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Poduction of Polyhydroxybutyrate (PHB) Using Batch and Two-stage Batch Culture Strategies

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Abstract: Batch and two-stage batch culture of *Ralstonia eutropha* ATCC 17697 (*Alcaligenes eutrophus)* and *Alcaligenes latus* ATCC 29712, were investigated for producing the intracellular bioplastic poly-β-hydroxybutyric acid (PHB) using shake flasks technique. The highest growth and PHB production of *Ralstonia eutropha* ATCC 17697 and *Alcaligenes latus* ATCC 29712 were recorded on (Kim *et al* 1994a) medium containing glucose or sucrose (as a carbon source), respectively. Ammonium sulfate was the best nitrogen source for PHB production by both strains. The productive medium which contain carbon source and ammonium sulfate in C/N ratio of 12.57 gave the highest PHB either by *Ralstonia eutropha* ATCC 17697 and *Alcaligenes latus* ATCC 29712. Washed cells of Alcaligenes strains produced PHB concentration and content higher than crude cells at different limiting nutritional treatments. Applying the two stage batch fermentation with nitrogen limitation increased the PHB content (%) of *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 cells about 48.43 % and 14.29 %, respectively, as compared with that obtained in batch culture using shake flasks technique.

Key words: Poly-B-hydroxybutyrate (PHB), *Ralstonia eutropha*, *Alcaligenes eutrophus*, *Alcaligenes latus*, Batch culture, Two-stage batch culture.

INTDRODUCTION

Poly- β-hydroxybutyrate (PHB) is an intracellular microbial thermoplastic that is widely produced by many bacteria (Braunegg *et al.*, 1998). In terms of molecular weight, brittleness, stiffness, melting point and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical-derived thermoplastics such as polypropylene. Therefore in certain application, PHB can directly replace some more traditional, nonbiodegradable polymers (Poirier *et al.*, 1995).

Alcaligenes eutrophus, non-growth-associated PHB producer, require the limitation of an essential nutritional element such as N, P, Mg, K, O or S in the presence of an excess of carbon source for the efficient synthesis of PHB. *Alcaligenes latus*, a-growth-associated PHB producer, accumulates PHB up to 80 % of dry cell weight without limitation of any nutrient. Various carbohydrates in the growth media including glucose, sucrose, lactic acid, butyric acid, valeric acid and various combinations of butyric and valeric acids are utilized as carbon sources for the production of bioplastic by some bacterial strains (Kim *et al.*, 1994b;Yu *et al.*, 1998 and Mitomo *et al.*, 1999). The presence of inorganic chemicals such as ammonia or ammonium salts 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. The best growth of *A.eutrophus* and PHB production was obtained with ammonium sulfate in a synthetic medium containing 3 % glucose at pH 7 (Beaulieu *et al.*, 1995). Yu *et al.* (1998) reported that, higher C/N ratio (deficiency of nitrogen in the medium) would promote the production of the polymer by microorganisms. Grothe *et al.* (1999) reported that, the C/N ratio in *A.latus* medium is wide (21.5) and probably explained by the large amount of PHB in the cells which does not contain nitrogen.

The production cost of PHB is quite as compared with that of synthetic nondegradable plastic, and much effort has recently been devoted to make this process economically more feasible by improving the productivity and developing a new separation process (Hankermeyer and Tjeerdema, 1999). So, the present investigation was designed to determined the optimum carbon & nitrogen sources, C/N ratio as well as optimum nutrient limitation for maximizing the accumulation of PHB by *Ralstonia eutropha* ATCC 17697 and *Alcaligenes latus* ATCC 29712 using shake flask as a batch and two-stage batch culture.

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MATERIALS AND METHODS

Bacteria Used:

A lyophilized cultures of *Ralstonia eutropha* ATCC 17697 (*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 5ºC and transferred monthly on fresh slants.

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 inoculum preparation of alcaligenes strains for shake flasks 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 in addition to 1 ml trace elements solution. The pH of the medium was adjusted to 6.8 with NaOH.
Med.3:- [Kim *et al.,* 1994 a] was used for production of bioplastic polymer 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 elements 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}): $FeSO_4.7H_2O$, 10; $ZnSO_4.7H_2O$, 2.25; $CuSO_4.5H_2O$, 1.0; $MnSO_4.4H_2O$, 0.5; $CaCl_2.2H_2O$, 2.0; $Na_2B_4O_7.10H_2O$, 0.23; $(NH_4)_6Mo_7O_{24}$, 0.1; 35 % HCl, 10ml .

Standard Inoculum:

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

Factors Affecting Accumulation of PHB:

A. Carbon Sources:

This experiment was designed to study the effect of different carbon sources on polymer (PHB) production by *Ralstonia eutropha* ATCC 17697 and *Alcaligenes latus* ATCC 29712. Therefore, trials were done to replace the glucose as a carbon source by fourteen sources of carbon in amounts equal to that present in the original medium in order to avoid the error which may be resulted from the differences in carbon concentration in each source. Carbon sources applied were starch, sucrose, lactose, maltose, galactose, mannose , mannitol, fructose , glycerol , ethanol, lactic acid, malic acid, acetic acid and butyric acid. The propagation was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium (Med.3). These flasks were inoculated with 1 ml standard inoculum. The inoculated flasks were then incubated at 30ºC using rotary shaker (150 rpm). Samples (10 ml) were taken from the growing cultures periodically every 24 hours under aseptic conditions. The fermented cultures were centrifuged at 15000 X *g* for 4 min at 4°C and the sediment was washed twice with distilled water, then dried to constant weight (90°C). The polymer (PHB) content was extracted from cells and determinate according to Grothe *et al.* (1999). The relation between time and cell dry weight (growth curve) was then plotted on semi-log paper. The parameters of growth and PHB production were calculated.

B. Nitrogen Sources:

Fourteen nitrogen sources were applied namely peptone, malt extract, beef extract, yeast extract, casein, proteose peptone, treptone, ammonium sulfate, ammonium molibidate, ammonium nitrate, ammonium phosphate, ammonium chloride, sodium nitrate, and sodium nitrite. The amount of nitrogen compound added was calculated to give the same nitrogen concentration as the original source. The previous procedures of propagation were used. Parameter of growth and polymer production were also calculated

C. C/N Ratio:

Six levels of C/N ratios ranged from 9.43 to 25.15 were prepared by using different ammonium sulfate concentration ranged between 1.5 - 4.0 gl^{-1} in productive medium. The propagation was carried out as mentioned before.

Using Two-stage Batch Culture for PHB Production:

The cells of the tested bacterial strained were first grown in 250 ml Erlenmeyer flasks containing 100 ml med.3 (complete medium). The inoculated flasks were incubated at 30°C using rotary shaker (150 rpm) for 96 hours. Samples (10 ml) were taken from the grown cultures to estimate the cell dry weight and polymer content. The remaining content of these flasks (90 ml) were used as crude cells or washed cells to be inoculated the second-step flasks which containing 100 ml productive media. Different nutrient limitation treatments were constructed by omission or reducing the concentration of nitrogen or/and phosphorus in med.3 to the tenth as follows: -

1- sugar solutions (2%)
2- nitrogen free med.3
3- med.3 containing low nitrogen concentration (0.4 g/L) $(NH_4)_2SO_4$
4- med.3 containing low phosphorus concentration (1.33 g/L) KH_2PO_4
5- nitrogen free med.3 containing low phosphorus concentration
6- med.3 containing low concentration of nitrogen and phosphorus
7- phosphorus free med.3
8- phosphorus free med.3 with low nitrogen concentration
9- nitrogen and phosphorus free med.3

The inoculated flasks of second step were incubated at 30°C for 96 h. Samples (10 ml) were taken from the growing cultures periodically every 24 h under aseptic condition. The cell dry weight and PHB content were estimated.

Fermentation Period:

This experiment was carried out to detect the proper time for maximum PHB production by tested bacterial strains. The inoculated medium was incubated at 30°C for different period from 2to 3 days using two-stage culture. Samples were taken as latterly mentioned in the text in order to determine the accumulated PHB.

Determination of PHB:

PHB was extracted from paste cells, precipitated and determined as dry weight (gl^{-1}) according to the method recommended by Grothe *et al.* (1999).

Calculations:

The specific growth rate (μ) and doubling time (t_d) were calculated from the exponential phase according to Painter and Marr (1963). Number of generation was calculated according to Stanier *et al.* (1970)

Specific production rate (μ_p) was calculated according to Painter and Marr (1963). PHB yield coefficient relative to cell dry weight ($y_{p/x}$) was calculated according to Grothe *et al.* (1999). Productivity (P) and PHB content (percentage of PHB dry weight per cell dry weight) were calculated according to Wang and Lee (1997).

RESULTS AND DISCUSSION

Effect of Some Nutritional Requirements on PHB Production in Batch Culture:

Carbon Source:

Data illustrated by Fig. (1) show that *R. eutropha* ATCC 17697 and *A.latus* ATCC 29712 grow exponentially through 96 h incubation on all the tested different carbon sources in med.3. The highest growth of *R. eutropha* ATCC 17697 being 6.21,5.83 and 4.02 grams dried cells /L were recorded on media containing glucose, fructose and sucrose, respectively, after 96 h incubation period. The highest growth of *A. latus* ATCC 29712 were 7.92, 5.43 and 4.71 grams dried cells /L on media containing sucrose, glucose and fructose, respectively . The growth parameters of these strains gave the same trend, where the highest figures of specific growth rate (μ) & number of generation (N) and lowest doubling time (td) were recorded by *R.eutropha* ATCC 17697 on glucose medium being 0.053 h^{-1}, 7.34 and 13.08 h, respectively. The corresponding figures obtained by *A.latus* ATCC 29712 on sucrose medium were 0.055 h^{-1}, 7.62 and 12.60 h, respectively. These results are in line with those obtained by Gomez *et al.* (1996) who stated that the best growth of *A.eutrophus* and *A.latus* were obtained in media containing glucose and sucrose, respectively.

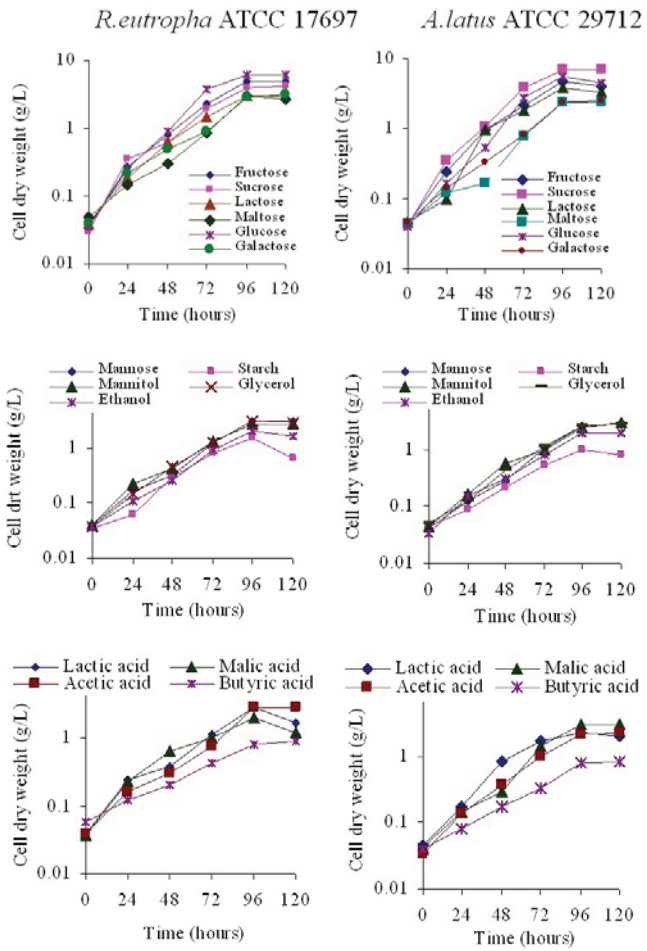

Fig. 1: Growth curves of R. eutropha ATCC 17697 and A. latus ATCC 29712 as affected by different carbon sources in med.3 during 120 hours incubation at 30oC using shake flasks as a batch culture.

With respect to PHB production, data illustrated by Fig (2) clearly show that, the PHB production by *A.latus* ATCC 29712 increased with increasing incubation period to reach the maximum after 96 h, whereas it was not observed by *R.eutropha* ATCC 17697 during the first 48 h of incubation (phase of growth). The highest PHB concentrations (gl^{-1}) by *A.latus* ATCC 29712 were observed on sucrose (3.64 gl^{-1}), glucose (2.18 gl^{-1}) and fructose (1.87 gl^{-1}). The highest PHB concentrations being 0.37, 0.23 and 0.19 gl^{-1} were attained by *R.eutropha* ATCC 17697 on medium containing glucose, fructose or sucrose, respectively. Also, the highest figures for specific production rate(μ_p), PHB yield coefficient ($y_{p/x}$), PHB content (%) and productivity (P) were recorded by *R.eutropha* ATCC 17697 (0.088 h^{-1}, 0.059, 5.88 % & 0.0038 $gl^{-1}h^{-1}$) and *A.latus* ATCC 29712 (0.092 h^{-1}, 0.46, 45.96 % & 0.0379 $gl^{-1}h^{-1}$) on glucose & sucrose media, respectively.

Therefore, the best these sugars namely sucrose, fructose and glucose while be use separately as a carbon source in med.3 either for cultivating *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 in order to maximize their PHB accumulation in the further studies.

Nitrogen Source:

The influence of different nitrogen sources on the growth and PHB production by alcaligenes strains in media containing glucose, sucrose or fructose (as a carbon sources) have shown that, the organic sources gave higher growth than inorganic sources after 96 h, but PHB production was increased in cells grown on inorganic sources than organic sources except ammonium nitrate and sodium nitrite. The highest value of PHB concentration (gl^{-1}) for both strains was attained in different media supplemented with ammonium sulfate after

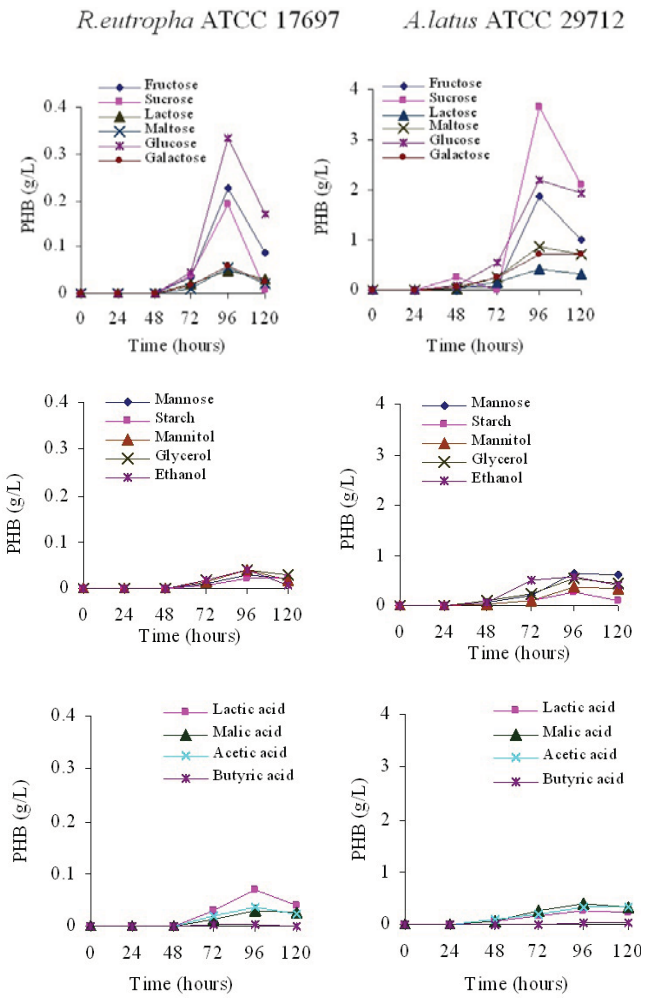

Fig. 2: Polyhydroxybutyrate production by R. eutropha ATCC 17697 and A. latus ATCC 29712 as affected by different carbon sources during 120 hours incubation at 30oC using shake flasks as a batch culture.

96 h incubation. The highest figures of PHB concentration (gl^{-1}), productivity ($gl^{-1}h^{-1}$) and PHB content (%) were observed by *R.eutropha* ATCC 17697 growing on glucose medium being 0.32 gl^{-1}, 0.0033 $gl^{-1}h^{-1}$ & 4.79 %, while they were 3.56 gl^{-1}, 0.0371 $gl^{-1}h^{-1}$ and 45.52 % by *A.latus* ATCC 29712 on sucrose medium (Table 1). Beaulieu *et al.* (1995) in a similar study reported that ammonium sulfate was the best nitrogen source for PHB production by *A.eutrophus* in synthetic medium containing 3 % glucose at pH 7.The lowest figures of these parameters by both strains were recorded in fructose medium containing ammonium nitrate as a nitrogen source (data not show). These results are in line with those obtained by Grothe *et al.* (1999). They stated that apparently with ammonium nitrate, only the nitrogen of the ammonium was bioavailable, and enzymes for assimilation of nitrate were not synthesized.

From the foregoing results, it could be recommended to use ammonium sulfate as nitrogen source for highest PHB production either by *R.eutropha* ATCC 17697 or *A.latus* ATCC 29712.

Carbon - to - Nitrogen Ratio (C/N Ratio):

Data plotted in Figs. (3 & 4) show that C/N ratio which gave the highest cell mass did not give the highest PHB production for both strains. The cell dry weight of both strains decreased with C/N ratio increase and the highest biomass was observed at C/N ratio of 9.43 being 6.51 gL^{-1} and 7.54 gL^{-1} by *R.eutropha* ATCC 17697 on glucose medium and *A.latus* ATCC 29712 on sucrose medium. On the contrary, the production of

Table (1): Effect of different nitrogen sources on growth and PHB production by *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 in med.3 containing glucose or sucrose (as a carbon source) and incubated at 30°C for 120 h using shake flasks as a batch culture.

Nitrogen sources	*R.eutropha* ATCC 17697									*A.latus* ATCC 29712								
	Glucose medium									Sucrose medium								
	Cell dry weigh gl^{-1}						PHB parameters			Cell dry weigh gl^{-1}						PHB parameters		
	0 h	24 h	48 h	72 h	96 h	120	gl^{-1}	P	%	0 h	24 h	48 h	72 h	96 h	120	gl^{-1}	P	%
Peptone	0.04	0.21	0.65	2.65	6.81	6.74	0.09	0.0009	1.32	0.04	0.35	0.89	3.64	8.82	7.51	1.74	0.0181	19.73
Malt extract	0.05	0.20	0.62	2.88	6.94	6.95	0.12	0.0013	1.73	0.05	0.36	0.76	3.43	8.49	7.01	2.02	0.0210	23.79
Beef extract	0.04	0.29	0.59	2.71	6.75	6.92	0.10	0.0010	1.48	0.03	0.37	0.89	3.47	8.91	7.11	1.93	0.0201	21.66
Yeast extract	0.06	0.27	0.63	3.01	6.98	6.02	0.11	0.0011	1.58	0.02	0.34	0.91	3.54	8.81	7.02	1.75	0.0182	19.86
Casein	0.07	0.21	0.63	3.81	7.12	6.52	0.10	0.0010	1.40	0.04	0.37	0.77	4.32	8.98	7.85	1.93	0.0201	21.49
Proteose peptone	0.02	0.24	0.54	2.87	6.89	5.11	0.12	0.0013	1.76	0.06	0.31	0.89	3.48	8.82	7.11	1.71	0.0178	19.39
Tryptone	0.04	0.23	0.59	3.97	7.21	6.87	0.15	0.0016	2.08	0.05	0.34	0.92	3.61	8.78	7.21	1.65	0.0172	18.79
Amm.sulfate	0.05	0.30	0.78	3.87	6.42	6.32	0.32	0.0033	4.79	0.05	0.39	1.18	3.24	7.82	6.64	3.56	0.0371	45.52
Amm.phosphate	0.05	0.20	0.53	2.54	5.94	5.85	0.22	0.0023	3.70	0.03	0.30	0.89	2.54	5.32	4.19	2.23	0.0232	41.92
Amm.chlorid	0.06	0.22	0.59	2.87	4.87	4.12	0.21	0.0022	4.31	0.05	0.25	0.97	2.64	6.98	5.32	2.42	0.0252	34.67
Amm.molibidate	0.08	0.27	0.54	2.95	4.75	4.93	0.21	0.0022	4.24	0.06	0.24	0.85	2.43	5.56	4.18	2.23	0.0232	40.11
Amm.nitrate	0.04	0.21	0.65	1.42	3.95	4.01	0.02	0.0002	0.51	0.04	0.11	0.54	1.01	2.71	2.04	0.46	0.0005	16.97
Sodium nitrate	0.05	0.25	0.64	3.21	4.92	4.11	0.22	0.0023	4.47	0.04	0.29	0.75	2.98	5.45	4.43	2.43	0.0253	44.59
Sodium nitrite	0.04	0.10	0.31	1.12	3.01	2.98	0..02	0.0001	0.66	0.07	0.31	1.01	1.78	3.56	4.67	0.65	0.0068	18.26

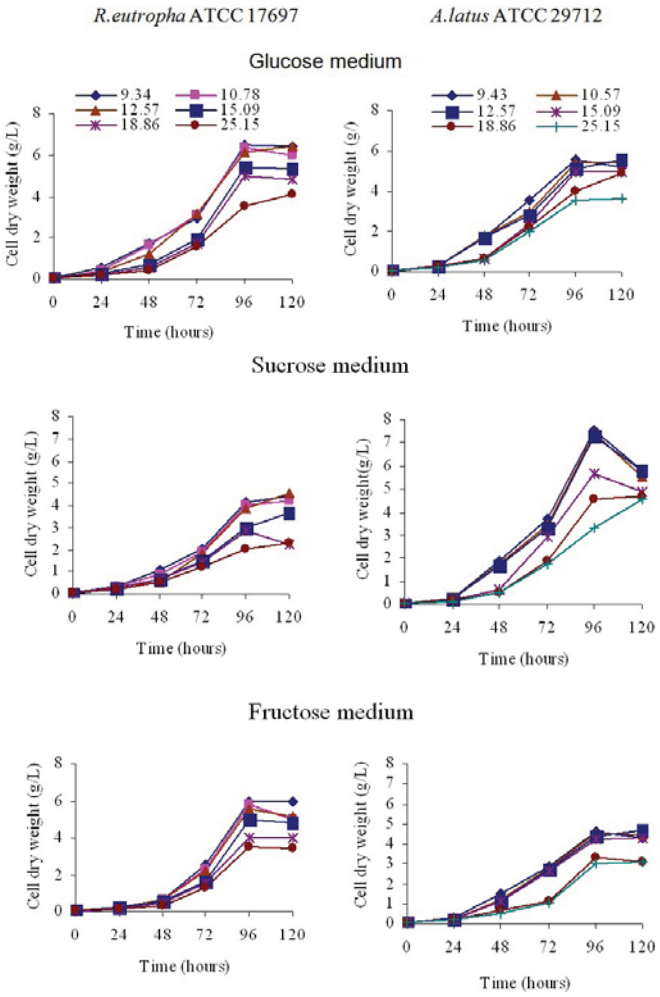

Fig. 3: Growth curve of R. eutropha ATCC 17697 and A. latus ATCC 29712 as affected by different C/N ratios in med.3 containing glucose, sucrose or fructose (as carbon sources) and incubated at 30°C for 120 hours using shake flasks as a batch culture.

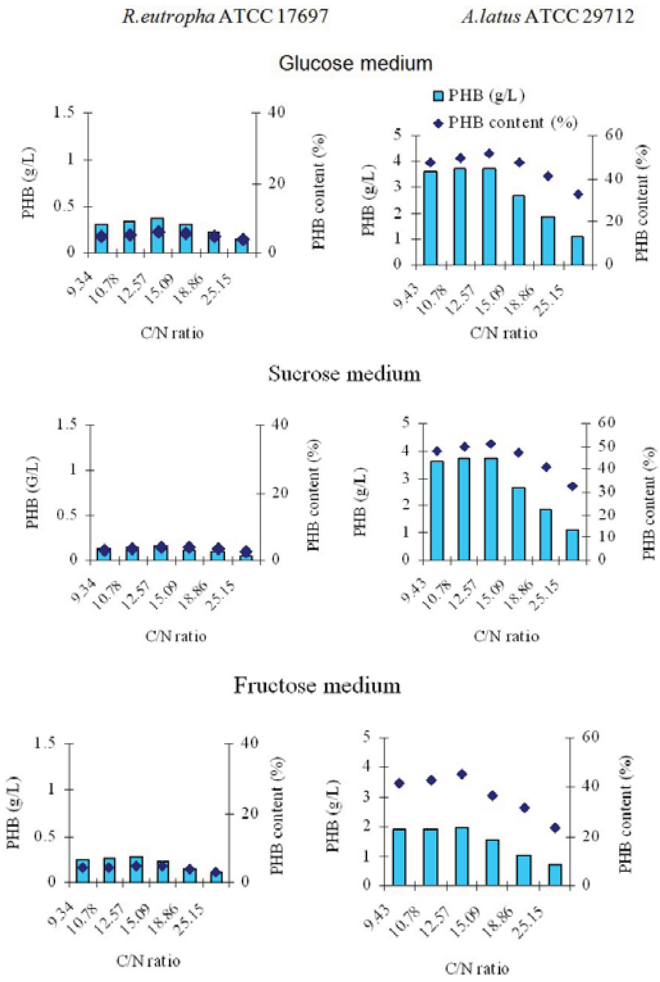

Fig. 4: Effect of different C/N ratio on PHB production by R. eutropha ATCC 17697 and A. latus ATCC 29712 in med.3 containing glucose, sucrose or fructose (as carbon sources) after 96 h using shake flasks as a batch culture.

PHB increased with increasing C/N ratio, till reaching the maximum values at C/N ratio of 12.57, then decreased gradually with increasing C/N ratio to 25.15. At C/N ratio of 12.57, the concentration of PHB (gl^{-1}) produced by *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 on glucose and sucrose medium were 0.37 and 3.74 gl^{-1}, while the corresponding figures of PHB content were 5.99 % and 51.30 %, respectively. These results are in agreement with those of Yu *et al.* (1998) and Grothe *et al.* (1999) who reported that the PHB production by *A.latus* was increased with increasing C/N ratio using sucrose as a carbon source.

In view of the previous results by one-stage batch culture, it could be concluded that, the PHB content (%) of *A.latus* ATCC 29712 (51.30 %) was higher than accumulated by *R.eutropha* ATCC 17697 (5.99 %) on sucrose and glucose media without limitation of any nutrient, respectively. Doi *et al.* (1988) and Mansfield *et al.* (1995) stated that, the pathway of P(3HB) synthesis by *A.eutrophus* from acetyl-CoA is inhibited by the presence of a nitrogen source, causing acetyl-CoA to enter the tricarboxylic acid cycle for energy generation and the formation of amino acids. This result led to the liberation of free reduced CoA, which inhibits the condensation reaction of acteyl-CoA to acetoacetyl-CoA by β-Ketothiolase. Also, Yamane *et al.* (1996) reported that this character in *A.latus* was due to the low activity of 3-Ketotholase, which was needed for acetoacetyl-CoA cleavage. So that *A.eutrophus*, non growth-associated PHB producer produce the PHB in the presence of a carbon source, but at the same time an essential growth component is absent. Generally, it could be stated that, two-stage process is necessary, which involves an initial cell growth step followed by a PHB production step.

Effect of Two-stage Batch Culture on Polymer Production:

The cells of alcaligenes strains were first grown in complete medium (med.3) for 96 hours, then the biomass was used as inoculum of med.3 under different limiting nutritional treatments for PHB production. At all treatments of nutrient limitation, slight increase in cell dry weight of both strains during the second stage of batch culture was observed on med.3 containing glucose or sucrose (as a carbon source). The highest growth of *A.latus* ATCC 29712 and *R.eutropha* ATCC 17697 were recorded at all different treatments of sucrose and glucose media, respectively, at the end of first stage and continued through second stage. These results are in line with those obtained by Wang & Lee (1997). Who stated that the overall cell mass in the nutrient-deficient stage, in batch flask culture, remained almost unchanged throughout operation period.

Results of PHB accumulated by crude cells of alcaligenes strains, reavel that they gradually increased during the fermentation period, till reaching the maximum after 72 h of incubation in second step, for all different treatments of nitrogen and phosphorus limitation on media containing glucose or sucrose as a carbon source. Data in Table (2) show that, the highest production of PHB by *R.eutropha* ATCC 17697 was obtained in cells growing on nitrogen free med.3 (treatment No.2) followed by medium containing low concentration of nitrogen and phosphorus ,0.4 gl^{-1} $(NH_4)_2SO_4$ & 1.33 gl^{-1} KH_2PO_4 (treatment No.6) glucose. The highest PHB content (%) and productivity produced by *R.eutropha* ATCC 17697 were recorded in cells grown on glucose media at treatment No.2 being 25.84 % & 0.0046 $gl^{-1}h^{-1}$, respectively. At treatment No.3, *A.latus* ATTC 29712 gave the highest PHB content (%) and productivity in cells growing in sucrose media being 55.78 % & 0.0115 $gl^{-1}h^{-1}$, respectively.

Table (2): PHB production by *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 as affected by different limitations of nitrogen & phosphorus in med.3 containing glucose sucrose (as a carbon source) inoculated with crude cells and incubated at 30 °C for 96 h using shake flasks as a two-stage batch culture.

Treatment NO.	*R.eutropha* ATCC 17697							*A.latus* ATCC 29712						
	Glucose medium							Sucrose medium						
	PHB gl^{-1}					PHB		PHB gl^{-1}					PHB	
	0 h	24 h	48 h	72 h	96 h	P	%	0 h	24 h	48 h	72 h	96 h	P	%
Treatment 1	0.17	0.21	0.26	0.30	0.21	0.0018	10.10	1.70	1.73	1.76	1.79	1.67	0.0107	52.03
Treatment 2	0.16	0.51	0.59	0.77	0.61	0.0046	25.84	1.74	1.78	1.82	1.87	1.65	0.0111	54.20
Treatment 3	0.18	0.33	0.47	0.62	0.50	0.0037	20.74	1.77	1.80	1.84	1.93	1.78	0.0115	55.78
Treatment 4	0.15	0.31	0.46	0.59	0.49	0.0035	19.60	1.72	1.75	1.78	1.79	1.66	0.0107	53.34
Treatment 5	0.16	0.20	0.32	0.47	0.35	0.0028	15.82	1.72	1.75	1.77	1.81	1.71	0.0108	52.92
Treatment 6	0.15	0.45	0.58	0.72	0.60	0.0043	23.76	1.74	1.76	1.78	1.89	1.72	0.0113	54.78
Treatment 7	0.16	0.23	0.33	0.47	0.35	0.0028	15.82	1.71	1.73	1.76	1.78	1.65	0.0106	51.45
Treatment 8	0.16	0.26	0.36	0.42	0.31	0.0025	14.05	1.71	1.74	1.77	1.79	1.63	0.0107	51.14
Treatment 9	0.15	0.22	0.31	0.38	0.25	0.0023	12.62	1.73	1.75	1.77	1.78	1.62	0.0106	51.30

Results in Fig. (5) show that the highest PHB concentration (gl^{-1}) and PHB content (%) were recorded by washed cells of *R. eutropha* ATCC 17697 after 72 h incubation period on glucose medium with nitrogen free (treatment No.2) followed by glucose medium containing low concentration of nitrogen and phosphorus (treatment No.6) being 4.47 gl^{-1} & 47.76 % and 3.99 gl^{-1} & 44.78 %, respectively. The corresponding figures obtained by *A. latus* ATCC 29712 on sucrose medium at treatments No. 3 & 2 were 4.87 gl^{-1} & 64.16 % and 4.62 gl^{-1} & 61.52 %, respectively.

From the foregoing results, it could be concluded that the PHB production increased under nitrogen or phosphorus limitation during the second stage of batch flask cultures, with crude or washed cells of the first stage. At these limitation treatments, washed cells of *A. latus* ATCC 29712 gave higher values of PHB concentration and content than *R. eutropha* ATCC 17697. Also, it could be recorded that using washed cells led to increase PHB content accumulated by *R. eutropha* ATCC 17697 and *A.latus* ATCC 29712 by about 26 % and 8.38 %, respectively, as compared with that obtained by crude cells in nitrogen free medium. This may be due to the effect of washing which eliminating the toxic materials from crude cells. These results are in line with those obtained by Kelley & Srienc (1999) and Hankermeyer &Tjeerdema (1999) who stated that nitrogen or phosphorus limitation stimulated the production of PHB by *A.eutrophus*. Also, Wang and Lee (1997) stated that the PHB content of *A. latus* increased very sharply from 52 % to 83 % after application of nitrogen limitation.

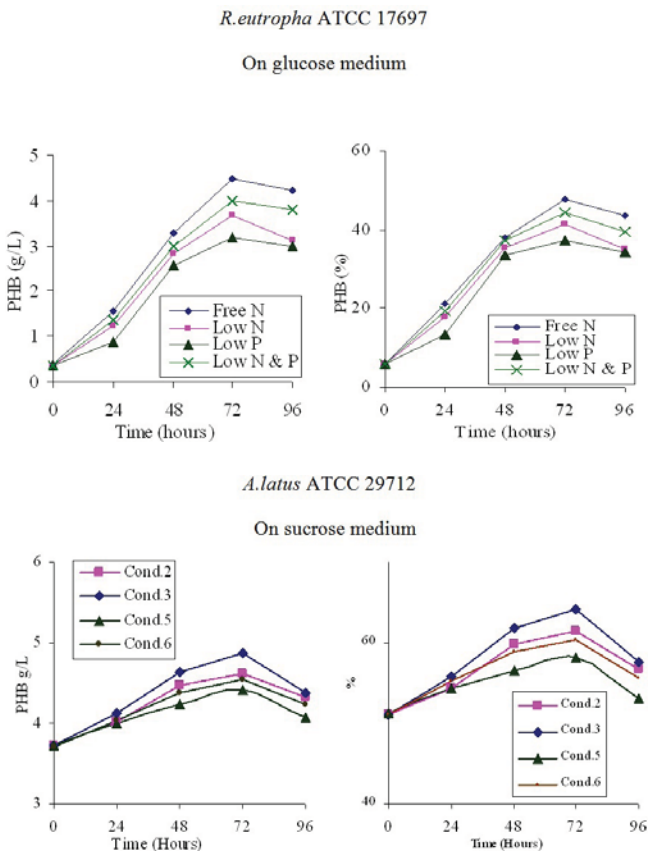

Fig. 5: Effect of different limitation treatments on PHB production by washed cells of R. eutropha and ATCC 17697 *A. latus* ATCC 29712 in med.3 containing glucose or sucrose (as a carbon sources) during 96 h incubation at 30°C using shake flasks as a two-stage batch culture.

Effect of Fermentation Period:

Data in Fig. (6) show that the PHB production was increased by increasing the incubation period till reaching the maximum by *A.latus* ATCC 29712 & *R.eutropha* ATCC 17697 after 60 h incubation on different limitation treatments on sucrose and glucose media, respectively. The highest figures of PHB concentration (gl^{-1}) and PHB content (%) were attained by *R.eutropha* ATCC 17697 on free nitrogen glucose medium (treatment No.2) being 4.83 gl^{-1} and 54.82 %. *A.latus* ATCC 29712 gave the maximum PHB concentration and PHB % being 5.48 gl^{-1} and 67.40 % on sucrose medium containing low nitrogen concentration (treatment No.3), while on free nitrogen sucrose medium (treatment No.2) being 5.32 gl^{-1} and 65.59 %. These results are in line with those obtained by Lee (1996) who noticed that *A.eutrophus* cells encounter nitrogen or phosphate limitation after 60 h, and accumulate P(3HB) during the next 40 to 60 h from supplied glucose.

From the above mentioned results, it could be stated that applying the two stage batch fermentation with nitrogen limitation in second stage increased the PHB content (%) of *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 cells about 48.83 and 14.29 %, respectively, as compared with that obtained in batch culture using shake flasks technique. The aforementioned results are in line of those obtained by Yamane *et al.* (1996) who stated that non-growth associated PHB producers as *A.eutrophus* accumulated PHB in the presence of a carbon source, but at the same time an essential growth component is absent. Thus, a two-stage process is necessary, which involves an initial cell growth step followed by a PHB production step. This process accordingly complicates the bioreactor operation and lower PHB content by *A.latus* (a growth-associated PHB producer) due to prolonged culture time.

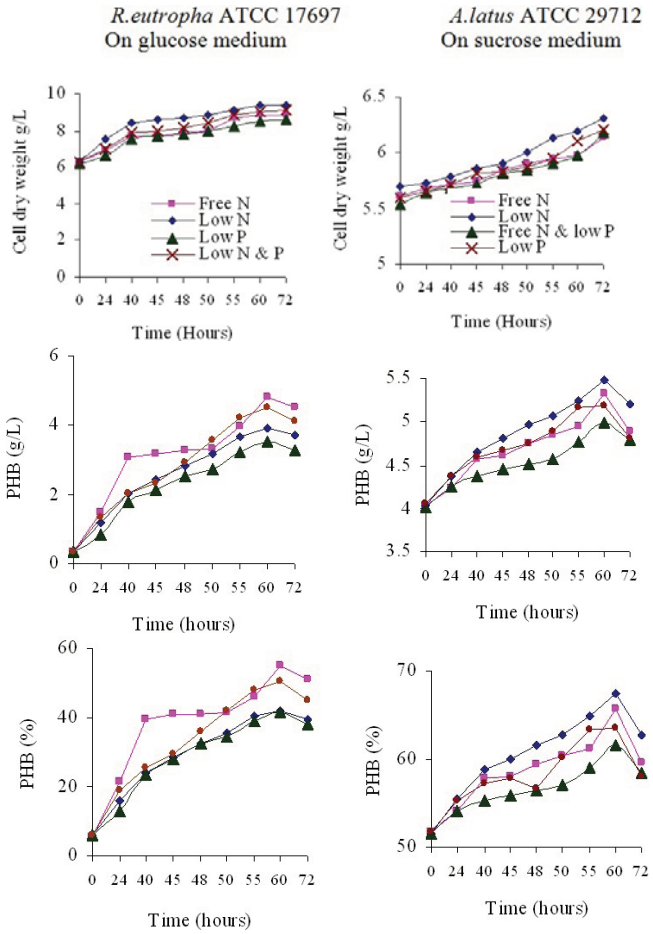

Fig. 6: Effect of fermentation period on growth and PHB production by washed cells of R. eutropha and ATCC 17697 A. latus ATCC 29712 in med.3 containing glucose or sucrose (as a carbon sources) at different limitation treatments during 72 h incubation at 30°C using shake flasks as a two-stage batch culture.

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