with 1.25 g/L NH_4NO_3 . Also at 1.25 g/L NH_4NO_3 biomass was 6.282 g/L, consumed total sugars concentration was 8.286 g/L, PHB % was 42.390 % and conversion coefficient was 41.191 %. Therefore, this concentration of ammonium phosphate (2.00 g/L) which proved to be the optimum, was used in the following experiments .

Similar results have been reported by **Cho *et al*., 2001** where they found that a higher nitrogen concentration increased cell growth and PHB production. These results indicate that a high nitrogen concentration could enhance PHB production as well as cell growth .

Toledo *et al*., 1995 reported that, *A. chroococcum* grew in NH_4^+-medium supplemented with 1 % glucose under aerobic conditions, it formed higher amount of PHB . It appears that under that culture condition *A. chroococcum* takes up carbon source and store then after conversion to PHB with higher than in nitrogen-fixing cultures. This interpretation could be supported by the fact that energy charge of the cells grown on NH4+-medium was lower than that of cells grown in N-free medium . Also, the ratios of ATP to ADP in cells grown in NH_4^+-medium were low when compared with cells grown in N-free medium .

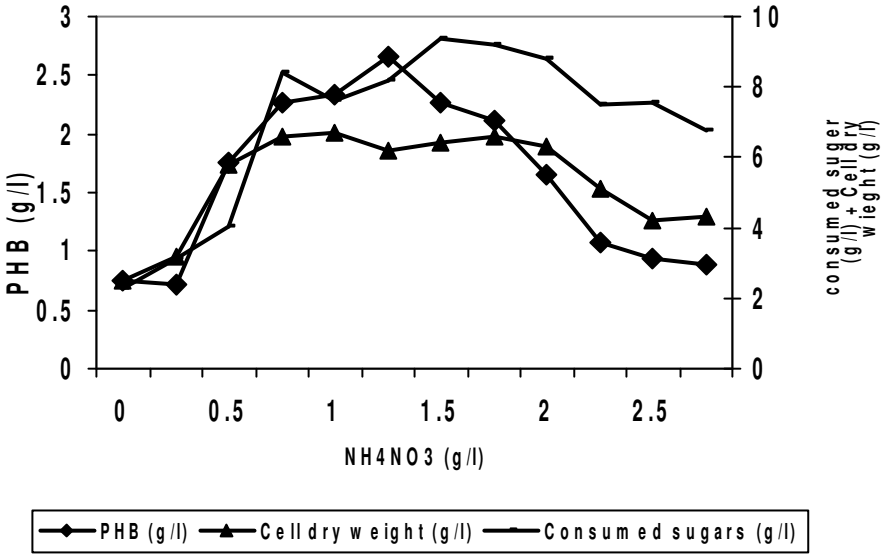

Fig (3): The effect of deferent concentrations of nitrogen source on PHB production

Effect of K_2HPO_4 concentrations on PHB production

The effects of K_2HPO_4 on the cell growth and PHB production were investigated by increasing or reducing K_2HPO_4 levels. The results on the effect of K_2HPO_4 on PHB production are in Table 4 . The PHB concentration was increased with the increasing of K_2HPO_4 then declined. The highest value of PHB concentration 3.030 g/L was obtained with 0.20 g/L K_2HPO_4. Also at 0.20 g/L K_2HPO_4 biomass was 8.233 g/L, consumed total sugars concentration was 9.100 g/L, PHB % was 36.803 % and conversion coefficient was 33.296 %. Therefore, this concentration of K_2HPO_4 (0.20 g/L) which proved to be the optimum, was used in the following experiments .

Table (4): The effect of deferent concentrations of K_2HPO_4 on PHB production

K_2HPO_4 G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.00	7.20	2.481	0.007	0.282	3.054	0.229	0.056
0.10	7.05	5.547	2.187	39.426	7.688	28.446	17.496
0.20	**6.85**	**8.233**	**3.030**	**36.803**	**9.100**	**33.296**	**24.240**
0.30	6.80	8.353	2.987	35.759	9.827	30.395	23.896
0.40	6.90	8.934	2.669	29.874	10.132	26.342	21.352
0.50	7.05	8.367	2.561	30.608	10.650	24.046	20.488
0.60	7.10	8.673	2.672	30.808	10.789	24.765	21.376
0.70	7.15	8.347	2.667	31.951	10.987	24.274	21.336
0.80	7.05	8.328	2.605	31.280	10.654	24.450	20.840
0.90	7.00	8.074	2.733	33.849	10.415	26.241	21.864
1.00	7.00	8.836	2.633	29.798	10.876	24.209	21.064
1.10	6.75	8.330	1.469	17.635	10.872	13.511	11.752
1.20	6.80	8.900	1.025	11.516	10.567	9.700	8.200
1.30	6.90	8.486	0.951	11.206	10.654	8.926	7.608

Cho *et al*., 2001 were studied the PHB production by *Azotobacter vinelandii* and they used 0.3 g/L of KH_2PO_4 . And **Khanna & Srivastava 2005** were used 1.5 g/L KH_2PO_4 and 4.0 g/L Na_2HPO_4 . But **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.64 g/L of K_2HPO_4.

The growth of the organism was maximal at 2·0 g/L K_2HPO_4, whereas the maxima of PHB yield and productivity were obtained at 1·0 g/L K_2HPO_4. This concentration of phosphate is suboptimal for cell growth but stimulated PHB accumulation. A further increase in K_2HPO_4 concentration suppressed the PHB yield and productivity **(Borah, *et al*., 2002**).

Similar results have been reported by **Borah, *et al*., 2002** where they found that, the growth of the organism was maximal at 2·0 g/L K_2HPO_4, whereas the maxima of PHB yield and productivity were obtained at 1·0 g/L K_2HPO_4. This concentration of phosphate is suboptimal for cell growth but stimulated PHB accumulation. A further increase in K_2HPO_4 concentration suppressed the PHB yield and productivity. These results are in good conformity with the findings of **Kim *et al*. (1996)** who hase studied the influence of phosphate and potassium on the accumulation of PHB by different bacteria.

Effect of KH_2PO_4 concentrations on PHB production

The effects of KH_2PO_4 on the cell growth and PHB production were investigated by increasing or reducing KH_2PO_4 levels. The results on the effect of KH_2PO_4 on PHB production are in Table 5 . The PHB concentration was increased with the increasing of KH_2PO_4 then declined. The highest value of PHB concentration 3.111 g/L was obtained with 0.15 g/L KH_2PO_4. Also at 0.15 g/L KH_2PO_4 biomass was 8.541 g/L, consumed total sugars concentration was 10.734 g/L, PHB % was 36.424 % and conversion coefficient was 28.982 %. Therefore, this concentration of KH_2PO_4 (0.15 g/L) which proved to be the optimum, was used in the following experiments .

Table (5): The effect of deferent concentrations of KH_2PO_4 on PHB production

KH_2PO_4 G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.00	7.05	3.612	0.438	12.126	5.114	8.5647	3.504
0.05	7.00	5.088	2.299	45.184	8.349	27.536	18.392
0.10	6.85	7.175	2.836	39.526	9.702	29.231	22.688
0.15	**6.80**	**8.541**	**3.111**	**36.424**	**10.734**	**28.982**	**24.888**
0.20	6.50	8.249	2.948	35.737	10.890	27.070	23.584
0.25	6.50	8.328	2.433	29.214	10.735	22.664	19.464
0.30	6.55	8.207	2.408	29.340	10.145	23.735	19.264
0.35	6.30	7.132	2.605	36.525	9.347	27.869	20.840
0.40	6.45	6.876	1.122	16.317	9.387	11.952	8.976
0.45	6.75	6.423	1.138	17.717	8.467	13.440	9.104
0.50	6.90	6.485	0.827	12.752	8.399	9.846	6.616

Cho *et al*., 2001 were studied the PHB production by *Azotobacter vinelandii* and they used 0.3 g/L of KH_2PO_4 . But **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.16 g/L of KH_2PO_4.

Cho *et al*., 2001 found that, the maximum PHA production was obtained at 165 : 1 of C : P ratio, and the dry cell mass and PHA content were 8.0 g/L and 72%, respectively. At phosphorus concentrations higher than the optimum value, the cell mass and PHA production decreased. In particular, a large amount of phosphorus inhibited PHA formation.

Effect of NaCl concentrations on PHB production

The effects of NaCl on the cell growth and PHB production were investigated by increasing or reducing NaCl levels. The results on the effect of NaCl on PHB production are in Table 6 . The PHB concentration was increased with the increasing of NaCl then declined. The highest value of PHB concentration 3.553 g/L was obtained with 0.10 g/L NaCl. Also at 0.10 g/L NaCl biomass was 7.667 g/L,

consumed total sugars concentration was 10.361 g/L, PHB % was 46.341 % and conversion coefficient was 34.292 %. Therefore, this concentration of NaCl (0.10 g/L) which proved to be the optimum, was used in the following experiments .

Table (6): The effect of deferent concentrations of NaCl on PHB production

NaCl G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.00	6.15	6.016	1.387	23.055	8.115	17.091	11.096
0.05	6.00	7.667	2.416	31.511	9.973	24.225	19.328
0.10	**6.05**	**7.667**	**3.553**	**46.341**	**10.361**	**34.292**	**28.424**
0.15	6.15	8.533	3.454	40.478	11.852	29.142	27.632
0.20	6.85	8.773	3.158	35.996	11.667	27.067	25.264
0.25	6.80	8.472	3.129	36.933	11.522	27.156	25.032
0.30	6.85	8.670	3.075	35.467	10.701	28.735	24.600
0.35	6.90	7.853	2.439	31.058	10.044	24.283	19.512
0.40	6.90	7.279	2.278	31.295	10.689	21.311	18.224
0.45	6.90	6.707	2.353	35.082	10.645	22.104	18.824
0.50	7.25	6.453	2.634	40.818	10.005	26.326	21.072

Pozo *et al*., 2002 were studied the PHB production by *Azotobacter chroococcum* and they used 0.20 g/L of NaCl.

Effect of $MgSO_4.7H_2O$ concentrations on PHB production

The effects of $MgSO_4.7H_2O$ on the cell growth and PHB production were investigated by increasing or reducing $MgSO_4.7H_2O$ levels. The results on the effect of $MgSO_4.7H_2O$ on PHB production are in Table 7 . The PHB concentration was increased with the increasing of $MgSO_4.7H_2O$ then declined. The highest value of PHB concentration 3.747 g/L was obtained with 0.25 g/L $MgSO_4.7H_2O$. Also at 0.25 g/L $MgSO_4.7H_2O$ biomass was 7.228 g/L, consumed total sugars concentration was 10.321 g/L, PHB % was 51.840 % and conversion coefficient was 36.304 %. Therefore, this concentration of $MgSO_4.7H_2O$ (0.25 g/L) which proved to be the optimum, was used in the following experiments .

Table (7): The effect of deferent concentrations of $MgSO_4.7H_2O$ on PHB production

$MgSO_4.7H_2O$ G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.00	6.55	3.167	1.387	43.795	6.121	22.659	11.096
0.05	6.35	5.546	1.416	25.531	8.161	17.350	11.328
0.10	6.35	6.212	2.458	39.568	9.778	25.138	19.664
0.15	6.25	7.361	3.110	42.249	9.001	34.551	24.880
0.20	6.10	7.333	3.548	48.384	10.960	32.372	28.384
0.25	**6.05**	**7.228**	**3.747**	**51.840**	**10.321**	**36.304**	**29.976**
0.30	6.15	7.125	2.800	39.298	11.661	24.011	22.400
0.35	6.45	6.468	2.488	38.466	11.542	21.556	19.904
0.40	6.55	6.987	2.456	35.150	10.654	23.052	19.648
0.45	6.35	5.753	2.915	50.669	9.617	30.310	23.320
0.50	6.25	5.154	1.793	34.788	9.355	19.166	14.344

Khanna & Srivastava 2005 reported that **,** the trace metal solution had a positive effect on residual biomass production presumably because they are needed for the growth of the cells. Growth factors present in CSL may be responsible for its positive effect on growth. Fructose, KH_2PO_4 and $MgSO_4.7H_2O$ had a positive effect

on PHB production, whereas rest of the components had a negative effect. This can be easily explained by the fact that the limitation of any amongst N or P will lead to production of PHB by the culture.

Cho *et al*., 2001 were studied the PHB production by *Azotobacter vinelandii* and they used 0.3 g/L of $MgSO_4.7H_2O$. And **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.20 g/L of $MgSO_4.7H_2O$. But **Khanna & Srivastava 2005** were used 0.51 g/L $MgSO_4.7H_2O$.

Effect of $CaSO_4.2H_2O$ concentrations on PHB production

The effects of $CaSO_4.2H_2O$ on the cell growth and PHB production were investigated by increasing or reducing $CaSO_4.2H_2O$ levels. The results on the effect of $CaSO_4.2H_2O$ on PHB production are in Table 8 . The PHB concentration was increased with the increasing of $CaSO_4.2H_2O$ then declined. The highest value of PHB concentration 3.812 g/L was obtained with 0.08 g/L $CaSO_4.2H_2O$. Also at 0.08 g/L $CaSO_4.2H_2O$ biomass was 8.003 g/L, consumed total sugars concentration was 10.120 g/L, PHB % was 47.632 % and conversion coefficient was 37.667 %. Therefore, this concentration of $CaSO_4.2H_2O$ (0.08 g/L) which proved to be the optimum, was used in the following experiments . **Cho *et al*., 2001** were studied the PHB production by *Azotobacter vinelandii* and they used 0.015 g/L of $CaSO_4.2H_2O$. But **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.05 g/L of $CaSO_4.2H_2O$. But **Khanna & Srivastava 2005** were used 0.02 g/L CaCl2.

Table (8): The effect of deferent concentrations of $CaSO_4.2H_2O$ on PHB production

$CaSO_4.2H_2O$ G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.00	6.50	2.166	0.957	44.182	5.176	18.489	7.656
0.02	6.25	4.147	1.333	32.143	7.308	18.240	10.664
0.04	6.00	7.114	2.879	40.469	9.660	29.803	23.032
0.06	5.95	7.625	3.677	48.222	9.651	38.099	29.416
0.08	**5.90**	**8.003**	**3.812**	**47.632**	**10.120**	**37.667**	**30.496**
0.10	6.05	8.228	3.800	46.183	11.654	32.606	30.400
0.12	6.15	8.125	3.749	46.141	11.351	33.027	29.992
0.14	6.20	8.468	2.146	25.342	11.202	19.157	17.168
0.16	5.55	7.455	1.973	26.465	10.946	18.024	15.784
0.18	5.85	7.403	0.929	12.548	10.465	8.877	7.432
0.20	5.20	7.560	0.57	7.539	10.659	5.347	4.560

Effect of $NaMoO_4.2H_2O$ concentrations on PHB production

The effects of $NaMoO_4.2H_2O$ on the cell growth and PHB production were investigated by increasing or reducing $NaMoO_4.2H_2O$ levels. The results on the effect of $NaMoO_4.2H_2O$ on PHB production are in Table 9 . The PHB concentration was increased with the increasing of $NaMoO_4.2H_2O$ then declined. The highest value of PHB concentration 3.807 g/L was obtained with 0.01 g/L $NaMoO_4.2H_2O$. Also at 0.01 g/L $NaMoO_4.2H_2O$ biomass was 8.115 g/L, consumed total sugars concentration was 11.113 g/L, PHB % was 46.913 % and conversion coefficient was 34.257 %. Therefore, this concentration of $NaMoO_4.2H_2O$ (0.01 g/L) which proved to be the optimum, was used in the following experiments .

Cho *et al*., 2001 were studied the PHB production by *Azotobacter vinelandii* and they used 0.00036 g/L of $Na_2MoO_4.2H_2O$. But **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.01 g/L of $Na_2MoO_4.2H_2O$.

Table (9): The effect of deferent concentrations of $NaMoO_4.2H_2O$ on PHB production

$Na_2MoO_4.2H_2O$ G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.000	6.65	0.544	0.072	13.235	3.646	1.974	0.576
0.002	6.50	3.147	0.914	29.043	7.321	12.484	7.312
0.004	6.65	5.114	1.458	28.509	9.015	16.173	11.664
0.006	6.75	7.625	2.531	33.193	9.460	26.754	20.248
0.008	6.55	8.018	3.456	43.103	10.531	32.817	27.648
0.010	**6.00**	**8.115**	**3.807**	**46.913**	**11.113**	**34.257**	**30.456**
0.012	6.15	8.125	3.749	46.141	11.320	33.118	29.992
0.014	6.65	8.468	3.146	37.151	10.878	28.920	25.168
0.016	6.70	7.455	2.973	39.879	9.212	32.273	23.784
0.018	6.50	7.43	2.29	30.821	9.400	24.361	18.320
0.020	6.20	7.56	2.14	28.306	9.414	22.732	17.120

Effect of Ferric citrate concentrations on PHB production

The effects of Ferric citrate on the cell growth and PHB production were investigated by increasing or reducing Ferric citrate levels. The results on the effect of Ferric citrate on PHB production are in Table 10 . The PHB concentration was increased with the increasing of Ferric citrate then declined. The highest value of PHB concentration 3.810 g/L was obtained with 0.02 g/L Ferric citrate . Also at 0.02 g/L Ferric citrate biomass was 8.140 g/L, consumed total sugars concentration was 10.233 g/L, PHB % was 46.805 % and conversion coefficient was 37.232 %. Therefore, this concentration of Ferric citrate (0.02 g/L) which proved to be the optimum, was used in the following experiments .

Table (10): The effect of deferent concentrations of Ferric citrate on PHB production

Ferric citrate G/L	Final pH	Cell dry weight (g/l)	PHB (g/l)	PHB (%)	Consumed sugars	Conversion coefficient (%)	Yield of PHB (%)
0.000	6.50	2.154	0.178	8.263	3.140	5.668	1.424
0.005	6.45	5.288	0.741	14.012	7.015	10.563	5.928
0.010	6.15	7.017	1.587	22.616	9.251	17.154	12.696
0.015	6.45	8.736	2.489	28.491	9.444	26.355	19.912
0.020	**6.00**	**8.140**	**3.810**	**46.805**	**10.233**	**37.232**	**30.480**
0.025	6.20	7.814	3.111	39.813	11.465	27.134	24.888
0.030	6.10	7.125	3.753	52.673	10.312	36.394	30.024
0.035	6.90	7.468	3.155	42.246	9.249	34.111	25.240
0.040	6.95	6.475	2.474	38.208	9.639	25.666	19.792
0.045	6.20	6.439	2.667	41.419	8.654	30.818	21.336
0.050	6.05	6.444	2.58	40.037	8.053	32.037	20.640

Cho *et al*., 2001 were studied the PHB production by *Azotobacter vinelandii* and they used 0.01029 g/L of ferric citrate and 0.0075 g/L $FeSO_4.7H_2O$. But **Pozo *et al*., 2002** were studied the PHB production by *Azotobacter chroococcum* and they used 0.02 g/L of ferric citrate .

The physical properties of PHB plymer:

Analysis of the spectrum of PHB was observed at 3400, 1639, 3018, 2978, 2842, 1216, 1044 and 669-765 δ were respsctively , for (-OH) broad peak, (C=C) double bond, (≡C-H) acetylenic bond, ($-CH_3$) methyl group, (-S-H) thiol weak

adsorption, (≡C-O-C≡) ether, sulfoxide and (F-Cl) haloalkanes. Similar results were obtained by **Saha** *et al*., 2007 and **McCormack** *et al*., 2005.

REFERENCES

Abdel Hafez, A. M.; Abdelhady, H. M.; Sharaf, M. S. and El-Tayeb, T. S. (2007). Bioconversion of Various Industrial By- Products and Agricultural Wastes into Pullulan . Journal of Applied Sciences Research, 3(11): 1416-1425.

Abd-El-Malek, Y. and Y. Z. Ishac (1968). Evaluation of methods used in counting *Azotobacter*. J. Appl. Bact., **31**: 267-275.

Ahn, W. S; Park, S. J; Lee, S. Y. (2001) . Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. Biotechnol. Lett. 23:235–40.

Beaulieu, M.; Y. Beaulieu; J. L. Me´Linard; S. Pandian and J. Goulet. (1995). Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. Appli. Environ. Microbiol. **61(1)**: 165–169.

Bitar, A. and Underhill, S. (1990) . Effect of ammonium supplementation of poly-b-hydroxybutyric acid by *Alcaligenes eutrophus* in batch culture. Biotechnol. Lett. **12:**563–568.

Borah, B.; P. S. Thakur and J. N. Nigam. (2002). The infuence of nutritional and environmental conditions on the accumulation of poly-b-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. J. Appli. Microbiol. **92:** 776 – 783.

Cho, K. S.; Ryu, H. W.; Park, C. H. and Goodrich, P. R. (2001). Utilization of Swine Wastewater as a Feedstock for the Production of Polyhydroxyalkanoates by *Azotobacter vinelandii* UWD . Journal of Bioscience and Bioengineering. **91 (2):** 129-133.

Choi, J. and Lee, S. Y. (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol . 51:13–21.

Dubois, M.; K. A. Gilles; J. K. Hamilton; P. A. Revers and F. Smith (1956). A colorimetric method for determination of sugars and related substances. Anal. Chem. **28 (3)**: 350-356.

Khanna, S. and Srivastava, A. K. (2005). Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process Biochem. **40:** 2173–2182.

Khanna, S.; Srivastava, A. K. (2006). Optimization of nutrient feed concentration and addition time for production of poly(-hydroxybutyrate). Enzyme and Microbial Technology. **39:** 1145–1151.

Khardenavis, A. A.; Kumar, M. S.; Mudliar, S. N. and Chakrabarti, T. (2007). Biotechnological conversion of agro-industrial waste waters into biodegradable plastic, poly b-hydroxybutyrate. Bioresource Technology. In press.

Khodair, T. A. (2003). Microbial production of degradable polymers as substitute for synthetic plastics. Ph. D. Thesis, Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ., Egypt.

Kim, B. S. (1992). Production of poly(3-hydroxybutyrate) from inexpensive substrates. Enzyme Microb Technol 2000;27:774–7. Appl Microbiol. Biotechnol. 38:117–21.

Kim, B. S. (2000). Production of poly(3-hydroxybutyrate) from inexpensive substrates. Enzyme and Microbial Technol. **27:** 774–777.

Koutinas, A. A.; Xu, Y.; Wang, R. and Webb, C. (2007). Polyhydroxybutyrate production from a novel feed stock derived from a wheat-based biorefinery. Enzyme and Microbial Technology. **40 (5):** 1035–044.

Lee, S. Y. and J. I. Chol (1998). Polyhydroxyalkanoates biodegradable polymer. In Manual of Industrial Microbiology and Biotechnology (2nd Ed.), A. L. Demain and J. E. Davies(eds.) . pp 616-627. ASM Press, Washington, D. G.

Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 49:1–14.

McCormack, D. N.; Clyburn, V. L. and Pittman, D. W. (2005). Detection of Free Fatty Acids following a Conditioned Taste Aversion in Rats. Chief Physiology and Behavior Journal. 21 : 1-34.

Nelson, N. (1944). A photometric adaptation of the somogyi method for the determination of glucose. J. Biol. Chem. **153**: 375-380.

Pettinari, M. J.; G. J. Vazquez; D. Silberschmidt; B. Rehm; A. Steinbuchel and B. S. Mendez. (2001). Poly(3-hydroxybutyrate) synthesis genes in *Azotobacter* sp. strain FA8. Appli. Environ. Microbiol. **67(11):** 5331–5334.

Pozo, C.; M. V. Martinez-Toledo; B. Rodelas and J. Gonzalez-Lopez. (2002). Effects of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpechin (wastewater from olive oil mills) as primary carbon source. J. Biotechnol. **97:** 125–131.

Reddy, C. S. K.; R. Ghai and R. V. C. Kalia. (2003). Polyhydroxyalkanoates: an overview**.** Bioresource Technol. **87:** 137–146.

Saha, S. P.; Patra, A. and Paul A. K. (2007). Studies on intracellular degradation of polyhydroxyalkanoic acid–polyethylene glycol copolymer accumulated by *Azotobacter chroococcum* MAL-201. Journal of Biotechnology. 132: 325–330

Suriyamongkol, P.; Weselake, R.; Narine, S.; Moloney, M. and Shah, S. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants. Biotechnology Advances. **25:** 148–175.

Toledo, M. V. M.; J. G. Lopez; B. Rodelas; C. Pozo and V. Salmeron. (1995). Production of poly-b-hydroxybutyrate by *Azotobacter chroococcum* H23 in chemical defined medium and alpechin medium. J. Appli. Bacteriol. **78:** 413–418.

Wang, Y. J.; Hua, F. L.; Tsang, Y. F.; Chan, S. Y.; Sin, S. N.; Chua, H.; Yu, P. H. F. and Ren, N. Q. (2007). Synthesis of PHAs from waster under various C:N ratios. Bioresource Technology. **98:** 1690–1693.

الملخص العربى

تأثير الظروف الغذائية على تراكم مادة البولى بيتاهيدروكس بيوتيرات فى *Azotobacter chrococcum*

تم استخدام طريقة المزرعة المهتزة فى دوارق مخروطية وباستخدام حضان هزاز بسرعة ٢٠٠ لفة / دقيقة على درجة حرارة ٣٠°م لدراسة بعض العوامل المؤثرة على إنتاج مادة PHB من *Azotobacter chroococcum* ، وكان أفضل وقت بعد ٥٤ ساعة وأفضل pH كان ٧,٤ ، كما تمت دراسة تأثير بعض مصادر الكربون والنيتروجين المختلفة وكان أفضل مصدر كربون هو شراب الجلوكوز بتركيز ١٢,٥ % (محسوبة كسكريات كلية) وأفضل مصدر نيتروجينى كان نترات الأمونيوم بتركيز ١,٢٥ جرام / لتر . أيضاً تم دراسة تأثير كل من فوسفات بوتاسيوم أحادية ، فوسفات بوتاسيوم ثنائية ، كلوريد صوديوم ، كبريتات ماغنسيوم ، كبريتات كالسيوم ، موليبيدات صوديوم وسترات حديديك .