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Biosynthesis of Polyhydroxyalkanotes in Wild type Yeasts

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Abstract

Biosynthesis of biodegradable polymers polyhydroxyalkanotes (PHAs) have been studied extensively in wild type and genetically modified prokaryotic cells, however the content and structure of PHAs in wild type yeasts is not well documented. The purpose of this study was to screen yeast isolates collected from different ecosystems for their ability to accumulate PHAs. Identification of the isolates and characterization of PHAs produced by the positive isolates were investigated. One positive isolate (strain Y4) was identified by both API20C system and 18S rDNA sequencing. The data revealed that isolate Y4 belongs to the yeast genus *Rhodotorula* and exhibits 18S rDNA similarity value >99% to the species *Rhodotorula minuta*. Quantification of PHAs yield of strain Y4 in glucose, oleic acid and tween 60 containing medium for over a growth period of 96 h gave 2% of PHAs in biomass. The nature of produced PHAs was analyzed by infrared spectroscopy and nuclear magnetic resonance (^{1}H and ^{13}C NMR) and found to contain polyhydroxybutyrate and polyhydroxyvalerate (PHBV).

Key words: *Rhodotorula minuta*, NMR, polyhydroxyalkanotes, spectroscopic analysis, wild type yeasts

Introduction

Among various biodegradable plastics available in the market, there is a growing interest in the group of polyhydroxyalkanotes (PHAs). These are natural polymers produced by a variety of bacteria and they are 100% biodegradable (Doi and Abe, 1990; Holmes, 1988; Kalia *et al.*, 2000). PHAs have been shown to occur in over 90 genera of Gram-positive and Gram-negative bacteria species (Steinbuchel, 1991). Bacteria synthesize and accumulate PHAs as carbon and energy storage materials or as a sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon sources (Steinbuchel and Valentin, 1995).

The major commercial drawback of the so-called bacterial PHAs is their high production cost, making them substantially more expensive than synthetic plastics (Steinbuchel, 1991). Consequently, looking for eukaryotic cell systems like yeast able to accumulate PHAs seems to be a beneficial alternative to the production of PHAs in bacteria. PHA-producing yeast may have potential commercial applications. First large scale fermentation and separations technology are well developed for this organism. Another consideration is that yeast may perform better than bacteria on cheap carbon sources such as molasses. In addition, niche PHA-producing yeast might be found as part of a co-generation scheme in which cells are cultivated for ethanol as another primary product.

The introduction of PHAs biosynthetic genes into yeast is of interest not only from production point of view but also from a metabolic engineering standpoint because it represents the transfer of a foreign metabolic pathway into a host organism to produce a new product. *Saccharomyses cerevisiae* is the model system used in genetic engineering experiments to host PHAs biosynthetic genes and it is not known to accumulate PHAs (Leaf *et al.*, 1996). However, as a eukaryotic cell, *S. cerevisiae* adds a dimension of complexity to the problem of PHAs synthesis because, in contrast to bacteria, biochemical pathways are compartmentalized. Introducing multi-step pathways into yeast is also complicated by the requirement of

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a separate promoter or each gene, as compared to bacteria where operons can be expressed (Abd-El-Haleem *et al*., 2007; Abuelhamd *et al.*, 2007).

On the other hand, looking for wild type yeasts and confirm their ability to accumulate PHAs naturally was the main target of this study. This will introduce us into a new area of research to isolate and characterize the original yeast PHAs biosynthetic genes, which will help in solving the problem of complexity, came from using prokaryotic PHAs biosynthetic genes not only in yeasts but also in higher eukaryotic systems.

Experimental

Materials and Methods

Yeast isolates and screening for PHAs accumulation. Forty yeast strains were isolated from the following samples; molasses, foodstuffs, concentrated juices and soils. Samples were seeded by dilution in YEPD medium (containing 2% (w/v) yeast extract, peptone and dextrose) and the strains were incubated at 30°C for 24h according to Sanni and Lonner (1993). PHA accumulation was screened in yeast isolates on YEPD agar plates containing 25 µg Nile-Red (Sigma) per liter as described elsewhere (Spiekermann *et al.*, 1999). Three lighted isolates were recorded positives; subsequently isolates were subjected for identification using the standard biochemical test API 20C Aux system (BioMerieux Vitek, Inc., Hazelwood, Mo.)

PHAs production. For PHAs production, a stationary-phase culture grown in YEPD medium was harvested by centrifugation and cells were washed once in water and re-suspended in mineral salts medium (MSM) (Schlegel *et al.*, 1961) supplemented with 0.1% glucose, 0.5% of the detergent Tween 60 (Sigma, St. Louis, Mo.), and 0.1% fatty acid (oleic acid). Cells were grown at 30°C for an additional 1 to 6 days before harvest of the cells for PHAs analysis. The pH of the growth media was 6.0.

PHAs extraction. PHAs extraction procedure was performed according to Findlay and White (1983). Lyophilized yeast cell sediments were placed in a Soxhlet extractor lined with glass wool and wrapped with a resistance strip heater. Enough chloroform to amply cover the sample was added, and the sample was sonicated for 10 min. The sample was extracted for overnight in a total of 125 ml of chloroform. The extraction thimble of the Soxhlet extractor was heated so that the chloroform present boiled, maintaining solubility of the polymers. The chloroform was recovered and removed in a rotary evaporator in vacuum. Subsequently, the polymer was redissolved in hot chloroform and PHAs was recovered from the chloroform by nonsolvent precipitation and filtration. Methanol was used as the nonsolvent (4–6 volumes). To determine PHAs yield, the correlation between production of PHAs and dry cell weight was determined (Conover and Iman, 1981).

Spectroscopic analysis of PHAs. The Infra Red (IR) spectroscopic analysis was taken with IR (Bruker). The NMR spectra were recorded on a JOEL ECA 500 spectrometer. The 500 MHz ^{1}H-NMR spectra were recorded from a $CDCl_3$ solution of the PHA (30 mg/ml) at 20°C, 1.30809856 s acquisition times and 12.5250501 KHz spectral width. The ^{13}C-NMR spectra were recorded from a $CDCl_3$ solution of the samples using ^{1}H-decoupling, 0.69206016 s acquisition time and 47.348485 KHz spectral width. ^{1}H-NMR and ^{13}C-NMR chemical shifts were referred to $CHCl_3$ and $CDCl_3$ ($c = 7.24$ ppm and $c = 77.0$ ppm, respectively).

Phylogenetic analysis of strain Y4. The primers used for the amplification and sequencing of 18S-rRNA-encoding genes were those described by Suh and Nakase (1995) and Suh *et al.*, (1996). The PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The sequences were analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences. Selected rDNA sequences were aligned using the ClustalW program (Shingler, 1996). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using ClustalW by distance matrix analysis and the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

Results and Discussion

Yeast isolates and screening for PHAs accumulation. It is established that a considerable number of both Gram-negative and Gram-positive bacteria are able to accumulate PHAs and can grow on different carbon sources. According to the number of carbon atoms in the monomeric units of the PHAs, PHAs-producing bacteria can be generally classified into two groups (Steinbuchel and Valentin, 1995). The first class of bacteria, including *Ralstonia eutropha*, produces short chain length PHA with monomer units ranged from C3 to C5, while the other class, including *Pseudomonas oleovoran*s, produces medium chain length PHB with monomer units from C6 to C14 (Anderson and Dawes, 1990).

In contrast, PHAs in wild type yeast have received brief mention in the literature. Nuti and Lepidi (1974) and Safak *et al.*, (2002) reported the presence of PHAs in wild type yeasts, this is not in agreement with our

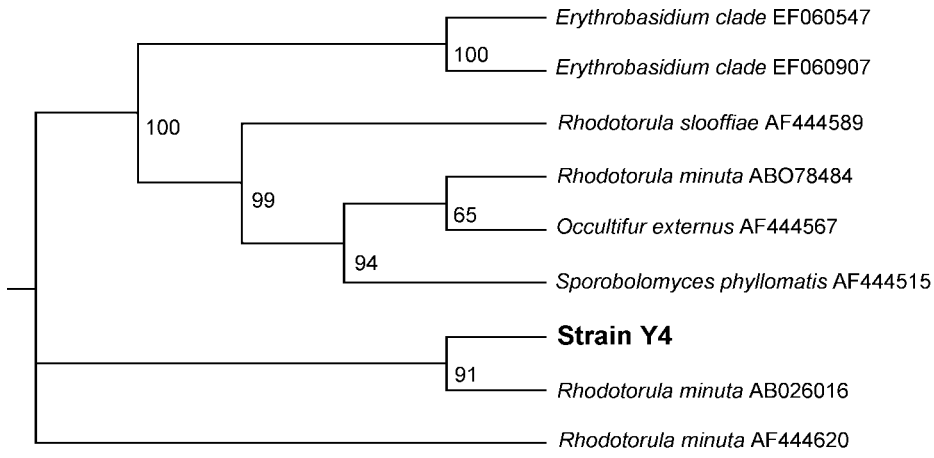

Fig. 1. Phylogenetic tree showing the relationships among selected strains (in boldface) and published 18S rDNA sequences (their GenBank accession numbers are putted in the tree); bootstrap values per 100 bootstrap analyses are also presented.

experience, and it appears that the methods employed by theses authors could not distinguish between PHB and monomeric 3HB moieties. Oligomeric PHB of 120–200 subunits has been reported in yeast at about 0.0002% of dry weight (Safak *et al.*, 2002), and is believed to function not as a storage material but as a membrane transport channel in complex with calcium ion and polyphosphate in all living cells.

In the present work, screening among forty yeast isolates for presence/absence of PHAs accumulation was performed using the Nile-red staining assay. Under the UV transilluminor, three isolates (Y1, Y4 and Y8) exhibited strong fluorescence signals in comparing to other yeast isolates. It is known that the Nile-red stain emitted strongly positive red fluorescence signals only with hydrophobic compounds like PHAs and lipids. Nile-red intended to show any lipid particles inside the cells and it did not react with any tissue constituent except by solution and could be detected by fluorescence spectroscopy (Gorenflo *et al.*, 1999; Spiekermann *et al.*, 1999).

To confirm the Nile red assay' results, cells of each strain were grown in 250 ml Erlenmeyer flasks containing 50 ml MSM medium at 30°C on a rotary shaker at 200-rev/min. All grown flasks were exposed to the same conditions, and three separate flasks were inoculated from the same culture in each case. Data from shaken flasks indicated that strains Y1 and Y8 did not contain measurable PHAs (data not shown); while strain Y4 contained 2.01% PHAs of the cell dry weight.

Subsequently, the Y4 isolate was subjected for biochemical identification using API 20C Aux system. The results showed correct identification of the isolate without the need to supplement extra tests and placed it under the yeast species *Rhodotorula minuta*. As well as, partial 18S ribosomal RNA nucleotide sequence (532 bp) was determined of the strain Y4. As shown in Figure 1, phylogenetic analysis indicates that strain Y4 is belonging to the yeast species *Rhodotorula minuta* exhibiting similarity value >99%. The phylogenetic relationships of this yeast correlate well with the biochemical identification assay. The GenBank accession number for strain Y4 nucleotide sequence is FJ222373.

Spectroscopic analysis of PHAs. The determination of the monomer composition of Poly(3HB) and

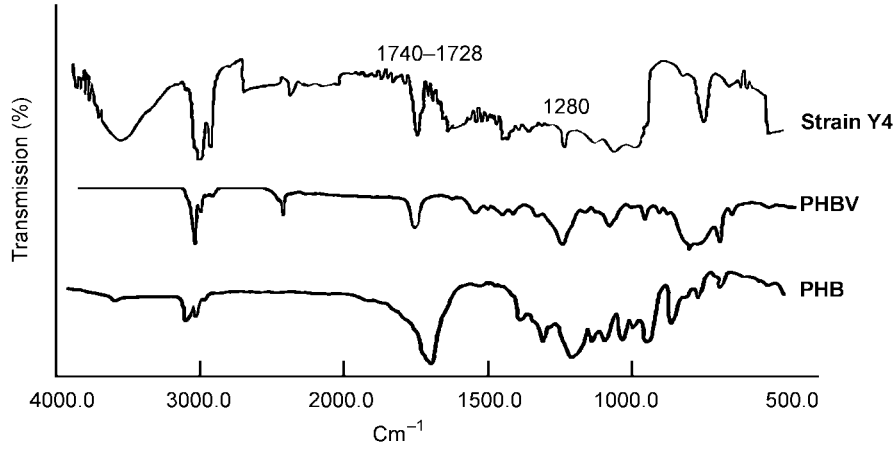

Fig. 2. Infrared-spectra (IR) of PHAs: Standard PHB; Standard PHB-co-PHV; PHA polymers produced in strain Y4.

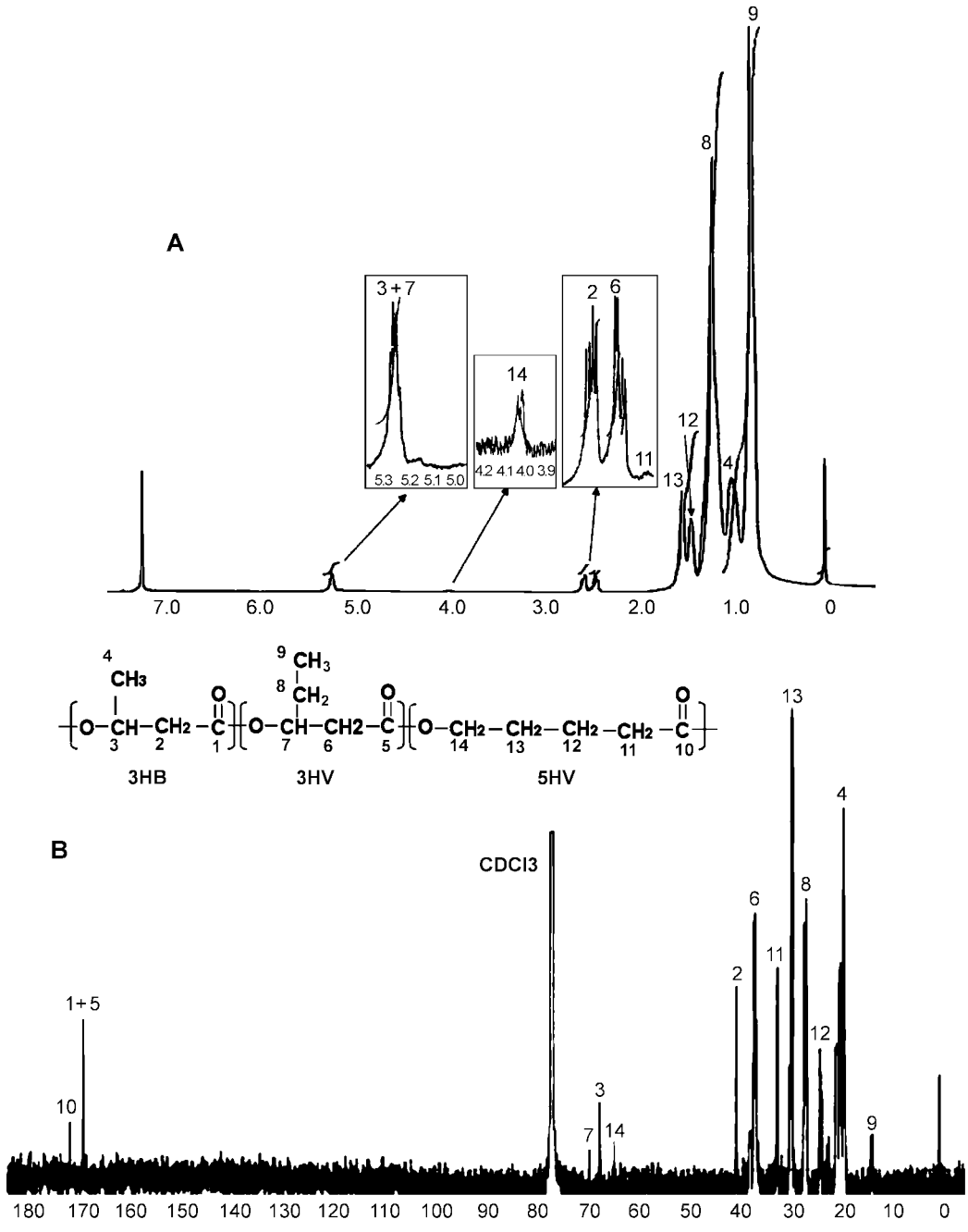

Fig. 3. ^{1}H NMR (A) and ^{13}C NMR (B) spectra analysis of the polymer produced by the wild type yeast strain Y4.

Poly(3HB-co-3HV) is relatively simple, and can easily be acquired by means of GC, GC-MS, and HPLC. However, with these methods it is not possible to detect the presence of two or more distinctive polymers, because the polymers are hydrolyzed before analysis (Anderson and Dawes, 1990; Doi, 1995). It is, however, possible to determine this by means of NMR: the complexity of the carbonyl signals in the ^{13}C-NMR spectrum can be used to determine whether PHA consists of homopolymers or a copolymer. In addition, NMR has also been used in the determination of the chain dynamics, the crystallinity and the pathways involved in PHA synthesis (Anderson and Dawes, 1990; Doi, 1995; Doi *et al.*, 1986; Williams and Fleming, 1996).

Therefore, the extracted PHA-like polymers from strain Y4 were subjected for IR and NMR (^{1}H and ^{13}C) spectroscopic analysis. IR spectroscopy showed intense absorptions typical to PHA at 1728–1740 cm^{-1} and at 1280 cm^{-1} corresponding to C = O and C-O stretching groups, respectively (Fig. 2). IR analysis indicated also that the polymer is mostly polyhydroxybutyrate (PHB) with polyhydroxyvalerate (PHV). As shown in Figure 3, both ^{1}H and ^{13}C Nuclear magnetic resonance spectra confirmed poly(3HB-co-3Hv-co-5HV) copolymer production in strain Y4. The

spectra demonstrated the presence of signals indicating 3-PHB, 3-HV and 5-HV side chains.

Out of the above results, it appears that yeast isolate Y4 produce a mixed polymer and that their yields and types may possibly be varied using various other substrates and culture conditions. Thus, these results attempted for the first time monomer detection by spectroscopic analysis of PHA polymers synthesized in wild type yeasts. PHAs in wild type yeast were originally reported by Reusch (1999) followed by Seebach *et al.* (1992) and Leaf *et al.* (1996). Theses authors have not attempted monomer detection by ^{1}H and/or ^{13}C NMR, though such species may be relatively insoluble in chloroform. However, Reusch (1999) suggests that native low molecular mass PHB may be complexes with various lipids or proteins, making its solubility behavior unpredictable. The molecular cloning of PHAs biosynthetic genes to identify the pathway of synthesis by isolated strain Y4 is currently underway.

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