Genetic engineering of *Schizosaccharomyces pombe* to produce Bacterial Polyhydroxyalkanotes

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

1 & 2 Environmental Biotechnology Department & Protein Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, New Burg-Alarab City, Alexandria, Egypt.

3 Microbiology Department, Faculty of Science, Alazhar University, Cairo, Egypt.

ABSTRACT: A commercial use of microbial produced products, like polyhydroxyalkanotes (PHAs), in the sense of an environmental precaution appears meaningful and necessary. In order to more economically produce microbial products, this investigation was focused on suitable producers, like the yeast *Schizosaccharomyces pombe*. Since it is not capable of the PHA synthesis, easily cultured and they must be modified genetically. Therefore, the genes of the PHB biosynthesis pathway of *Ralstonia eutropha* (phaB; and PHA synthase,phaC) in *Schizosaccharomyces pombe* (fission "yeast") is almost as easily cultured and manipulated as yeast, it is well characterized as to classical and molecular genetics, its nuclear genome has been sequenced, and it is an alternative fungal model system, comparable to that of the yeast, *Saccharomyces cerevisiae*. Based on a dominant marker and a target for multiple integration of an expression cassette into the genome, strain Q01 has been reported to produce Bacterial Polyhydroxyalkanotes (PHA) biosynthetic pathway of *Ralstonia eutropha* (3-ketothiolase,phaA or bktB; acetoacetyl-CoA reductase,phaB; and PHA synthase, phaC) in *Schizosaccharomyces pombe* strain Q01 through an integrative shuttle vector pYIplac128.

MATERIALS AND METHODS

**Strains and culture conditions**: Plasmids and yeast shuttle vectors were maintained and propagated in *E. coli* (XL1-blue) according to Sambrook et al. (1989). *Schizosaccharomyces pombe* strain Q01 was obtained from Stratagene (La Jolla, CA, USA). *Schizosaccharomyces pombe* harboring plasmids were maintained and grown in either leucine deficient medium (0.67% Yeast nitrogen base without amino acids [Sigma, St. Louis, Mo. USA]; 0.05% ammonium sulfate, 2% glucose and 0.4% of the appropriate amino acids dropout supplements per liter) or in EMM thiamine agar plates obtained from Stratagene (La Jolla, CA, USA). For routine analysis, 0.002 vol of a solution of 0.25 mg Nile-red or Nile blue A (Sigma, St. Louis, Mo. USA) per ml dimethylsulfoxide (DMSO) was added to the sterilized medium to give a final concentration of 0.5 µg dye per ml medium. The agar plates were exposed to Ultra violet light (312 nm) after appropriate cultivation periods to detect accumulation of PHAs and other lipid storage compounds (Spiekermann et al. 1999). For PHB accumulation up to 9.018 % PHB. The presence of heterologous DNA in the transgenic yeast was examined by means of Western blot analysis. In addition, both PHA synthase activity and kinetics were determined. The U/V, 1H and 13C NMR spectral analysis have confirmed that the polymer produced by the yeast *S. pombe* strain Q01/PHB is a pure homopolymer of 3-hydroxybutyric acid.
production, a stationary-phase culture of transgenic yeast was grown in YPOD broth medium containing 0.5% yeast extract, 0.5% Bactopeptone, 0.1% oleic acid, 0.1% glucose and 0.5% Tween 40 at pH 7. Growth was carried out in 250 ml Erlemeyer flasks containing 50 ml culture medium at 30°C on a rotary shaker at 200-rev/min for 96 hours. Yeast dry weight was measured as described by Riis and Mai (1988).

Plasmid construction: Plasmid pBBR68 (Spiemann et al. 1999) carries the entire PHB operon of Ralstonia eutropha, served as the source of PHB genes. Plasmid pBBR68 was digested by BamHI and EcoRI, and the 5.2 kb fragment harboring the entire genes of PHB was ligated to the cohesive ended pYIplac128 integrated vector (Gietz and Sugino, 1988) to create pYI-PHB. The pYI-PHB plasmid equipped with the expression cassettes phbC, phbA and phbB, was linearized by digestion with EcoRV and transformed into S. pombe Q01 by Lithium acetate procedure (Gietz et al. 1992). Transformants were recovered on leucine deficient medium.

Western blot analysis: Protein concentration were determined by Bradford method (Bradford 1976), using bovine serum albumin as a standard. Western blotting was performed using the Semidry Fastblot apparatus (Biometra, Germany). Protein was detected applying the respective monospecific, polyclonal anti-PhaC antiserum and alkaline-phosphatase-antibody conjugate as second antibody. The SDS-PAGE gel, Whatman paper and membrane, SDS-PAGE gel, Whatman paper and cathode (Kyhse-Andersen, 1984). The chromogenic visualization of the bound antibodies was done by immuring the membrane in a 10 ml solution of 5-bromo-4-chloro-3-indolyl phosphate tablets solution for 10-30 min until the reaction complex was visualized clearly. The color reaction was stopped by rinsing the membrane with distilled water.

In vitro PhaC Enzyme assay and kinetic: PhaC synthase activity was determined spectrophotometrically by monitoring the release of CoA at 412 nm (Valentin and Steinbuchel, 1993). The standard assay contained 1 mM DTNB dissolved in Tris/HCl buffer 50 mM Ph 7.5), 20 mM MgCl2 and 0.75, 1.25, 2.5, 3.7 and 5 µM of DL-DTNB dissolved in Tris/HCl buffer 50 mM Ph 7.5), 20 mM MgCl2 and 0.75, 1.25, 2.5, 3.7 and 5 µM of DL-DTNB dissolved in Tris/HCl buffer, (150 mM, pH 7.5) at 37°C. The kinetic parameter for the release of CoA was 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 was defined as the amount which produced 1 µmol product per minute under assay conditions. In addition, kinetic parameters for release of CoA at different substrate condition were determined. The apparent kinetic parameters were estimated from Lineweaver-Burk plots; $K_m$ and $V_{max}$ were calculated for each enzyme.

Production and analysis of PHB: All experiment and results were compared with PHB standard authentic sample obtained from wild type Ralstonia eutropha and confirmed as a pure P(3-β-hydroxybutyric acid) by GC/MS. For production of PHB, transgenic yeast was grown in YPOD broth medium in 250 ml Erlemeyer flasks containing 50 ml culture medium at 30°C on a rotary shaker at 200-rev/min for 96 hours. 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 (Findlay and White 1983). 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 filtered through a Pasteur pipette plugged with glass wool and PHB was recovered from the chloroform by non solvent 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 order to investigating the absorption behavior of the PHB product in the UV/Vis spectral range, according to the protocol described by Ugur and Sahin (2002), precipitates prepared above were collected by centrifugation, deride and converted to crotonic acid by treatment with concentrated sulfuric acid. Absorbance spectra were determined by scanning the samples between 200 and 300 nm with a UV/Vis spectrophotometer (Spekol 1100, analytikjena, Germany). 1H NMR and 13C NMR spectra were recorded in CDC13 solutions on a JOEL GX5 500 spectrometer (500 MHz for both 1H and 13C), using CDC13 as internal standards.

RESULTS AND DISCUSSION

Cloning and Expressing of PHB in transgenic yeast: As shown in Figure 1, the expression cassette phbC, phbA and phbB, was cloned into the integrative yeast shuttle vector Yiplac128 (Gietz and Sugino, 1988) and then the EcoRV-linearized pYI-PHB plasmid, carrying the entire operon of PHB, was attempted to clone that complements the leu2 phenotype by transformation by using LEU2 as the selectable marker. Schizosaccharomyces pombe strain Q01, which contained the leu2 mutation resulting in leucine auxotrophy, was used as the recipient. The pYI-PHB vector was digested with EcoRV, which cuts once in LEU2 gene, and transformed into the yeast strain S. pombe, Q01 for high frequency targeted integration (Orr-Weaver et al. 1983). The deletion allele leu2-Δ1 is a partial deletion of the LEU2 gene between the EcoRI and ClaI sites leaving about 110 bp of homology between the break point and the EcoRI site (Gietz and Sugino. 1988). This amount of homology is apparently sufficient to produce numbers of LEU2 transformants recovered when S. pombe strain Q01 is transformed with EcoRV-cut pYI-PHB vector. The linearized PHB plasmid was transferred to Schizosaccharomyces pombe strain Q01, by Lithium acetate procedure and the transformants were recovered on media without leucine. Leucine-prototrophic transformants were selected from YNB agar or EMM thiamine agar plates as a leucine deficient medium and restricted onto the same leucine deficient medium supplemented with 0.5µg/ml Nile-red. The agar plates were exposed to 312 nm UV light after appropriate cultivation to detect the accumulation of PHAs. Plasmid free-Schizosaccharomyces pombe, Q01 host strain was also cultivated on EMM agar plates.
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supplemented with 0.5 µg/ml Nile red and appropriate amount of leucine and then exposed to UV light to ensure that *S. pombe*, Q01 can not accumulate PHA. UV-visible transformants were selected and the cosmids of these colonies were isolated and analyzed by restriction analysis. An approximately 9.5-kbp plasmid was isolated, and this plasmid corresponded to an estimated 5.2-kbp entire PHB DNA insert.

**Fig 1:** Diagram of plasmid YIplac128. Details of construction are given in the text. The linear map represents the PHB operon of *R. eutropha* derived from plasmid pBHR68.

**Western blot analysis and enzyme assay:** Recombinant yeast transformed with pYI-PHB designated *Schizosaccharomyces pombe* Q01/PHB was tested for PHA synthase expression by Western blotting using the specific anti-serum toward the C-terminal oligopeptide of PhbCRe (Fig. 2). After the cells were grown to the stationary phase in YPOD broth medium, whole cell extracts of the transgenic strain *S. pombe* Q01/PHB, along with the plasmid free strain *S. pombe* Q01 as a negative control, were disrupted by sonication (TOMY UD-200). As shown in Figure 2, extracts from the transgenic yeast *S. pombe*, Q01/PHB reveal a protein band at approximately 66 kD (Lane 1, Fig. 2), the size of PhbCRe protein that does not appear at extracts from the plasmid free host strain Q01 (Lane 2, Fig. 2). Western blot analysis indicated that the recombinant yeast cells contain PhbC protein present in *Ralstonia eutropha* cells.

**Fig 2:** Detection of PhaC polymerase by Western blot analysis of the recombinant *Saccharomyces pombe* phb-1. (Lane 1), and the Wild type strain Y-51 (Lane 2). Western blotting was performed with polyclonal antibodies raised against PhaC1
In vivo enzyme activities and kinetics of PhaC synthase were determined spectrophotometrically at 412 nm (Valentin and Steinbuchel, 1994), using crude extracts of strain S. pombe Q01/PHB and plasmid free strain S. pombe Q01. The activities against different substrates concentration were plotted using Lineweaver Burk plot model. The apparent \( K_m \) and \( V_{max} \) for the plasmid free strain S. pombe Q01 were 0.33 ± 0.05 mM and 2.7 ± 0.02 nmol/min/mg, respectively, compared to 6.45 ± 2 mM and 175.7 ± 12 nmol/min/mg for the transgenic yeast S. pombe strain Q01/PHB. This further confirms that the PhaC synthase activity in the transgenic S. pombe strain Q01/PHB was well expressed in the cytoplasm with a presence of factors like natural inhibitors could effect on the \( K_m \) value. Kinetic parameters for release of CoA at different substrate condition were determined as in Fig 3.

**PHB production:** For production of PHA from the transgenic yeast strain S. pombe Q01/PHB, cells were grown in 250 ml Erlenmeyer flasks containing 50 ml YPOD culture medium at 30°C on a rotary shaker at 200-rev/min. Since the PHA synthase is well expressed in yeast grown in media containing either Tween alone or Tween supplemented with oleic acid (Poirier et al. 2001), cells were grown in YPOD broth medium supplemented with 0.1% oleic acid and 0.5% Tween 40 and allowed to grow for up to 120h incubation period. The optical densities of the cultures were monitored and cells were harvested for analysis after 96 hours, which corresponds to a culture age 24 h after stationary phase. All grown flasks were exposed to the same conditions, and three separate flasks were inoculated from the same culture in each case.

The amount of PHB in the transgenic yeast strain S. pombe Q01/PHB was 0.002 - 0.112 g/l and the percentage of PHB in these cells was between 0.511509 - 9.017713 % of the dry cell weight (Table 1). While the PHB productivity percentage in the transgenic yeast was the highest after 96h incubation period, the lowest PHB productivity was found after 24h incubation. As shown in Figure (4), the amount of the PHB in cells was monitored over 6 days and the PHB accumulated in cells with increase the incubation period until it reaches the maximum PHB productivity (9.017713 %) at day 4. At days 5 and 6, PHA was found 0.032% and 0.022 % because PHB can be degraded by intercellular depolymerases and subsequently metabolized as carbon and energy source.

**Table 1:** The PHB content of the transgenic S. pombe, Q01/PHB

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Dry Cell weight (g/l)</th>
<th>*PHB (g/l)</th>
<th>**Yield of PHB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.391 ± 0.01</td>
<td>0.002 ± 0.02</td>
<td>0.511509</td>
</tr>
<tr>
<td>48</td>
<td>1.316 ± 0.06</td>
<td>0.082 ± 0.01</td>
<td>6.231003</td>
</tr>
<tr>
<td>72</td>
<td>1.323 ± 0.01</td>
<td>0.106 ± 0.01</td>
<td>8.012094</td>
</tr>
<tr>
<td>96</td>
<td>1.242 ± 0.11</td>
<td>0.112 ± 0.02</td>
<td>9.017713</td>
</tr>
<tr>
<td>120</td>
<td>0.986 ± 0.05</td>
<td>0.032 ± 0.00</td>
<td>3.245436</td>
</tr>
<tr>
<td>144</td>
<td>0.941 ± 0.20</td>
<td>0.022 ± 0.01</td>
<td>2.337938</td>
</tr>
</tbody>
</table>

Analysis of PHB: As shown in Figure 5, the highest UV scanning intensity was recorded at the PHB wavelength (235 nm). The IR spectroscopic analysis gave further insights into the chemical structure without a previous hydrolysis of the polymer and reflects the monomeric units. However, NMR is an important and very sensitive method for determining the domain size and miscibility, which is not easy to identify using conventional microscopic or thermal analysis (Doi et al. 1995) As shown in Figure 6, the IR spectrum of the polymer revealed the presence of marked peaks at wave numbers 3440, 2920–2980, 1720 and 1240–1370 cm⁻¹ representing the presence of O–H bending, two bands of C–H stretch, strong absorption band of aliphatic carbonyl C=O of RCOÀ and C–H band of aliphatic compound respectively. The \(^1^H\) NMR spectral analyses revealed the presence of 3, 2 and 1 protons at chemical shifts 1.2, 2.4–2.6 and 5.3 respectively (Figure 7B). The molecular composition of the polyester as indicated by chemical shifts, generates a structure of (CH2–CH) backbone and assigned the presence of (CH3) group. The \(^1^3^C\) NMR spectrum (at 500 MHz) of the polymer on the other hand showed chemical shifts at 19.87, 40.86, 67.70 and 169.26 ppm, which assigned the presence of (CH3), (CH2), (CH) and (C=O) groups respectively (Figure 7B). The values of chemical shifts as well as the assignments of the signals are in good agreement with those of Ralstonia.

*Corresponding author current address: Biological Sciences Department, College of Arts and Sciences, Qatar University, Doha, Qatar, Post Code 2713. Phone: (+974) 4852702 Fax: (+974) 835061 E-mail: abdelhalacemm@yahoo.de*
eutropha. The characteristic signals for other hydroxyalkanoic acids, however, were totally lacking and confirmed the homopolymeric nature of the compound isolated from the transgenic yeast *S. pombe* strain Q01/PHB.

Recently, metabolic engineering approaches were taken to develop several recombinant microorganisms and transgenic plants for more efficient production and recovery of PHB either by heterologous expression or modifying the pathway. Recombinant *Escherichia coli* strains harboring the *Ralstonia eutropha* PHA biosynthesis genes have been reported to have several advantages as PHA producers compared with wild-type PHA-producing bacteria (Choi et al. 1998). Recombinant strains of *Ralstonia eutropha* PHB 4, which harbored *Aeromonas caviae* polyhydroxyalkanoates (PHA) biosynthesis genes under the control of a promoter for *R. eutropha* phb operon, were produced PHA production from various alkanolic acids including hexanoate, octanoate, pentanoate and nonanoate (Kichise et al. 1999). The recombinant strain of the *R. eutropha* PHB-4 harboring the phaC1Ps and the phaGPs genes of *Pseudomonas sp.* 61-3 produced PHA copolymers consisting of 3-hydroxybutyrate (3HB) and medium-chain-length 3-hydroxyalkanoate (mcl-3HA) units of 6-12 carbon atoms from sugars (Matsumoto et al. 2001). The genes encoding the polyhydroxyalkanoates (PHA) biosynthetic pathway in *Ralstonia eutropha* (3-ketothiolase, phaA or bktB; acetoacetyl-CoA reductase,phaB; and PHA synthase,phaC) were engineered for plant plastid targeting and expressed using leaf (e35S) or seed-specific (7s or lesquerella hydroxylase) promoters in *Arabidopsis* and *Brassica* (Valentin et al. 1999). Recombinant *Escherichia coli* strains that synthesized PHAs with altered monomer compositions by engineering a new potential PHA synthetic pathway, in which ketoacyl-coenzyme A (CoA) intermediates derived from the beta-oxidation cycle are accumulated and led to the PHA polymerase precursor R-3-hydroxyalkanoates in *E. coli* hosts (Ren et al. 2000).

*Ralstonia eutropha* H16 harboring the gnd gene encoding 6-phosphogluconate dehydrogenase (6PGDH) and the tktA gene encoding the transketolase (TK) in PP pathway of *E. coli* were used to modify the metabolic flux of gluconate to the PHB biosynthesis (Lee et al. 2003).

Fig 4: Time course of PHA accumulated in *S. pombe*, Q01/PHB host cells. Values represent the mean and standard deviations of three measurements.

Fig 5: UV spectra of PHB isolated from transgenic Yeast (lane indicates $\lambda_{max} = 235$ nm).
Generally, the potential of *S. pombe*, Q01 strain as a heterologous gene expression system has been considered due to the availability of a genetic transformation system which is based on a dominant marker and a target for multiple integration of an expression cassette into the genome (Kondo et al. 1995). The present work was focused on the synthesis of PHA in *S. pombe*, Q01 expressing the bacterial PHA biosynthetic pathway of *R. eutropha*. The data obtained by expressing the entire PHA pathway in *Schizosaccharomyces pombe*, Q01 indicated that our transgenic yeast *Schizosaccharomyces pombe* strain Q01/PHB accumulates a considerably higher amount of PHB when compared with other workers. Cells of *Saccharomyces cerevisiae* transformed with the synthase plasmid accumulated up to 0.5% of cell dry weight as PHB (Leaf et al. 1996). The maximum amount of PHA accumulated in recombinant *S. cerevisiae* growing in media containing fatty acids was 0.45% of the dry weight (Poirier et al. 2001). Similarly, the maximum amount of PHB found in *S. cerevisiae* growing on glucose was 6.7% (Breuea et al. 2002) while under the same cultivation conditions, *Arxula adeninivorans* accumulated a maximum amount of 0.107% PHB and 0.128% PHV (Terentiev et al. 2004). Thus, Recombinant *Schizosaccharomyces pombe*, Q01 strain harboring the *R. eutropha* PHA biosynthesis genes have been one of the most successful examples in the aspect of metabolic engineering. Through its large-scale production, *S. pombe*, Q01 has become a promising source for PHA formation.

*Corresponding author current address:  Biological Sciences Department, Collage of Arts and Sciences, Qatar University, Doha, Qatar, Post Code 2713. Phone: (+974) 4482702 Fax: (+974) 835061 E-mail: abdelhalceem@yahoo.de*
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