Isolation of PHA–Producing Bacteria from Date Syrup Waste

Seyed Ahmad Ataei,[1] *Ebrahim Vasheghani-Farahani,*[*1] *Seyed Abbas Shojaosadati,*[1] *Hossein Abdul Tehrani*[2]

Summary: Since the major problem associated with the industrial production of Polyhydroxyalkanoates (PHAs) is their high production cost, this study was carried out using date syrup as the major carbon source to decrease the production cost and also help to supply other nutrient requirements. To isolate PHA–producing bacteria for this purpose, microorganisms were isolated from the syrup waste of a local date factory. These purified colonies were screened for intracellular granules by staining with Sudan Black. The positive-staining strains were grown for production of PHAs in 5% date syrup as carbon source supplemented with mineral salt medium. The culture was incubated at 30 °C with shaking at 140 rpm for 60 h. Among positively stained bacteria, the best PHA producers were selected on the basis of cell growth, cell dry weight, PHA content and the monomer composition of PHA. One of them could utilize date syrup for growth and produce the homopolymer of Polyhydroxybutyrate (PHB) with a cell density of about 5.1 g/L and maximum concentration of PHB equal to 3.6 g/L which is 71% of cell dry weight. Another one produces copolymer of Poly (hydroxybutyrate-hydroxyvalerate) in date syrup media with a maximum concentration of 2.2 g/L containing 10 wt % valerate in shake flask cultivation.

Keywords: biopolymer; cheap substrate; date syrup waste; isolation of bacteria; PHB/PHV

Introduction

Polyhydroxyalkanoates (PHAs) are carbon-energy storage materials that are accumulated intracellulary in a variety of microorganisms under controlled concentrations of nutrients such as nitrogen, oxygen and/or mineral ions.[1]

Due to its biodegradability, biocompatibility and other extraordinary properties, PHAs could be ideal for use in medicine (e.g. for hard and soft tissue implants, dressing and sewing material, tissue growth materials), pharmacy (controlled-release drug delivery systems), food packaging and agriculture.[2]

A major problem in commercializing PHAs is their high production cost as compared with those of plastics based on petrochemicals. Much effort has been devoted to lower the production cost of PHA by developing better bacterial strains and efficient strategies for fermentation and recovery of PHAs.[3]

Evaluation of the process for the production of PHA suggested that the major contributor to the overall PHA production cost was carbon substrate cost (up to 50%).[4] Therefore it is desirable to produce PHA from agricultural residual carbon sources or even from waste product such as molasses, date syrup, whey and corn steep liquor. To the best of our knowledge only one report is available for production of PHA using date syrup as carbon source. It was reported that at the best conditions with 5% of date syrup, 3.3 g/L of cell dry weight with 50% PHB (1.65 g/L polymer) was obtained.[5]

[1] Biotechnology Group,Chemical Engineering Department, Tarbiat Modares University, P.O. Box 14115-143,Tehran, Iran
Fax: (+98) 21 82883381; E-mail: evf@modares.ac.ir
[2] Biotechnology Department, School of Medical Sciences, Tarbiat Modares University

This study was carried out to isolate PHA producing bacteria from date syrup waste and evaluate their potential application for PHA production from this cheap raw material supplemented with other renewable nutrients.

Materials and Methods

Isolation of Bacterial Strains

Liquid sample from wastewater of ''Salwa'' Date Factory Ltd. (Kerman, Iran) were taken and serially diluted with sterile distilled water, and then 200 μL of the dilution was spread on a sterile nutrient agar medium in Petri dish. The plates were incubated at pH = 7 and 30 °C for 5 days. Colonies which developed on the agar were differentiated by color, form and edge appearance. Pure bacterial isolates were obtained by re-culturing individual colonies several times on fresh agar medium to produce single colonies. Agar slants of these colonies were preserved at 4 °C for one month.

Visualization of PHA In Vivo

Each of the purified isolates was stained with Sudan Black and observed for the granules under light microscope (pictures not shown).[6]

Screening

After Purification, positively stained colonies were screened for PHA accumulation by the following method. The isolate was first grown in seed culture of MSM supplemented with 10 g/L fructose as carbon source in order to produce large amount of cells. After 24 hours, 5% of seed culture was transferred to fermentation medium contained 5% (w/v) date syrup as carbon source. The culture was incubated at 30 °C for 60 hours while shaking at 140 rpm.

Analytical Methods

Cell Growth

Cell growth was monitored by measuring the absorbance of culture broth at 600 nm in a UV/visible Varian Cary 50 Conc spectrophotometer (Italy) after suitable dilution with water. The biomass concentration was evaluated using a calibration curve.

Cell Dry Weight

Cell suspension (50 mL) was centrifuged at 8000 rpm for 20 min in a centrifuge B16 B. Braun Biotech International (Germany) and washed with distillated water. The sediment was dried at 90 °C for 24–36 hrs until its weight remained constant.

PHA Content

The PHA content was determined by gas chromatography. The type of hydroxyalkanoates in the copolymer was analyzed by GC-MS method. Data was supported by FTIR spectroscopy.

Gas Chromatography

Cell suspension (5 mL) was placed in polytetrafluroethylen-lined screw-cap test tube and centrifuged at 8000 rpm for 20 min. The pellet was washed with distilled water and re-centrifuged. Two mL of chloroform, 0.85 mL of methanol containing approximately 0.4 mg/cm^3 of benzoic acid as an internal standard and 0.15 mL of concentrated sulfuric acid were added to it and then heated for 10 minutes at 100 °C. The resulting solution was sonicated for approximately 10 min until the solution was completely homogenized and reheated for 2 hours at 100 °C then cooled rapidly. One mL of distilled water was added to the solution and vortexed for 1 minute. The organic and aqueous layers were allowed to separate. The bottom organic phase (methyl ester of alkanoates) was transferred into a fresh tube. One μL of this solution was analyzed with a Phillips gas chromatograph. The chromatograph was operated with DP-5 capillary column (50 m length, 0.25 I.D., 0.25 μm film), a split injection ratio of 1:15 and Argon as the carrier gas (1.5 mL/min). A flame ionization detection (FID) unit was operated at 280 °C with an injection temperature of 250 °C. The oven temperature was set at 75 °C for 2 min, increased at 10 °C/min to 100 °C, and

then to 230 °C at 35 °C/min and held for 2 min. The external standards were PHB and P(3HB/3HV) cotaining12% valerate (Sigma). The retention times of the methyl esters of 3HB, 3HV and benzoic acid were 1.1, 1.9 and 3.6 min, respectively.

GC-MS

The GC-MS system incorporated a column (HP-5MS, 50 m length, 0.2 I.D, 0.33 μm film) coupled with a mass spectrometer GC-MS-5973 mass selective detector (Aligent,Germany). The oven temperature was set at 70 °C for 1 minute, increased at 10 °C/min to 100 °C, then to 230 °C at 30 °C/min and held for 10 min. One μL of methyl ester phase was injected into GC-MS system.

FTIR Spectroscopy

The PHAs of the cell pellets were extracted and the resulting PHAs were purified and lyophilized.[7] Tow mg of lyophilized PHA were thoroughly mixed with 100 mg KBr (spectroscopic grade) using mortar and pestle. From this mixture 15 mg was used for making KBr pellets. The pellets were dried at 100 °C for 4 h. FTIR spectrum was taken using Perkin Elmer (USA) model 1720 Fourier transform IR spectrometer.

Results and Discussions

Growth of Positively Stained Isolates

Among positively stained isolates which were identified as *Bacillus* sp., the PHA content of six isolates was considerable

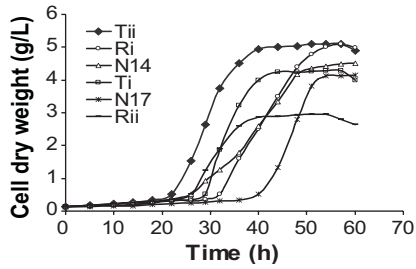

Figure 1.
Growth curve of 6 isolates with high PHA content.

(Table 1). Figure 1 shows the growth curve of these isolates on 10 g/L fructose as carbon source and mineral salt medium for a period of 60 h.

Preliminary experiments show that the PHA synthesis begins gradually at post-exponential phase and reaches the maximum at the early stationary phase. As shown in Figure 2, the post-exponential phase of Tii was 36 h after inoculation and the amount of its biomass reached to a maximum of 5.1 g/L after 50 h. The maximum cell dry weight of Ri strain was the same as that of Tii, but the post-exponential phase of this strain started after 51 h of inoculation. Since Tii strain reached to its maximum production with highest PHA content in a shorter time compared to the Ri, it was selected as the best PHA producer.

N14 strain was also selected because of its ability to produce small amounts of hydroxyvalerate in the copolymer backbone. This strain was able to produce 2.2 g/L of PHA.

The growth curves in Figure 1 and 2 show long lag period which may be due to

Table 1.
Cell dry weight, PHA% and polymer composition of different isolates grown in date syrup as carbon source.

Isolate code	Cell dry weight (CDW g/L)	PHA content (g/L)	PHA %	Monomer Composition of PHA
Tii	5.1	3.6	71	HB[a)]
N17	4.1	1.5	37	HB/HV[b)] (4% HV)
Ri	5.1	3.5	69	HB
N14	4.5	2.2	49	HB/HV (10% HV)
Rii	3.1	2.1	68	HB
Ti	4.3	2.1	49	HB
Ralstonia eutropha	2.8	0.98	35	HB
Bacillus megaterium	3.3	1.72	52	HB

[a)] hydroxybutyrate.
[b)] hydroxyvalerate.

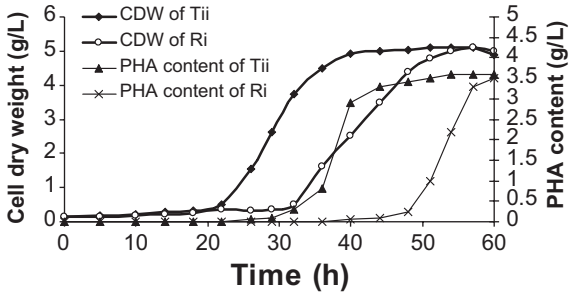

Figure 2.
Growth curve and PHA content of Tii and Ri isolates (CDW: Cell dry weight).

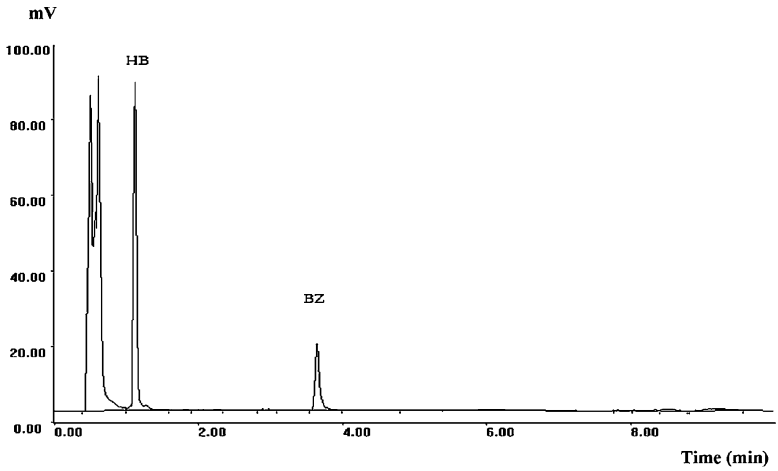

Figure 3.
GC analysis of the methyl ester extracted from Tii (HB: hydroxybutyrate; BZ: Benzoic acid).

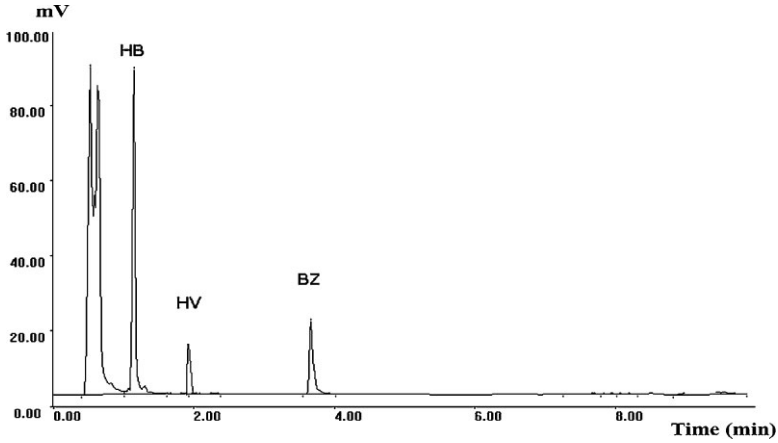

Figure 4.
GC analysis of the methyl ester extracted from N14 (HB: hydroxybutyrate; HV: hydroxyvalerate; BZ: Benzoic acid).

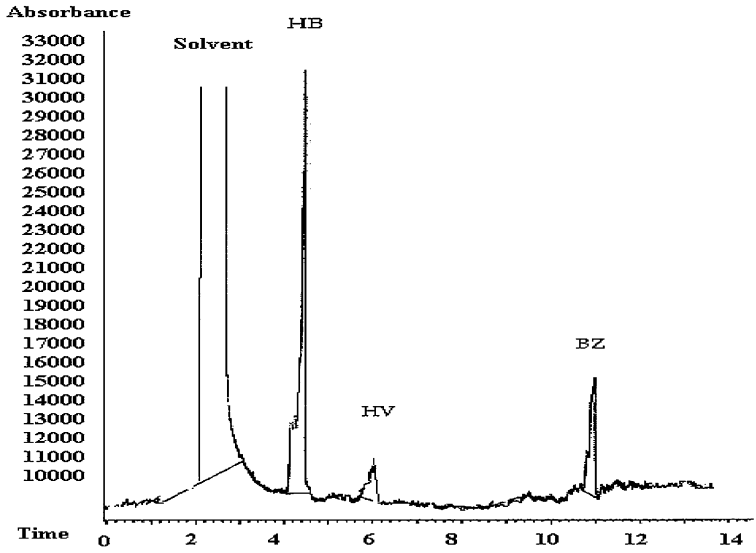

Figure 5.
GC-MS analysis of the methyl ester extracted from N14 (HB: hydroxybutyrate; HV: hydroxyvalerate; BZ: Benzoic acid).

using different medium for seed culture and fermentation medium.

PHA Content and PHA%

Concentration of PHA in the fermentation medium and PHA% of cell dry weight obtained for different positively stained isolates, are shown in Table 1.

This table also includes cell dry weight and composition of the building blocks (monomers) of PHAs. For comparison, corresponding results for *Ralstonia eutropha* and *Bacillus megaterium*[5] are given. Among positively tested isolates, Tii and N14 were selected, because of their relatively high PHA% and type of monomer

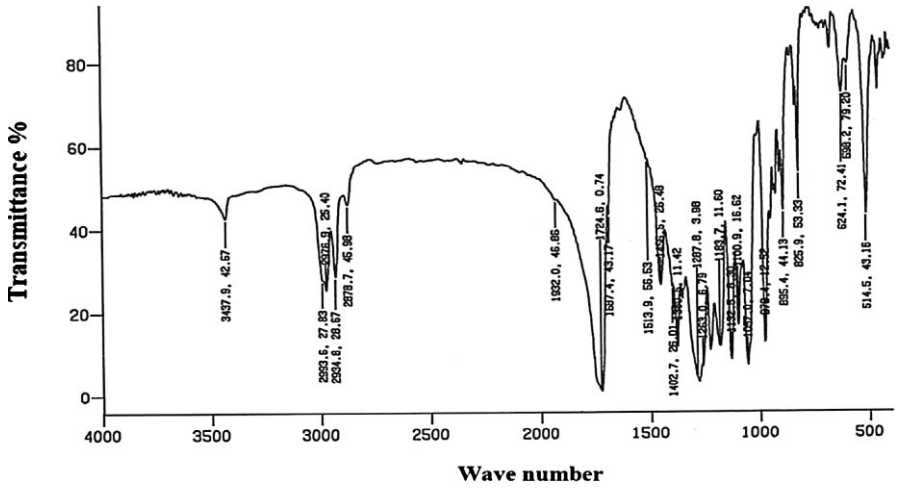

Figure 6.
FTIR spectrophotometer of the PHA extracted from N14.

composition. Between these two isolates, Tii is superior to N14 in terms of PHA content and PHA %, but strain N14 could utilize date syrup for PHB/PHV production with 10 mole% valerate, whereas Tii produces homopolymer of PHB as shown in Figure 3 and 4. *Ralstonia eutropha* as a well-studied microorganism in the literature was inoculated in this medium to compare its performance with isolated strains of this study. Table 1 shows that PHB production of Tii, isolated in this study, is higher than that of both *Ralstonia eutropha* and *Bacillus megaterium* using date syrup as renewable substrate.

Composition of PHA

As shown in Figure 4, the GC chromatograph of PHA produced by N14 indicates that this polymer is a copolymer of β-hydroxybutyrate and hydroxyvalerate P(3HB/3HV) with 10% valerate. This finding was supported by GC-MS and FTIR analysis given in Figure 5 and 6.

Conclusion

Different bacteria strains were isolated from date syrup waste and tested for polyhydroxyalkanoate production using date syrup as carbon source. Based on their performance for PHA production from date syrup, two of them with desirable performance were selected. One of them, named Tii, could produce 5.1 g/L biomass with 71% w/w PHB in cell dry weight, which is higher than the corresponding values of 3.3 g/L biomass with 52% PHB reported in literature.[5] The other one, named N14, could utilize date syrup to produce 2.2 g/L copolymer of PHB/HV with 10 mol% valerate.

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