Journal of Science and Technology ***55*** *(2) (2017) 195-201*
DOI: 10.15625/0866-708X/55/2/8677

DIFFERENT FRUCTOSE FEEDING STRATEGIES FOR POLY(3-HYDROXYBUTYRATE) PRODUCTION BY*Yangia* sp. ND199

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Received: 8 September 2016; Accepted for publication: 24 November 2016

ABSTRACT

Yangia sp. ND199 is a halophilic bacterium isolated from mangrove soil sample. This strain was able to produce polyhydroxyalkanoate (PHA) from different carbon sources. Only homopolymer poly(3-hydroxybutyrate) (PHB) was synthesized when fructose was used as carbon source. The bacterium could accumulate high PHB content during the exponential phase. Maximum cell dry weight (CDW) of 7.8 g/l and PHB content of 49 wt% were obtained after 27 h of cultivation in batch fermentation. High CDW and PHB content were achieved by using fed-batch fermentation with different fructose feeding strategies. The highest CDW of 78.5 g/l, PHB content of 67.5 wt%, and PHB productivity of 1 g/l/h were obtained by using two-stage fed-batch fermentation, is among the highest reported so far for PHB production by halophilic bacteria.

Keywords: Yangia sp. ND199, fed batch fermentation, PHB production, fructose.

1. INTRODUCTION

Plastic is a kind of material that is commonly used in everyday life in many forms. Most of plastics are made from petroleum oil, they are normally non-biodegradable materials. The use of petroleum-based plastics leads to big problem, because they accumulate in landfills and threaten the environment. The ecological drawbacks of petroleum-based plastics have pushed scientists to investigate and develop biodegradable plastics [1].

Polyhydroxyalkanoates (PHA) are polyester of hydroxyalkanoic acid that accumulated by various bacteria as intracellularly carbon and energy storage [1, 2]. Since first founded by Lemoigne [3], more hundred PHAs have been discovered and it's still increasing now. PHAs are thermal and elastic plastics, biocompatible, nontoxic and low immunogenicity that suitable for many applications including medical field. Biosynthesis of PHA is known to occur normally if carbon source is provided in excess while another nutrient such as nitrogen, sulfur, phosphorus,

iron, or magnesium is limited. For PHA production, two-stage fed-batch culture is commonly used: biomass production in initial stage and PHA accumulation in second stage [2, 4].

Yangia sp. ND199 is a halophilic bacterium isolated from mangrove forest soil. The bacterium was able to produce PHA from various substrates such as glucose, fructose, glycerol, sugarcane molasses, and high fructose corn syrup [5]. Fed batch cultivation for production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) using pure glycerol, crude glycerol and high fructose corn syrup (HFCS) was investigated. However, the cell density was low resulted in low PHA concentration and PHA volumetric productivity [5, 6]. In order to improve cell density and PHA volumetric productivity, fed-batch fermentation with different feeding strategies was investigated.

2. MATERIALS AND METHODS

2.1. Bacterial strain and media

Yangia sp. ND199 was maintained in HM medium (medium for halophile) as described in previous studies [5, 6]. The basal medium for fermentation in bioreactor contains (g/l): fructose, 20; peptone, 3.0; yeast extract, 6.0; $MgSO_4.7H_2O$, 1.7; $CaCl_2.2H_2O$, 0.18; KCl, 1.0; KH_2PO_4, 1.1; KBr, 0.12; NaCl, 30; and 1 ml trace element solution. Trace element solution was prepared by dissolving $NiCl_2.6H_2O$, 200 mg; $CuSO_4.4H_2O$, 100 mg; $FeSO_4$, 100 mg; $CoCl_2.6H_2O$, 200 mg; H_3BO_3, 300 mg; Na_2MoO_4, 100 mg; $MnCl_2.4H_2O$, 200 mg; $ZnSO_4.7H_2O$, 100 mg in one liter of water. The pH of medium was adjusted to 7.0 before sterilization. Fructose was separately sterilized and added to bioreactor before start the fermentation.

2.2. Batch fermentation

Batch fermentation was conducted in a 10 liters bioreactor (MDL-100, BE. Marusbishi, Tokyo, Japan) containing three liters of culture medium. The bacterium was cultivated in HM medium for 15 h (OD_{600nm} 7±0.5), after that 300 ml seed culture was added to the bioreactor. The fermentation process was carried out at 32 ^oC, pH was maintained at 7.0 by using NaOH (5N) and HCl (5N) as adjusted solutions. The initial agitation speed and aeration volume were set at 200 rpm and 1L/min, respectively. Samples were collected interval 3 h for cell dry weight (CDW), fructose concentration, and PHA content analysis.

2.3. Fed-batch fermentation

Fed-batch fermentation was carried out in 10 L bioreactor with three liters of initial volume. All feed solutions were prepared with similar components as basal medium above but higher concentration. For two-stage fed-batch fermentation: nitrogen-feed solution was used for first stage, and then free nitrogen solution was used for second stage. Fructose solution (70%, w/v) was prepared and sterilized separately.

The initial conditions of fermentations were set at 200 rpm of speed and 1.5 L/min of air. Dissolved oxygen was kept at above 20% by adjustment of speed and air flow. pH was maintained at 7.0 and controlled automatically using 5M NaOH/HCl. Different fructose feeding strategies were carried out in first and second stages of fermentation process. Samples were collected interval 1.5 h for CDW, fructose concentration, and PHA content analysis.

2.4. Analytical methods

Bacterial cells were harvested by centrifuging at 13000 rpm for 10 min, washed twice with distilled water, the CDW was determined after lyophilized by using Flexi Vaccum Dryer (USA). Fructose concentration was analysed offline by using p-dinitrosalysilic acid (DNS) method [7]. PHB content was determined by gas chromatography (GC) analysis. GC samples were prepared as described by Huijberts et al. [8], methyl ester was analysed using HP-7890A system (Hewlett Packard CO, USA) equipped with HP5 capillary column [9].

3. RESULTS AND DISCUSSION

3.1. PHB production by batch cultivation

Based on results of batch culture in flask experiments, batch fermentation process with three liters volume was performed in a 10 liter bioreactor. Figure 1 depicted the batch fermentation profile of strain *Yangia* sp. ND199. The growth curve of *Yangia* sp. ND199 can be divided to three phases: first 15 h of cultivation was the time of the exponential phase with increasing of CDW from 0.2 to 7.8 g/l, the stationary phase started from 18 h of cultivation, and the cells entered the death phase after 30 h of cultivation. The results of GC analysis indicated that *Yangia* sp. ND199 synthesized homopolymer poly(3-hydroxyalkanoate) (PHB) when fructose was used as carbon source. The PHB accumulation by strain *Yangia*sp. ND199 occurred early in batch fermentation, PHB content of 8 wt% was obtained after 3 h of cultivation and then increased to maximum value of 49 wt% after 27 h of cultivation. High fructose concentration in culture medium had trigged the PHB accumulation of this strain even there were no nutrient limitations. It means that the strain *Yangia* sp. ND199 can be classified to the second PHA producing bacteria group which require no such nutrient limitations for polymer accumulation [4].

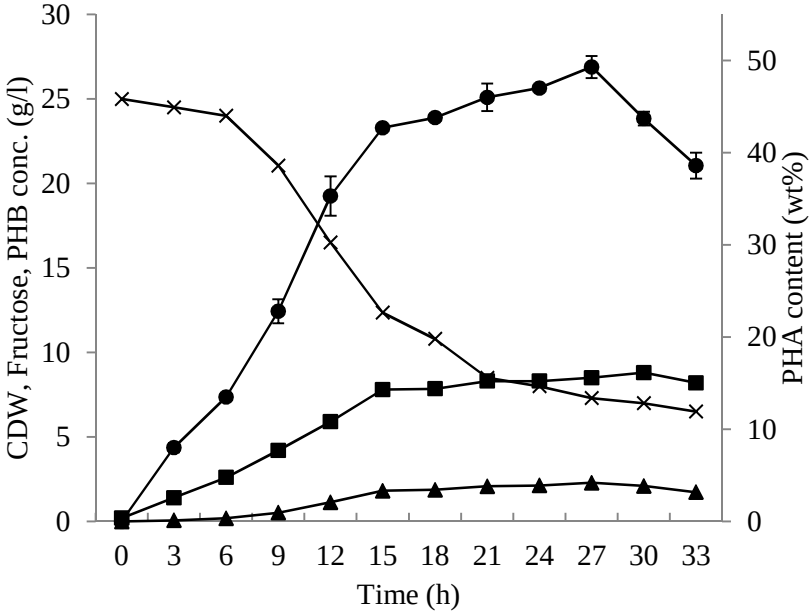

Figure 1. Batch cultivation for PHB production by *Yangia* sp. ND199. The symbols represent CDW (■), fructose (×), PHB content (●), and PHB concentration (▲).

As can be seen from the Figure 1, the rate of growth and PHB accumulation was high at the beginning of fermentation process (first 15 h) when the concentration of fructose and also other nutrients in culture medium were high. After that when fructose concentration in the culture medium reduced to lower than 10 g/l, cell growth and PHA accumulation rates were decreased (Figure 1). Hence, in order to obtain high CDW and PHA content, high concentration of nutrients need to be maintained during the cultivation process. For that reason, fed-batch fermentation for PHB production by *Yangia* sp. ND199 was designed.

3.2. Fed batch cultivation for PHB production by *Yangia* sp. ND199 with different carbon feeding strategies

Fed-batch cultivation was conducted in 10 L bioreactor, fed solutions were added to the bioreactor to avoid nutrient limitations, and fructose concentration was keep around 20 g/L during the cultivation process. The results of CDW, PHB content and PHB concentration showed in Figure 2. Maximum PHB content of 45 wt% was achieved after 33 h of cultivation, slightly lower than that obtained in batch fermentation (49 wt% after 27 h of cultivation). However, CDW of 20.8 g/l was obtained after 33 h of cultivation, 2.4 folds higher than that obtained in batch cultivation (8.8 g/l). The addition of fed solutions to the culture medium helped to improve CDW. But the CDW obtained here (20.8 g/l) is still lower than that obtained by this strain in previous study [6]. The accumulation of PHB during early stage of cultivation may inhibit the growth of bacterium because most of acetyl-CoA converts to PHB through PHB synthesis pathway, only small amount of acetyl-CoA enters to Krebs cycle for the production of ATP that need for bacterial cells growth (Figure 3). Thus change fructose concentration in medium may help to improve CDW and PHB accumulation.

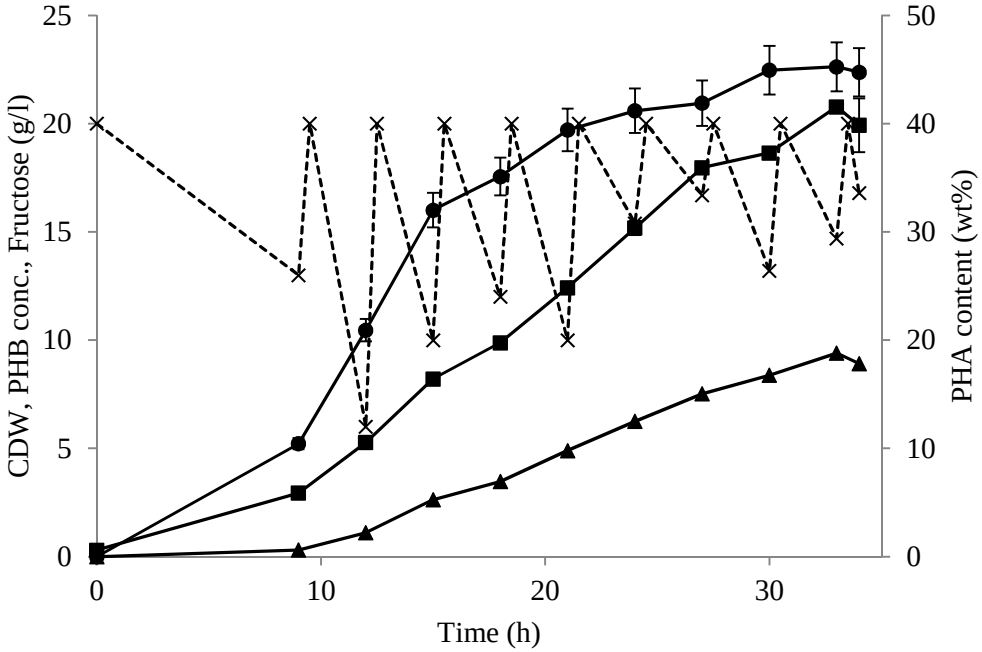

Figure 2. PHB production in fed batch fermentation with 20 g/l of fructose. The symbols represent CDW (■), fructose (×), PHB content (●), and PHB concentration (▲).

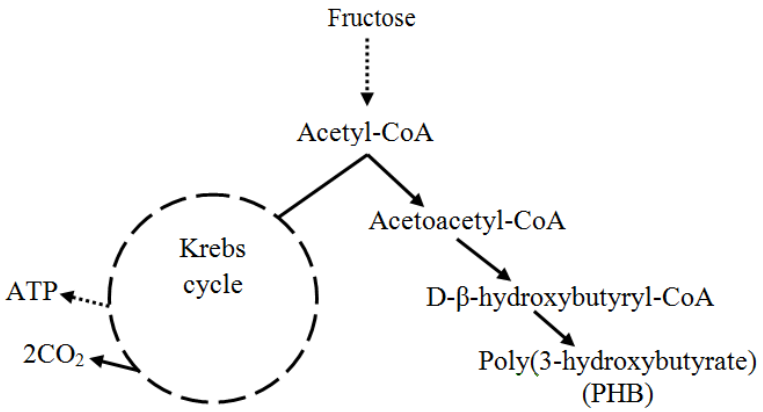

Figure 3. Pathways for ATP production and poly(3-hydroxybutyrate) biosynthesis from fructose (Adapted from Shudesh et al. [2]).

In order to get high CDW, fed-batch fermentation with a modification of fructose feeding strategy was designed. Fructose concentration of around 10 g/L was maintained during first stage and then it was increased to around 20 g/L during second stage. As showed in Figure 4, CDW was increased and reached maximum value of 45 g/l after 34 h of cultivation, 2.2 folds higher than last fed-batch fermentation (20.8 g/l). However, PHB content obtained in this fed-batch fermentation was 46.6 wt%, similar to that obtained in last fed-batch fermentation (45 wt%). The results of PHB analysis showed that the synthesis of PHB was still occurred during early stage of cultivation process, PHB content of 25.5 wt% was reached after 10 h of cultivation (Figure 4). To avoid that, the concentration of fructose in the culture medium needs to be reduced.

Previous studies have shown that the synthesis of PHA of bacteria can be increased if a nutrient limitation is applied, even with PHA producer bacteria belong to second group (require no such nutrient limitations for PHA synthesis) [4]. For example, *Alcaligenes latus* is a PHB producer bacterium belonging to the second group, in fed-batch culture under nutrient-sufficient conditions the PHB content obtained was always 50 wt%, however, when nitrogen limitation was applied, the PHB content could be increased to 87 wt% [10].

Two-stage fed-batch fermentation was then designed: the first stage for biomass production and second stage for PHA accumulation. During first stage (first 18 h of cultivation) fructose was kept lower than 5 g/l and fed solution with nitrogen was added to the bioreactor, fructose concentration was then increased to 20 g/l (step by step) and fed solution without nitrogen was used for second stage. Figure 5 showed that during first stage when fructose concentration was low and the requirement nutrient was provided, the synthesis of PHB was inhibited and only about 10 wt% of PHB was accumulated. PHB content was rapidly increased during second stage (after 18 h) and reached maximum value of 67.5 wt% after 54 h of cultivation. The results obtained here demonstrated that the accumulation of PHB by this bacterium is very sensitive with the concentrations of carbon and nitrogen sources in the culture medium. High concentration of the carbon source and low concentration of the nitrogen source could trigger the formation of PHB in *Yangia* sp. ND199.

CDW was increased during fermentation process and achieved maximum value of 78.5 g/l after 45 h of cultivation (10 times higher than that obtained in batch fermentation). The increment of CDW in first stage was mostly due to the increasing of bacterial cells number, but

it was mainly due to the increment of PHB content in bacterial cells during the second stage. Overall, the maximum PHB productivity of about 1 g/l/h was reached after 45 h of cultivation. The results obtained from this experiment are comparable with those of the highest reported so far for PHB production by halophilic bacteria. An equivalent of PHB productivity but lower CDW (44 g/L) and higher polymer content (81 %wt) were produced by *Halomonas boliviensis* LC1 using glucose as carbon source [11]. Using open fed batch fermentation, *Halomonas* TD1 could produce as high as CDW of 80 g/L with higher PHB content of 80% on glucose [12].

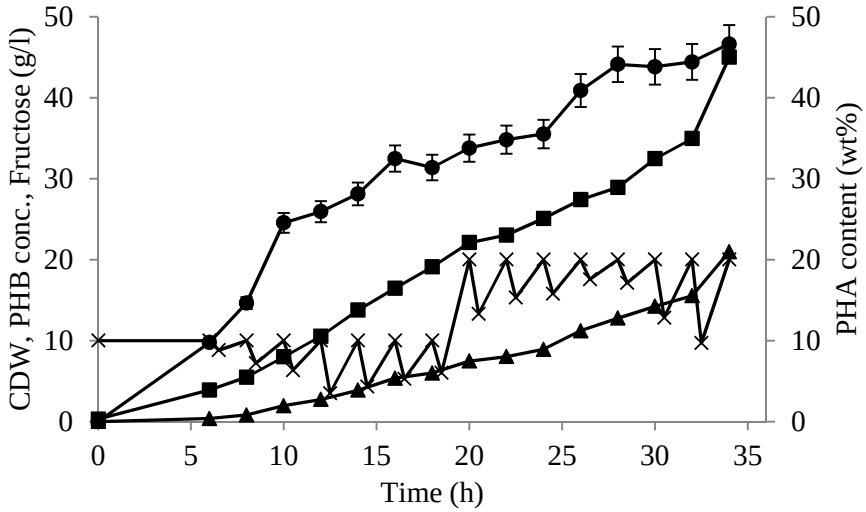

Figure 4. Fed batch cultivation for PHB production by *Yangia*sp. ND199 with two different fructose concentrations (10 and 20 g/l). The symbols represent CDW (■), fructose (×), PHB content (●), and PHB concentration (▲).

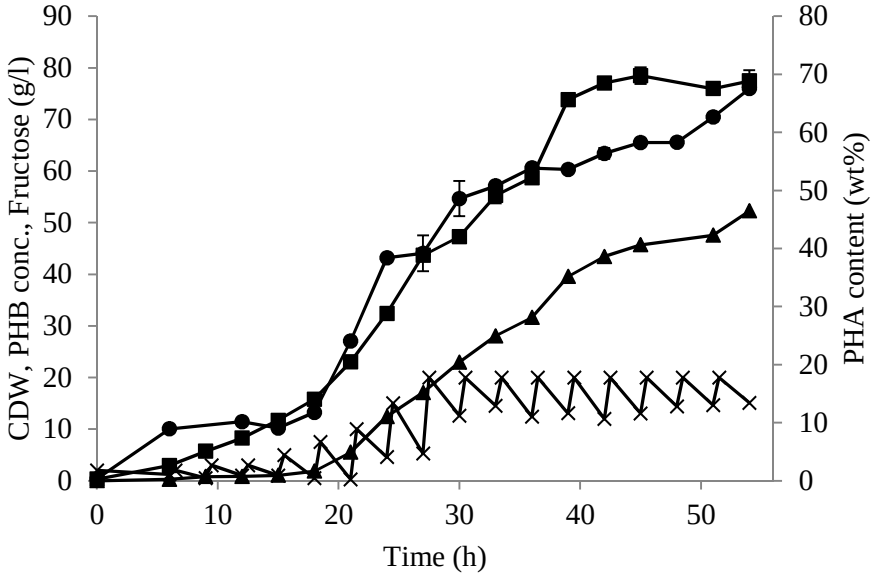

Figure 5. Two-stage fed-batch fermentation for PHB production by *Yangia*sp. ND199. The symbols represent CDW (■), fructose (×), PHB content (●), and PHB concentration (▲).

4. CONCLUSION

The study showed that the strain *Yangia* sp. ND199 belongs to second PHA producer group that canaccumulate PHB together with cell growth. CDW of 7.8 g/l and PHB content of 49 wt% were achieved in batch fermentation. High CDW of 78.5 g/l and PHB content of 67.5 wt% were obtained by using two-stage fed-batch fermentation in which the first stage was carried out at optimum conditions for bacterial cell growth and the second stage was carried out under the conditions of excess carbon and nitrogen limitation for inducing high PHB accumulation.

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