Optimization of nitrogen and phosphorus limitation for better biodegradable plastic production and organic removal using single fed-batch mixed cultures and renewable resources

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Abstract The process for the production of biodegradable plastic material (polyhydroxyalkanoates, PHAs) from microbial cells by mixed-bacterial cultivation using readily available waste (renewable resources) is the main consideration nowadays. These observations have shown impressive results typically under high carbon fraction, COD/N and COD/P (usually described as nutrient-limiting conditions) and warmest temperature (moderate condition). Therefore, the aim of this work is predominantly to select mixed cultures under high storage responded by cultivation on a substrate – non limited in a single batch reactor with shortest period for feeding and to characterize their storage response by using specific and kinetics determination. In that case, the selected-fixed temperature is 30 °C to establish tropical conditions. During the accumulated steady-state period, the cell growth was inhibited by high PHA content within the cells because of the carbon reserve consumption. From the experiments, there is no doubt about the PHA accumulation even at high carbon fraction ratio. Apparently, the best accumulation occurred at carbon fraction, 160 $\pm$ 7.97 g COD/g N (PHA_{mean} = 44.54% of dried cells). Unfortunately, the highest PHA productivity was achieved at the high carbon fraction, 560 $\pm$ 1.62 g COD/g N (0.152 $\pm$ 0.17 g/l. min). Overall results showed that with high carbon fraction induced to the cultivation, the PO_4 and NO_3 can remove up to 20% in single cultivation.

Keywords COD/N and COD/P; feast–famine regimes; mixed cultures; palm oil mill effluent (POME); polyhydroxyalkanoates (PHAs); renewable resources

Introduction

PHAs have attracted attention as the candidates for biodegradable polymers, because they possess similar material properties as the common petrochemical-based synthetic thermoplastics and elastomers currently in use (Durner *et al.*, 2001; Serafim *et al.*, 2004). Additionally, PHAs are also capable of being produced from a wide variety of carbon sources, such as renewable resources (sucrose, starch, cellulose or triacylglycerols), fossil resources (methane, mineral oil, lignite or hard coal), byproducts (molasses, whey or glycerol), chemicals (propionic acid, 4-hydroxybutyric acid) and carbon dioxide (Marazioti *et al.*, 2003). Since the 1900s, extensive studies have been conducted on PHA production in batch or fed-batch operation for most carbon and fatty acid-based (Kim *et al.*, 1994; Tanaka and Ishizaki, 1994). Therefore, this study has been conducted on PHA production in fed-batch operation for certain reasons; feasibility studies, high cell density cultivation and high polymer content.

Under new development of fed-batch cultivation, PHB production is particularly operated under a two-step process in which a large amount of biomass is first produced

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(at non-limiting nutrient conditions) followed by PHA accumulation by the cells under restricted nutrient adaptation. Metabolic engineering is being extensively explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Therefore, this paper consequently reported the operating factor of PHB formation under limiting carbon-to-nitrogen (COD/N) and carbon-to-phosphorus (COD/P) including the specific rates of substrate utilization, PHA production and biomass concentration (C_x).

One of the reasons for the enhanced PHA production using renewable resources because of the defined medium (substrates) was the availability of more acetyl-CoA and NADPH, a cofactor required for the synthesis of PHB in these media (Ganduri *et al.*, 2005). Additionally, with an aim to lower the production cost of PHB, a waste generation especially from palm oil mill effluents (POME) must fully be utilized to attain a zero discharge.

A kind of mixed cultures was proposed lately by various strategies due to the lack of pure culture and substrates. Furthermore, most of microorganisms can accumulate PHB as intracellular compounds – polymers (carbon and energy storage) – during transient periods (feast/famine regime). These polymers will produce PHAs to levels as high as 90% of the cells' dry weight (CDW). Hence, the mixtures of sewage and POME sludge are adequate to induce most of the significant microorganisms. Since more prokaryotes (microorganisms) are capable of PHA formation (Chua *et al.*, 2003), this study predominantly assumed the cultivation solely depends on culture-enrichment. Previously, we examined the accumulation of PHB under limited carbon-to-nitrogen (using sunflower oil as a substrate), and almost no interesting results were obtained (Md Din *et al.*, 2004a). However, the favourable factors (e.g. temperature and harvesting time) have been made in the next stage to induce the PHA production (Md Din *et al.*, 2004b).

Materials and methods

Experiment set-up and fed-batch operation

A sequencing batch reactor (SBR) with working volume of 6 l (with 50% discharge level) has been used to establish the biomass performance in POME and sewage sludge. This will allow the quick modification of enzyme activity, which will enable slow growing factor (best accumulation period). The initial mixed culture was developed using 10% activated sludge from the sewage treatment plant and 90% from palm oil mill effluent (POME). The cultivation was kept maintained in a single fed-batch reactor and operated in two steps: growth and accumulation stage. Firstly, the system will allow an extensive growth (using nutrient medium) and then the limiting nutrient (no nutrient medium adaptation) will be introduced in the next step. Five different runs of cultivation were conducted. Usually, the typical steady state was applied from 3 to 4 weeks, after the acclimatization of dynamic feeding. The schematic diagram of the SBR is given in Figure 1. A high concentration of POME has been proposed for the system in order to generate higher autotrophic bacteria rather than heterotrophic, especially for assimilation activities. Almost all of the process is conducted in turbulence regime to ensure good mass transfer and mixture using 1,000 rpm approximately. The reactor was equipped with a pH and O_2 electrode to monitor DO and pH profiles. pH was controlled at the desired value (7.00 ± 0.1) using 2 mol l^{-1} HCl or 2 mol l^{-1} NaOH, while temperature was controlled using a water-jacketed thermostat bath. Each operating condition was conducted with the same cycle length. However, the other operating experiments were fixed, such as temperature (30 °C) and air diffuser (1.5 l/min).

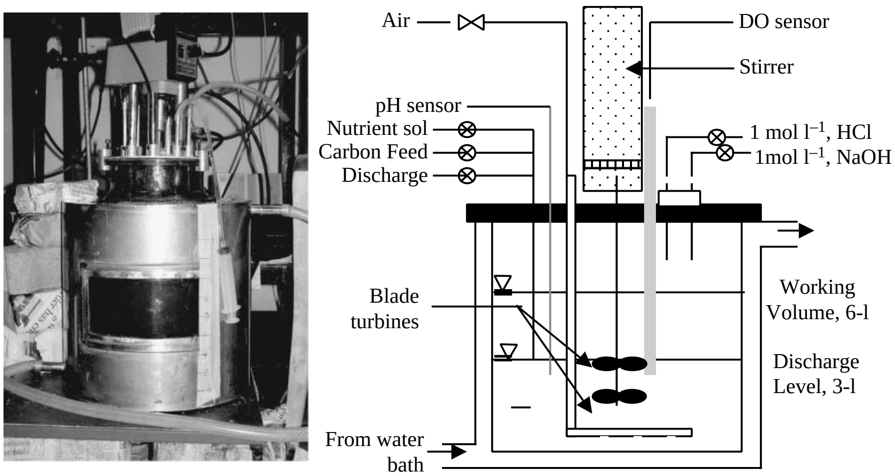

Figure 1 Left: Picture of SBR during cultivation process; Right: Schematic diagram for details SBR system

Analytical procedures

Samples taken from the reactor for analysis of NH_4-N, PO_4-P, TOC and COD and volatile fatty acids (VFAs) were immediately centrifuged and filtered using 0.45 μm filters to separate the bacterial cells from the liquid (APHA, 1995). The TOC and VFAs in the supernatant were measured by gas chromatography (GC), while NH_4-N and PO_4-P concentration in the supernatant were measured at 630 nm and 520 nm respectively with auto analyzers (Skalar 5010). The cell concentration was determined by measuring CDW, VSS and ash. This quantification of CDW and ash content was done according to the Dutch Standard Method (NEN6621) (NNI, 1982 NEN). The PHA content of the washed and dried biomass was determined by extraction, hydrolyzation, and esterification in a mixture of hydrochloric acid, 1-propanol, and dichloroethane at 100 °C. The resulting organic phase was extracted with water to remove any free acids. The PHA content (%) was defined as the percentage of the ratio of PHA concentration to CDW.

Results and discussion

Overall observation

Generally, the mole ratio of COD/N and COD/P in the medium was controlled by setting the total chemical oxygen demand (COD) and nitrogen concentration of ammonium and phosphate in the feed medium to the desired values. This definitely performed using certain types of discharging and feeding regime. The details of the method are shown in Table 1. In this study, all of the sludge must be processed with an adequate sequence of metabolic measurement (i.e. TOC or COD reduction) to ensure the unbalanced growth which occurred during the feeding phase. Although the system was bound to perform the limiting condition of PO_4-P, we believed that the function was still the same as the NH_4-N experiments. Simultaneous achievement of effective wastewater treatment and

Table 1 Computing the COD/N in each cycle measurement

Experiment code	COD/N ratio (g COD/g N)	COD/P ratio (g COD/g P)	Feast:Famine (%:%)	Fixed configuration
CNP_{pome} − 110	110 ± 8.96	130 ± 4.29	48:52	Cycle = 6 h
CNP_{pome} − 150	150 ± 2.27	170 ± 7.47	57:43	HRT = 12 h
CNP_{pome} − 260	260 ± 7.97	180 ± 0.49	67:33	Temp = 30 °C
CNP_{pome} − 490	490 ± 4.35	160 ± 2.23	50:50	DO = 1.5 l/min
CNP_{pome} − 560	560 ± 1.26	160 ± 6.98	67:33	

enrichment of PHA accumulating microorganisms is vital to ensure the success of the PHA production system by activated sludge (Du *et al.*, 2000; Van Loosdrecht and Heijnen, 2002).

The residual biomass value was used to express cell growth during PHA accumulation. As shown in Table 2, it was found that cell growth (residual biomass) in the CNP_{pome}-560 system decreased when NH_4 was limited. This should be theoretically impossible because there was still substrate utilization and no sludge wasting during the PHA accumulation phase; thus the value should remain steady or increase. The only reasonable explanation was that the biomass was lost with the effluent. Unfortunately, the lowest residual biomass concentration indicated that the system was found to have a high PHA component (total biomass minus residual biomass). The best productivity of PHA occurred in CNP_{pome}-490 with almost 0.152 ± 0.17 g/l.min obtained at the end of the feast phase. Meanwhile, the PHA content lowered to at least 4.0 g/l when compared with CNP_{pome}-260. After approximately 30 days of steady-state operations, activated sludge content under COD/N cycling conditions was less than 20% of CDW. Also, the biomass residual in this reactor gradually decreased from 7.45 ± 0.11 g/l to 1.25 ± 0.13 g/l due to the unbalanced growth rate, which was probably because acetyl-CoA cannot enter the TCA cycle to obtain energy for cells, because of a high concentration of NADH (Madison and Huisman, 1999).

Statistical analysis on PHA production and organic removal

According to the statistical analysis, the overall PHA production was not as good as the other variables (Table 3). Using ANOVA single factor, there is no significant difference (p-value) for all of the carbon-to-nitrogen-to-phosphorus ratio (COD/N/P) systems under PHA production. It was also found that only CNP_{pome}-260 occurred in the best conditions for PHA production because the mean value appeared at a higher value (44.54 ± 8.44%) as compared with CNP_{pome}-110, CNP_{pome}-150, CNP_{pome}-490 and CNP_{pome}-560. TOC removal revealed the most favourable condition when compared to the others, due to the $p < 0.0001$ (confidence level (cf) > 95%) and $R^2 = 0.831$. The sequences of these performances appeared from the best to the least favourable condition i.e. CNP_{pome}-110 > CNP_{pome}-560 > CNP_{pome}-490 > CNP_{pome}-260. However, the exposition of PO_4 removal depicted that CNP_{pome}-260 performed the best acclimation, which constituted up to 13.81 ± 7.62% ($R^2 = 0.58$ and cf > 95%). The unfavourable system for PO_4 removal was observed at CNP_{pome}-560, which was not friendly for P elimination, but the results show that the systems (experiment conditions) tend to release it rather than consuming up to percentage as high as −12.77 ± 4.05% ($R^2 = 0.083$ and cf < 95%). The lowest ratio (COD/N/P) shows an unfavourable condition for NO_3 removal, while the highest ratio (CNP_{pome}-490 and CNP_{pome}-560) gave the different situations. However, CNP_{pome}-560 revealed the best removal 23.80 ± 8.14% ($P < 0.01$, cf > 95%) as compared to the others.

Table 2 Biomass component and PHA accumulation during feast period at limiting of N and P condition

Experiment	Residual Biomass (g/l)	Biomass composition (g/l)	PHA productivity (g/l.min)	PHA content (g/l)
CNP_{pome}-110	7.45 ± 0.11	23.44 ± 1.27	0.081 ± 0.05	12.99 ± 1.29
CNP_{pome}-150	6.34 ± 0.06	25.87 ± 2.08	0.060 ± 0.05	9.53 ± 0.99
CNP_{pome}-260	2.16 ± 0.22	24.47 ± 1.06	0.127 ± 0.09	20.28 ± 1.07
CNP_{pome}-490	0.88 ± 0.17	20.12 ± 3.44	0.152 ± 0.17	24.24 ± 1.13
CNP_{pome}-560	1.24 ± 0.13	27.66 ± 0.98	0.137 ± 0.01	21.85 ± 0.86

Note: Residual biomass = Biomass-PHA-Poly-P, PHA content = PHB + PHV + PHH, Biomass composition = PHA content + Active biomass

Table 3 Summary on statistical analysis for limitation of N and P system

Parameter	Experiment	Mean	Std Dev	*p*-value	Conf. Level	R^2	Std Error	*p*-value	F > Fcri
%PHA production	CNP_{pome}-110	32.60	7.15	0.0160	>95%	0.083	2.155	0.5186	No (ah)
	CNP_{pome}-150	32.35	5.59	0.0006	>95%	0.152	1.685		
	CNP_{pom}e-260	44.54	8.44	0.0608	<95%	0.732	2.546		
	CNP_{pome}-490	33.04	6.95	0.0006	>95%	0.650	2.096		
	CNP_{pome}-560	32.41	6.99	0.0029	>95%	0.133	2.106		
%TOC removal	CNP_{pome}-110	0.23	1.34	0.2512	<95%	0.246	0.405	<0.0001	Yes (rnh)
	CNP_{pome}-150	8.07	5.85	0.0000	<95%	0.831	1.763		
	CNP_{pome}-260	−7.18	19.26	0.0049	>95%	0.543	5.808		
	CNP_{pome}-490	19.41	8.12	0.0001	>95%	0.345	2.447		
	CNP_{pome}-560	15.03	11.67	0.6282	>95%	0.672	3.519		
%PO_4-P removal	CNP_{pome}-110	5.73	1.61	0.0007	>95%	0.009	0.486	<0.0001	Yes (rnh)
	CNP_{pome}-150	0.08	1.38	0.1990	>95%	0.237	0.417		
	CNP_{pome}-260	13.81	7.62	0.0002	<95%	0.580	2.297		
	CNP_{pome}-490	−11.63	14.72	0.0034	<95%	0.447	4.437		
	CNP_{pome}-560	−12.77	4.05	0.0003	>95%	0.083	1.221		
%NO_3-N removal	CNP_{pome}-110	−1.42	7.61	0.2750	<95%	0.236	2.295	<0.0001	Yes (rnh)
	CNP_{pome}-150	−41.94	9.69	0.0001	>95%	0.001	2.922		
	CNP_{pome}-260	−25.88	20.70	0.0025	>95%	0.336	6.241		
	CNP_{pome}-490	21.60	5.53	0.0002	>95%	0.007	1.668		
	CNP_{pome}-560	23.80	8.14	0.0082	<95%	0.337	2.453		

Note: ah = accept hypothesis, rh = reject null hypothesis

Conclusions

Glycogen was also quantified, but values were always very low and did not change significantly along the cycle (data not shown). Due to the Pearson coefficient, the best conditions for simultaneous PHA production and other organic removals were depicted in CNP_{pome}-490, followed by CNP_{pome}-260, CNP_{pome}-110, CNP_{pome}-560 and CNP_{pome}-150. The average production of PHA could only reach up to 44% of CDW, indicating that the optimization of PHA sludge content must be carried out by varying oxygen rate, feeding regime or transient conditions.

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