FERMENTATIVE PRODUCTION OF POLYHYROXYBUTYRATE (PHB) BY HIGH CELL DENSITY CULTURE OF *R.EUTROPHA* AND *A.LATUS* IN pH-STAT FED-BATCH CULTURE

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ABSTRACT

Different concentrations of *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 cells ranged from 40 to 70 gl^{-1} were obtained under optimal condition and used for PHB production under nitrogen limitation in productive medium using pH-stat fed-batch culture technique. Applying nitrogen limitation led to increase the PHB concentration, PHB synthesis rate & productivity by *A.latus* ATCC 29712 about 1.33, 2.37 & 1.33 fold than that attained under nitrogen sufficient. At all high cell density fed-batch culture, the PHB concentration and PHB content increased rapidly during early stage of the application of nitrogen limitation whereas residual cells were decreased. The highest figures of PHB concentration, PHB content, PHB synthesis rate and productivity were recorded by applying nitrogen limitation after 23.5 and 37 h when cell concentration of *A.latus* ATCC 29712 and *R.eutropha* ATCC 17697 reached to about 70 gl^{-1}. These figures were 74.07 gl^{-1}, 71 %, 0.064 $gg^{-1}h^{-1}$ & 1.93 $gl^{-1}h^{-1}$ for the first strain and 59.23 gl^{-1}, 53.28 % 0.018 $gg^{-1}h^{-1}$ & 0.91 $gl^{-1}h^{-1}$ for the second strain, respectively. The pH-stat fed-batch culture under nitrogen limitation conditions increased the PHB productivity by *A.latus* ATCC 29712 and *R.eutropha* ATCC17697 about 4.39 and 2.76 fold, respectively, as compared with that obtained in fed-batch culture with pulsed feeding of sugar.

Key words : Poly-B-hydroxybutyrate, pH-state fed-batch culture, *Ralstonia eutropha* ATCC 17697, *Alcaligenes latus* ATCC 29712, Nitrogen limitation – High cell density fed- batch culture.

INTRODUCTION

In fermentation processes where cell growth and/or product formation is inhibited by high substrate concentration or by the accumulation of a byproduct, substrate is intermittently fed to the culture system in order to maintain the substrate concentration below a certain level for enhancement of biological and metabolic activity. This can be achieved by using various feeding strategies including DO stat (Wang & Lee, 1997 and Lee *et al*, 1997) and pH stat (Yamane *et al*, 1996; Wang & Lee,1998 and Kobavashi *et al*, 2000) or by monitoring the glucose concentration with an on-line glucose analyzer when glucose is used as a substrate (Kim *et al*,1994 a & b). Wang and Lee (1997) demonstrated that, nitrogen limitation could significantly enhance PHA production for *A.latus*. Cells were first cultured by the DO-stat feeding strategy without nitrogen limitation. Nitrogen limitation was applied at a cell concentration of 76 gl^{-1} and the sucrose concentration was maintained within 5 to 20 gl^{-1}. After 8 hours of nitrogen limitation, the cell concentration, P (3HB) concentration and P (3HB) content reached 111.7 gl^{-1}, 98.7 gl^{-1} and 88 %, respectively, resulting in a productivity of 4.94 g of P (3HB) per liter per hour. In this respect, Yamane *et al* (1996) reported that a PHB concentration of 68.49 gl^{-1} could be obtained in 18 hours with a pH-stat fed- batch culture of *A.latus* using a high inoculum concentration (13.7 gl^{-1} dried cell), resulting in high PHB productivity of 3.97 $gl^{-1}h^{-1}$. Also, Wang and Lee (1998) studied the production of a high concentration of PHB (104 gl^{-1}) with high productivity by the pH-stat fed-batch culture of the filamentation-suppressed recombinant *E.coli* in a defined medium. Kim *et al* (1994b) reported that, P (3HB) concentrations of 71 and 92 gl^{-1} could be obtained by limiting nitrogen at the *A.eutrophus* cell concentrations of 30and 55gl^{-1}, respectively. By further delaying the nitrogen limitation, until the cell concentration reached 70 gl^{-1}, a final P (3HB) concentration of 121 gl^{-1} could be achieved. However, delaying nitrogen limitation until the cell concentration reached 90 gl^{-1} resulted in lower PHA concentration and productivity.

Kim (2000) and Nonato *et al* (2001) reported that *Ralstonia eutropha* or *Bhurkolderia* sp. are grown aerobically to a high cell density in a well balanced medium consisting of cane sugar and inorganic nutrients. Cell growth is then shifted to PHB synthesis by limiting nutrients other than the carbon source, which is continually fed as a high concentration sugar syrup. After 45-50 h, the fed-batch fermentation process is stopped, with a final dry cell mass of 125-150 kg/m^3, containing nearly 65-70 % PHB.

In the present work *A.latus* ATCC29712 and *R.eutropha* ATCC 17697 were grown in bioreactor as pH-stat fed-batch culture under nitrogen limitation with different cell concentrations in order to detect the optimum treatment for PHB production.

MATERIALS AND METHODS

1-Bacteria used

A lyophilized cultures of *Ralstonia eutrophus* ATCC17697 (formerly *Alcaligenes eutrophus*) and *Alcaligenes latus* ATCC 29712 were obtained from American Type Culture Collection, University Boulevard, Manassas, Virginia U.S.A. Both strains were subcultured on nutrient agar slants, maintained at 5°C and transferred monthly on fresh slants.

2-Media used

- Med. 1: -Nutrient agar medium (Difco Manual, 1977) was used for preservation of alcaligenes cultures.
- Med. 2: Kim *et al* (1994b) was used for standard inoculums preparation of alcaligenes strains for bioreactor experiments. It consists of (gl^{-1}): glucose, 10; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.5; $Na_2HPO_4.12H_2O$, 9.0; Mg $SO_4.7H_2O$,0.2 Trace element solution, 1 ml. The pH of the medium was adjusted to 6.8 with NaOH.
- Med. 3: Kim *et al* (1994a) was used for production of bioplastic by alcaligenes cultures. It consists of (gl^{-1}):glucose,20; $(NH_4)_2SO_4$,4.0; KH_2PO_4, 13.3;$MgSO_4.7H_2O$,1.2; citric acid, 1.7;Trace element solution, 10 ml. Glucose and $MgSO_4$-$7H_2O$ were autoclaved separately then added aseptically to the medium. The pH of the medium was adjusted to 6.8 with NaOH. (The trace element solution contained (gl^{-1}):$FeSO_4.7H_2O$,10g;)
- Med. 4: Nutrient-rich- medium **(Chua *et al* 1998).** This medium was used for seed culture of tested strains in fed-batch experiments. It

consists of (gl^{-1} tap water) : yeast extract, 10; polypeptone, 10; meat extract, 5.0; and ammonium sulfate, 5.0. The pH was adjusted to 6.8 with NaOH.

3-Standard inoculum

Standard inoculum was prepared by inoculation of 250 ml conical flasks, each containing 100 ml med.2 with a loop of tested culture. The inoculated flasks were incubated on rotary shaker (150 rpm) for 24-48 hours at 30°C. The content of these flasks were used for preparation of standard inoculum (1ml contained 3.5: 6 X 10^5 viable cells) for all experiments.

4-Bioreactor experiments

In the present work 3L dished bottom bioreactor Z6110/Coob (Cole-Parmer Instrument) was used, which consists of 3 liter vessel equipped with lipseal stirrer assembly, automatic pH controller, automatic dissolved O_2 controller, CO_2 controller, automatic temperature controller, foam controller and multi-channel peristaltic pump (for feeding). The PHAs producing bacteria were grown in the bioreactor as pH-stat fed batch culture. Moreover the effect of different cell concentrations on PHB production were studied under nitrogen limitation conditions.

4.1. pH-stat fed-batch culture

In these experiments, feeding solutions were automatically fed to the bioreactor and maintain the pH at 6.8 by a pH controller. So, two different feeding solutions were used namely, ammonia solution (NH_4OH, 28 %) and sugar solution which contained 700 gl^{-1} sugar, for cultivation of tested strains (Yamane *et al*, 1996 and Kim *et al*, 1994b). Ammonia solution was replaced by 5 *N* NaOH solution during the period of nitrogen limitation. Both solutions were supplied simultaneously by the single pump, but with different tubings under the control of a pH stat. Samples (10 ml) were taken from the growing culture periodically under aseptic condition to determine cell dry weight, PHB produced and residual sugar. PHB parameters were also calculated.

4.2. High-cell-density fed-batch culture

Four fermentations of pH-stat fed-batch culture were carried out using med.3 which was free from nitrogen source to study the effect

of different cell concentrations, which were obtained by delaying the time of applying nitrogen limitation, on PHB production by tested strains. Therefore, trials were done to replace ammonia water by 5 *N* NaOH solution after 28, 31, 34 & 37 or 15.5, 18.5, 21 & 23.5 h of incubation period of pH-stat fed batch culture for *Ralstonia eutropha* ATCC 17697 or *Alcaligenes latus* ATCC 29712 in order to obtain approximately 40, 50, 60 & 70 gl^{-1} cell dry weight, respectively. Samples (10 ml) were taken from the growing culture periodically under aseptic conditions to determine cell dry weight, PHB produced and residual sugar. PHB parameters were calculated.

5. Chemical determinations

Total sugars were determined in the fermented liquor according to the method of Flood & Prestly (1973). PHB was extracted from paste cells precipitated and determined as dry weight (gl^{-1}) according to the method recommended by Grothe *et al* (1999).

6. Calculation

Specific production rate of PHB (μ_p) was calculated according to Painter and Marr (1963). PHB content (percentage of PHB dry weight per cell dry weight), Productivity (P) and PHB synthesis rate were calculated according to Lee & Chol (1998), Lee (1996) and Wang & Lee (1997), respectively.

RESULTS AND DISCUSSION

I-pH-stat fed-batch culture:

PH-stat fed-batch culture is a powerful technique for the achievement of high cell density which has many advantage over a low cell density culture as a higher product concentration, increased productivity and a decreased recovery cost. Therefore, it is a preferred mode of operation in industrial application. Thus, a pH-stat substrate feeding system was used in which feeding solution was fed automatically to maintain the pH of culture broth at 6.8. By feeding a solutions containing 70 % sucrose and 28 % ammonia water during The fermentation period of *A.latus* ATCC 29712. The concentration of sucrose in broth was ranged from 7.62 – 10.61 gl^{-1} during PHB accumulation phase. The cell dry weight and residual cells (gl^{-1}) of *A.latus* ATCC 29712 increased during 72 h fermentation period of pH-stat fed-batch culture (with nitrogen sufficient) whereas the values

of cell productivity increased to record the maximum value being 2.98 $gl^{-1}h^{-1}$ after 30 h incubation period as shown in Table (1). With respect to PHB production, data clearly show that the concentration of PHB (gl^{-1}) and content (%) increased to reach the maximum value being 55.79 gl^{-1} and 51.16 % at 38 h, respectively, resulting in PHB synthesis rate, productivity and specific production rate of 0.027 $gg^{-1}h^{-1}$, 1.45 gl^{-1} h^{-1} and 0.12 h^{-1}, respectively. Also, it could be noticed that a higher cell productivity values, ranged from 2.18 to 2.98 gl^{-1} h^{-1}, were attained during the first 15-30 h incubation period under nitrogen sufficient culture. The corresponding figures of cell dry weight at this period were ranged from 39.78 to 96.51 gl^{-1}. So, different cell concentrations with highest productivity will be obtained during this period to apply nitrogen limitation for securing high PHB production in further experiments.

Table (1): Growth of *A.latus* ATCC 29712 and PHB production during 72 h incubation at 30°C under nitrogen sufficient condition on med.3 using bioreactor as pH-stat fed- batch culture.

Time (hours)	Cell dry weight (gl^{-1})	Residual cell (gl^{-1})	PHB concentration (gl^{-1})	PHB content (%)	PHB synthesis rate ($gg^{-1}h^{-1}$)	PHB productivity ($gl^{-1}h^{-1}$)	μ_p (h^{-1})	Cell productivity ($gl^{-1}h^{-1}$)	Residual sugar (gl^{-1})
0	7.01	6.37	0.64	9.13	-	-	-	-	20.0
5	12.57	10.61	1.96	15.59	0.025	0.26	0.22	1.11	13.62
10	25.56	21.00	4.56	17.84	0.019	0.39	0.20	1.86	9.57
15	39.78	30.01	9.77	24.56	0.020	0.61	0.18	2.18	7.62
20	55.54	35.75	19.79	35.63	0.027	0.96	0.17	2.43	9.78
25	76.78	44.45	32.33	42.11	0.029	1.27	0.16	2.79	9.87
30	96.51	50.95	45.56	47.21	0.029	1.50	0.14	2.98	9.64
35	105.31	52.95	51.36	49.72	0.028	1.48	0.13	2.81	8.78
37	108.72	53.27	55.45	51.00	0.028	1.48	0.12	2.75	10.61
38	109.06	53.27	55.79	51.16	0.027	1.45	0.12	2.69	8.85
40	109.43	54.36	55.07	50.32	0.025	1.36	0.11	2.56	9.53
48	113.31	62.20	51.11	45.11	0.017	1.05	0.09	2.21	18.31
72	113.41	65.10	48.31	42.60	0.010	0.66	0.06	1.48	29.21

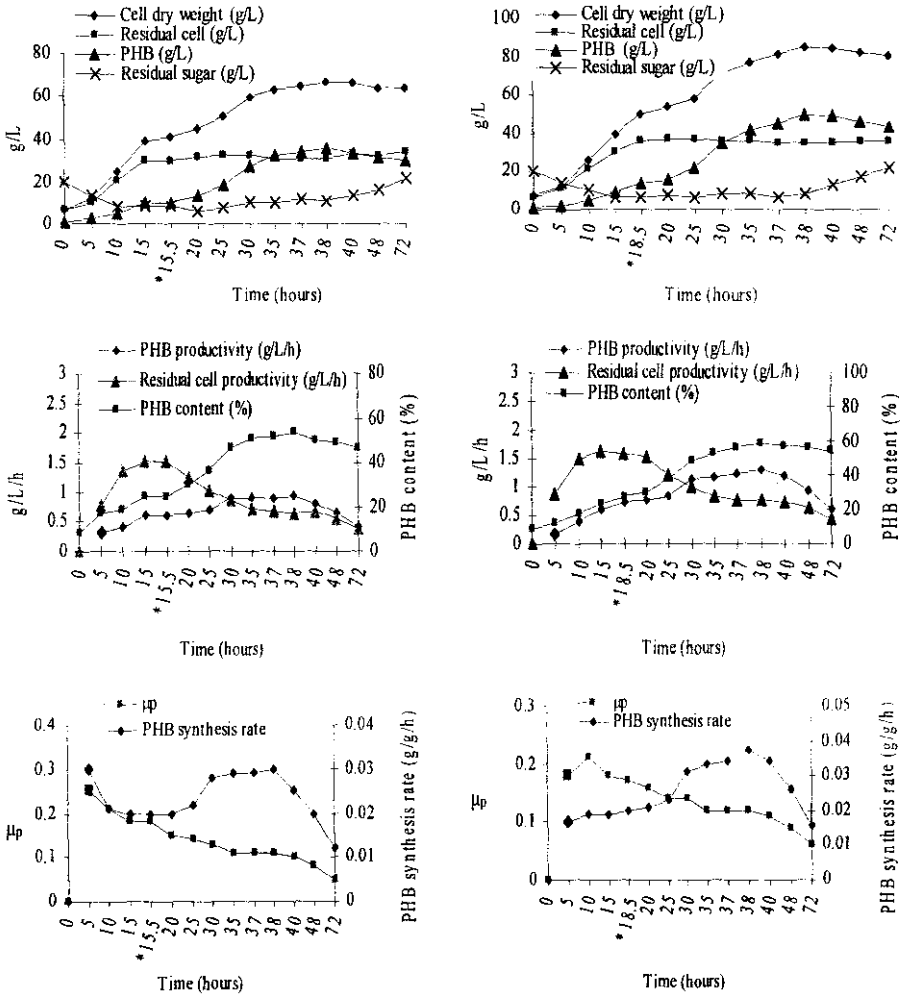
μ_p = Specific production rate (h^{-1})

II-High-cell density fed-batch culture

In this experiments, different pH-stat fed-batch cultures, with varying cell concentration for starting the nitrogen limitation, were carried out to increase PHB productivity by tested strains. Data illustrated by Figs (1&2) show that the different cell concentrations of *A.latus* ATCC 29712 being 40.12, 49.98, 60.48 & 72.35 gl^{-1} were obtained by delaying the time to apply nitrogen limitation to 15.5, 18.5, 21 and 23.5 h, respectively. At all high cell density fed-batch cultures, the PHB concentration (gl^{-1}) as well as PHB content (%) increased rapidly during the early stage of the application of nitrogen limitation. The residual cell concentration did not increase but rather decreased to give the lowest value at 38 h of fermentation period. Also, the specific production rate of PHB (μ_p) was decreased to reach the minimum value after 72 h fermentation period. Applying nitrogen limitation when *A.latus* ATCC 29712 cell concentration reached to 40.12 or 49.98 gl^{-1} at 15.5 h or 18.5 h, *A.latus* ATCC 29712 gave the concentration of cell and PHB, after 38 h, lower than that obtained under nitrogen sufficient. The highest figures of PHB concentration, PHB content, PHB synthesis rate and productivity were obtained after 38 h of incubation and increased by increasing the cell concentrations than 49.98 gl^{-1} (at starting the apply of nitrogen limitation) to reach the maximum at cell concentration of 72.35 gl^{-1}. Recorded PHB parameters, after 38 h at cell concentration 72.35 gl^{-1}, were 74.07 gl^{-1}, 71.00 %, 0.064 $gg^{-1}h^{-1}$, 0.12 h^{-1} and 1.93 $gl^{-1}h^{-1}$ for PHB concentration. PHB content, synthesis rate, specific production rate and productivity.

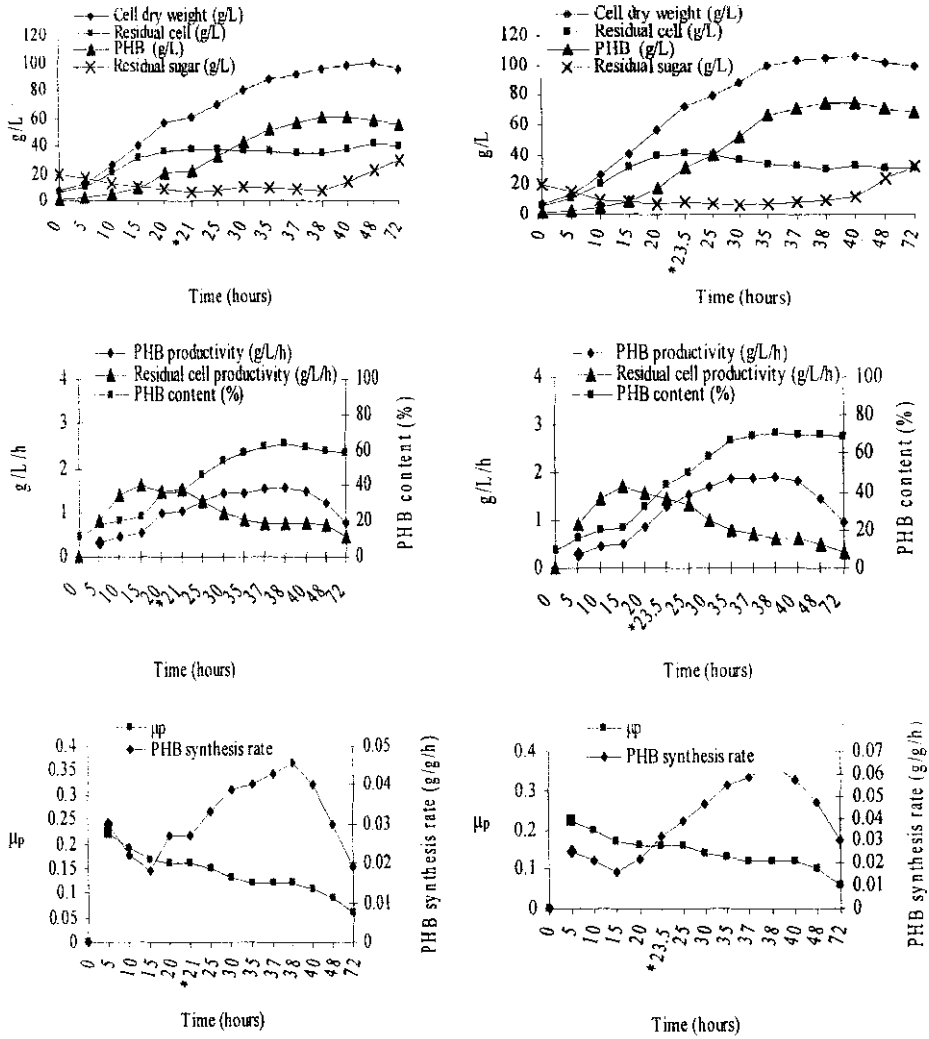
Comparing the previous data of the maximum PHB production, with that obtained under nitrogen sufficient of pH-stat fed-batch culture, it is obvious that applying nitrogen limitation (after 23.5 h) led to increase the PHB concentration, PHB synthesis rate & productivity about 1.33, 2.37 & 1.33 fold, respectively, and decrease the residual cell productivity from 1.23 to 0.63 $gl^{-1}h^{-1}$. These results are in line with those obtained by Wang & Lee (1997), who stated that the PHB content could be increased by applying nitrogen limitation by *A.latus* and ammonia feeding must be stopped at higher cell productivity to trigger enhanced PHB synthesis. They added that, the residual cell concentration did not increase but rather decreased under nitrogen limiting condition. Also, PHB content could be

decreased after reaching the highest values, this seems to be due to the reduced metabolic activity of cells containing a large amount of PHB.



(ammonia feeding was stopped at15.5 h) (ammonia feeding was stopped at18.5 h)

Fig.(1) : Growth of *A.latus* ATCC 29712 and PHB production during 72 h incubation at 30°C on med.3 under nitrogen limitation condition (ammonia feeding was stopped at 15.5 h & 18.5h) using bioreactor as a pH-stat fed-batch culture. (μ_p= specific production rate).

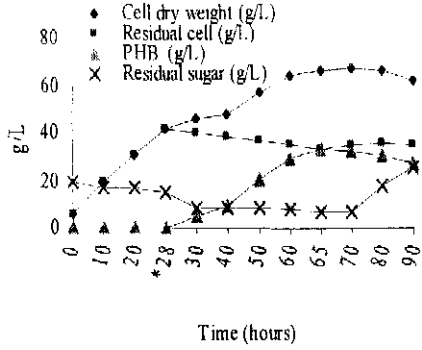


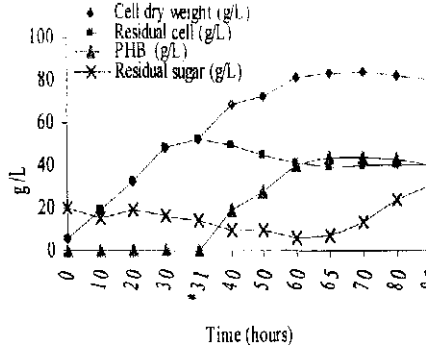
(ammonia feeding was stopped at 21 h) (ammonia feeding was stopped at 23.5 h)

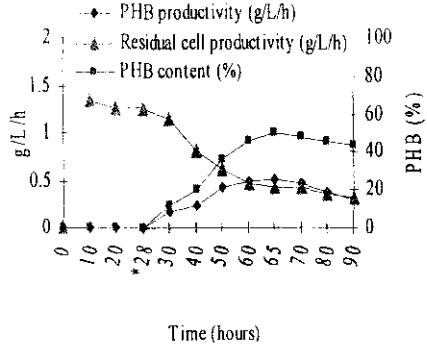
Fig.(2) : Growth of *A.latus* ATCC 29712 and PHB production during 72 h incubation at 30°C on med.3 under nitrogen limitation condition (ammonia feeding was stopped at 21 h & 23.5 h) using bioreactor as a pH-stat fed-batch culture (μ_p=specific production rate).

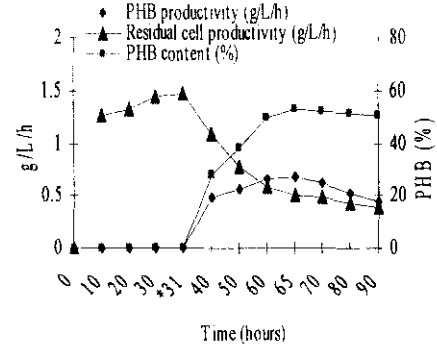
With respect to PHB production by different concentrations of *R.eutropha* ATCC 17697 cells (at starting the apply of nitrogen limitation) during 90 h incubation period on med.3 under nitrogen limitation using pH-stat fed-batch culture technique, results in Figs (3&4) show that increasing cell concentrations from 41.31 to 71.73 gl^{-1}, obtained after delaying the time of apply nitrogen limitation from 28 to 37 h, led to increase the concentrations of cell dry weight and PHB as well as PHB parameters after 65 h incubation period. The increase in PHB synthesis rate resulted in the rapid increase of PHB concentration and PHB content. Delaying the nitrogen limitation until the *R.eutropha* ATCC 17697 cell concentration reached 71.73 gl^{-1} at 37 h, after a short time (2 h) where ammonium in the culture broth became depleted, PHB was started to accumulate in the cells. The PHB content increased very sharply from 11.33 to 47.23 % during 20 h after application of nitrogen limitation then slightly increased to record the highest value at 65 h fermentation period. During the first 20 h of nitrogen limitation, gradual decrease in residual cell was observed at residual glucose concentrations ranged from 2.67 to 12.79 gl^{-1}. The PHB synthesis rate reached a maximum value being 0.018 $gg^{-1}h^{-1}$ at 65 h and then decreased when PHB content reached a value of 52.57 %. This seems to be due to the reduced metabolic activity of cell containing large amount of PHB as was also observed by Kim *et al* (1994b) in *A.eutrophus*. At 65 h, the concentration of cell and PHB were 111.16 and 59.23 gl^{-1}, respectively, resulting in 53.28 % PHB content and 0.91 $gl^{-1}h^{-1}$ PHB productivity. These results are in line of those obtained by Kim *et al* (1994b) who stated that, a higher PHB concentration (121 gl^{-1}) and productivity (2.42 $gl^{-1}h^{-1}$) could be achieved by delaying nitrogen limitation until the cell concentration reached 70 gl^{-1} at 30 h.

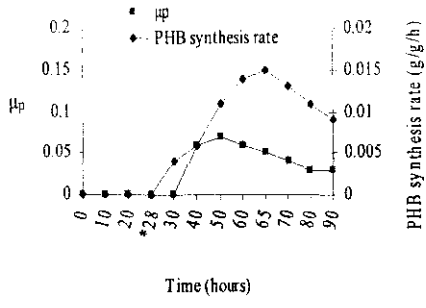
Generlly it could be noticed that the pH-stat fed-batch culture under nitrogen limitation with high cell density was the best strategy for PHB production by *A.latus* ATCC 29712 and *R.eutropha* ATCC 17697 and increased PHB productivity about 4.39 and 2.76 fold by *A.latus* ATCC 29712 and *R.eutropha* ATCC 17697, respectively, as compared with that obtained in fed-batch culture with pulsed feeding of sugar by El-Sayed *et al* (2008).

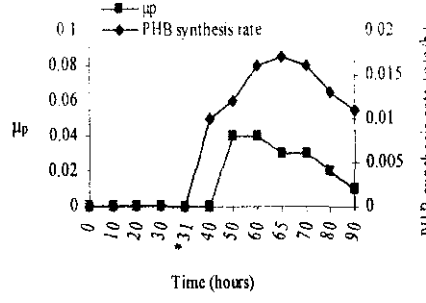






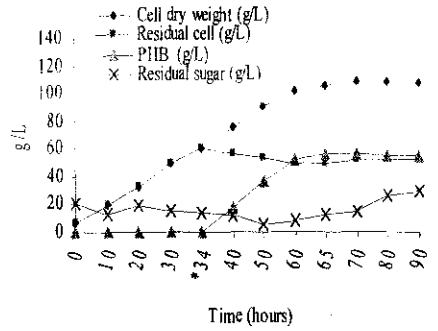


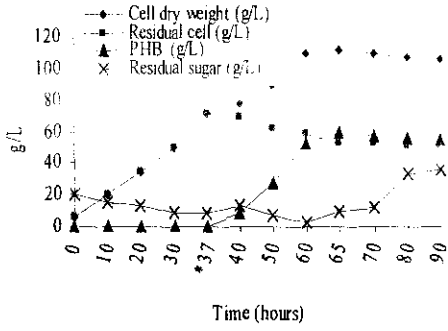


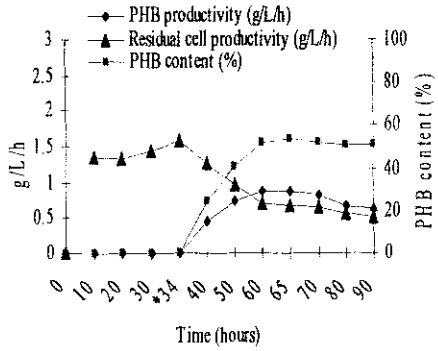


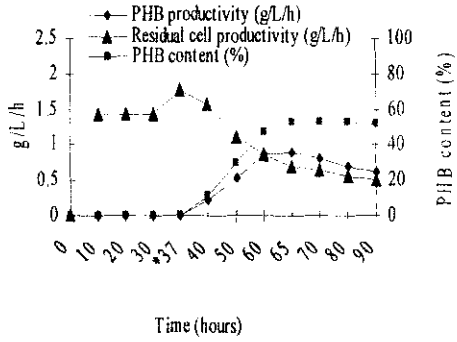
(ammonia feeding was stopped at 28 h) (ammonia feeding was stopped at 31 h)

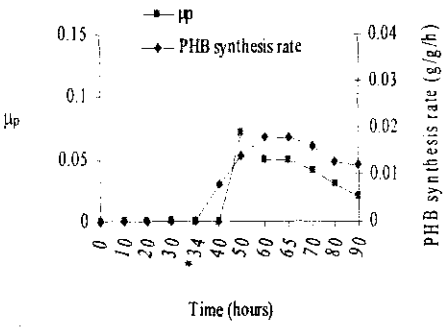
Fig.(3) : Growth of *R.eutropha* ATCC 17697 and PHB production during 90 h incubation at 30°C on med.3 under nitrogen limitation condition (ammonia feeding was stopped at 28 h & 31h) using bioreactor as a pH-stat fed-batch culture(μ_p= sepcific production rate).

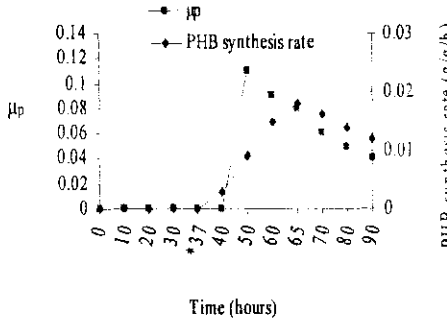












(ammonia feeding was stopped at 34 h) (ammonia feeding was stopped at 37h)

Fig.(4) : Growth of *R.eutropha* ATCC 17697 and PHB production during 90 h incubation at 30°C on med.3 under nitrogen limitation condition (ammonia feeding was stopped at 34 h & 37 h) using bioreactor as a pH-stat fed-batch culture(μ_p= sepcific production rate).

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إنتاج مركب البولى بيتا هيدروكسى بيوتيرات(PHB) تخميرياً بواسطة تركيزات مختلفة من خلايا,*R.eutropha* ATCC 17697, *A.*latus ATCC29712 فى مزرعة الـ H-state fed batch

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تمت دراسة تأثير تركيزات مختلفه من خلايا

A.latus ATCC 29712 , *R.eutropha* ATCC 17697

تتراوح من ٤٠ ـ ٧٠ جرام/لتر والتى يتم الحصول عليها بتأخير وقت بداية ظروف نقص النتروجين على إنتاج PHB فى البيئة باستخدام مزارع الدفعة الواحدة المغذاه تبعاً لإنخفاض درجة pH (pH-state-Fed-batch) ووجد أن تطبيق ظروف نقص النتروجين أثناء تنمية السلالة الأخيرة يؤدى إلى زيادة تركيزPHB ومعدل تخليقه وكذلك إنتاجيته حوالى ١,٣٣ ، ٢,٣٧ ، ١,٣٣ ضعف عن تلك التى يتم الحصول عليها تحت ظروف توفر النتروجين ووجد أن تركيز مركب PHB ومحتوى الخلايا منه فى جميع مزارع الدفعة الواحدة المغذاه يزيد بصورة سريعة خلال مرحلة مبكرة بعد تطبيق ظروف نقص النتروجين بينما يحدث نقص تركيز بقية محتويات الخلايا . كما لوحظ أن تطبيق ظروف النتروجين عندما يعمل تركيز الخلايا إلى ٧٠ جرام/لتر لكل السلالتين يؤدى إلى الحصول على أعلى تركيز من مركب PHB وكذلك محتوى الخلايا منه ، معدل تخليقه وإنتاجيته والتى وصلت إلى ٧٤,٠٧ جرام/لتر ، ٧١% ، ٠٦٤. ٠ جرام/جرام/لتر ، ١,٩٣ جرام/لتر/ساعة بواسطة *A.latus* ATCC 29712 بينما كانت القيم المتحصل عليها بواسطة *R.eutropha* ATCC 17697 ٥٩,٢٣ جرام/لتر ، ٥٣,٢٨% ، ٠١٨. ٠ جرام/جرام/ساعة ، ٩١. ٠ جرام/لتر/ساعة على الترتيب .