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Production of Hydroxybutyrate Monomers by *Pseudomonas mendocina* Biodegraded Poly (3-hydroxybutyrate-co-4-hydroxybutyrate)

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The production conditions of hydroxybutyrate monomers using *Pseudomonas mendocina* DS04-T-degradedpoly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)[poly(3HB-*co*-4HB)] in submerged culture were optimized.The optimum culture medium (w/v)contained 0.15% poly(3HB-*co*-4HB), 0.1% $(NH_4)_2SO_4$, 1.6% $Na_2HPO_4 \cdot 12H_2O$, 0.4% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$,and 0.0075% $ZnSO_4 \cdot 7H_2O$. The optimum parameters for liquid fermentation were as follows: cultivation time, 28 h; temperature, 30 °C; rotary speed, 150 rpm; initial pH, 7.3; liquid-medium volume, 120 mL; and inoculum size, 1.5%.Under the optimum conditions, thehydroxybutyrate(HB) monomer yieldwas 43.2% ± 0.42%, the polyhydroxyalkanoatedepolymerase activity was39.6 ± 0.37U/mL, and theHB monomer yieldwas3.5times that under basic culture medium and initial conditions.

Key words: Poly(3-hydroxybutyrate-co-4-hydroxybutyrate); Hydroxybutyrate; Biodegradation; *Pseudomonas mendocina.*

Polyhydroxyalkanoates (PHAs) are storage compounds accumulated by many bacteria as a carbon and energy reserve material under imbalanced growth conditions. PHAs degraded and metabolized when the limitation is removed[1-4]. PHAs are popular in the biomaterial market because of their biodegradability, apparent biocompatibility, similar material properties to conventional plastics, and ability to be manufactured from renewable resources[5-7]. PHAs with numerous useful properties and wide-ranging applications are used in different packaging materials, disposable personal hygiene products, medical applications including fabrication of biodegradable body implants and control of drug delivery, veterinary medicine, and agricultural applications [2,6,8,9]. PHAs are also potentially low-cost, competitive, and environmentally benign replacements for synthetic, biologically inert polyester plastics[10]. Meanwhile, biodegradable plastics with excellent properties as aforementioned are not yet commonly. Their widespread application is still not economically attractive compared with conventional nonbiodegradable plastic. From an industrial point of view, the production cost of biodegradable plastics is higher than that of synthetic plastics. Furthermore, current disposal systems cannot recover the production cost of biodegradable plastics because almost all biodegradable plastics used are incinerated with other garbage, and the recycling of biodegradable plastics such as synthetic ones has not been developed, although several biodegradable plastic degradation studies have been performed. The increased incidence of using PHAs may pose risks to the natural environment because of their slow rates for complete degradation or formation of intermediates during degradation. PHA materials must be urgently applied reasonably to improve the

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properties of materials and research the ability of biodegradation. Therefore, recovering plastic hydrolysates can help develop cost-effective biodegradable-plastic recycling systems[11].The degradability of extracellular PHAs in vitro depends on specific extracellular PHA depolymerases released by microorganisms[12,13], and the end-products of PHA degradation are generally monomers, dimers, or a mixture of oligomers [14]. PHAs are sources of optically active 3-hydroxyalkanoic acid (3-HB) monomers used in biomedical and pharmaceutical fields and as starting materials to obtain other new polyesters[6,15, 16, 17]. Essentially, these synthons may serve as a platform for the production of various industrial materials, including fungicides, flavors, pheromones, amino acids, vitamins, as well as antimicrobial and antiviral agents[18,19]. Thus, the production of hydroxybutyrate (HB) monomers is meaningful and constructive to explore because the wide-ranging applications of HB monomers can enable the development of new recycling methods. The potential commercial value of HB monomers may drive a significant part of research on this field. Indeed, the development of a cost-effective industrial process for the production of HB monomers is of considerable interest.

Pseudomonas mendocina DS04-T with significant PHA degradability has already been isolated in a previous study[20].Accordingly, this study aimed to identify the factors influencing HB monomer production by *P. mendocina* DS04-T-degraded poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [poly(3HB-*co*-4HB)] and determine the suitable parameters for liquid fermentation.

MATERIALS AND METHODS

Materials

Poly(3HB-*co*-4HB) powder containing 12mol% 4-HB was obtained from Tianjin GreenBio Materials Co., Ltd. (Tianjin, China). 3-HB monomer was obtained from Sigma. Other biochemical reagents, solvents, and chemicals were obtained from Beijing DingguoChangsheng Biotechnology Co., Ltd. (Beijing, China) and used without further purification.

P. mendocina DS04-T was obtained from the microbiology laboratory of the Northeast Normal University (Changchun, China). The basal medium and LB medium contained the following (in w/v): poly(3HB-*co*-4HB) powder, 0.15%; $Na_2HPO_4 \cdot 12H_2O$, 1.2%; KH_2PO_4, 0.55%; NH_4Cl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; $CaCl_2 \cdot 2H_2O$, 0.0005%; tryptone, 1.0%; yeast extract, 0.5%; and NaCl, 1.0%. The pH was adjusted to 6.8 and 7.0. For a solid medium, 2.0% (w/v) agar was added[21].

Inoculum preparation

After incubation in LB medium slant at 30 ºC for 24 h, the strain was collected with sterile water and the cell concentration was adjusted from 1.0×10^8 cell/mL to 1.5×10^8 cell/mL. This bacterial suspension was used as the seed for the next liquid culture.

Optimization of culture medium for HB production

The concentrations of the carbon source [poly(3HB-*co*-4HB)] and nitrogen were selected and screened. Various substitute carbon and nitrogen sources were used in basic medium. Subsequently, a seven factor–two level orthogonal test was used to optimize the inorganic salts of medium composition for HB monomer production, and the orthogonal design is shown in Table 1. After determining the optimum carbon and nitrogen sources, as well as inorganic salts, a six factor–five level orthogonal test was used to optimize the culture medium constituents. The orthogonal design of the culture medium with variable sources and levels is shown in Table 2. Approximately 1% (v/v) of the bacterial suspension was inoculated and cultured in a 250 mL flask containing 100 mL of culture medium in a rotary shaker (150 rpm) at 30 °C for 24 h in the aforementioned experiments.

Optimization of cultivation conditions for HB monomer production

The cultivation time (4, 8, 12, 16, 20, 24, 28, 32, and 36 h), cultivation temperature (25, 30, 35,37, and 40 ºC), rotary speed (120, 140, 160, 180, and 200 rpm), culture-medium loading volume (80, 100, 120,140, and 160 mL/250 mL), culture medium initial pH (5.8, 6.3, 6.8, 7.3, 7.8, and 8.3), and inoculum size (0.5, 1.0, 1.5,2.0, 2.5and 3.0%, v/v) were investigated for HB production. All trials were conducted in the optimum liquid medium obtained according to the previous section.

Poly(3HB-co-4HB) depolymerase activity assay and HB monomer assay

The fermentation medium was

centrifuged at 12 000 rpm for 15 min, and the supernatant was analyzed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS, Waters, USA). The working parameters of the instrument were as follows: mass range, *m/z* 0–800; exclusion voltage, 30 kV; attraction voltage, 9.3 kV; detection voltage, -4.75 kV; and vacuum degree, 1.9×10^{-4} Pa.

Poly(3HB-*co*-4HB) emulsion (2 mg/mL) in 20 mM phosphate buffer (pH 6.8) was used as the substrate. The PHA depolymerase activity was assayed by adding 1 mL of the supernatant to 3 mL of the substrate, followed by incubation at 50 °C for 20 min. The decrease in turbidity of the poly(3HB-*co*-4HB) emulsions was measured at a wavelength of 650 nm using a UV-vis spectrophotometer (Unico UV2600, USA). One unit of PHA depolymerase activity was defined as a 0.001 OD decrease in the absorbance per minute at 650 nm under previously described assay conditions.

The supernatant was concentrated with a centrifugal ultrafiltration pipe (Pall Filtration Co., USA) having a molecular weight cutoff of 3 kDa. The protocol for analysis was a modification of a previously reported method[22]. The filtrate was analyzed by an HPLC system (HITACHI L2000, Japan) after acidification using 1 M H_2SO_4 to pH 2.0 to facilitate analysis using an Inertsil C18 column (4.6 × 250 mm) (SHIMADZU Inc., Japan). The HPLC parameters were as follows: mobile phase, H_2O (adjusted to pH 2.8 with HCl)–acetonitrile (85:15, v/v); flow rate, 1 mL/min; detection wavelength, 210 nm; and column temperature, 10 °C.

RESULTS AND DISCUSSION

Fig. 1 shows the MS results of the fermentation supernatant of poly(3HB-*co*-4HB) by *P. mendocina* DS04-T. When the degradation time was 28 h, the degradation products of poly(3HB-*co*-4HB) were only identified as HB monomers without any other oligomer. Fig. 2a shows that the HPLC retention time of the 3-HB standard monomer was 2.52 min. Fig. 2b shows the HPLC results of the poly(3HB-*co*-4HB)-hydrolysis product of *P. mendocina* DS04-T. Only one peak with the same retention time as the HB standard monomer was observed. Figs. 1 and 2 clearly show that the HB monomer as the product was obtained after poly(3HB-*co*-4HB) hydrolysis by *P. mendocina* DS04-T when the degradation time was greater than 28 h. Therefore, *P. mendocina* DS04-T can be used to degrade poly(3HB-*co*-4HB). No other oligomer with similar properties was observed during HB monomer purification.

Medium optimization for HB monomer production

Fig. 3 shows the changes in PHA depolymeraseacticity and HB monomer production with increased poly(3HB-*co*-4HB) content. When the added amount ofpoly(3HB-*co*-4HB) was 0.2% (w/v), the highest yield of HB monomer was 15.9% ± 1.1%. However, PHA depolymerase activity decreased with increased poly(3HB-*co*-4HB) content in basic culture medium. Basic medium was suitable for PHA depolymerase production but did not completely compensate for the HB monomer yield.

Fig. 4a shows the effect of nitrogen source on PHA depolymerase activity and monomer production. When $(NH_4)_2SO_4$ was used as a nitrogen source in the medium, PHA depolymerase

Table 1. Orthogonal design of the inorganic salts of medium

Variable (g/L)	Levels	
	1	2
(A)KH_2PO_4	0	5
(B)$Na_2HPO_4 \cdot 12H_2O$	0	10
(C)$MgSO_4 \cdot 7H_2O$	0	0.5
(D) $FeSO_4 \cdot 7\ H_2O$	0	0.1
(E)$MnSO_4 \cdot H_2O$	0	0.1
(F) $ZnSO_4 \cdot 7\ H_2O$	0	0.1
(G)$CaCl_2 \cdot 2\ H_2O$	0	0.05

Table 2. Orthogonal design of the culture medium optimization

Variable (g/L)	Levels				
	1	2	3	4	5
(A) poly(3HB-*co*-4HB)	0.5	1.0	1.5	2.0	2.5
(B) $(NH_4)_2SO_4$	0.5	1.0	1.5	2.0	2.5
(C) KH_2PO_4	3	4	5	6	7
(D) $Na_2HPO_4 \cdot 12H_2O$	8	10	12	14	16
(E) $MgSO_4 \cdot H_2O$	0.3	0.4	0.5	0.6	0.7
(F) $ZnSO_4 \cdot 7\ H_2O$	0.05	0.075	0.1	0.125	0.15

Table 3. Results of orthogonal tests forthe inorganic salts of medium

No.	Variable							Yield of HB (%)	PHAs depolymerase activity (U/mL)
	A	B	C	D	E	F	G		
1	1	1	1	1	1	1	1	21.6	20.2
2	1	1	1	2	2	2	2	15.1	16.5
3	1	2	2	1	1	2	2	22.1	21.6
4	1	2	2	2	2	1	1	20.4	21.3
5	2	1	2	1	2	1	2	19.1	20.1
6	2	1	2	2	1	2	1	25.5	26.3
7	2	2	1	1	2	2	1	21.7	21.1
8	2	2	1	2	1	1	2	22.9	18.9
k_1	19.80	20.33	20.33	21.13	23.03	21.00	22.30	Σ=168.4	
k_2	22.30	21.78	21.78	20.98	19.08	21.90	19.80		
R[a]	2.50	1.45	1.45	-0.15	-3.95	0.10	-2.50		
k_1'	19.90	20.78	19.18	20.75	21.75	20.13	22.23		Σ=166.0
k_2'	21.60	20.73	22.33	20.75	19.75	21.38	19.28		
R'[b]	1.70	-0.05	3.15	0	-2.00	1.25	-2.95		

[a]Yieldof HB.
[b]PHAsdepolymerase activity.

activity and HB monomer production were significantly higher than when other nitrogen sources were added. This result indicated that ammonium salt promoted depolymerase activity and monomer production. Nitrate affected the growth rate of bacteria, thereby influencing the HB monomer yield and depolymerase activity. In particular, when peptone was used as a nitrogen source in the medium, PHA depolymerase activity and HB monomer yield were significantly lower than when inorganic nitrogen sources were used. Organic nitrogen sources were generally more beneficial to bacterial growth but not to monomer generation. Fig. 4b shows the changes in PHA depolymerase activity and HB monomer yield with increased amount of $(NH_4)_2SO_4$ added to the culture medium. Moreover, when the $(NH_4)_2SO_4$ content of the culture medium was about 0.15%, PHA depolymerase activity and HB monomer

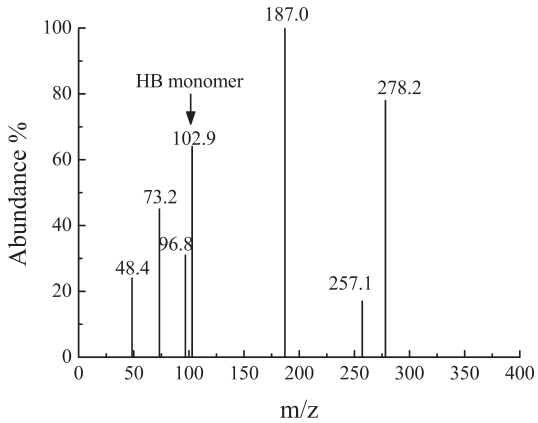

Fig. 1. MS of poly(3HB-*co*-4HB) hydrolysis products by *P. mendocina* DS04-T

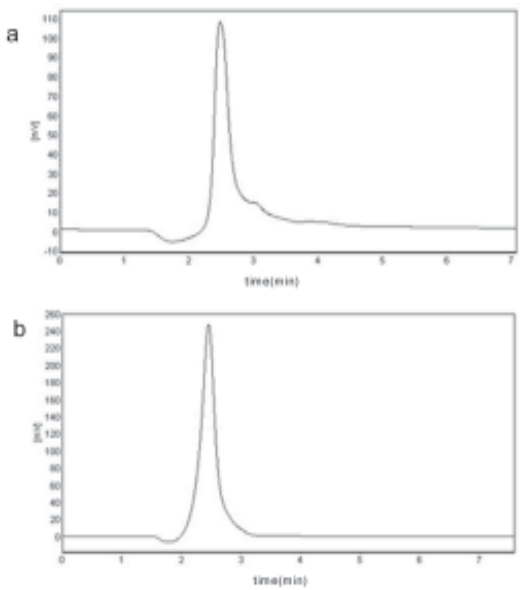

Fig. 2. HPLC analysis of poly(3HB-*co*-4HB) hydrolysis product. (a) 3-HB monomer standard solution, 6 mg/mL. (b) poly(3HB-*co*-4HB) hydrolysis product by *P. mendocina* DS04-T.

production were considerable. The results and analysis of the orthogonal design of inorganic salts in medium composition are shown in Table3. KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$,$MgSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$ were found to promote the HB monomer yield, but adding $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $CaCl_2 \cdot 2H_2O$ inhibited the HB monomer yield. However, $Na_2HPO_4 \cdot 12H_2O$ inhibited PHA depolymerase activity. Considering the acid–base balance of the fermentation broth, $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 were integrant contents of the medium. Analysis revealed that the inorganic salt components of the culture medium were confirmed as $Na_2HPO_4 \cdot 12H_2O$,KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$. The results and analysis of the

Table 4. Results of orthogonal tests forthe culture medium optimization

No.	Variable						Yield of HB (%)	PHAs depolymerase activity (U/mL)
	A	B	C	D	E	F		
1	1	1	1	1	1	1	20.3	23.2
2	1	2	2	2	2	2	31.7	26.0
3	1	3	3	3	3	3	33.7	22.7
4	1	4	4	4	4	4	23.2	16.2
5	1	5	5	5	5	5	30.3	26.3
6	2	1	2	3	4	5	23.9	23.2
7	2	2	3	4	5	1	12.9	23.6
8	2	3	4	5	1	2	28.5	23.1
9	2	4	5	1	2	3	17.9	30.1
10	2	5	1	2	3	4	19.2	19.1
11	3	1	3	5	2	4	26.8	22.2
12	3	2	4	1	3	5	39.5	23.3
13	3	3	5	2	4	1	26.2	23.4
14	3	4	1	3	5	2	38.5	26.6
15	3	5	2	4	1	3	36.2	26.6
16	4	1	4	2	5	3	26.1	22.2
17	4	2	5	3	1	4	26.6	21.6
18	4	3	1	4	2	5	25.4	23.1
19	4	4	2	5	3	1	29.4	32.8
20	4	5	3	1	4	3	22.7	26.1
21	5	1	5	4	3	2	27.6	22.6
22	5	2	1	5	4	3	36.5	22.1
23	5	3	2	1	5	4	21.6	22.8
24	5	4	3	2	1	5	25.4	21.9
25	5	5	4	3	2	1	19.3	33.6
k1	27.84	24.94	27.98	24.40	27.40	21.62	Σ=669.4	
k2	20.48	29.44	28.56	25.72	24.22	31.58		
k3	33.44	27.08	24.30	28.40	29.88	28.85		
k4	26.04	26.88	27.32	25.06	26.50	23.48		
k5	26.08	25.54	25.72	30.30	25.88	28.90		
Ra	12.96	4.50	4.26	5.90	5.66	9.96		
k1'	22.88	22.68	22.82	25.10	23.28	27.32		Σ=604.4
k2'	23.82	23.32	26.28	22.52	27.00	24.58		
k3'	24.42	23.02	23.30	25.54	24.10	24.97		
k4'	25.16	25.52	23.68	22.42	22.20	20.38		
k5'	24.60	26.34	24.80	25.30	24.30	23.56		
R'b	2.28	3.66	3.46	3.12	4.80	6.94		

aYield of HB
bPHAsdepolymerase activity

Table 5. Effects of culture conditions on the yield of HB monomer and PHAs depolymerase activity

Cultivation condition	Yield of HB monomer(%)	PHAs depolymerase activity(U/mL)
Cultivation time(h)		
4	6.91 ±0.08**	1.6±1.35**
8	13.99 ±0.13**	2.5±1.00**
12	17.25 ± 0.19*	3.7 ± 0.86**
16	19.51 ± 0.47*	9.3 ± 0.83**
20	21.89 ± 0.99	19.8 ± 1.54*
24	23.35 ± 1.21	24.2 ± 1.67
28	24.50 ± 1.29	25.8 ± 1.73
32	19.98 ± 1.36*	27.2 ± 0.90
36	17.81 ± 1.36*	27.1 ± 0.99
Cultivation temperature(°C)		
25	14.90 ± 0.90**	18.9 ± 1.95*
30	31.22 ± 1.15	23.0 ± 1.51
35	28.13 ± 1.08	21.5 ± 1.36
37	28.16 ± 1.03	20.5 ± 1.46*
40	25.76 ± 1.22*	14.3 ± 1.49**
Shaking speed(rpm)		
120	29.68 ± 1.48*	20.1 ± 1.01*
140	32.91 ± 1.30	22.9 ± 1.15
150	33.41 ± 1.42	23.4 ± 1.17
160	31.47 ± 1.02	23.8 ± 1.19
180	27.25 ± 1.31*	24.4 ± 1.22
200	26.07 ± 1.20*	24.6 ± 1.23
Volume of medium (mL/250mL)		
80	21.62** ± 2.08	19.3** ± 0.97
100	28.91 ± 1.45	25.6 ± 1.28
120	31.38* ± 1.27	27.1 ± 1.36
140	27.70 ± 1.28	24.8 ± 1.24
160	25.05 ± 1.30	23.6 ± 1.08
200	21.07** ± 1.20	22.6* ± 1.23
Initial pH		
5.8	16.73 ± 1.34**	3.2 ± 0.16**
6.3	20.00 ± 1.25*	4.3 ± 0.22**
6.8	26.45 ± 1.07	18.8 ± 0.94
7.3	32.73 ± 0.98*	24.6 ± 1.23*
7.8	27.94 ± 0.90	17.1 ± 0.86
8.3	24.74 ± 1.04	11.6 ± 0.58**
Inoculum content(%)		
0.5	36.52 ± 1.83	20.0 ± 1.0
1	37.57 ± 1.48	22.6 ± 1.13
1.5	40.26 ± 1.51*	23.0 ± 1.15
2	39.87 ± 1.49	21.5 ± 1.08
2.5	36.60 ± 1.23	20.9 ± 1.05
3	36.18 ± 1.41	20.08 ±1.04

Culture conditions: 1% (v/v) of the bacterial suspension was inoculated and cultured in a 250mL flask containing 100mL of culture medium (6.8) in a rotary shaker (150rpm) at 30 °C for 24h.* P<0.05; ** P<0.01

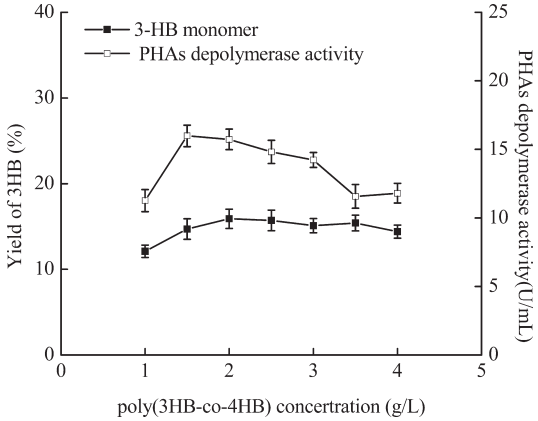

Fig. 3. Effects of carbon source content on PHAs depolymerase activity and HB monomer production.

orthogonal design of the culture medium with variable sources and levels are shown in Table 4. The *R* value in Table 4 shows that the effects of these variables decreased in the order of A > F > D > E > B > C. The poly(3HB-*co*-4HB) was a significant factor and should be controlled at high levels. The optimum culture medium was the combination $A_3F_2D_5E_3B_2C_2$, which was composed of the following (in w/v): 0.15% poly(3HB-*co*-4HB), 0.1% $(NH_4)_2SO_4$, 1.6% $Na_2HPO_4 \cdot 12H_2O$, 0.4% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.0075% $ZnSO_4 \cdot 7H_2O$.

Optimization of cultivation conditions for HB monomer production

Table 5 shows the effects of cultivation conditions on the HB monomer yield and PHA depolymerase activity. With increased incubation time, PHA depolymerase activity and HB monomer production initially increased slowly (because the strain required time to adapt to the new environment), rapidly increased from 12 h to 28 h, and then stabilized after 28 h of cultivation. Both PHA depolymerase activity and HB monomer production initially increased, became significantly higher at 30 °C culture temperature, and then gradually decreased. Excessively high or low culture temperature is known to affect the growth of bacteria and enzyme production, as well as cause enzyme dentauration and loss of function. The shaking speed and liquid volume medium in the flask can affect the amount of dissolved oxygen in the medium, thereby influencing bacterial growth. The effects of rotary speed on PHA depolymerase activity and HB monomer production were not

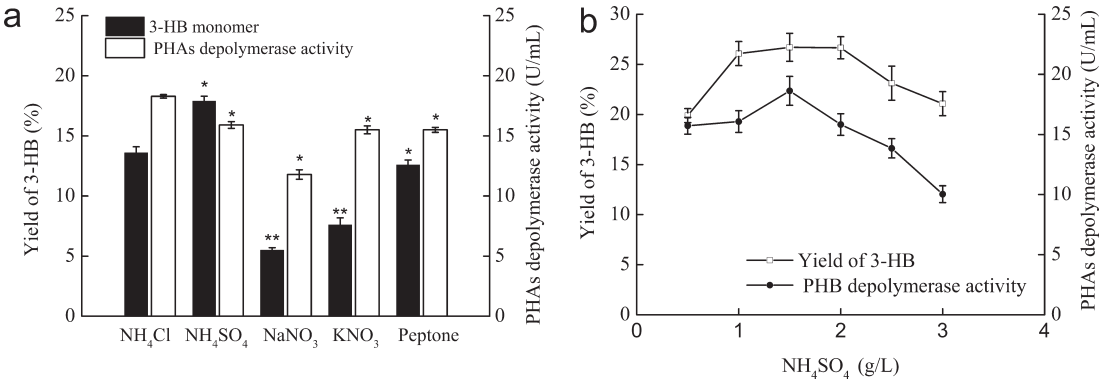

Fig. 4. Effects of nitrogen source type (a) and nitrogen source content (b) on PHAs depolymerase activity and HB monomer production.

apparent. However, PHA depolymerase activity and HB monomer production were slightly higher when the shaking speed was 150 rpm. Both PHA depolymerase activity and HB monomer yield were significantly higher when the liquid-medium volume was 120 mL, and then gradually decreased in large liquid-medium volumes (>120 mL). The effects of initial pH on PHA depolymerase activity and HB monomer production both showed a downward trend after an initial increase and then peaked when the initial pH was 7.3. Moreover, the effect of the inoculum size on PHA depolymerase activity was not apparent. However, HB monomer production initially increased and then decreased with increased inoculum content. When the inoculum size was 1.5%, PHA depolymerase activity and HB monomer production reached peak values. The optimum cultivation conditions were determined as follows: cultivation time, 28 h; temperature, 30 °C; initial pH, 7.3; rotary speed, 150 rpm; liquid-medium volume, 120 mL; and inoculum size, 1.5%.

Comparison of culture medium and conditions

P. mendocina DS04-T was cultured under initial and optimum conditions. The HB monomer yield and PHA depolymerase activity under the initial conditions were 12.1% ± 0.71% and 11.3 ± 0.81 U/mL, respectively, compared with 43.2% ± 0.42% and 39.6 ± 0.37 U/mL, respectively, under optimimum conditions. The HB monomer production obtained under optimum conditions was 3.5 times that obtained under basic culture medium and initial conditions.

CONCLUSIONS

The current study on PHA degradation mainly focused on the isolation of PHA-degrading strains under certain conditions, the purification of PHA-degrading enzymes, and the mechanism of PHA degradation. Optimization of HB monomer production has not yet been reported; thus, conditions for HB monomer production by *P. mendocina* DS04-T were optimized in this study. The strain-induced poly(3HB-*co*-4HB)-degradation process was analyzed, and results provided a reference for future studies on HB mass production. A valid method of reducing unfavorable effects on the environment was established, and this method can realize the recycling of poly(3HB-*co*-4HB) with sustainable development.

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