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SHORT COMMUNICATION

Production of poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) having a high hydroxyvalerate content with valeric acid feeding

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Abstract The capability of different organic acids to produce a derivative of PHB [poly(3-hydroxybutyric-co-3-hydroxyvaleric acid), P(3HB-co-3HV)] was examined in shake flask cultivations. Propionic and valeric acids demonstrated the potential to produce P(3HB-co-3HV) under nitrogen limiting conditions at 30°C. The addition time and the initial concentration of valeric acid needed for a high cellular HV content were identified by extensive experimentation. Fed-batch cultivation in 7-l bioreactor with valeric acid feeding resulted in the production of PHA containing 54% HV units.

Keywords Valeric acid · P(3HB-co-3HV) · Propionic acid · *Wautersia eutropha* · PHB

Introduction

Increasing concern about environmental pollution caused by waste petrochemical plastics has led to extensive research on biodegradable polymers such as poly(hydroxyalkanoates) (PHAs). These PHAs (represented mostly by poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) [P(3HB-co-3HV)]), are accumulated as intracellular polymers by a variety of microorganisms under specific nutrient limitation conditions [6]. These polymers have physical properties similar to commercially produced polypropylene (PP) [6, 10]. However, PHB is a crystalline and stiff material and this lack of elasticity limits the range of its practical applications. A random copolyester of (*R*)-3-hydroxybutyric acid (3HB) and (*R*)-3-hydroxyvaleric acid (3HV), P(3HB-co-3HV), is more ductile, easier to mold and tougher than PHB homopolyester [11]. With increasing HV content, the melting point of the polymer decreases allowing better thermal processing and even better biodegradation [11]. Several species of bacteria accumulate P(3HB-co-3HV) when organic acids [12, 13] are added in addition to a carbon source. In the present study, *Wautersia eutropha* was chosen because of its high polymeric yield and production rates.

Although some reports are available indicating the feasibility of P(3HB-co-3HV) production, the effect of various carbon sources for its production by this strain of *W. eutropha* is not very well elucidated in literature. Therefore, the potential of adding all possible carbon sources to produce P(3HB-co-3HV) was examined in the present investigation. Studies revealed the incorporation of HV units with valeric and propionic acids only. Later on, the effect of addition time and initial acid concentration of valeric acid for producing a polymer containing high HV content was established. Based on these studies, fed-batch cultivation with the addition of fructose and valeric acid was designed and implemented to mass produce P(3HB-co-3HV) having a high%HV content.

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Experimental methods

Effect of addition of various organic acids on P(3HB-co-3HV) production

Culture and inoculum development

The strain used for the present study was *W. eutropha* NRRL B 14690. Inoculum development conditions were similar to those reported in our earlier studies [7].

Effect of addition of different organic acids

Accumulation of P(3HB-co-3HV) by *W. eutropha* was investigated in optimized medium [7] with the addition of various organic acids (50 g/l, stock solution): acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, caprylic acid, lactic acid, oleic acid, sodium pyruvate, succinate, citrate and malate at 2 and 4 g/l concentrations. The experiment was started with the growth of the culture in fructose (40 g/l) and the addition of different concentrations (v/v) of these carbon sources was done after 20 h of growth. The above acids were added in two equal pulse installments at an interval of 10 h (such as: 1 g/l at 10th h and another 1 g/l at 20th h = 2 g/l). Biomass and PHA content was estimated in the samples after 60 h.

Effect of addition time and concentration of valeric acid on P(3HB-co-3HV) production

The effect of addition of different concentrations of valeric acid (Table 2) was investigated at 0, 10, 20 and 30 h. Biomass, PHB and P(3HB-co-3HV) content were estimated in all the samples harvested after 60 h.

Fed-batch cultivation with valeric acid feeding

Production of P(3HB-co-3HV) with valeric acid feeding was conducted in a 7-l bioreactor. The initial fructose and nitrogen concentration were 40 and 0.5 g/l. The reactor was operated as a batch for 20 h after which a feeding of 100 g/l valeric acid was started at a flow rate of 20 ml/h. This was continued for 12 h followed by the batch mode of cultivation for the consumption of residual nutrients (if any). Samples were withdrawn at 5–6 h interval and analyzed for biomass, PHA concentration and residual nutrients.

Analytical methods

Cell growth was monitored by measuring optical density of the culture broth at 600 nm after appropriate dilution. The supernatant obtained by centrifugation (5810R Centrifuge, Eppendorf AG, Hamburg) of the culture broth at 9,000×*g* for 10 min (at 4°C) was used for residual substrate analysis as per the protocol referred earlier [7]. The estimation of copolymer(s) was carried out using gas chromatographic method [17]. Riis and Mai [15] method was used for sample preparation. Residual valeric acid was detected by HPLC with an Aminex HPX-87H column (300 mm X 7.8 mm, Biorad, USA) [9]. The pH of the samples was adjusted to 2/3 with conc H_2SO_4 [18].

Results and discussion

Effect of the addition of different organic acids

Several reports are available in the literature which demonstrate the production of PHAs [1, 4] but the effect of different acids that can be used for the production of P(3HB-co-3HV) is not elucidated very well. Thus, shake flask cultivation for the production of P(3HB-co-3HV) was attempted with the addition of a number of organic acids (Table 1). Addition was done at the time when nitrogen limitation occurs and active polymer accumulation starts (20 h). Addition was carried out in two equal installments of 10 h interval to prevent the growth inhibition that might be caused due to addition of entire amount of acids in a single pulse. However, even in installments their acidic nature resulted in the growth suppression as compared to control samples (Table 1). Among all the different substrates investigated, the production of copolymer was achieved only with propionic and valeric acid addition. Earlier also it has been reported that *W. eutropha* requires odd-numbered carbon substrates such as propionic acid or valeric acid to incorporate the 3HV monomer [1, 3, 16].

Effect of valeric acid addition on P(3HB-co-3HV) production

The effect of valeric acid addition on the copolymer production was investigated in detail with respect to its addition time and the initial acid concentration as it resulted in a high%HV content. With propionic, as a substrate, there is a limitation in obtaining a higher HV fraction [8] which may be due the lower substrate specificity of the enzyme 3-ketothiolase for propionyl-CoA [3, 5].

The addition of valeric acid was carried out at 0 h (to see the effect at the beginning), 10 h (culture is in log phase), 20 h (nitrogen limitation has occurred and

Table 1 Growth, PHB and P(3HB-co-3HV) with different organic acids

	Biomass (g/l)	HB (g/l) (A)	HV (g/l) (B)	Total PHA (g/l) (A + B)
Control	14.5	8.8	–	8.8
Acetic acid (2 g/l)	6.6	3.65	–	3.65
Acetic acid (4 g/l)	7.3	4.02	–	4.02
Propionic acid (2 g/l)	9.8	6.4	0.7	7.1
Propionic acid (4 g/l)	8.2	4.6	1.4	6
Valeric acid (2 g/l)	9.3	3.4	1.5	4.9
Valeric acid (4 g/l)	7.2	1.1	1.8	2.9
Butyric acid (2 g/l)	12.1	9.5	–	9.5
Butyric acid (4 g/l)	8.9	6.2	–	6.2
Caproic acid (2 g/l)	10.8	7.5	–	7.5
Caproic acid (4 g/l)	5.3	2.5	–	2.5
Caprylic acid (2 g/l)	8.8	1.8	–	1.8
Caprylic acid (4 g/l)	2.5	0.55	–	0.55
Oleic acid (2 g/l)	13.8	9.4	–	9.4
Oleic acid (4 g/l)	13.8	9.5	–	9.5
Lactic acid (2 g/l)	12	9.4	–	9.4
Lactic acid (4 g/l)	9.7	7	–	7
Sodium pyruvate (2 g/l)	13.1	8.6	–	8.6
Sodium pyruvate (4 g/l)	11.9	8.8	–	8.8
Malate (2 g/l)	8.5	6.8	–	6.8
Malate (4 g/l)	6.9	5.3	–	5.3
Citrate (2 g/l)	9.1	6.7	–	6.7
Citrate (4 g/l)	11.5	7.1	–	7.1
Succinate (2 g/l)	8.8	5.2	–	5.2
Succinate (4 g/l)	10.9	7.6	–	7.6

PHB accumulation had initiated) and 30 h (active PHB accumulation phase). As the initial concentration of acid was increased there was a decrease in the amount of biomass formed due to inhibitory effect of toxic acids (Table 2). The same has been reported earlier also [13]. With the increase of valeric acid concentration, HV unit in polymer increased. Copolymer with HV unit fraction varying from 14 to 93 wt% was obtained at different valeric acid concentrations.

When valeric acid was added at 20 h, nitrogen in the medium was almost exhausted and polymer synthesis started substantially. Thus, a high P(3HB-co-3HV)

Table 2 Effect of initial valeric acid addition on biomass (g/l) and total PHA (g/l) formed

Time of addition	Initial valeric acid (g/l)	Biomass (g/l)	Total PHA (g/l)	%HV content	g PHA/g biomass
Control	–	13.7	6.9	–	0.8
0 h	2	8.05	3.1	14.6	0.58
	4	1.7	0.63	93.8	0.54
	6	0.22	0	0	0
	8	0.16	0	0	0
	10	0.06	0	0	0
10 h	2	10.6	5.7	20.5	0.53
	4	7.8	3.9	34.8	0.5
	6	5.7	4.2	84.6	0.73
	8	4.6	1.8	92.3	0.39
	10	3.3	1.6	92.6	0.48
20 h	2	11.05	5.96	24.8	0.54
	4	10.06	5.12	45.3	0.51
	6	6.7	3.8	73.4	0.57
	8	6.7	2.5	64.2	0.37
	10	4.6	1.1	66.4	0.24
30 h	2	12.1	6.6	23.2	0.54
	4	10.9	4.6	34.2	0.42
	6	8.3	3.9	36.1	0.47
	8	7.9	4.03	28	0.51
	10	7.1	4.2	30.6	0.59

concentration and high HV unit fraction were obtained at the same time. When added at 30 h, a large amount of PHB had already been accumulated in the cells and this slowdown of polymer synthesis led to the poor utilization of valeric acid. Consequently, the HV units in P(3HB-co-3HV) was very low.

Cultivation with valeric acid feeding for P(3HB-co-3HV) production

Since the addition of 6 g/l valeric acid at 20 h gave a significantly higher HV% (Table 2) with considerable biomass, the start up fed-batch cultivation with the feeding of valeric acid was designed. A highly concentrated inlet valeric acid stock solution (100 g/l) was used for nutrient feeding to ensure that the total acid concentration added in the reactor is 6 g/l with minimal broth volume increase. The feeding was continued for 12 h (20–32 h). Figure 1 shows the kinetics of P(3HB-co-3HV) production with the addition of valeric acid. Cultivation was carried out for a period of 72 h which resulted in the accumulation of 15 g/l biomass containing 7.5 g/l PHAs. Till 20 h, the PHA polymeric chain contained only HB units. As soon as valeric acid feeding was started, accumulation of HV units started in the polymer chain (Fig. 1a). At the same time%HB (wt/wt) in the polymeric chain started decreasing. However, the concentration of HB units in the polymeric chain was constant which again started increasing after complete removal of valeric acid from the broth (Fig. 1b). The maximum content of HV units in the chain (54%) was obtained at 39.5 h. After this period, there was no increase in the HV content of the chain due to negligible residual valeric acid in the broth. The incorporation of HB units in the polymeric chain again started increasing after this period of time which became almost constant after 67 h presumably due to very less quantity of residual fructose in the media.

Fed-batch cultivation for the production of P(3HB-co-3HV) by *W. eutropha* from glucose and propionic acid has been investigated earlier, presumably because it is relatively inexpensive. However, in most of the above co-cultivations the 3HV unit fraction in copolymer is in the range of 5–30 mol% [4]. On the other hand, valeric acid, as compared to propionic acid, demonstrated the superior convertibility to 3HV units [2]. Previously, P(3HB-co-3HV) with an HV fraction of 20.5 mol% has been obtained in the fed-batch culture of *R. eutropha* from glucose and valeric acid; however, the detailed feeding protocol of valeric acid was not reported [8]. Rhee et al. [14] obtained only 3 mol% 3-HV fraction using glucose as the only carbon source.

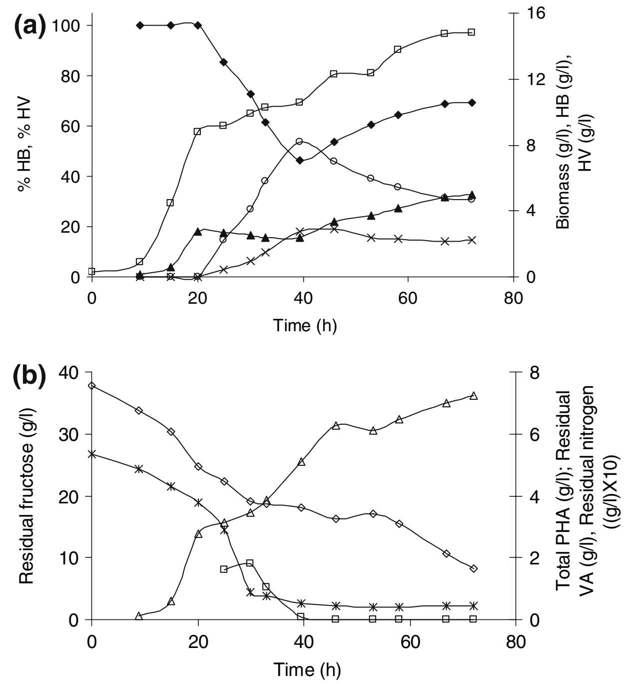

Fig. 1 Production of P(3HB-co-3HV) with the addition of valeric acid. **a** *Filled triangle* HB g/l, *filled diamond*% HB, *open circle*% HV, *open square* biomass, *multi symbol* HV g/l. **b** *Open square* residual valeric acid, *open triangle* total PHA, *open diamond* residual fructose

Thus, in the present study, a detailed study for the effect of different organic acids on production of P(3HB-co-3HV) by *W. eutropha* has been reported. Fed-batch cultivation was carried out which resulted in a polymer production having high% HV content. Future investigations need to focus on improvement in the productivity of the copolymer produced by altering the selective nutrient(s) feeding strategies at appropriate time interval(s).

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