Genetic engineering of *Schizosaccharomyces pombe* to produce Bacterial Polyhydroxyalkanotes

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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* [beta-ketothiolase ($phbA_{Re}$); acetoacetyl-CoA reductase ($phbB_{Re}$); as well as PHB synthase ($phbC_{Re}$), located onto the plasmid pBHR68 were cloned into the cohesive ended pYIplac128 integrated vector that transformed into the chromosome of the yeast *Schizosaccharomyces pombe* strain Q01. Under the optimized cultivation conditions, the transgenic yeast *S. pombe* strain Q01/PHB was able to produce PHB and accumulated 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 UV/Vis, ^{1}H and ^{13}C 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. @JASEM

Engineering of novel pathways in eukaryotic cell systems seems to be a beneficial alternative to the production of PHAs in bacteria. Yeast cells can be used as models to gain information on PHAs synthesis in eukaryotes. In addition, yeasts as hosts for synthesis of PHAs have certain advantages over bacteria. First, yeasts have been intensively studied from physiology, molecular biology and biotechnology points of views. Second, yeasts are physiologically flexible and they are larger than bacteria (Terentiev et al. 2004). Moreover, yeast like, *Saccharomyces cerevisiae*, *Klyveromyces marxianus*, *Candida utilis* and others, has been approved as a GRAS microorganism by Food and Drug Administration (Boze et al. 1994). *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, *Schizosaccharomyces pombe* strain Q01 has been reported as a good transformation host and expression system (Stratagene, La Jolla, CA, USA). It is established that, one of the huge problems of producing bioproducts in recombinant organisms is the stable and constant expression of their genes during fermentation (Makrides 1996, Summers 1998, Siegele and Hu 1997). At the same way, a lot of P(3HB) production by recombinant organisms is hampered by the loss of plasmid from the majority of the bacterial population (Lee 1996, Wang and Lee 1997). Such stability problems may be attributed to the metabolic load exerted by the need to replicate the plasmid and synthesize P(3HB), which diverts acetyl-CoA to P(3HB) rather than to biomass. In addition, plasmid copy numbers often decrease upon continued fermentation because only a few copies provide the required antibiotic resistance or prevent cell death by maintaining *par*B. For these reasons, transgenic

yeasts contain the genes required for PHA formation integrated into the chromosome might have advantages in PHA production processes over the use of the episomal cloning systems. First, no plasmids need to be maintained, generally obviating the required use of antibiotics or other stabilizing pressures. Second, no plasmid loss occurs, thereby stabilizing the number of gene copies per cell throughout the fermentation process, resulting in homogeneous PHA product formation throughout production process.

Therefore, in the present work, we were interested in expressing the genes encoding the polyhydroxybutyrate (PHB) biosynthetic pathway in *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 litter) 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 dimethylsulfooxide (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

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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 Erlenmeyer 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 pHBR68 (Spiekermann et al. 1999) carries the entire PHB operon of *Ralstonia eutropha,* served as the source of PHB genes. Plasmid pBHR68 was digested by *Bam*HI and *Eco*RI, 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 *phb*C, *phb*A and *phb*B, was linearized by digestion with *Eco*RV 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 were all incubated in transfer buffer for 10 min and afterwards put into the semi-dry transfer apparatus according to this sequence: anode, Whatman paper, 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 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°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 yeasts was grown in YPOD broth medium in 250 ml Erlenmeyer 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 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 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). ^{1}H NMR and ^{13}C NMR spectra were recorded in CDCI$_3$ solutions on a JOEL GSX 500 spectrometer (500 MHz for both ^{1}H and ^{13}C), using CDCI$_3$ 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 *Eco*RV-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 *Eco*RI and *Cla*I sites leaving about 110 bp of homology between the break point and the *Eco*RI 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 *Eco*RV-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 0.5μg/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.
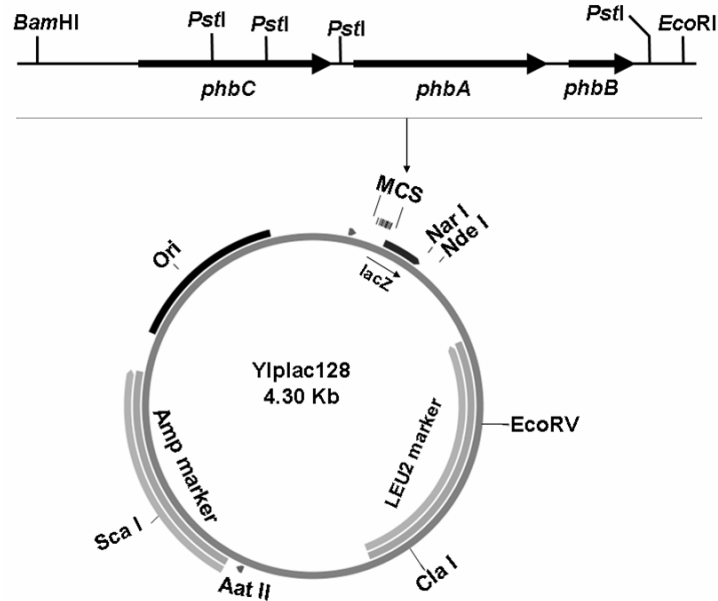

Fig 1: Diagram of plasmid YIplac128. Details of construction are given in the text. The linear map represents the PHB operon of *R. eutropha* derived from plasmid pBHR68.

Western blot analysis and enzyme assay: Recombinant yeast transformed with pYI-PHB designated *Schizosaccharomyces pombe* Q01/PHB was tested for PHA synthase expression of by Western blotting using the specific anti-serum toward the C-terminal oligopeptide of $PhbC_{Re}$ (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 $PhbC_{Re}$ 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.
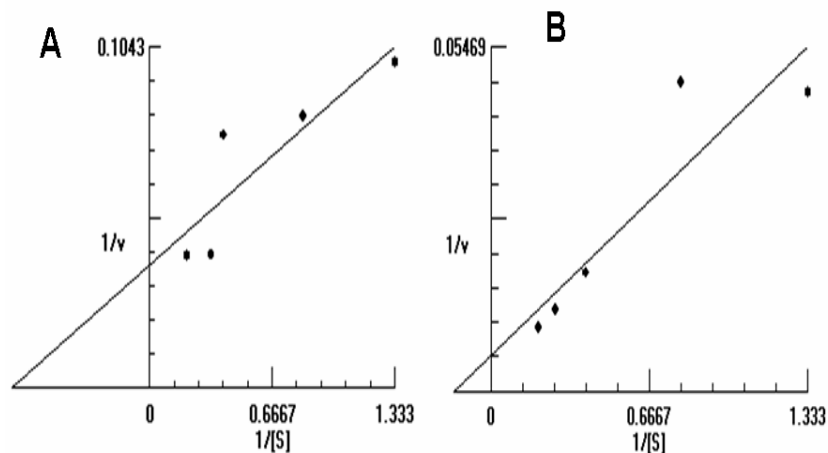

Fig 2: Detection of *PhaC* polymerase by Western blot analysis of the recombinant *Saccharomyces pombe* phb-1. (Lane 1), and the Wild type strain Y-51 (Lane 2). Western blotting was performed with polyclonal antibodies raised against PhaC1

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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 Linweaver 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.
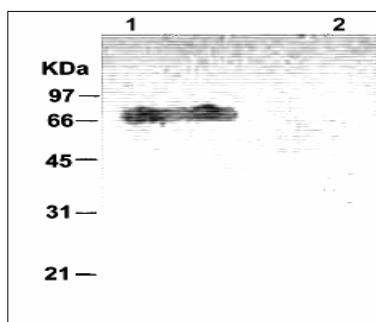

Fig 3: Kinetics of PhaC enzyme activity in the plasmid free *S. pombe* strain Q01 (A) and the transgenic *S. pombe* strain Q01/PHB (B). S refers to substrates in μM l^{-1}, while v refers to the specific activity in μM min^{-1} mg^{-1}.

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 were 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

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

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 ^{13}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*

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eutropha. The characteristic signals for other hydroxyalkanoaic acids, however, were totally lacking and confirmed the homopolymeric nature of the compound

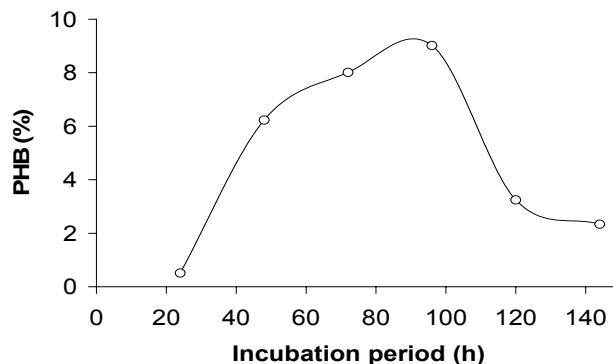isolated from the transgenic yeast *S. pombe* strain Q01/PHB.

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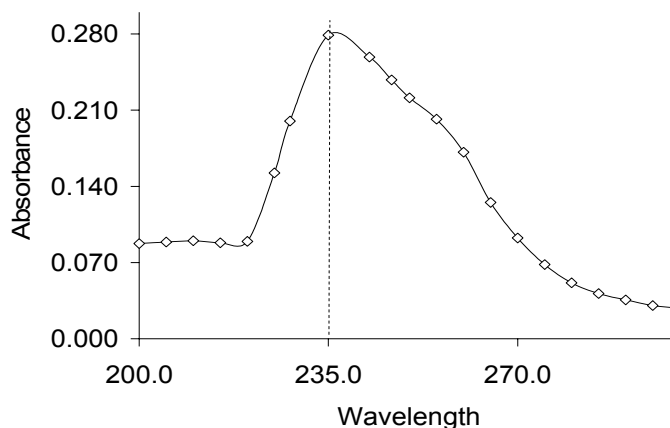

Fig 5: UV spectra of PHB isolated from transgenic Yeast (lane indicates λ_{max} = 235 nm).

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 alkanoic 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).
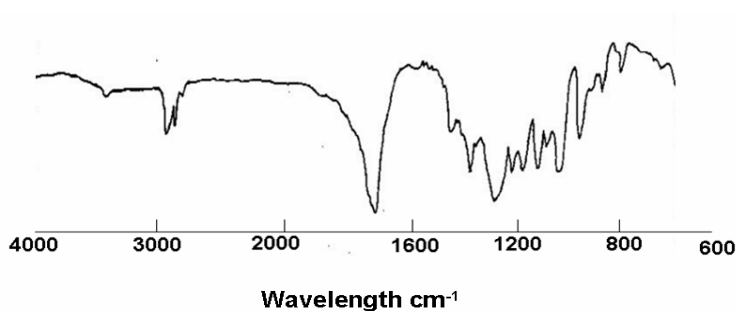
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Wavelength cm-1

Figure 6: Infrared absorption spectrum of
P(3HB) isolated from *S. pombe*, Q01/PHB.

Generally, the potential of *S. pombe*, Q01 strain as heterologous gene expression system has been considered due to the availability of a genetic transformation system which 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 considerable 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.
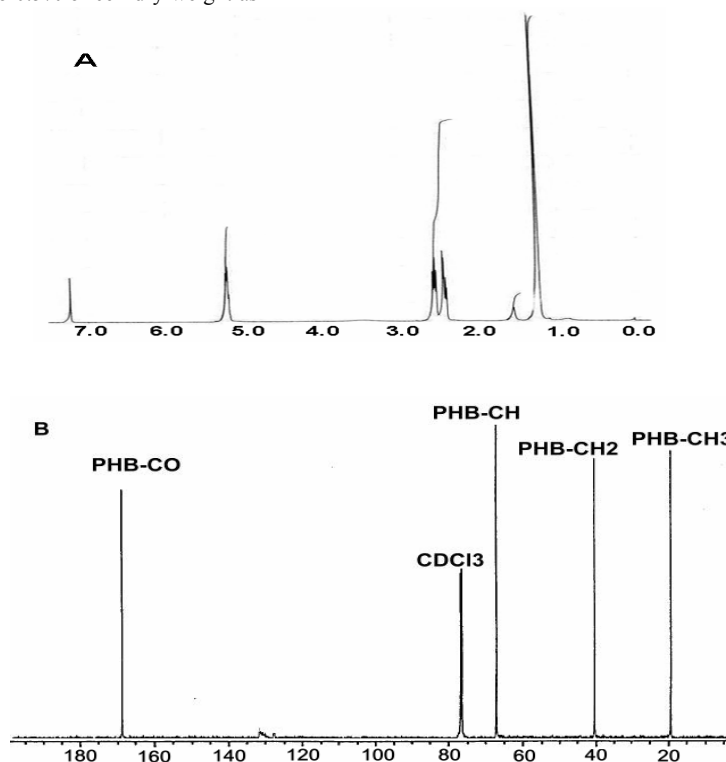

Fig 7: A) ^{1}H NMR spectrum and **B)**, ^{13}C NMR spectrum of isolated P(3HB) in CDCl3 solution.

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