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Some investigations on PHA production using activated sludge as inoculum

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Abstract
This paper indicates that activated sludge under certain conditions can achieve levels of polyhydroxyalkanoates (PHA) accumulation which would enable the economic recovery of this precursor of biodegradable plastics. The effects of C/N ratio and temperature on polyhydroxyalkanoates production were evaluated. Acetate uptake, ammonium consumption, oxygen uptake and PHA production were studied under aerobic, growth rate limiting conditions. Sludge fed with acetate showed the capability to accumulate PHA to about 6% and 20.5% of cell dry weight at 30 ºC and C/N =21.74 after 42 and 72 h, respectively. The accumulation of PHA strongly depends on temperature, with less PHA formation at higher temperature. The average values of maximum growth rate ($q_{max}\cdot C_x$), affinity constant (K_{O2}) and k_La were determined 0.266 $mgl^{-1}s^{-1}$, 0.556 mgl^{-1} and 0.1422 s^{-1}, respectively.

Keywords: *Mixed culture; polyhydroxyalkanoates (PHA); activated sludge*

Introduction
Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as carbon/energy or reducing-power storage materials in various microorganisms. PHAs have been attracting considerable attention as biodegradable substitutes for conventional polymers. To reduce their production costs, a great deal of effort has been devoted to developing better bacterial strains and more efficient fermentation/recovery processes. The use of mixed culture and cheap substrates can reduce the production cost of PHA. The idea of PHA production using mixed culture arose from recognition of the PHA's role as a metabolic intermediate in microbial processes for wastewater treatment. Activated sludge, a well-known mixed culture, is able to store PHA as carbon and energy storage material under unsteady conditions arising from an intermittent feeding regime and variation in the presence of an electron acceptor [1,7,8,11,14,17,21,].
Biological wastewater treatment usually occurs under dynamic conditions. Under these conditions, growth of biomass and storage of polymers occur simultaneously when there is an excess of external substrate (feast period). When all the external substrate is consumed, stored polymer can be used as a carbon and energy source during the famine period. The microorganisms involved experience rapidly changing conditions of nutrient availability and can adapt continuously to changes in substrate. Microorganisms which are able to quickly store available substrate and consume

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the storage to achieve a more balanced growth have a strong competitive advantage over organisms without this capacity [21]. Activated sludge accumulates PHA to ca. 20% of dry weight under anaerobic conditions. The PHA content of activated sludge can be increased to 62% in a micro-aerophilic–aerobic sludge process [14,17]. When compared with a pure culture (>88% of cell dry weight)[8], the merits of PHA production in open mixed culture would be enhanced economy, simpler process control, no requirement of monoseptic processing, and an improved use of wastes [14]. Considerable effort has gone into producing PHA using mixed culture [20, 12, 5, 19, 4, 18, 2, 22, 16, 17, 13].

Materials and methods

Experimental set up

A 2-L SBR (sequencing batch reactor) was used and operated continuously in cycles of 6 h. One SBR cycle consisted of several phases: influent addition, reaction, and effluent withdrawal (Table 1). The reactor was aerated and mixed continuously except during the effluent withdrawal. Sludge from a conventional activated sludge wastewater treatment plant in the Netherlands was used as inoculum. The temperature of the reactor was adjusted using a thermostat bath, the pH was kept at 7.0 using 1 M NaOH and HCl solutions. The reactor was well aerated with an airflow of 1 L/min controlled by a mass-flow controller and stirred with two standard geometry six-blade turbines. The residence time was 13.3 h. Under steady-state conditions, the process was extensively monitored (pH, DO) and sampled (acetate, NH_4^+, PO_4^{3-}, PHA, ash content, CDW) during several cycles.

Inoculum

Activated sludge was sampled from a municipal wastewater treatment plant in the Netherlands and used as a model population in which to attain PHA accumulation.

Medium

A synthetic wastewater with the following composition was used: HAc 160, KH_2PO_4 6.4, NH_4Cl 1.4, $MgSO_4$ 0.2 (gl^{-1}), trace element solution according to Vishniac and Staner media 5 ml/L [23].

Analytical procedures

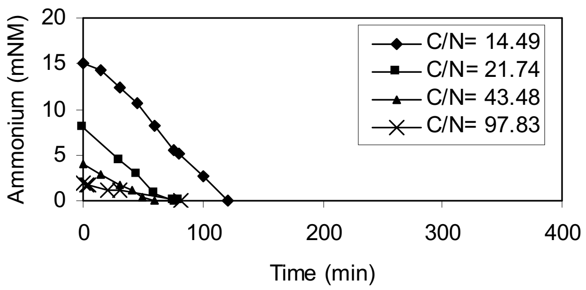
The dissolved oxygen concentration in the reactor was measured online as a percentage of air saturation using a DO electrode. Samples taken from the reactor were centrifuged for 10 min at 10000 rpm at 4 ^{o}C to separate the bacterial cells from the liquid. The acetate concentration of the supernatant was measured by gas chromatography. NH_4^+ and PO43- were analyzed spectrophotometrically with an autoanalyser (Skalar 5010). The biomass concentration and ash content of biomass were determined according to standard methods [10]. The PHA content of the washed (KP buffer solution) and freeze-dried biomass was determined by extraction, hydrolyzation, and esterification in a mixture of hydrochloric acid, 1-propanol and dichloroethane at 100 ^{o}C. Respiration experiments were carried out using an online respirometer [15]. The respirometer is a stirred, non-aerated, thermostated, 25 ml vessel with a DO electrode, connected with the SBR. When the pump was stopped, the decrease in oxygen concentration in the respirometer due to respiration could be measured.

Results and discussion

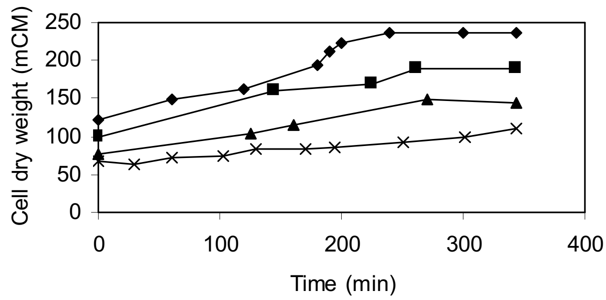
An analysis of the experimental results indicates the strong influence of C/N ratio and temperature on PHA formation in mixed culture. In this work, acetate uptake, ammonium consumption, biomass concetra-tion and PHA production were measured at different C/N ratios (14.49, 21.74, 43.47 and 97.83), and temperatures (20, 30, 34 ºC).

Table 1. Time sequence of one cycle (6 h)

Successive phases	Time in cycle (min)
Feeding (HAc, 100 ml)	0-60
Effluent withdrawal (0.9 L)	345-355
Filling (Mineral media, 0.8 L)	355-360
Mixing & Aeration	0-345



(a)



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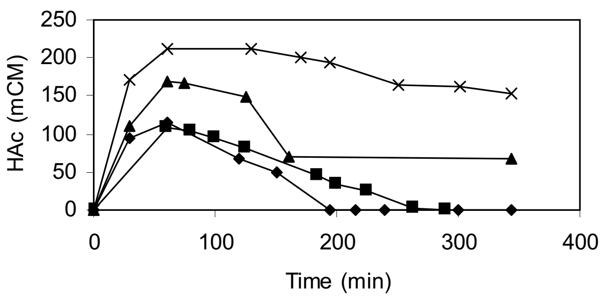

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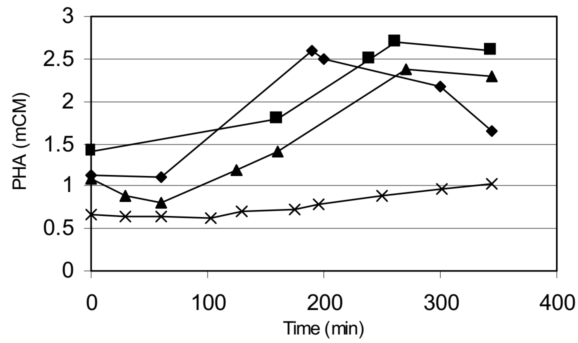

(d)

Figure 1.
(a) Effect of C/N ratio on ammonium consumption in selection/growth reactor. C/N=14.49, ♦; 21.74, ■; 43.48, ▲; 97.83, ×.
(b) Effect of C/N ratio on biomass concentration in selection/growth reactor. C/N=14.49, ♦; 21.74, ■; 43.48, ▲; 97.83, ×.
(c) Effect of C/N ratio on acetate uptake in selection/growth reactor. C/N=14.49, ♦; 21.74, ■; 43.48, ▲; 97.83, ×.
(d) Effect of C/N ratio on PHA production in selection/growth reactor. C/N=14.49, ♦; 21.74, ■; 43.48, ▲; 97.83, ×.

Looking to the Figures 1-(a, b, c), it becomes clear that with increasing C/N ratio, the ammonium and acetate uptake rates decrease due to the poor biomass concentration. The PHA production cycle is clearly divided in two phases. First, a feast phase when acetate can be taken up and used for growth. This phase is followed by famine phase (without external acetate). Acetate uptake is completed only for C/N 14.49 and 21.74, and there is no clear feast phase for other C/N ratios, because of insufficient biomass concentration or low growth resulting from nitrogen source limitation (Figures 1-b,c).

The accumulated PHA is consumed as a carbon/energy source during the famine phase for C/N 14.49 and 21.74. Since the accumulated PHA is enough to satisfy the maintenance energy demand, biomass will not be broken down for maintenance in the famine phase (Figures 1-c, d). As shown in Figure 1-(d), the maximum growth rate is observed at the limiting C/N ratio 21.74 in the selection/growth reactor. Table 2 compares specific acetate uptake rate ($-q_{Ac}$), specific PHA production rate (q_{PHA}), biomass production rate (r_{Bio}) and PHA production rate (r_{PHA}) for various C/N ratios. The oxygen consumption of biomass during a cycle was measured online at optimum C/N ratio and T= 34 ºC and the results of these respiration experiments presented in Figure 2.

Table 2. Comparison of biomass production rate (r_{Bio}), PHA production rate (r_{PHA}), specific acetate uptake rate ($-q_{Ac}$), specific PHA production rate (q_{PHA}), and their ratio in selection/ growth reactor (T= 34 ºC, SRT=13.3 h).

C/N ratio (mg/mg)	r_{Bio} (mCM/h)	r_{PHA} (mCM/h)	$-q_{Ac}$ (mCM/mCM h)	q_{PHA} (mCM/mCM h)	$q_{PHA}/-q_{Ac}$ (mCM/mCM)
14.49	29.88	0.68	0.458	0.00325	0.022758
21.74	20.39	0.529	0.331	0.0028	0.025944
43.48	15.99	0.287	0.299	0.0019	0.017949
97.83	7.52	0.0803	0.223	0.00056	0.010678

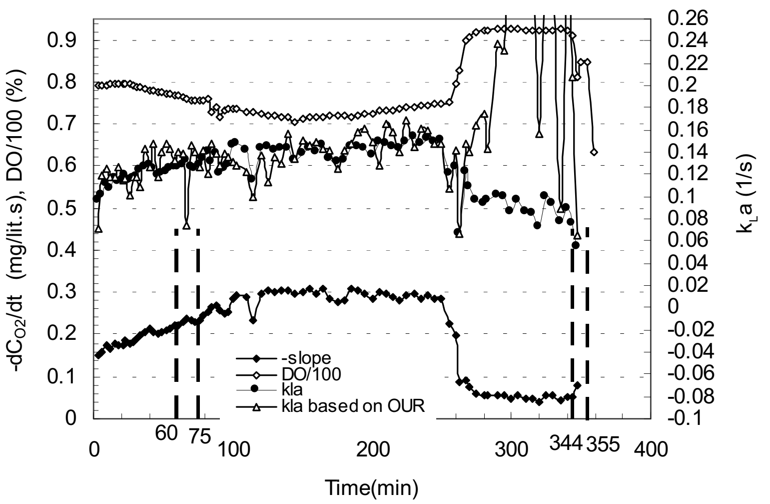

Figure 2. SBR steady state cycle respiration measurements. –Slope, ♦; Dissolved oxygen (DO%), ◊; k_La, •; k_La based on OUR, Δ.

An analysis of data indicates that at the end of the feast phase (t=262 min), there is a sharp rise in the DO curve, when the acetic acid concentration declines to zero. The oxygen uptake rate (OUR) increases due to growth and acetate uptake, achieves a constant value and then suddenly decreases at the end of the feast phase. The oxygen transfer coefficient (k_La) based on O_2 probe measurement was determined as ca. 0.1422 s^{-1} in accordance with the calculated k_La value based on OUR (0.1433 s^{-1}). For mixed culture, the average value of the maximum growth rate ($q_{max} \cdot C_x$) and the affinity constant (K_{O2}) were calculated to be 0.266 $mgl^{-1}s^{-1}$, 0.556 mgl^{-1}, respectively (Figure 3).

Table 3 shows that the bacteria accumulate PHA up to ca. 4.25% under growth rate limiting condition. However, PHA accumulation is relatively low under growth rate limitation conditions. The effect of temperature on biological nutrient removal and PHA storage has been studied during recent years [3,6,9]. In agreement with other reports, the accu-mulation of PHA increases with decreasing temperature in an accumulation reactor. The maximum PHA accumulation observed was about 20.5% at 30 ºC after 72 h. Maybe at high temperatures the acetate uptake is the limiting step instead of the maximum growth rate (Table 4).

Table 3. Effect of C/N ratio on PHA storage in the accumulation reactor (T= 34 ºC, pH=7).

Time (h)	MLVSS (mCM)	PHA (mCM)	PHA_{max} % (Based on cell dry weight)
C/N= 14.49			4.252
0	115.853	1.12	
24	318.2927	9.269	
48	334.9593	8.650	
72	203.6585	5.527	
96	232.9268	5.827	
C/N= 21.74			4.251
0	95.12	1.4	
24	293.29	9.396	
50	221.95	7.564	
52	219.92	7.372	
78	215.04	6.251	
C/N= 43.48			2.98
0	73.577	1.09	
24	130.85	3.039	
38	180.49	2.540	
48	128.60	2.796	
C/N= 97.83			2.94
0	64.878	0.67	
60	105.28	2.060	

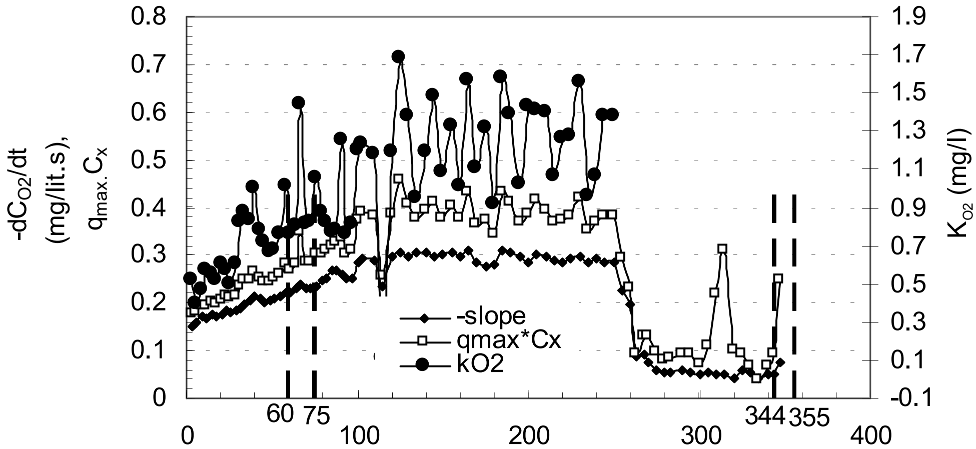

Figure 3. Variation of maximum specific growth rate and affinity constants. –Slope, ♦; qmax.C_x, □; K_{O2}, •.

Table 4. Effect of temperature on PHA storage in the accumulation reactor (C/N= 21.74, pH=7).

Temperature/ Time(h)	**MLVSS** (mCM)	**PHA** (mCM)	**PHA(%)** Based on cell dry weight
T= 34 ºC			
0	95.12	1.4	0
24	293.29	9.396	3.527
50	221.95	7.564	4.251
52	219.92	7.372	4.183
T= 30 ºC			
0	121.54	2.18	0
42	197.80	7.434	6.021
72	145.04	7.702	20.537
T=20 ºC			
0	167.886	3.231	0
24	172.215	3.527	5.981

Conclusion

In this research, a municipal activated sludge with good PHA accumulating characteristics was used as a model population. An overview of results proved that PHA accumulation by activated sludge feeding with acetate, reaches about 20.5% after 72 h under certain conditions (T=30 ºC and C/N=21.74) in an accumulation reactor. The accumulation of PHA strongly depends on the temperature and C/N ratio. The growth and PHA accumulation rates decrease when PHA accumulates in the cells. The accumulation levels are significantly lower than pure cultures grown under fed-batch conditions. Possible process economies can be made which may make this product relatively acceptable (economically?).

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