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Sphaerotilus natans Isolated from Activated Sludge and Its Production of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

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Abstract

Sphaerotilus natans is a sheathed bacterium existing in the activated sludge of wastewater treatment plants. It is one of the filamentous bacteria causing the bulking and foaming difficulties of activated sludge. Isolating the strain and culturing it in an axenic environment could not only provide the metabolic knowledge of the strains that would be useful in the development of wastewater treatment methods, but also could enable us to gain an understanding of the mechanism by which poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (poly[3-HB-co-3-HV]) is produced by this strain. This article reports the screening and isolation of the strain from the activated sludge using the Nile blue staining method together with Fourier transform infrared analysis. We investigated the ability of the selected strain to produce poly(3-HB-co-3-HV) copolymer using glucose and peptone, or by adding valeric acid or sodium propionate as precursor. Proper precursor feeding could dramatically enhance its 3HV content in the copolymer P(3HB-co-3HV). By controlling the different feeding times in fed-batch fermentation, different desired copolymers were obtained with 15, 40, and 70% 3HV mole fraction of the copolymer. Polymer properties were analyzed by gas chromatography, differential scanning calorimetry, thermo-gravimetry, and nuclear magnetic resonance analysis.

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Index Entries: Activated sludge; copolymer; poly(3-hydroxybutyrate-co-3-hydroxyvalerate); polymer properties; *Sphaerotilus natans*.

Introduction

Polyhydroxyalkanoates (PHAs) are thermoplastics completely biodegradable in a natural environment. They are produced as intracellular storage material by a large number of bacterial species. Polyhydroxybutyrate (PHB) and its copolymer poly(3-hydroxybutyrate)-co-(3-hydroxyvalerate) (P[3HB-co-3HV]) are typical types of PHAs. The copolymer is more valuable than the homopolymer, PHB, because it has more practical properties, particularly in terms of its crystal growth rate, melting point, and other physical properties and biodegradability *(1–3)*. Therefore, it has attracted much attention from researchers because of its widespread potential application as a biodegradable plastic and as a source of valuable chiral synthons. The copolymer, P(3HB-co-3HV), could provide a wide range of morphologies and physical properties when it contains different amounts of hydroxyvalerate (3HV) or is blended with some other additives *(4,5)*.

A sheathed bacteria, *Sphaerotilus natans*, is considered to be one of the specific trichome-forming bacteria. It causes activated sludge bulking and foaming in wastewater treatment systems *(6)*. *S. natans*'s ability to produce PHB is one physiologic characteristic used in its classification *(7)*. As a culture medium for the production of P(HB-co-HV) by microorganisms, an odd carbon fatty acid, such as propionic acid or valeric acid, was needed as a supplement as a precursor of the HV unit in addition to other carbon sources, such as glucose, fructose, lactose, and sucrose.

S. natans is justifiably considered an excellent candidate for P(HB-co-HV) synthesis from glucose and propionate mixtures, because it is extremely tolerant to propionic acid: cells grow even when 6 g/L of sodium propionate is added to the medium. It allows easy operation in a copolymer production process without strict control of propionic acid throughout fermentation. Moreover, *S. natans* forms flocs and the cell broth settles down immediately. It results in an easy separation of the biomass from the culture fluid, which was one of the most energy- and time-consuming processes in the production of PHAs *(8)*. Therefore, *S. natans* should be a potential strain to be developed for industrial application. However, few studies report the production of P(3HB-co-3HV) by *S. natans*. Much effort has been devoted to the investigation of relationships between different fatty acids, such as C4:C5 ratio, and 3HV mole fraction *(9–12)*, which is not a unique control for the different HV fractions incorporated into the P(3HB-co-3HV) copolymer during the production of P(3HB-co-3HV).

The present study, using *S. natans*, which was isolated from activated sludge by the Nile blue staining method and selected by Fourier transform infrared (FTIR) analysis, reaveled a new relationship between the HV fraction in the P(3HB-co-3HV) copolymer and the precursor: the mixture of glucose and sodium propionate. Different 3HV contents of copolymers

were achieved by fermentation feeding control. The properties of these copolymers were also analyzed.

Materials and Methods

Isolation of S. natans Strain by Nile Blue Staining Method and FTIR Analysis

Activated sludge was obtained from municipal wastewater treatment plants at Shatin and Yuanlang, Hong Kong. Activated sludge was streaked on a petridish using tryptone soya agar (Oxoid) as a medium at 30°C for 2 to 3 d. The white, round, smooth single colonies were picked out to inoculate on the same medium plates with duplication. The *Sphaerotilus*-like (observed under microscope) colonies were transferred into a selected medium plate at 30°C for 1 to 2 d. The composition of the selected medium was 6 g of glucose, 3 g of peptone, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$, 1 g of sodium propionate, and 1000 mL of tap water. The pH of the medium was 7.0. The Nile blue staining method *(13)* was used to select the PHA-producing bacteria, together with FTIR *(14)* analysis to choose a higher productive strain for this study. The selected strain was determined and characterized as *S. natans* by the South China Agriculture University, Guangzhou, China.

Production of P (3-HB-co-3-HV)

The selected *S. natans* LY2000 was employed. The strain was stored at –20°C in the presence of 10% (v/v) glycerol. Preincubation was at 30°C with rotary shaking in a 500-mL flask containing 100 mL of tryptone soy broth (Oxiod) medium for 24 h. The production of the copolymer PHAs, P(3HB-co-3HV), was carried out in a 500-mL shake flask and in a 10-L jar fermentor (B. Braun, Germany) using the medium with the following composition: 10 g/L of glucose, 3 g/L of peptone, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of $CaCl_2$, 1 g/L of $NaH_2PO_4 \cdot 2H_2O$, 2.6 g/L of K_2HPO_4, 0.01 g/L of $FeCl_3$, 0.005 g/L of boric acid, and 1.5 g/L of sodium propionate. The volume of the medium was 100 mL in a 500-mL shake flask or 6.5 L in a 10-L fermentor. Initial inoculation of the preculture was 1% in both the shake flask and the jar fermentor. The fermentation condition was usually at 30°C with an initial pH at 7.0, and 180 rpm for shake flask and 300 rpm with 0.5 (v/v) aeration for the jar fermentor.

Fed-batch experiments were conducted using a 10-L jar fermentor (B. Braun). The composition of the feeding medium to the fermentor was 5 g/L of glucose and 1.5 g/L of sodium propionate unless otherwise stated.

Extraction of Biopolymer from Fermentation Broth

The copolymer P(3HB-co-3HV) was extracted using the same method as for PHB *(15)*.

Analytical Method

Cell culture broth was periodically sampled and analyzed for optical density (OD) at 600 nm and cell dry wt (CDW). The glucose concentration in the culture broth was determined by using a YS1 Biochemistry Analyzer. The 3HV fraction in the copolymer and the composition of P(3HB-co-3HV) copolymer was analyzed using a gas chromatograph (Hewlett 5890, with a 10% CW 20M on Chromosorb W-AW 80/100 column, 6 ft). The working temperature of the column, injector, and the flame ionization detector was 135, 260, and 300°C, respectively. The nitrogen gas flow rate was set at 20 mL/min.

Thermoanalytical Techniques

The thermoproperties of the copolymer P(3HB-co-3HV) were determined using thermoanalytical techniques, thermogravimetry, and different scanning calorimetry (DSC). Thermogravimetry was performed with a Perkin-Elmer TAC7/DX thermogravimetry analyzer. The sample weight was 5–10 mg. The dynamic test was performed at 10 and 20°C at temperature intervals of 30–500, and 500–700°C , respectively. The sample amount was 3–10 mg. DSC was performed with a Mettler DSC30 analyzer with a 5°C /min heating or cooling rate at a temperature interval of –60–200°C.

The nuclear magnetic resonance ^{1}H-NMR analysis was carried out on a Bruker DPX-400 MHZ spectrometer according to the method reported by Yu et al. *(16)*.

Results and Discussion

Acquisition of Strain and Its Selection by FTIR Analysis

When the stained colonies were irradiated with a short-wave (254 nm) ultraviolet (UV) light in a dark room, the colonies with PHA fluoresced bright orange, but the other colonies without PHA remained unchanged (Fig. 1).

The ability of the PHA to accumulate in the selected strains by the Nile blue staining method was determined by FTIR analysis. Figure 2 shows that the ratios of the PHA peak (at 1736.56 cm^{-1}) and protein peak (at 1639.26 cm^{-1}) were different when the two strains were analyzed by FTIR. Because the ratio value is linked to the ability of the strain to produce PHA *(14)*, the higher potential productivity strain *S. natans* LY 2000 was selected.

Use of Different Carbon and Nitrogen Sources

Instead of glucose, several common carbon sources were added to the medium at the same concentration of 10 g/L. Cultivation was done in shake flasks for 48 h. Table 1 shows the results of the cell yield and PHA content in the cells obtained by utilizing the different carbon sources. *S. natans* grew better in glucose, sucrose, fructose, maltose, glycerol, and

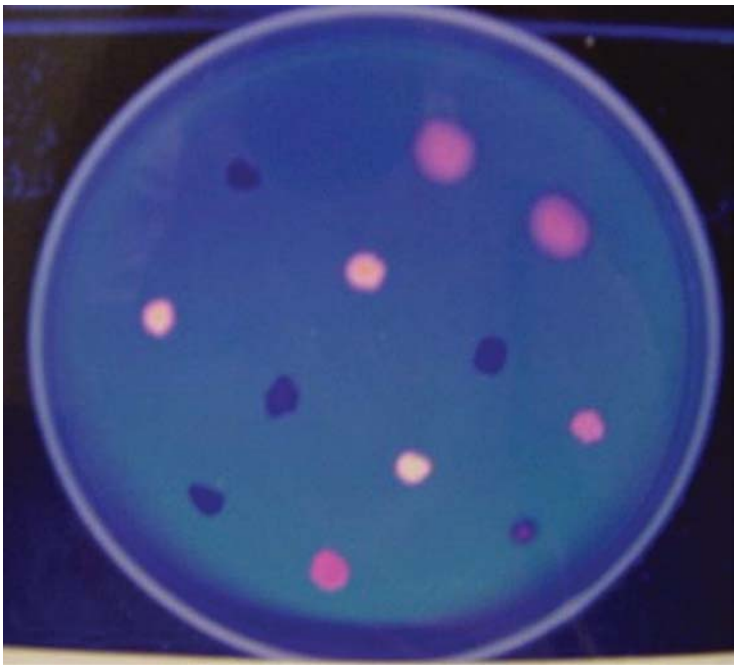

Fig. 1. Nile Blue–stained colonies of *Sphaerotilus*-like strains with and without PHA on irradiation of UV light. UV light was irradiated from the upper side.

NaAc, but only obtained a higher accumulation of PHAs in cell in glucose and NaAc media. Regarding the lower cost of production of PHAs, glucose was chosen to be the best carbon source.

Similar experiments choosing proper nitrogen source at the same concentration of 3 g/L as peptone in the medium were carried out (see Table 2). The results supported peptone as the proper nitrogen source by *S. natans* LY2000.

Ability of Selected S. natans *to Produce P(3HB-co-3HV)*

The selected *S. natans* LY2000 could produce the copolymer P(3HB-co-3HV), whereas other researchers had declared that *S. natans* only accumulated the homopolymer of HB when glucose was used as the sole carbon source. Although the 3HV fraction in the copolymer was very low, 3HV content was only 1.9% (data shown as control in Fig. 3.) when our strain was cultured in the medium without the presence of propionic acid or valeric acid or other odd carbon fatty acid. This provided the evidence that *S. natans* could synthesize the 3HB-3HV copolymer by the pathway that 3HV synthesizes from carbohydrate.

However, odd carbon fatty acid did enhance the HV content in copolymer (Fig. 3), and with a certain supplement of sodium propionate or valeric acid (1.5 g/L), HV incorporation into the copolymer was dramatically increased, and the 3HV content was 22.7, and 8.2% , respectively.

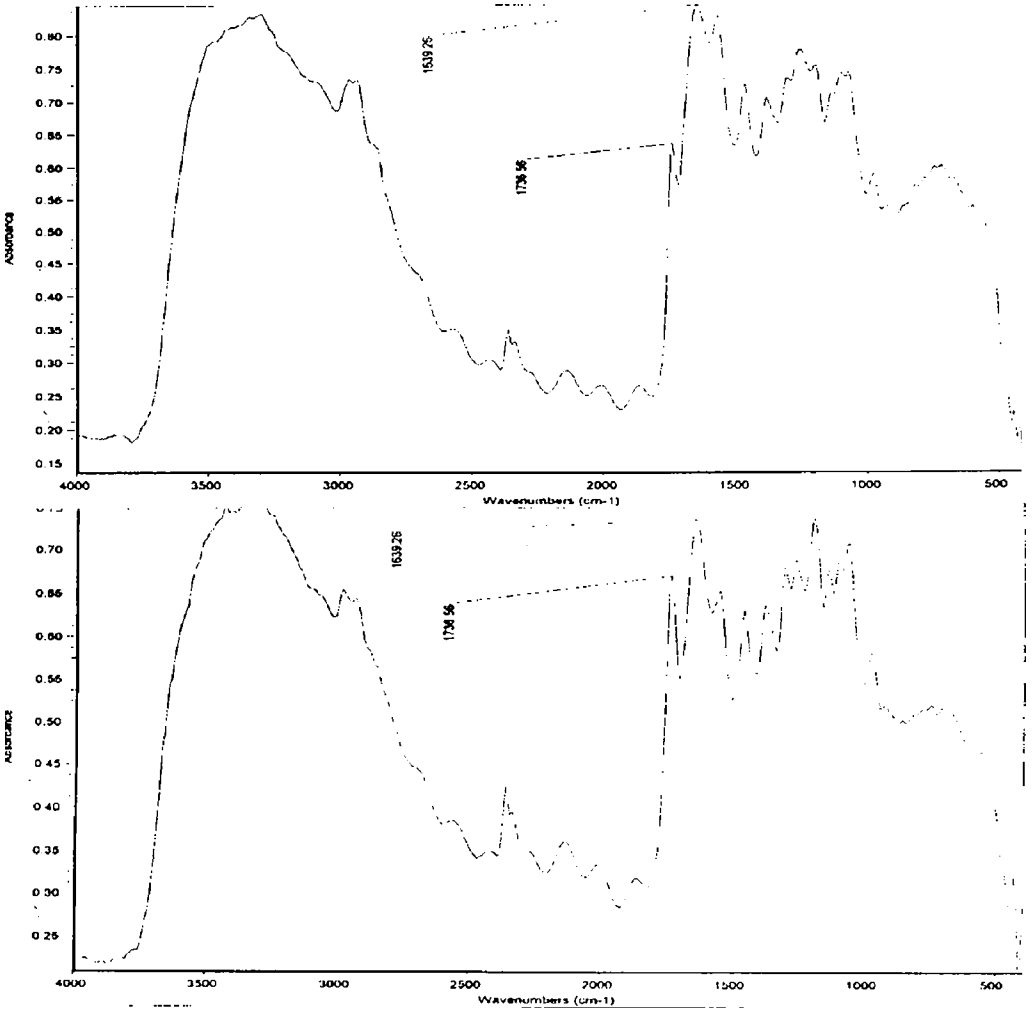

Fig. 2. FTIR results of the two different strains.

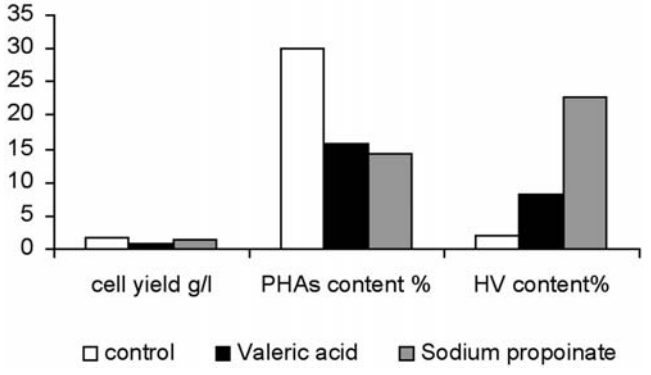

Fig. 3. Effect of valeric acid or propionate on accumulation of copolymer and 3HV content of copolymer.

Table 1
Synthesis of Copolymer from Several Carbon Sources

Carbon source	Cell yield (g/L)	PHA content (%)
Glucose	1.59	25.2
Sucrose	1.26	13.6
Fructose	1.12	11.7
Maltose	1.31	19.3
Lactose	0.65	13.8
Glycerol	1.01	14.6
NaAc	1.22	21.4

Table 2
Synthesis of Copolymer from Several Nitrogen Sources

Nitrogen source	Cell yield (g/L)	PHA content (%)
Tryptone	1.45	25.5
Peptone	1.62	26.8
Beef extract	1.27	16.5
Yeast extract	1.73	18.3
$(NH_4)_2SO_4$	1.37	16.4
Urea	1.49	15.0
NH_4Cl	1.19	14.3

Copolymer Production in Batch Experiments

Glucose concentration, cell concentration, and monomer composition of the copolymer were determined with respect to culture time (Fig. 4). A batch experiment was conducted in a 10-L fermentor. The pH was maintained between 6.5 and 6.8 by a pH controller during cultivation. Glucose was consumed within 20 h, while cell growth increased in inverse proportion to the concentration of carbon source and reached a maximum of 1.54 g/L after 23 h of cultivation. During the same period, polymer also accumulated gradually to 12.7%. By contrast, the content of 3HV monomer was dramatically decreased to about 20% after it jumped up to >60% in the early growth period phase, and then remained constant when the cells continued to grow. The final cell yield, PHA content in the cells, and 3HV monomer content in the copolymer were almost the same as when the cells were cultivated in a shake flask for 48 h.

Fed-Batch Experiments with a Desired HV Content in P(3HB-co-3HV) Copolymer

Figure 5 represents the time course of the concentration of glucose, HV content, PHA content, and cell yield while feeding was done at three different time points (A, B, and C). A is the time when HV content was at

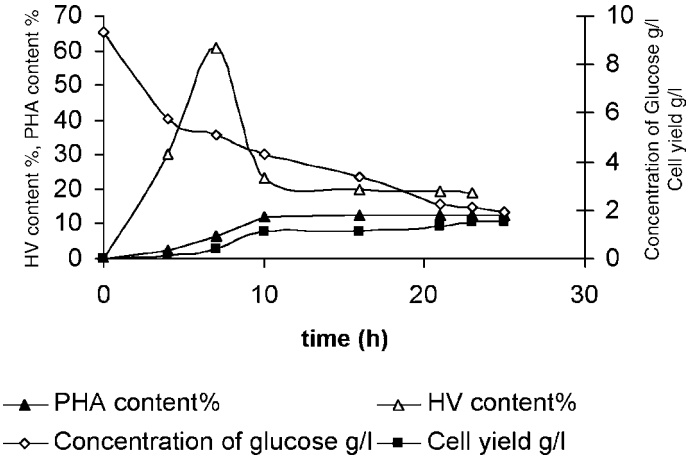

Fig. 4. Production of P(HB-co-HV) by *S. natans*.

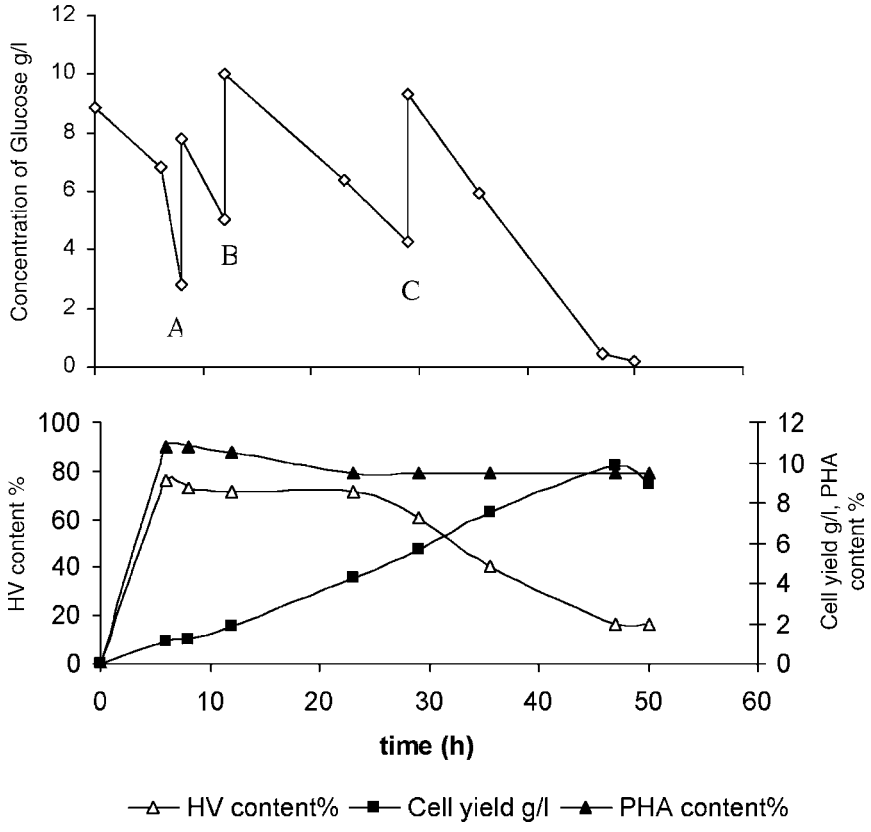

Fig. 5. Effect of feeding time on 3HV content in copolymer and production of a lower 3HV content of copolymer.

its growth phase, B is when HV content was at its stationary phase, and C is when HV content was at its decrease phase. Time points A, B, and C were 8, 12, and 29 h, respectively. Between 0 and 8 h, cell grow, PHA content, 3HV content, and glucose concentration had similar variety as in the batch experiment. Between 8 and 12 h, which was the period during the first time feeding, glucose concentration was kept above 5 g/L, and 3HV content did not decrease like that in batch fermentation after it reached its maximum of 76%. Moreover, after the second feeding, between 12 and 29 h, 3HV content could keep a constant value of about 70% for a while before the concentration of glucose decreased below 5 g/L. Then, 3HV content dropped continuously even after the third feeding; a high concentration of glucose could only support cell growth but not the accumulation of 3HV. Cell yield was 9.4 g/L while the final 3HV content was about 15%. Regardless of the different feeding time, PHA content remained constant at about 10% in the cells.

Different time points of feeding with a mixture of glucose and sodium propionate was assumed to be a key factor in producing different 3HV contents in the polymer. When feeding was introduced at the 3HV growing phase, high 3HV content of copolymer was produced. Harvesting of the cell culture broth should be done while the concentration of glucose is still high.

If a lower 3HV content copolymer was desired, the concentration of glucose could be allowed to drop to a very low value after the cell has reached its maximum yield. If a medium 3HV content copolymer was the objective, fermentation should be ended before the concentration of glucose drops to about 5 g/L. This hypothesis is supported by the later experiments shown in Figs. 6 and 7.

Figure 6 represents the fermentation process when a higher content 3HV copolymer was desired. The concentration of glucose in the feeding medium was changed to 10 g/L with the intention of gaining a higher cell growth. Feeding at the 3HV content growing phase did maintain the high 3HV level, as shown in Fig. 5. However, between 12 and 25 h, a higher concentration of glucose (10 g/L) feeding did not make the cells grow better than 5 g/L (Fig. 5). On the contrary, a high concentration of glucose inhibited cell growth. Cell yield, PHA content, and 3HV content of copolymer reached 4.2 g/L, 15.0%, and 70.2%, respectively.

Figure 7 shows that fermentation should end before the concentration of glucose decreases below 5 g/L, in order to get a medium-level 3HV content. Feeding was changed to pumping the 10 g/L of glucose into the medium, starting from the 16-h point and ending at 29 h. After ongoing cell growth, the concentration of glucose dropped to about 5%. When fermentation ended, cell yield, PHA content, and 3HV content reached 6.2 g/L, 11.63%, and 40.4%, respectively.

S. natans can biosynthesize P(3HB-co-3HV) using glucose as the carbon source without propionate, valerate, or other C_{0dd} fatty acids. Propionyl-CoA here is formed through the methylmalonyl-CoA pathway, which originates

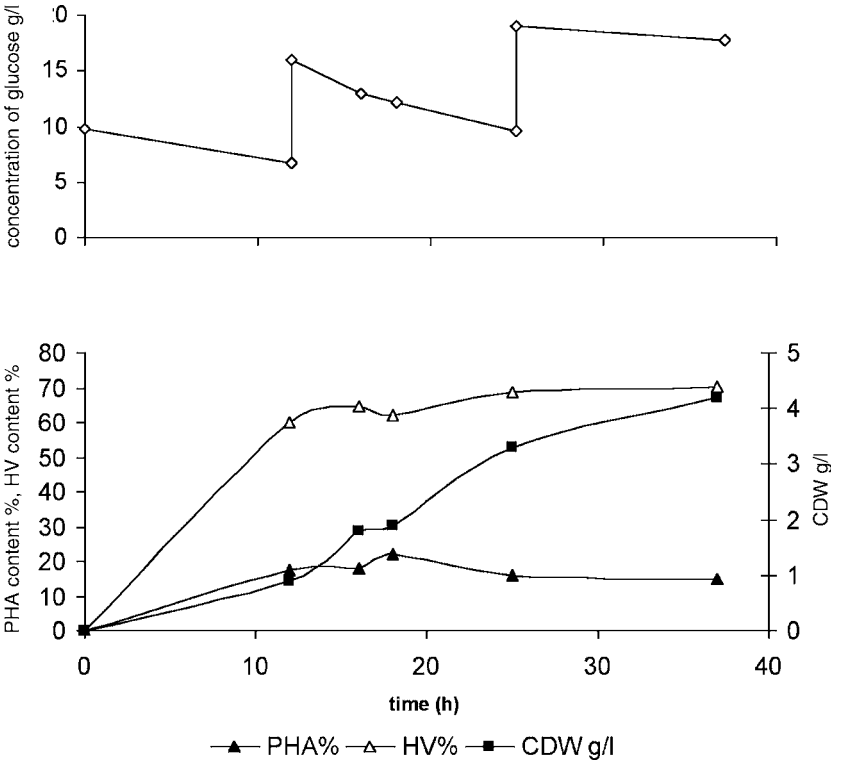

Fig. 6. Production of a higher 3HV content copolymer.

from succinyl-CoA in the tricarboxylic acid (TCA) cycle *(17)*. It could be assumed that even when propionate was added to the medium, this pathway still existed. The same amount of propionate was introduced in fermentation, but a different 3HV content was incorporated into the copolymer. At the 3HV content growing phase, besides supporting cell growth, glucose was used to produce succinyl-CoA for propionyl-CoA, which was the precursor of 3HV. Hence, glucose may play an important metabolic role in the pathway of 3HV synthesis, and also in the pathway of TCA cycle. However, when the 3HV content was in its reducing phase, glucose was mainly responsible for cell growth. This could explain why in the 3HV content decreased phase cells still grow with a higher transfer yield from glucose than in the 3HV content growing phase.

Analysis of Polymer Properties

Different 3HV content copolymers produced by *S. natans* were extracted from freeze-dried cells. Gas chromatography and NMR analysis indicated that 3HV content in the copolymer maintained the same content as it was incorporated into the cells. The standard P(3HB-co-3HV) copolymer (purchased from Fluka), was selected for comparison.

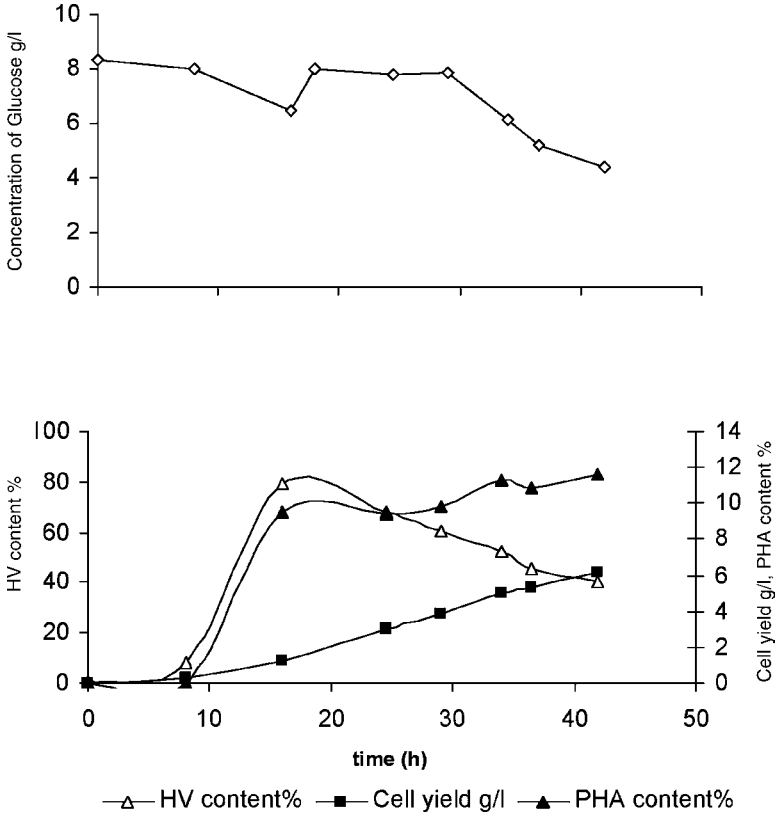

Fig. 7. Production of medium 3HV content copolymer.

Table 3 shows the melting temperature (*Tm*) and the temperature at the maximum rate of weight loss by therogravimetric analysis (*Tp*), which stands as the totally degradable temperature. PHB with a melting point at 177.6°C was of relatively high crystallinity, resulting in high stiffness and brittleness. With an increase in 3HV content in P(3HB-co-3HV), the *Tm* of the polymer decreased while the *Tp* did not show a big difference. The increase in 3HV monomeric units in the copolymer had reduced the crystallinity of the copolymer and this may affect the mechanical properties of the copolymers such as tensile, shear, compressive, and flexural strength.

Figure 8 shows the appearance of these different 3HV mole fraction copolymers. Samples A, B, and C are copolymers containing 70, 40, and 15% 3HV content, respectively. As to the flexibility of these three copolymers, A was the best, then B, and finally C. The results were in agreement in that different 3HV contents in P(3HB-co-3HV) copolymer changes the polymeric properties. Therefore, these copolymers can be developed to meet the needs for application in a variety of different fields. DSC analysis also investigated detailed crystallization behavior in different 3HV copolymers (data not shown).

Table 3
Thermoproperties of Different 3HV Content Copolymers

3HV content (%)	*Tm* (°C)	*Tp* (°C)
15	155.3	334.1
40	101.2	333.9
70	68.2	329.7

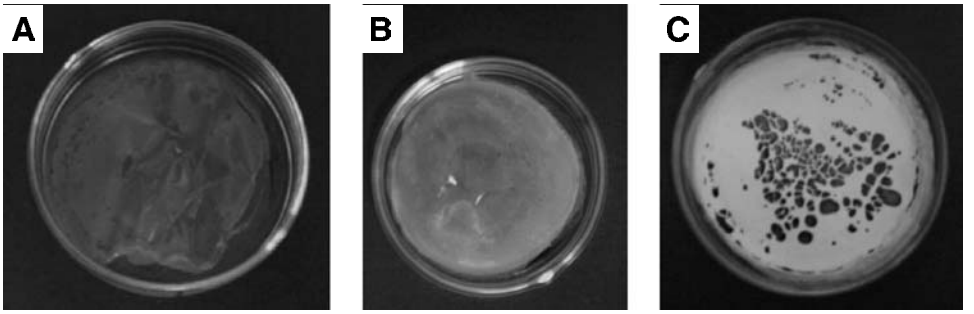

Fig. 8. Samples of different 3HV mole fractions in copolymer: **(A)** 70% 3HV; **(B)** 40% 3HV; **(C)** 15% 3HV.

Conclusion

The selected *S. natans* LY2000 from activated sludge produced a desired 3HV mole fraction in P(3HB-co-3HV) by controlling the feeding time points and ending time points during the fermentation process. The highest 3HV content of the copolymer was 70% and was first obtained by the biosynthesis pathway using glucose and sodium propionate as precursor. Furthermore, thermal properties of 15, 40, and 70% 3HV content in P(3HB-co-3HV) copolymer were analyzed. Differences in the contents of 3HV units in copolymers affected the physical properties of the copolymer. Future studies on the increased production of copolymers by *S. natans* as well as on the development of their application are needed.

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