JASEM ISSN 1119-8362

Full-text Available Online at www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *September*, 2007
Vol. 11(3) 5 - 10

Biosynthesis of polyhydroxyalkanotes in wildtype yeasts

[1*]DESOUKY A.M. ABD-EL-HALEEM; [1]SAHAR A. ZAKI; [2]ASHRAF T. ABUELHAMD; [3]AMRO AMARA; [1]GADALLAH M.S. ABOELREESH

[1 & 3] Environmental Biotechnology Department & Protein Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, New Burg-Elarab City, Alexandria, Egypt.
[2] Microbiology Department, Faculty of Science, Alazhar University, Cairo, Egypt.

ABSTRACT: Biosynthesis of the biodegradable polymers polyhydroxyalkanotes (PHAs) are studied extensively in wild type and genetically modified prokaryotic cells, however the content and structure of PHA in wild type yeasts are not well documented. The purpose of this study was to screen forty yeast isolates collected from different Egyptian ecosystems for their ability to accumulate PHAs. Identification of the isolates and characterization of PHAs produced by the positive strains in the Nile-red staining assay was envisaged. One positive isolates which was identified using the API 20C yeast identification system as *Rhodotorula minuta* strain RY4 produced 2% of PHA in biomass, in glucose, oleic acid and tween 60 containing medium, over a growth period of 96 h. The nature of the PHA thus produced was analyzed by infrared spectroscopy and nuclear magnetic resonance (^{1}H and ^{13}C NMR) and found to contain polyhydroxybutyrate and polyhydroxyvalerate. This study shows for the first time monomer detection by ^{1}H and/or ^{13}C NMR of PHA polymers synthesized in wild type yeasts. @ JASEM

Plastic materials have become an integral part of contemporary life because they possess many desirable properties, including durability and resistance to degradation. Over the past 10-20 years, their widespread uses have been increasingly regarded as a source of environmental and waste management problems (Anderson and Dawes 1990). These problems have created much interest in the development and production of biodegradable plastics. Estimates of the current global market for these biodegradable plastics range up to 1.3 billion kg per year (Kalia et al. 2000, Song et al. 1999). Among the various biodegradable plastics available, 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). 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-produced bacterial PHAs is their high production cost, making them substantially more expensive than synthetic plastics (Poirier et al. 1995). Therefore, 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. Finally 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. Therefore, the purpose of this study was to screen and characterize wildtype yeast isolates for their ability to accumulate PHAs.

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% (wt/v) yeast extract, peptone and dextrose) and the strains were incubated at 30°C for 24h according to Sanni and lonner (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.).

PHA production and extraction: For PHA production, a stationary-phase culture grown in YEPD medium was harvested by centrifugation and cells were washed once in water and resuspended in mineral salts medium (MSM) (Schlegel et al. 1961) supplanted 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 PHB analysis. The pH of the growth media was 6.0.

Current address of the corresponding author: Biological Sciences Department, Collage of Arts and Sciences, Qatar University, Doha, Qatar, Post Code 2713. Phone: (+974) 4852702 Fax: (+974) 835061 E-mail: abdelhaleemm@yahoo.de

PHB 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 PHA was recovered from the chloroform by nonsolvent precipitation and filtration. Methanol was used as the nonsolvent (4–6 volumes). To determine PHB yield, the correlation between production of PHB and dry cell weight was determined by Spearman's test (Conver 1971).

In vitro PhaC enzyme assays: PhaC synthase activity was determined spectrophotometrically by monitoring the release of CoA at 412 nm (Valentin and Steinbüchel. 1994). The standard assay contained 1 mM DTNB dissolved in Tris/HCl buffer 50 mM Ph 7.5), 20 mM $MgCL_2$ and 0.75, 1.25, 2.5, 3.7 and 5 µM of DL-β-Hydroxybutyryl CoA respectively in Tris/HCl buffer, (150 mM, pH 7.5) at 37^{o}C. The kinetic parameters for the release of CoA were determined using Lineweaver-Burk plots. In each assay, blank tubes (without enzyme) were measured to correct for spontaneous hydrolysis of substrate. One U of enzyme is defined as the amount which produced 1 µmol product per minute under assay conditions.

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).

RESULTS AND DISCUSSION

Yeast isolates and screening for PHA accumulation: It is established that a considerable number of both Gram-negative and Gram-positive bacteria are able to accumulate PHA and can grow on different carbon sources. According to the number of carbon atoms in the monomeric units of the PHA, PHA-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, PHA in wild type yeast has received brief mention in the literature. Nuti and Lepidi (1974) and Safak et al. (2002) reported the presence of PHA in wild type yeasts, this is not in agreement with our 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 (Seebach et al. 1994), 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 yeast isolates for presence/absence of PHA accumulation was performed using the Nile-red staining assay. Under the UV transilluminor, three isolates (Y1, Y4 and Y8) exhibited a strong fluorescence in comparing to other yeast isolates. This finding is considered an evidence for the specificity of the Nile-red staining approach to screen new microbial isolates for PHAs production. 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 or flow cytometery (Gorenflo et al. 1999, Spiekermann et al. 1999).

Subsequently, the three positive isolates were subjected for biochemical identification using API 20C Aux system. The results showed correct identifications of the isolates without the need to supplement extra tests. The isolates identified as *Kebeckera* spp. (K-Y1), *Rhodotorula minuta* (R-Y4) and *Candida norvegensis* (C-Y8), respectively.

In vivo PhaC enzyme assays and PHA production: Afterward, biochemical identified isolates K-Y1, R-Y4 and C-Y8 were subjected for *In vivo* PhaC enzyme assay using crude extracts of each strain. The apparent K_m and V_{max} for the strains is presented in Table 1.

Table 1: PhaC synthase activity in strains KY1, RY8 and CY4

Strain	K_m	V_{max}
R-Y4	63.99	329.8
C-Y8	4.62	102.4
K-Y1	1.037	70

The results revealed that PhaC synthase is highly expressed in strain R-Y4 in compare to the other two isolates. Kinetic parameters for release of CoA at different substrate condition were determined as in Figure 1. It is known that PHA synthase requires a phosphopantheine prosthetic group for activity (Gerngross et al. 1994). Thus, the results obtained indicated that RY4 cells could perform this process.

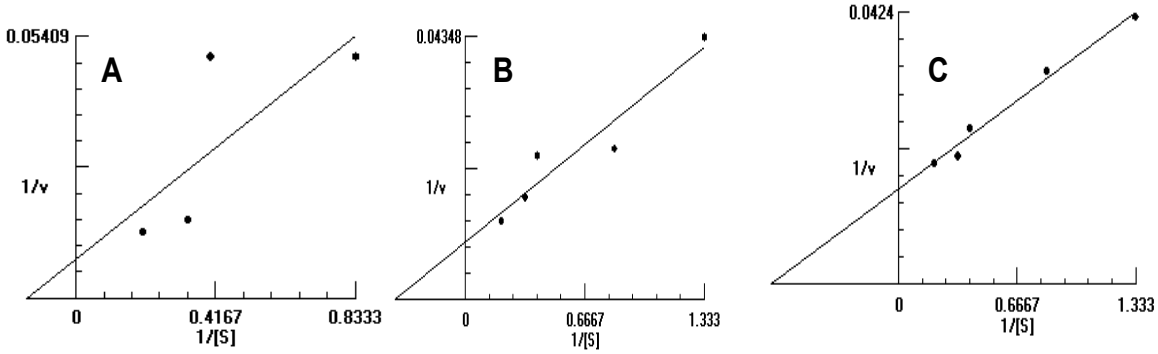

Fig 1: Kinetics of PhaC enzyme activity in strains RY4 (A), CY8 (B) and KY1 (C). S refers to substrates in μM l^{-1}, while v refers to the specific activity in μM min^{-1} mg^{-1}.

To confirm these 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 shack flasks indicated that strains K-Y1 and C-Y8 did not contain measurable PHA. In contrast, strain R-Y4 that grown under the same conditions contained 2% PHA of the cell dry weight; therefore, PHA produced by *Rhodotorula minuta* strain R-Y4 was subjected for further spectroscopic analysis.

*Spectroscopic analysis of PHAs***:** The determination of the monomer composition of Poly(3HB) and 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 (Doi et al. 1985, Anderson and Dawes 1990, Doi 1995, Williams. and Fleming 1996). Therefore, the extracted PHA-like polymers from strain RY4 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 R-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 R-Y4 produce a mixed polymer and that their yields and types may possibly be varied using various other substrates and cultural 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 (1989) 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 (1989) suggests that native low molecular mass PHB may be complexes with various lipids or proteins, making its solubility behavior unpredictable.

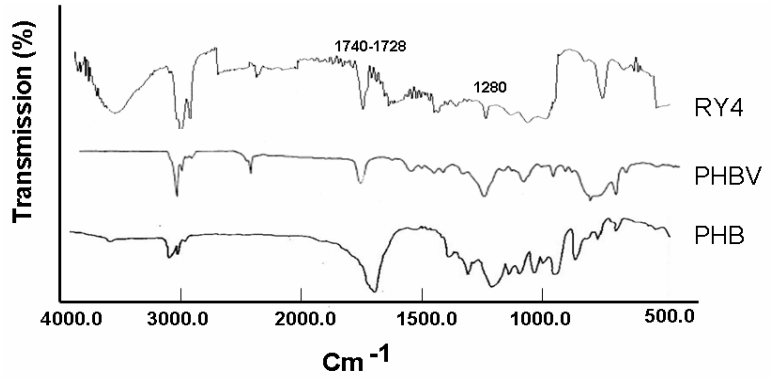

Fig 2: Infrared-spectra (IR) of PHA: Standard PHB; Standard PHB-co-PHV; PHA polymers produced in strain RY4.

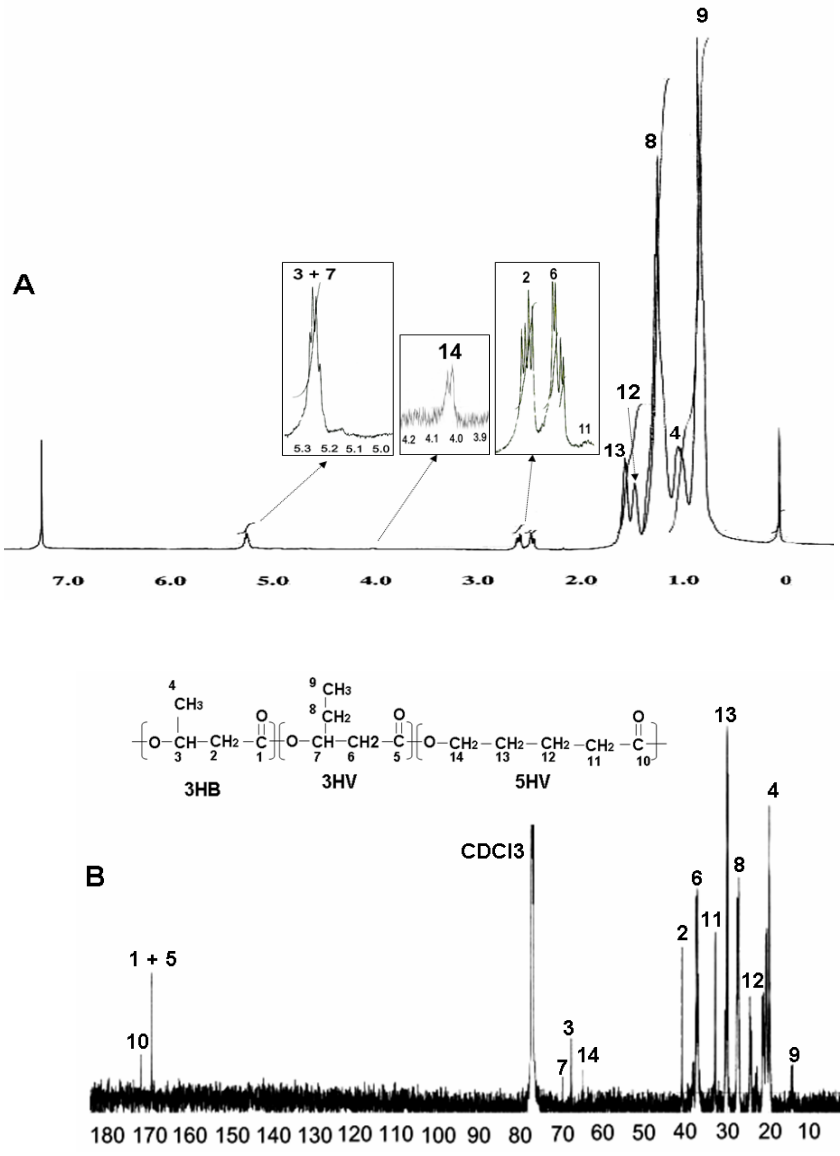

Fig 3: ^{1}H NMR (A) and ^{13}C NMR (B) spectra analysis of the polymer produced by the wildtype yeast strain RY4

Acknowledgement: This research project is carried out under the program of the Egyptian Strategy for Biotechnology and Genetic Engineering contract number 83. The program is administrated by the Science and Technology Center at the Academy of Scientific Research and Technology.

REFERENCES

Anderson, A. J. and Dawes, E. A. (1990), Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54: 450–472.

Conver, W. J. (1971), Practical non parametric statics, John Weleyard and Sonc. Inc, New York.

Doi, Y. (1995), Microbial synthesis, physical properties, and biodegradability of polyhydroxyalkanoates. Macromol. Symp. 98: 585-599.

Doi, Y. and Abe, C. (1990), Biosynthesis and characterization of new bacterial copolyester of 3-hydroxyalkanoates and 3-hydroxy-ω-chloroalkanoates. Macromolecules 23: 3705–3707.

Doi, Y., Kunioka, M., Nakamura, Y. and Soga, K. (1986), Nuclear magnetic resonance studies on poly(L-hydroxybutyrate) and a copolyester of L-hydroxybutyrate and L-hydroxyvalerate isolated from Alcaligenes eutrophus H16. Macromolecules 19: 2860-2864.

Findlay, R.H. and White D.C. (1983), Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. Environ. Microbiol. 45: 71-78.

Gerngross, T.U., Snell, K.D., Peoples, O.P., Sinskey, A.J., Csuhai, E., Masamune, S. and Stubbe, J. (1994), Overexpression and purification of the soluble polyhydroxyalkanoates synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry 33: 9311–9320.

Gorenflo, V., Steinbuchel, A., Marose, S., Rosenberg H. and Scheper, T. (1999), Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining. Appl. Microbiol. Biotech. 51, 765–772.

Holmes, PA. (1988), Biologically produced PHA polymers and copolymers. In: Bassett DC, editor. Developments in crystalline polymers. London: Elsevier. 2: 1–65.

Kalia, V.C., Raizada N. and Sonakya, V. (2000), Bioplastics. J. Sci. Ind. Res. 59: 433–445.

Leaf, T. A., Peterson, M. S., Stoup, S. K., Somers, D. and Srienc, F. (1996), Saccharomyces cerevisiae expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiology 142: 1169-1180.

Lee, S. Y. (1996) Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1– 14.

Nuti, M.P. and Lepidi, A.A. 1974. Polyhydroxybutyrate in *Saccharomyces cerevisiae* and its significance in the fermentation process. Proc Fourth International symposium on Yeasts, Part 1: 123-124.

Poirier, Y., Nawrath, C. and Somerville, C. (1995) Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Biotechnology 13: 142–150.

Reush, R. N. (1989), Poly-β-hydroxybuyrate/calcium Polyhydroxyphosphate, a biodegradable thermoplastic produced in transgenic plants. Science 256: 520-523.

Safak, S., Mercan, N., Aslim, B. and Beyatli, Y. (2002), A study on the production of Poly-Beta-Hydroxybutyrate by some eukyryotic microorganisms. Turk. Elect. J. Biotech. 11-17.

Sanni, A.I and Lonner, C. (1993), Identification of yeast isolates from Nigerian traditional alcoholic beverages. Food Microbiol. 10: 517-523.

Schlegel, H.G., Kaltwasser, H.and Gottschalk, G. (1961), Ein Submersverfahren zur Kultur wasserstofoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38: 209-222.

Seebach, D., Beck A.K., Breitschuh, R. and Job, K. (1992) Direct degradation of the biopolymer poly[(*R*)-3-hydroxybutyric acid] to (*R*)-3-hydroxybutanoic acid and its methyl ester. Org. Synth. 71: 39–47.

Song, S.S., Hein, S. and Steinbuchel, A. (1999), Production of poly(4- hydroxybutyric acid) by fed-batch cultures of recombinant strains of *Escherichia coli*. Biotechnol. Lett. 21: 193–197.

Spiekermann, P., Reham, B. H., Klascheuer, R., Baumeister, D. and Steinbuchel, A. (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanolic acids and other lipid storage compounds. Arch. Microbial. 171: 73-80.

Steinbuchel, A. (1991) Polyhydroxyalkanoic acids, p.123-216.In D. Byrom (ed.), Novel biomaterials from biological sources. MacMillan, New York, N.Y.

Steinbuchel, A. and Valentin, H. E. (1995), Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128: 219–228.

Valentin, H. E. and Steinbüchel, A. (1994), Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thiolase for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. 40: 699-709.

Williams, D. H. and Fleming, I. (1996), Spectroscopic methods in organic chemistry, 5th ed. McGraw-Hill International (UK) Ltd., London, United Kingdom.