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A FED-BATCH FERMENTATION PROCESS FOR POLY (3-HYDROXYBUTYRATE-co-3-HYDROXYVALERATE) PRODUCTION BY *Yangia* sp. ND199 USING MOLASSES AS SUBSTRATE

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ABSTRACT: A locally isolated halophilic bacterium, *Yangia* sp. ND199 was able to use molasses as substrate for copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-co-3HV)] production. Cell dry weight (CDW) of 6.37 g/l, P(3HB-co-3HV) content of 43.1 wt% and P(3HB-co-3HV) concentration of 2.75 g/l were obtained by *Yangia* sp. after 60 h of cultivation in flask. In a batch cultivation mode in a fermentor, the CDW was increased to 9.1 g/l but P(3HB-co-3HV) content was decreased to 37 wt%. Fed-batch fermentation with two different nutrient feeding strategies was used. High CDW of 54.8 g/l was obtained after 54 h of cultivation but P(3HB-co-3HV) content was still low (39.8 wt%). Two-step fed-batch fermentation with two different nutrient feeding strategies was then designed. High CDW of 50 g/l and P(3HB-co-3HV) content of 52.9 wt% were obtained after 54 h of cultivation. The two-step fed-batch process designed here for the production of P(3HB-co-3HV) by *Yangia* sp. ND199 can be developed and used for further studies.

Keywords: *Yangia*, fed-batch fermentation, molasses, P(3HB-co-3HV).

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a group of biodegradable polyesters of biological origin, PHAs accumulate intracellularly as carbon and energy storage materials in many microorganisms, usually when grown under the limitation of a nutrient, such as oxygen, nitrogen, phosphate, sulphur, or magnesium and in the presence of excess carbon [1, 14]. The properties of PHAs are similar to those of common petrochemical-based synthetic thermoplastics and can hence potentially replace them [9]. Furthermore, these polymers are biocompatible and hence have several medical applications, such as bone plates, osteosynthetic materials, surgical sutures, vascular grafts, heart valves and drug delivery systems [3, 9, 17].

Poly(3-hydroxybutyrate) [P(3HB)] is the most common type of PHA and was first described by a French scientist in 1926 [7]. Since then, various bacterial, archaeal and fungal strains have been identified to accumulate P(3HB) both aerobically and anaerobically [2]. However, application of this homopolymer is limited as it is highly crystalline, stiff and brittle in nature [9]. These polymer properties can be improved through the incorporation of comonomers, such as 3-hydroxyvalerate (3HV) and 4-hydroxybutyrate (4HB). Copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-co-3HV)] is more flexible and tougher than PHB and can be applied in many different area [9].

One of the major bottlenecks in the commercial application of PHA is their high price as compared to the conventional petroleum-based plastic materials. Several factors can affect the overall economics of PHA production, these include the yield of PHA on a carbon source, PHA productivity, the price of raw materials, the fermentation technology and purification method employed. About 40-50% of the total production cost is attributed to the carbon source [94]. Hence, a promising solution for low-cost PHA production is to develop fermentation strategies that allow high PHA content and productivity from cheap carbon substrate.

Recently, several halophilic and halotolerant bacterial strains isolated from mangrove soil samples in Nam Dinh province, Northern Vietnam, were shown to have the ability to accumulate PHAs. Among them, strain *Yangia* sp. ND199 were found to synthesize the

copolymer P(3HB-co-3BV) when grown on glucose or sucrose as carbon source [15, 16]. In the present work, we undertaken to find strategies for improving the volumetric productivity of P(3HB-co-3HV) by *Yangia* sp. ND199 using molasses as the carbon source. For this, two-step fed-batch fermentation was designed to attain both high cell density and high P(3HB-co-3HV) content.

MATERIALS AND METHODS

Bacterial strain and maintenance

Yangia sp. ND199 was maintained on solid HM medium at 4°C, containing (g/l) as follows NaCl: 30, $MgSO_4.7H_2O$: 0.25, $CaCl_2.2H_2O$: 0.09, KCl: 0.5, NaBr: 0.06, peptone: 5, yeast extract: 10, glucose: 1, and granulated agar: 20. The pH of the medium was adjusted to 7.0 [10].

Molasses

Molasses from Nordic sugar (Lund, Sweden) was used as carbon source. The molasses contains about 560 g/l sucrose.

P(3HB-co-3BV) production in shake flasks

Yangia sp. ND199 was grown in 20 ml of HM medium in a 100 ml flask at 32°C with rotary shaking at 180 rpm for 13 h (OD_{600} = 5.0). Subsequently, 2.5 ml of each culture broth were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of HM1 medium (table 1). The pH of this medium was initially adjusted to 7.0 using 1 M NaOH. The cultures were incubated at 32°C with rotary shaking at 180 rpm. Samples were withdrawn at 12, 24, 36, 48 and 60 h of cultivation for cell dry weight (CDW) and P(3HB-co-3HV) content analysis.

Table 1. Composition of the culture media and feed solution used for P(3HB-co-3HV) production by *Yangia* sp. ND199

Component	Batch medium HM1 (g/l)	Fed-batch medium HM2 (g/l)	Feed solution I (g/l)	Feed solution II (g/l)	Feed solution III (g/l)
NaCl	30	30	30	30	30
$MgSO_4.7H_2O$	0.25	1.7	10.2	10.2	10.2
KCl	0.5	1	6	6	6
KBr	0.06	0.12	0.72	0.72	0.72
KH_2PO_4	0.25	1.1	6.6	6.6	6.6
$CaCl_2 \cdot 2H_2O$	0.09	0.18	1.08	1.08	1.08
Peptone	1	3	18	-	-
Yeast extract	1	6	36	-	-
Sucrose in molasses	22	22	130	130	-
Glucose	-	-	-	-	130

P(3HB-co-3BV) production in batch fermentation

Yangia sp. ND199 was grown in 200 ml of HM medium in 1000 ml flask at 32°C with rotary shaking at 180 rpm for 13 h (OD_{600} = 5.0). The 200 ml culture broth was used to inoculate in 5-L fermentor containing 1.8 l of HM1 medium. Temperature during the cultivation was maintained at 32°C while pH was kept constant at 7.0 using 5 M HCl/NaOH. Stirring velocity and aeration were initially set at 250 rpm and 1 l/min and increased during the fermentation to maintain the dissolved oxygen concentration above 20%. Samples were taken at different times for CDW and P(3HB-co-3HV) content analysis.

Production of P(3HB-co-3BV) by fed-batch cultures with two nutrient feeding strategies

Yangia sp. ND199 was grown in 50 ml of HM medium in 250 ml flask at 32°C with rotary shaking at 180 rpm for 13 h (OD_{600} = 5.0). The 300 ml culture broth was used to inoculate in 10-L fermentor containing 2.7 l of HM2 medium (table 1). Fed-batch cultivations were

performed under the following conditions: temperature of 32°C, pH 7.0 and the dissolved oxygen concentration was kept above 20%. Sucrose concentration was maintained at about of 20 g/l by adding the feed solution I during first 24 h and then the feed solution II until the end of fermentation. Samples were taken at different times for CDW and P(3HB-co-3HV) content analysis.

Two-step fed-batch fermentation for P(3HB-co-3BV) production

Yangia sp. ND199 was grown in fed-batch fermentation as described above and the feed solution I was used to maintain sucrose concentration at 20 g/l. After 24 h of cultivation, cells were harvested by filtration and re-grown in second fed-batch fermentation, feed solution III was used to maintain carbon source concentration around 20 g/l. Samples were taken at different times for CDW and P(3HB-co-3HV) content analysis.

Quantitative analysis

CDW was determined by centrifuging 3 ml of the culture samples at 6000 rpm for 15 min in a pre-weighed centrifuge tubes, the pellet washed once with 3 ml distilled water, centrifuged and dried at 105°C until constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

P(3HB-co-3HV) concentration analysis was performed using a gas-chromatographic method [5].

In our studies, P(3HB-co-3HV) content (wt%) was obtained as the percentage of the ratio of PHA concentration to CDW [6].

RESULTS AND DICUSSION

Cell growth and P(3HB-co-3HV) production in flask cultures using molasses as substrate

The capacities of cell growth and P(3HB-co-3HV) synthesis by *Yangia* sp. ND199 using molasses as substrate were first investigated in flask experiment. The time course of growth and polymer accumulation by *Yangia* sp. ND199 in HM1 medium revealed that polymer accumulation by the bacterium was initiated from the early stages of growth and was found proportional with cell growth. Maximum CDW of 6.37 g/l, P(3HB-co-3HV) content of 43.1 wt% and P(3HB-co-3HV) concentration of 2.75 g/l were obtained by *Yangia* after 60 h of cultivation. The results obtained from this experiment (when molasses was used as carbon source) are comparable with those of the previous report (when pure sugars, such as glucose, sucrose, fructose or glycerol as carbon substrate) [16], suggesting that molasses can be a good carbon substrate for P(3HB-co-3HV) production and the growth of *Yangia* sp. ND199.

P(3HB-co-3BV) production in fermentor

Based on the flask culture results, batch fermentation for P(3HB-co-3HV) production by *Yangia* sp. ND199 using molasses as substrate was performed in 5 l fermentor. The results of cell growth rate, PHA content, PHA concentration and sucrose consumption during fermentation are summarized in figure 2. The biomass and PHA accumulation were increased during the cultivation and reached a maximum CDW of 9.1 g/l, PHA content of 37 wt% and PHA concentration of 3.35 g/l after 33 h of fermentation. In constrast, the sucrose concentration was decreased during the cultivation and only 1.4 g/l of sucrose was remained in the culture medium after 33 h of fermentation. It was found experimentally that the growth yield coefficent was 0.44 g cell/g sucrose and the product yeild was 0.16 g PHA/g sucrose.

In the fermentor, the pH and dissolved oxygen concentration were monitored and controlled at optimal condition for bacterial cells growth. For that reason, the growth rate of bacterial cells in the fermentor experiment (9.1 g/l after 33 h growth) is faster than that obtained in the flask experiment (6.37 g/l after 60 h growth). However, optimum culture conditions may inhibit the accumulation of PHA [1, 12], for that reason the PHA content was decreased from 43.1 wt% (flask experiment) to 37 wt% (fermentor experiment).

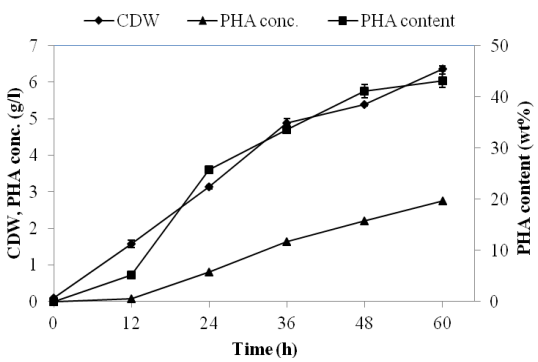

Figure 1. Profiles of CDW, P(3HB-co-3HV) content and concentration during cultivation of *Yangia* sp. ND199 in shake flasks

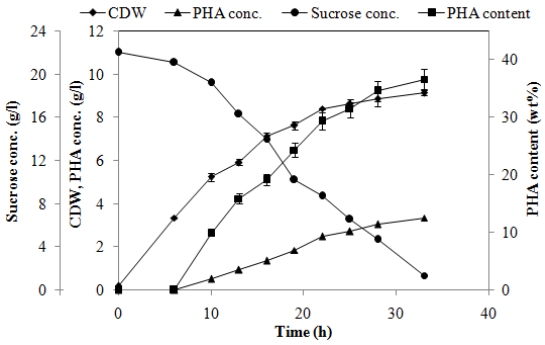

Figure 2. Profiles of CDW, P(3HB-co-3HV) content, P(3HB-co-3HV) concentration and sucrose concentration during cultivation of *Yangia* sp. ND199 in a fermentor

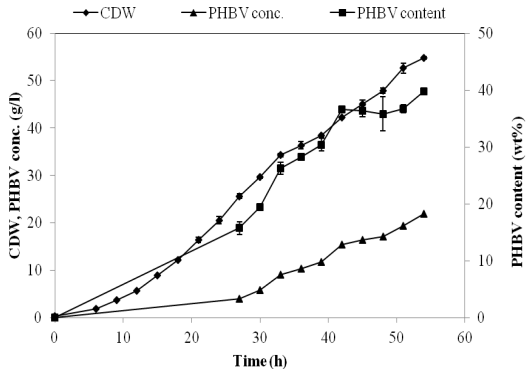

Figure 3. Profiles of CDW, P(3HB-co-3HV) content and concentration in a fed-batch fermentation mode using molasses as carbon substrate

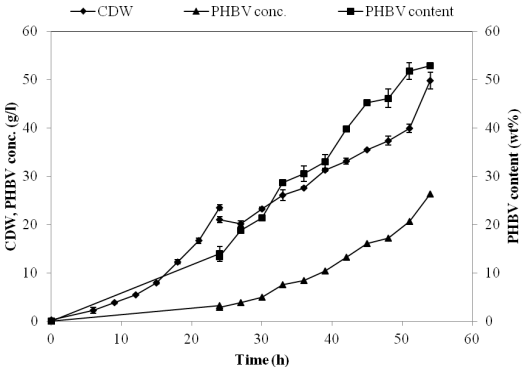

Figure 4. Profiles of CDW, P(3HB-co-3HV) content and concentration in a two-step fed-batch fermentation process using molasses and glucose as carbon substrates, respectively

Production of P(3HB-co-3HV) by fed-batch cultures with two nutrient feeding strategies

Being an intracellular product, PHA yield is related to that of the biomass. However, the conditions of optimal production of cell mass and PHA are different with respect to the nutrients concentration of the culture medium. The supplement of balance nutrients will be favourable for bacterial cell growth. In constrast, PHAs are usually synthesized within bacterial cells when growth is limited by the deplition of nutrients such as nitrogen, oxygen, and other essential emements but have an excess of carbon source [1, 14]. In order to get high productivity of PHA, fed-batch cultures of *Yangia* sp. ND199 were used to produce P(3HB-co-3HV) with two different nutrient feeding strategies. First, all nutrients required for bacterial cells growth are provided to enhance biomass production. Second, to promote the synthesis of polymer, nitrogen limiting conditions were imposed by eliminating yeast extract and peptone from the feed solution II. Figure 3 showed that the biomass and P(3HB-co-3HV) content were increased and reached maximum value of 54.8 g/l and 39.8 wt%, respectively, after 54 h of cultivation. The (3HB-co-3HV) productivity obtained after 54 h of cultivation was 0.4 g/l/h. The biomass obtained in this experiment (54.8 g/l) is more higher than that obtained in batch fermentation (9.1 g/l). However, the (3HB-co-

3HV) content (39.8 wt%) is similar to the batch fermentation (37 wt%).

Previous studies have been showed that the use of a complex subtrate, such as yeast extract makes it difficult to control the supply of nutrients for achieving high cell density as well as high PHA content [11, 13]. Our results obtained in this experiment are in agreement with previous studies, suggesting that the use of molasses (a complex substrat) as carbon substrate can be good for bacterial cell growth, however, it may inhibit the accumulation of PHA. For that reason, comparing the results obtained from these experiments with those of the highest reported so far for PHA production from molasses [8] we found that the PHA content obtained here by *Yangia* sp. ND199 (39.8 wt%) is lower than that obtained by a recombinant *Escherichia coli* strain (80 wt%); however, the biomass concentration (54.8 g/l) in case of *Yangia* sp. ND199 is higher than that obtained by *E. coli* (39.5 g/l).

Two-step fed-batch fermentation for P(3HB-co-3HV) production

In order to get high productivity of PHA, a process comprices two-step fed-batch culture was designed. The first fed-batch step was perfomed under optimal conditions for the growth of *Yangia* sp. ND199, in wich feed solution I containing molasses as carbon source was used. After 24 h of cultivation, the biomass (24 g/l) was fitrated to remove the inhibitor and transferred to a second fed-batch culture with fresh medium and the feed solution III (nitrogen free and containing glucose, a defined and suitable carbon source for *Yangia* sp. ND199) was used to promote the biosynthesis of P(3HB-co-3HV). The CDW, P(3HB-co-3HV) content and P(3HB-co-3HV) concentration reached after 54 h of cultivation (30 h of cultivation in second step) are summarized in figure 4. As can be seen from the results, the CDW of 50 g/l was achieved after 54 h of cultivation, lower than that obtained in fed-batch fermentation (54.8 g/l). However, the accumulation of the P(3HB-co-3HV) in the bacterial cells were significantly increased by using two-step fed-batch fermentation, P(3HB-co-3HV) content of 52.9 wt% and P(3HB-co-3HV) productivity of 0.48 g/l/h were obtained after 54 h of cultivation, 1.33 and 1.2 times higher than those obtained in fed-batch fermentation, respectively. The observation suggests that the use of two-step fed-batch fermentation with two different feed solutions can help to improve P(3HB-co-3HV) production by *Yangia* sp. ND199.

CONCLUSION

This study showed that the moderate halophile *Yangia* sp. ND199, isolated recently from the mangroves in Northern Vietnam, was able to use molasses as carbon source for P(3HB-co-3HV) production. Fed batch fermentation with two different nutrient feeding strategies or two separate steps was designed for P(3HB-co-3HV) production by *Yangia* sp. ND199. High CDW of 50 g/l, P(3HB-co-3HV) content of 52.9 wt% and P(3HB-co-3HV) productivity of 0.48 g/l/h were achieved after 54 h of cultivation using two-step fed-batch fermentation. The process designed here for the production of P(3HB-co-3HV) by *Yangia* sp. ND199 can be developed and used for further studies.

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SỬ DỤNG CHỦNG *Yangia* sp. ND199 TRONG QUI TRÌNH LÊN MEN BÁN LIÊN TỤC SẢN XUẤT POLY (3-HYDROXYBUTYRATE-co-3-HYDROXYVALERATE) TỪ RỈ ĐƯỜNG

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TÓM TẮT

Chủng vi khuẩn ưa mặn *Yangia* sp. ND199 phân lập tại Việt Nam có khả năng chuyển hóa rỉ đường thành poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)]. Khi nuôi cấy trong bình nón, chủng vi khuẩn này có thể tạo ra lượng sinh khối 6,37 g/l với hàm lượng P(3HB-co-3HV) tích lũy 43,1% và 2,73 g/l. Khi nuôi chủng này trong nồi lên men, lượng sinh khối tăng lên 9,1 g/l nhưng hàm lượng P(3HB-co-3HV) tích lũy lại giảm còn 37%. Phương pháp lên men bán liên tục với 2 chiến lược tiếp dinh dưỡng khác nhau đã được sử dụng. Lượng sinh khối đã tăng lên và đạt 54,8 g/l sau 54 h nuôi cấy, tuy nhiên, hàm lượng P(3HB-co-3HV) vẫn thấp (39,8%). Vì vậy, qui trình lên men 2 pha với 2 chiến lược tiếp dinh dưỡng khác nhau đã được thiết kế. Với qui trình mới này, lượng sinh khối và lượng P(3HB-co-3HV) thu được khá cao, lần lượt là 50 g/l và 52,9% sau 54 h nuôi cấy. Qui trình lên men được thiết kế để sản xuất P(3HB-co-3HV) bằng việc sử dụng chủng *Yangia* sp. ND199 có thể cải tiến và sử dụng cho các nghiên cứu tiếp theo.

Từ khóa: Yangia, P(3HB-co-3HV), lên men bán liên tục, rỉ đường, vi khuẩn ưa mặn.

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