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Fermentation strategy for the production of poly(3-hydroxyhexanoate) by *Aeromonas sp.* KC014

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Abstract−Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HHx)) was produced by *Aeromonas sp.* KC014 strain isolated from Taiwan in soil environment in the flask and fermentor cultures. The medium optimization, such as carbon source and nitrogen source, carbon-nitrogen ratio was conducted to obtain the optimum 3-hydroxyhexanoate content. The defined medium with dodecanoic acid as the carbon source and $(NH_4)_2SO_4$ as the nitrogen source was obtained as the main medium. When cells grown in medium containing 30 g/L dodecanoic acid, 15 g/L sodium gluconate and 1 g/L soytone (C/N was 30/1) as final PHA concentration, the cell dry weight and HB content of 5.16 g/L, 14.0 g/L and 36.0%, respectively, were obtained. The maximum HHx/PHA content increased from 0.1% to 1.3% nearly 12-fold when the dissolved oxygen was decreased from 40% to 20%. P(3HB-*co*-3HHx) biosynthesis was triggered by the addition of limited nitrogen, phosphorus and magnesium to get a maximum HHx/PHA content of 14% in 95 hours.

Key words: Polyhydroxyalkanoate, *Aeromonas*, Fermentation, P(3HB-*co*-3HHx)

INTRODUCTION

The petrochemical industry helps people in industry, investors and various links across the global industrial chain to garner more attention and comb the development track of related fields in biopolymers. Biopolymers are renewable, because they are made from plant materials which can be grown year after year indefinitely. These plant materials come from agricultural nonfood crops. Therefore, the use of biopolymers could create a sustainable industry. There are potential applications for biopolymers produced by micro-organisms within the agriculture, medical and pharmaceutical industries, primarily due to their biodegradability. Some biopolymers are biodegradable as they are broken down into CO_2 and water by microorganisms. Also, due to the its high strength, light weight, durability, and low degradability, plastic has become an important necessity in modern life. However, in the process of incinerating waste, plastics made of acrylonitrile may generate hydrogen cyanide, which is a harmful substance to human health. Despite the fact that recycling and reusing plastic products seems feasible, the implementation still requires support and cooperation of people, and the color and shape of recycled products will also limit the reusability. Thus, biopolymers will provide a solution to the treatment of plastic wastes [1-5]. Among the biopolymers, polyhydroxyalkanoates (PHAs) are linear polyesters produced in nature by bacterial fermentation of sugar which plays a vital role in this emerging field. PHAs are polymers generated by microorganisms as energy storage vehicles which are accumulated when essential nutrients such as nitrogen or phosphorus is available only in limited concentrations in the presence of excess carbon source. PHAs families inherently have a well defined structure. More than 150 different monomers can be combined within this family to give materials with extremely different properties. When the total number of carbon atoms in the HA monomer unit is equal to or less than five, the HA is known as a short chain-length (SCL) monomer, and the PHAs resulting from the polymerization of SCL monomers are known as SCL PHA. When the total number of carbon atoms in the HA monomer unit is at least six and up to 14, the HA is known as a medium chain-length (MCL) monomer, and the PHAs resulting from the polymerization of MCL monomers are known as MCL PHA. Accumulation of MCL-PHAs occurs under nitrogen limitation when cells are grown on MCL-alkanes, alkanols, or alkanoic acids. The composition of the accumulated PHA shows a relation towards the carbon source employed; this statement is once again proved by our study. When dodecanoic acid is used as a carbon substrate 3-hydroxyhexanoate is the main constituent of the PHA. PHA copolymer called 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx), a new member of PHAs family, has been developed for various potential uses. Recently, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx) has been reported to possess similar mechanical properties to one of the representative commercial polymers, namely, low-density polyethylene (LDPE) [6] and hence can be used in their place. This study focuses on synthesizing copolyesters of PHA containing scl monomers such as 3-hydroxybutyrate (HB) and mcl monomers such as 3-hydroxyhexanoate (HHx) which belongs to this novel class of biopolymers by KC014 strain (identified as *Aeromonas sp.*) isolated from Taiwan. It has been shown in the literature that this microorganism can produce P(3HB-*co*-3HHx). Kobayashi et al. [7] reported that P(3HB-*co*-3HHx) was produced by using *Aeromonas* sp. FA-440. The maximum HHx content in P(3HB-*co*-3HHx) reached to 30%, but the productivity was low when cultivated in the fermenter. Qiu et al. used metabolic engineering for the production of P(3HB-*co*-3HHx)

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by *Aeromonas hydrophila* [8-11] in which the HHx content in P(3HB-*co*-3HHx) is about 10%. Therefore, this research aims to improve the HHx content in P(3HB-*co*-3HHx) by *Aeromoans sp*. screened from the Taiwan environment by examining the effects of carbon source, nitrogen source, different nutrient limitation conditions and a simple fermentation strategy for the efficient large scale production of P(3HB-*co*-3HHx).

MATERIALS AND METHODS

1. Bacterial Strain and Culture Medium

Aeromonas sp. strain KC014 used in this study was isolated from the soil environment in Taiwan, and it is identified as a species of *Aeromonas* sp. strain by Professor S-S Kung and Professor C-C Chein of the Graduate School of Biotechnology and Bioengineering, Yuan Ze University, Taiwan [12]. P(3HB-*co*-3HHx) was purchased from the P&G Company. The composition of the standard defined medium, named as MM-Gnla, constitutes dodecanoic acid (15 g/L), sodium gluconate (15 g/L), yeast extract (1 g/L), $(NH_4)_2SO_4$ (44 g/L), $MgSO_4 \cdot 7H_2O$ (0.41 g/L), $Na_2HPO_4 \cdot 12H_2O$ (0.65 g/L), KH_2PO_4 (1.5 g/L), 10 mL/L solution of Fe(III)-NH_4-citrate (5 g/L) and $CaCl_2 \cdot 2H_2O$ (2 g/L), and 1 mL/L solution of $ZnSO_4 \cdot 7H_2O$ (100 g/L), $MnCl_2 \cdot 4H_2O$ (30 g/L), H_3BO_3 (300 g/L), $CoCl_2 \cdot 6H_2O$ (200 g/L), $CuSO_4 \cdot 5H_2O$ (10 g/L), $NiCl_2 \cdot 6H_2O$ (20 g/L) and $Na_2MoO_4 \cdot 2H_2O$ (30 g/L).

2. Culture Conditions

For the preculture, an appropriate amount of frozen culture of *Aeromonas sp*. strain KC014 was transferred to 100 mL LB medium, and the culture was incubated at 30 °C, 200 rpm agitation for 12 to 16 h. After cultivation 0.5 mL cultivated broth was added into 0.5 mL autoclaved glycerol, and mixed well before being frozen in the −80 °C freezer for subsequent studies. After preculture for cell revival, 5 ml of the preculture broth was inoculated into the 500 mL flask containing 100 mL sterilized main culture medium (culture media components varied with the design of experiments) at 30 °C and 200 rpm agitation. The fed batch and batch fermentation experiments were also conducted in fermenter with 2 L as working volume under optimized conditions.

3. Analytical Procedures

To measure the dry cell weight (DCW), PHA concentration and HHx content, 40 ml of the cell broth was centrifuged at 3,000 g and the collected cells were washed three times with distilled water and ethanol solution to remove dodecanoic acid. The washed cells were freeze dried and weighed, then the gradually dried cells were subjected to gas chromatography analysis.

4. Gas Chromatography Analysis

For methanolysis of P(3HB-*co*-3HHx), lyophilized cells were suspended in 1 ml chloroform and 1ml methanol containing 2.8 M H_2SO_4 in a screw-capped tube, and then incubated at 100 °C for 2 h. Internal standard benzoic acid was used for comparing the 3HHx peak for analyzing 3HHx content continued by demineralization (addition of demineralized water), and then the organic phase containing the resulting methyl esters of co-monomers was analyzed by GC (Focus GC, Thermo, U.S.A.).

RESULTS AND DISCUSSION

1. Fermentation in Shaker Flask

1-1. Effect of Carbon and Nitrogen Sources on P(3HB-*co*-3HHx) Production

In previous studies [13,14], *Aeromonas* strain was cultured in MM-GnLa medium with the dodecanoic acid and sodium gluconate as carbon source to produce P(HB-*co*-HHx). In this study, *Aeromonas sp.* KC014 strain was also cultured in MM-GnLa based medium, but the effect of three different kinds of carbon sources, namely carbohydrate, hydrocarbon and oil, were evaluated on increasing the content of HHx. Carbohydrates, such as glucose, sucrose, fructose, sodium gluconate, starch and gluconic acid, were tested in this study. Secondly, hydrocarbons such as hexanoic acid, nonanoic acid, decanoic acid and dodecanoic acid were also used. Finally, oil compounds such as glycerol, olive oil and soybean oil were used. Fig. 1a shows the results based on carbohydrates; only glucose, gluconic acid, sodium gluconate and starch hold the ability to synthesize both HB and HHx among them. Eventually, the hydrocarbon hexanoic acid and dodecanoic acid could also be able to produce both HB and HHx among these four hydrocarbons as shown in Fig. 1b. While considering the oil as the carbon source, olive oil and soybean oil show positive effects towards synthesizing HB and HHx (result shown in Fig. 1c). For the complex carbon sources, we chose two of the single carbon sources which could produce both HB and HHx. The cell dry weight, HB content, HHx content, PHA concentration and HHx/PHA are listed in Table 1 Based on the results, the complex carbon source, dodecanoic acid and sodium gluconate could pro-

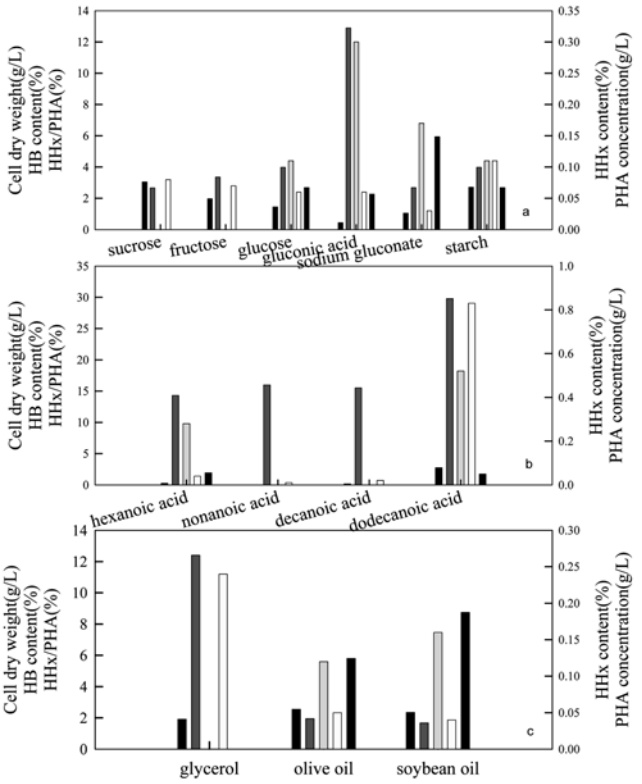

Fig. 1. Effect of carbon source (a) carbohydrate (b) hydrocarbon (c) oil Cell growth and PHA accumulation by *Aeromonas sp.* KC014 strain in MM-GnLa medium with carbon source =15 g/L in the flask. (▬) Cell dry weight, (▬) HB content, (▬) HHx content, (▭) PHA concentration, (▬) HHx/PHA.

Table 1. Effects of complex carbon source in the MM-GnLa medium on cell dry weight, HB content, HHx content, PHA concentration and HHx/PHA by KC014 strain

	Cell dry weight (g/L)	HB content (%)	HHx content (%)	PHA concentration (g/L)	HHx/PHA (%)
ab	3.41	1.47	0.03	0.05	2.03
ac	1.97	5.94	0.09	0.12	1.44
ad	4.02	2.38	0.08	0.10	3.12
ae	1.40	5.18	0.12	0.07	2.26
af	7.26	2.12	0.00	0.15	0.00
ag	1.76	2.63	0.03	0.05	1.24
ah	0.36	4.34	0.09	0.02	2.03
bc	10.5	20.8	0.44	2.22	2.05
bd	5.33	1.48	0.00	0.08	0.00
be	7.90	2.90	0.05	0.23	1.79
bf	4.90	0.12	0.00	0.01	0.00
bg	0.46	6.82	0.00	0.03	0.00
bh	0.36	6.15	0.11	0.02	1.75
cd	5.16	11.0	0.16	0.58	1.45
ce	6.60	10.2	0.14	0.68	1.40
cf	3.06	7.22	0.08	0.22	1.13
cg	3.52	1.85	0.00	0.07	0.00
ch	0.11	3.94	0.07	0.004	1.75
de	7.90	2.03	0.06	0.17	3.02
df	1.98	5.96	0.12	0.12	1.90
dg	0.79	15.8	0.34	0.13	2.11
dh	0.55	11.3	0.22	0.06	1.87
ef	1.35	6.68	0.10	0.09	1.50
eg	1.18	10.5	0.18	0.13	1.70
eh	5.87	3.87	0.05	0.23	1.22
fg	0.78	15.0	0.28	0.12	1.82
fh	0.37	13.9	0.29	0.05	2.04
gh	0.28	8.40	0.15	0.02	1.73

a: glucose; b: sodium gluconate; c: dodecanoic acid; d: olive oil; e: soybean oil; f: starch; g: gluconic acid; h: hexanoic acid.

duce the highest PHA concentration, 2.22 g/L. The HB content of most cases is less than 10%.Complex nitrogen sources were also discussed--three organic nitrogen sources such as peptone, tryptone and soytone cross with three inorganic nitrogen sources, namely $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and $(NH_4)_6Mo_3O_{24}\cdot 4H_2O$--to form complex nitrogen sources. For the complex nitrogen sources we chose two of the single nitrogen sources. Based on the results in Table 2, the complex nitrogen source of soytone and $(NH_4)_2SO_4$, could produce the highest PHA concentration, 2.17 g/L. All of these complex nitrogen sources used in this culture system could produce more than 3% HHx/PHA. Interestingly, the HB content shows a higher value of more than 30%.

1-2. Effects of Co-substrate on P(3HB-*co*-3HHx) Production

Ouyang et al. [11] reported that P(HB-*co*-HHx) was produced by *Aeromonas sp*. with the different concentration ratios of two carbon sources of MM-GnLa medium, e.g., dodecanoic acid and sodium gluconate. Therefore, we hoped to find the ratio and the concentrations of two carbon sources which could obtain the higher

Table 2. Effects of complex nitrogen sources in the MM-GnLa medium on cell dry weight, HB content, HHx content, PHA concentration and HHx/PHA by KC014 strain

	Cell dry weight (g/L)	HB content (%)	HHx content (%)	PHA concentration (g/L)	HHx/PHA (%)
ab	3.28	21.9	0.97	0.75	4.24
ac	2.84	30.9	1.78	0.93	5.43
ad	3.64	49.9	2.77	1.92	5.27
ae	3.40	42.4	2.16	1.51	4.86
af	2.52	28.3	1.43	0.75	4.80
bc	3.07	20.0	1.06	0.65	5.03
bd	2.09	35.1	1.55	0.77	4.22
be	5.89	34.1	1.72	2.11	4.80
bf	2.87	30.2	1.40	0.91	4.41
cd	3.28	36.2	2.02	1.25	5.29
ce	5.27	39.0	2.27	2.17	5.50
cf	2.61	21.9	0.92	0.60	4.03
de	4.90	32.0	1.50	1.64	4.49
df	3.63	38.5	2.21	1.48	5.44
ef	3.53	35.7	1.91	1.32	5.07

a: peptone; b: tryptone; c: soytone; d: $(NH_4)_2HPO_4$; e: $(NH_4)_2SO_4$; f: $(NH_4)_6Mo_3O_{24}\cdot 4H_2O$.

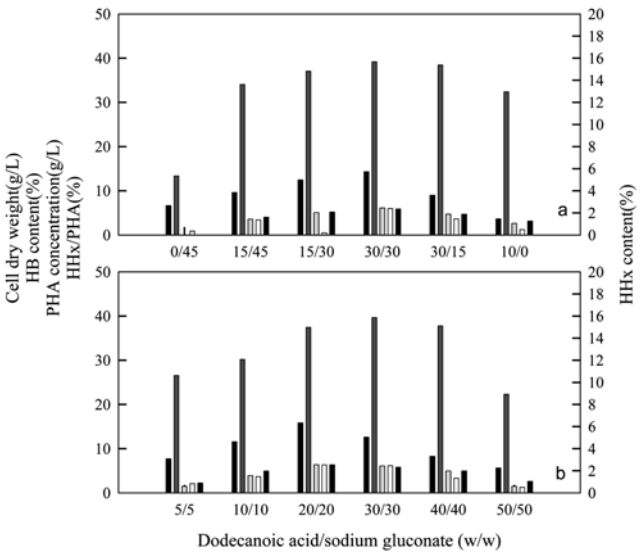

Fig. 2. Effect of weight ratio of dodecanoic acid to sodium gluconate on cell growth and PHA accumulation by *Aeromonas sp.* strain KC014 in 48 h flask cultures. (▬) Cell dry weight, (▬) HB content, (▬) HHx content, (▬) PHA concentration, (▬) HHx/PHA.

PHA concentration. The results are shown as Fig. 2a; different PHA concentration were obtained by the different concentration ratios of dodecanoic acid and sodium gluconate. From these six different ratios, a PHA concentration of 5.97 g/L would be the highest when the ratio of dodecanoic acid to sodium gluconate was 1 : 1. Based on this result, we changed the concentration of dodecanoic acid and sodium gluconate as the same proportion. We modulated each con-

centration of dodecanoic acid and sodium gluconate from 5 to 50 g/L and found the relationship between these two concentrations. The results are shown in Fig. 2b. From these six different concentrations, the PHA concentration, 6.34 g/L, would be the highest when the ratio of dodecanoic acid and sodium gluconate was 20/20 (g/g). And the cell concentration and PHA concentration of this KC014 strain would be inhibited by high concentration of the substrate. Therefore, the ratio of carbon to nitrogen was carried out by using 15/1, 20/1, 30/1 and 60/1. These results are shown in Fig. 3. The highest PHA concentration, the cell dry weight and HB content were 5.16 g/L, 14.0 g/L and 36.0%, respectively, when the ratio of carbon to nitrogen was 30/1.

2. P(3HB-*co*-3HHx) Fermentation in Fermenter

2-1. Effect of Dissolved Oxygen on P(3HB-*co*-3HHx) Production

The effects of dissolved oxygen (D.O) on PHA production were studied with the fermenter culture. The air flow was controlled at 1 L/min. The D.O. was controlled by the agitation rate at 40% and 20% saturated air. The culture conditions were 30 g/L dodecanoic acid, 15 g/L sodium gluconate and 1 g/L soytone (C/N was 30/1) at pH 7 and 30 °C. The results are shown in Fig. 4 that the PHA concentration became steady when D.O was 40% and PHA concentration was 2.42 g/L at the 54th hour, and when D.O. was 20% the PHA concentration was about 4.80 g/L at the 70th hour. The maximum PHA concentration increased nearly two-fold when the dissolved oxygen was decreased from 40% to 20%. It means that this strain preferred lower dissolved oxygen to produce PHA. The cell dry weight, PHA concentration and HHx/PHA for D.O. of 20% and 40% are 15.3 g/L, 4.8 g/L and 1.3%, and 9.27 g/L, 2.42 g/L and 0.1%, respectively.

2-2. Effect of Limiting Nutrients on P(3HB-*co*-3HHx) Production

Kobayashi et al. [7] reported that microorganisms would accumulate PHA as carbon and/or energy source under the condition of limiting nutritional elements such as N, P, S, O, or Mg in the presence of excess carbon source. Hence, we hope to utilize this nutrient limitation strategy to improve the PHA content. We decreased the concentrations of several components such as ammonium sulfate, di-sodium hydrogen phosphate and magnesium sulfate in the main culture medium to 1/3-fold of each original. The results showed that PHA content was increased substantially with a decrease in magnesium sulfate concentration. The PHA concentration was 3.73 g/L under the conditions of nutrient limitation, and the cell dry weights were lower than that of control (without nutrient limitation) culture. After activating and pre-culturing, 5% (w/v) culture broth was inoculated into the fermenter with initial working volume as 2 L. Inside the fermenter the temperature was thermostated thermostatted at 30 °C, dissolved oxygen is was 20%, air flow is 1 L/min, pH 7 and

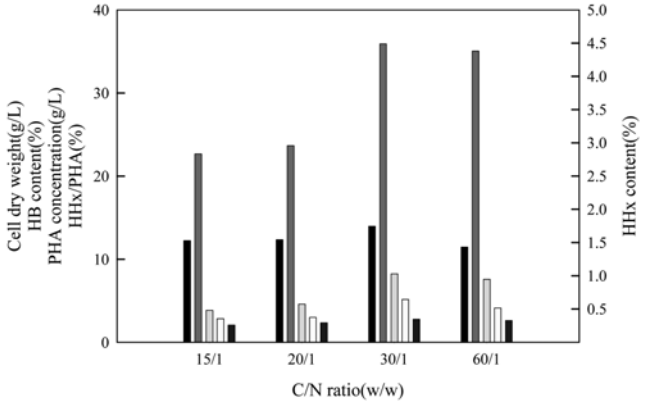

Fig. 3. Effect of C/N ratio on cell growth and PHA accumulation by *Aeromonas sp.* KC014 strain in the flask. (▬) Cell dry weight, (▬) HB content, (▬) HHx content, (▭) PHA concentration, (▬) HHx/PHA.

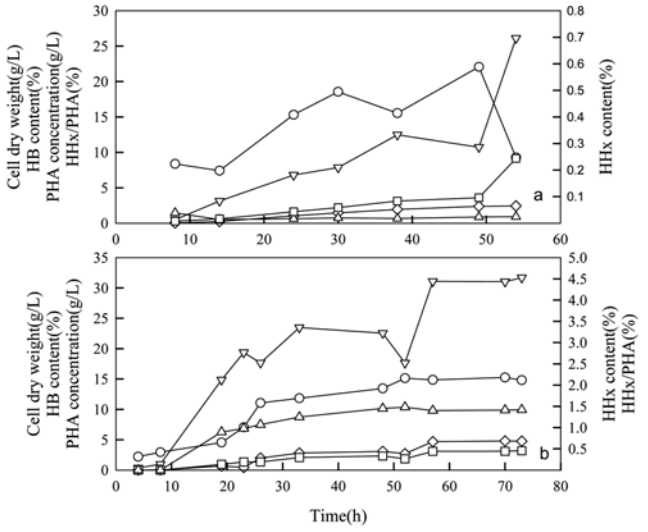

Fig. 4. Time course for dissolved oxygen (a) 40% (b) 20% on cell growth and PHA accumulation with of in the batch fermentor. (○) Cell dry weight; (▽) HB content; (□) HHx content; (◇) PHA concentration; (△) HHx/PHA.

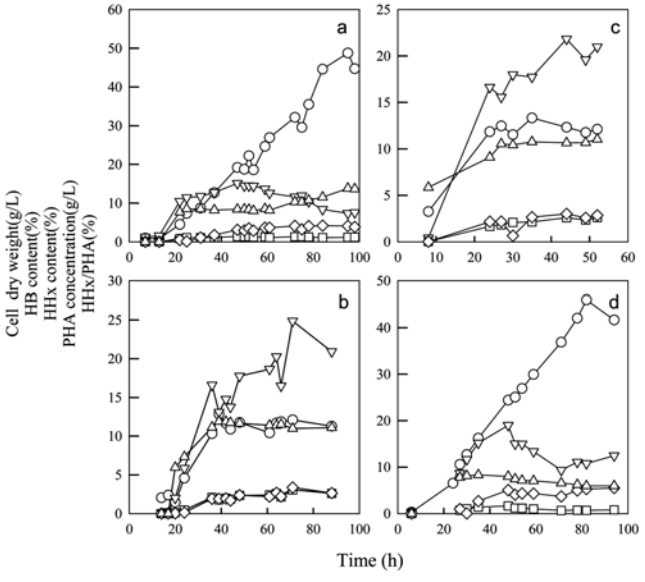

Fig. 5. Effect of feeding solution (a) $(NH_4)_2SO_4$ 53 g/L, (b) $Na_2HPO_4 \cdot 12H_2O$=127 g/L (c) $MgSO_4 \cdot 7H_2O$-5.5 g/L, (d) $(NH_4)_2SO_4$ 53 g/L and trace element solution 40 mL on cell growth and PHA accumulation in MM-GnLa medium with dodecanoic acid 200 g/L, sodium gluconate 200 g/L in the fed-batch fermenter. (○) Cell dry weight; (▽) HB content; (□) HHx content; (◇) PHA concentration; (△) HHx/PHA.

Table 3. Several important parameters of fed-batch fermentation with four different compositions of feeding solution

	$MgSO_4 \cdot 7H_2O$	$(NH_4)_2SO_4$	$Na_2HPO_4 \cdot 12H_2O$	$(NH_4)_2SO_4$, TE
Cell dry weight (g/L)	12.9	48.7	13.3	46.0
HB content (%)	23.1	8.37	27.7	10.8
HHx content (%)	1.77	1.08	2.14	0.68
PHA concentration (g/L)	1.91	4.21	2.65	5.27
HHx/PHA (%)	7.1	11.4	7.2	5.95
Productivity (g/L/h)	0.05	0.05	0.08	0.06
Yield (g/g carbon source)	0.05	0.02	0.06	0.02
μ (h^{-1})	0.13	0.03	0.07	0.04

TE: trace element solution, Fe(III)-NH_4-citrate=5 g/L, $CaCl_2 \cdot 2H_2O$=2 g/L, $ZnSO_4 \cdot 7H_2O$, 100 g/L, $MnCl_2 \cdot 4H_2O$=30 g/L, H_3BO_3=300 g/L, $CoCl_2 \cdot 6H_2O$=200 g/L, $CuSO_4 \cdot 5H_2O$=10 g/L, $NiCl_2 \cdot 6H_2O$=20 g/L, $Na_2MoO_4 \cdot 2H_2O$=30 g/L.

200 rpm. Dissolved oxygen was controlled by the agitation rate from 200-950 rpm. During fermentation the feeding components were added when the agitation rate arose rose and to became steady. After the first feeding, a 50 mL feeding solution was fed into the fermenter for every 3 hours. The 150 mL feeding solution consists consisted of 30 g dodecanoic acid, 30 g sodium gluconate and various compounds of MM-GnLa medium. In this study we discussed three compounds in the feeding solution: i.e. 8 g $(NH_4)_2SO_4$, 0.82 g $MgSO_4 \cdot 7H_2O$ and 19.3 g $Na_2HPO_4 \cdot 12H_2O$. Results for the first feeding regime are shown in Fig. 5a when the feeding solution was composed of 30 g dodecanoic acid, 30 g sodium gluconate and 8 g of $(NH_4)_2SO_4$. The cell dry weight and PHA concentration, 48.7 g/L and 4.21 g/L, would be the highest at the 95^{th} hour. Second results are shown in Fig. 5b. The culture procedures and culture conditions are identical to that in the first part. 150 mL of feeding solution included 30 g of dodecanoic acid, 30 g of sodium gluconate and 19.3 g of $Na_2HPO_4 \cdot 12H_2O$ was fed with constant feeding, the cell dry weight, 13.3 g/L, was the highest at the 35^{th} hour. PHA concentration was 3.01 g/L at the 44^{th} hour. Third case was that 150 mL of feeding solution included 30 g of dodecanoic acid, 30 g of sodium gluconate and 0.82 g of $MgSO_4 \cdot 7H_2O$, as shown in Fig. 5c. The cell dry weight was 12.9 g/L at the 39^{th} hour. PHA concentration 2.67 g/L was the highest at the 64^{th} hour. According to Fig. 5d, $(NH_4)_2SO_4$ could enhance cell growth and cause cell dry weight to be higher. In order to improve the cell concentration, the new components were added into the 150 mL feeding solution, which included 30 g dodecanoic acid, 30 g sodium gluconate and 8 g $(NH_4)_2SO_4$. Finally, 40 mL of trace element solution containing $Na_2HPO_4 \cdot 12H_2O_9$ (0.65 g/L), KH_2PO_4 (1.5 g/L), Fe(III)-NH_4-citrate (5 g/L), $CaCl_2 \cdot 2H_2O$ (2 g/L), of $ZnSO_4 \cdot 7H_2O$ (100 g/L), $MnCl_2 \cdot 4H_2O$ (30 g/L), H_3BO_3 (300 g/L), $CoCl_2 \cdot 6H_2O$ (200 g/L), $CuSO_4 \cdot 5H_2O$ (10 g/L), $NiCl_2 \cdot 6H_2O$ (20 g/L), and $Na_2MoO_4 \cdot 2H_2O$ (30 g/L), was introduced into the feeding solution. The cell dry weight reached 46.0 g/L at the 82th hour. PHA concentration was 5.52 g/L in 94 hour.

CONCLUSIONS

The carbon source and nitrogen source of MM-GnLa medium to produce P(3HB-*co*-3HHx) with *Aeromonas sp*. KC014 strain studied in this work were dodecanoic acid, sodium gluconate and soytone, $(NH_4)_2SO_4$. In the fed-batch fermentation when $(NH_4)_2SO_4$ was used as a limiting component, it enhanced the cell dry weight. The highest cell dry weight was obtained when the components of feeding solution of 150 mL were 30 g of dodecanoic acid, 30 g of sodium gluconate, 8 g of $(NH_4)_2SO_4$ and 2 g of soytone. The HHx content could reach more than 10% when the phosphate concentration was limited in the medium.

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