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Original Research Article

Identification and Isolation of Acetoacetyl-CoA reductase (*phbB*) gene Involved in Poly (β-Hydroxybutyrate) Biosynthesis in *Ralstonia eutropha* (IMTCC 1954 strain)

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Keywords

Ralstonia eutropha, Poly-β-hydroxybutyrate (PHB), *E.Coli*

A B S T R A C T

Poly 3-hydroxybutyrate (PHB) is the most known degradable biopolymers, produced by some genera of bacteria under unfavorable growth conditions. Isolation, and cloning, of Acetoacetyl-CoA reducatase (*phbB*) gene from *Ralstonia eutropha* -IMTCC 1954 strains was achieved. Suitable primers were designed for the *phbB* PCR approach was used to clone the *phbB* gene. The *phbB* gene was successfully isolated, cloned and The PCR amplicon 742 bp corresponding to *phbB* gene was identified through the agrose gel and this fragments was further gel purified and sequenced and *phbB* gene yielded a 723 bp which matched with sequence of *phbB* gene from *Ralstonia eutropha* strain. The identity of the sequences was further validated by blasting the sequence (BLASTn) against all the reported nucleotide sequences in the NCBI GenBank and almost 96% identity was observed with both the forward and reverse run, thus confirming the *phbB* gene sequence

Introduction

The first member of Polyhydroxy alkanoates (PHA), the homopolymer poly (3-hydroxybutyrate) (PHB) was discovered by Maurice Lemoigne (1926) in the bacterium *Bacillus megaterium***.** Poly (3)-hydroxybutyrate (PHB) is a natural (non-toxic) biodegradable thermoplastic polymer synthesized by many gram-positive and gram-negative bacteria. In nature synthesis of PHB in different microorganisms, follows different metabolic pathways. Microorganisms also vary in the extent of PHB accumulation like in *Ralstonia eutropa* 70-90 % (w/v), *Azotobacter* 70 % (w/v) *Cyanobacterium* 27%, *Rhizobium* 40-50% (w/v) and synthesis of PHB has been also reported to vary in different legume crops like Lupinus lutenus 32%, Vicia faba 5.23%, Pisum sativum 1.4%, and glycine max 50% (Wong and Evans, 1971., Lee and Copeland, 1994., Kadouri *et al.*, 2003**.,** Imperlini, *et al.*, 2009., Kumari *et al.*, 2013., Seva Nayak,D and Singh, B.2017**).** The synthesis of PHB is considered the simplest biosynthetic pathway which involves three enzymes namely β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase that are encoded by *phbA, phbB and phbC* respectively (Peoples and Sinskey, 1989a and b). *phaA* gene encodes β-ketothiolase, the first enzyme involved in the condensation of two acetyl-CoA molecules

to form acetoacetyl-CoA. The next step is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA catalyzed by the acetoacetyl-CoA reductase (Steinbüchel and Schlegel *et al.*, 1991). The last reaction is the polymerization of (*R*)-3-hydroxybutyryl-CoA monomers catalyzed by PHB synthase (Rehm. 2003, Stubbe and Tian, 2003). Poly (3)-hydroxybutyrate (PHB) has been used as a substitute to petroleum-based synthetic plastics which is the main reason of environmental pollution.

The use of biodegradable plastics will help to clean environment by dropping off non-biodegradable solid wastes and will help to beauty the environment (Sudesh and Iwata, 2008, Mukesh *et al.*, 2016). Use of bio plastics is much more important in near future; molecular level of studies is not yet studied in *Ralstonia eutropha* IMTCC 1954 strain. In the present investigation we used *Ralstonia eutropha* IMTCC 1954 strain for isolation, cloning and sequence and validation of PHB gene (s) Acetoacetyl-CoA reducatase (*phbB*) gene

Materials and Methods

Bacterial strains and plasmids

Ralstonia eutropha strains MTCC-1954 was procured from Institute of Microbial Technology (IMTECH), Chandigarh, and India and also from (*Ralstonia eutropha* strain) Japan, and E.Coli DH5 alpha strain was obtained from the division of biochemistry. Indian Agricultural Research Institute (IARI), New Delhi, India.

Media and growth conditions

Ralstonia eutropha and Escherichia coli were cultivated in Luria-Bertani (LB) medium **(**Bertani, 1951**)** at 30^0C and 37^0C respectively in an incubator shaker.

Chemicals and enzymes

All restriction endonucleases, DNA markers, and phage T4 DNA ligase (EC 6.5.1.1) were obtained from New England Biolabs, Beverly, Mass. *Thermus aquaticus* (*Taq*) DNA polymerase (EC 2.7.7.7) and shrimp alkaline phosphatase (EC 3.1.3.1) were obtained from Roche Molecular Biochemicals, Indianapolis, Ind. All DNA oligonucleotides, antibiotics and fine chemicals were obtained from Sigma Biochemicals, St. Louis, Mo.

Genomic DNA extracted

Genomic DNA was extracted from *R.eutropha* following with modifications previously by described Smith *et al.,* (1989). Transfer the 1.5 ml of overnight bacterial culture to a micro centrifuge tube and spun at 5000 x rpm for 2 min. Supernatant and was decanted, pellet was resuspended in 467 µl TE buffer (pH 8.0) by repeated pipetting. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added and thoroughly mixed followed by incubation at 37 ^{0}C for the 1 hr. So it an equal volume of phenol/chloroform was added by inverting the tube until the phases was completely mixed. The DNA/phenol mixture was carefully transferred into another tube and spun for 2 min at 7000 rpm. The upper aqueous phase was transferred to a new tube. The complete phenol/chloroform added an equal volume of step was repeated again. To the aqueous phase 1/10 volume of sodium acetate and 0.6 volumes of isopropanol were added and gently mixed until the DNA precipitated. The above solution was centrifuged at 10000 rpm for 10 min and pellet was collect and washes with 70% ethanol, and air dried. DNA pellet was transferred in 100-200 µl TE buffer (pH 8.0) and DNA yield was measured by diluting 10 µl of DNA into 1 ml of TE

(1:100 dilution) and measuring the absorbance at 260 nm. Extracted DNA yield calculated in µg/ ml and at -20^{0}C until further use

Amplification of genomic DNA by Polymerase Chain Reaction (PCR)

DNA isolated from from *R.eutropha was* used for PCR amplification as per the protocol given by Murray and Thompson (1980), with some modifications. In a clear 0.2 ml microcentrifuge tube,10µlDNA (~10ng) 8µl dNTPs (250µL of dATP/dTTP/dGTP/dCTP each), 5µl 10x Taq polymerase buffer (500mM KCl,100mM Tris-HCl (pH 8.3), 15mM $MgCl_2$, 0.1% gelatin), 2µl (10 pmoles) each of forward primer and reverse primers (Table-6.1), 0.5 µl Taq polymerase (5U/µl) and sterile water were added to make up the volume to 50 µl. The tubes were spun briefly and placed in a 9600 Perkin-Elmer Centus thermal cycler. The PCR conditions were as follows: duration steps for 5 min at 94^{o}C, followed by 30 cycles of duration 2 min at 94^{o}C, primer annealing 2 min at 55 ^{o}C and extension for 2 min at 72 ^{o}C. After 30 cycles were over, a further extension reaction was carried out at 72^{o}C for 5 min followed by cooling at 4 ^{o}C. An aliquot (10µl) was run a 1 % agarose gel to check quality and the quantity of the PCR products.

Subcloning of PCR amplified inse

Ligation

The ligation reaction mixture containing, 3µl plasmid [pTZ57R/T (0.165µg, 0.18pmol ends)] vector, 4µl purified PCR fragment, (approx. 0.54pmol ends), 6µl 5X Ligation buffer, 29µl water (nuclease-free), 1µl T4 DNA Ligase (5u), was taken into 1.5 ml microcentrifuge tube and volume was made upto 50 µl upto with nuclease-free water. The mixture was incubated at 22°C for to overnight (Engler and Richardson., 1982)

Competent Cell Preparation

Competent cells were prepared according to the method of Mandel and Higa (1970). A single colony of DHα5 (*E coli*) strain was inoculated in 25 ml LB and shaken overnight at 37 ^{o}C at 200 rpm, till the O.D. reached 0.5-0.6. The cells were chilled for 10 min on ice before centrifugation at 200 rpm for 10 min at 4 ^{o}C in microcentrifuge. The pellet was resuspended in 10 ml ice cold 0.1 M $CaCl_2$ and kept on ice for 30 min. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4 ^{o}C and the pellet was again resuspended in 2 ml chilled 0,1M $CaCl_2$ and kept on ice for 2h. Aliquots of 100 µl were distributed in microcentrifuge tubes containing 100 µl of sterile 40% glycerol and stored at -70 ^{o}C till further use.

Transformation

For Transformation, 2µl of ligated mix was added to 100 µl DHα5 competent cells kept on ice for 30 min. A brief heat-shock at 42 ^{o}C for 35s was given followed by chilling on ice for 120 s. LB broth (400µl) was added to each tube and the tubes were incubated for 60 min at 37 ^{o}C. On LB agar plates containing ampicillin (100 µg/ml) 50 µl 2% X-Gal and 10 µl and 0.1M IPGT were spread and allowed to dry. The transformed cells were given a brief spin and the pellet was re suspended in 100 µl LB and spread on each plate. The plates were incubated for 16-18 h at 37 ^{o}C (Van *et al.*, 1983)

Plasmid preparation

The plasmid DNA was isolated from the white colonies. The white colony was streaked on a fresh LB plates and then a

single colony was inoculated in 5ml LB containing ampicillin (100µg/ml) and incubated overnight at 37 ^{o}C with vigorous shaking. Plasmid was isolated essentially by the method of Stephen *et al.,* (1990).

The bacterial culture (1.5 ml) was spun down in a microcentrifuge at 13,000rpm for 2 min. The pellet was resuspended in 150µl solution I (50mM glucose. 25mM Tris-HCl (pH 8.0), 10mM EDTA with lysozyme@5mg/ml) and freshly prepared 300 µl solution II (0.2N NaoH, 1% SDS) was added to the tube, inverted several times and kept on ice for 5 min. Then, 225µl solution III (3M potassium acetate pH 4.8) was added to the tube, inverted several times and kept on ice for 10 min. The sample was centrifuged at maximum speed for 5 min and the clear supernatant was transferred to a fresh tube. Ethanol (675µl) was added to the supernatant, and the tube was vortexed and spun at maximum speed for 5 min in a micro-centrifuge. The pellet obtained was washed with 70% ethanol, dried and dissolved in 20µl sterile water.

Fig.1 Sequence result of *Ralstonia eutropha* strain (MTCC 1954) *phbB* gene

PHB-B (723bp)

CCGNNCNGGNCGCCGGCCGGATGCACAGCGAGGCCAGGCCCTTCTTCGCGTCACGGCGCTTCATCTCGTGCAGCAGCCCC
ACCAGGATACGGCAGCCCGACGCGCCGATCGGGTGGCCGATGGCGATGGCGCCGCCGTTCACATTGACCTTGGAGGTGT
CCCAGCCCATCTGCTGGTGCACCGCCAGCGCCTGCGCGGCAAAGGCCTCGTTGATCTCCATCAGGTCCAGGTCTTGCGGG
GTCCACTCGGCGCGCGACAGGGCGCGCTTGGAGGCCGGCACCGGGCCCATGCCCATCACCTTGGGATCGACACCGGCGT
TGGCATAGCTCTTGATCGTGGCCAGCGGGGTCAGGCCCAGTTCCTTGGCCTTGGCCGCCGACATCACCACCACCGCGGCG
GCGCCGTCGTTCAGGCCCGAGGCGTTGGCCGCGGTCACCGTGCCGGCCTTGTCNAAGGCGGGCTTGAGGCCGGACATGC
TGCCCAGCGTGGCGCCCTGGCGCACGAACTCNTCGGTCTTGAAGGCCACCGGNTCCCCCTTGCGCTGNGGGATCAGCACC
GGGACGATCTCTTCCTCAAACTTGCCGGCCTTCTGCGCGGCTTCGGCCTTGTTCTGCGAGCCAACGGCGAACTCCTCCTG
AACCTCGGGCGTGATGCCATACTCCTTGNACTTTTCTCGGCGGTGATGTCTTGCGGTACTTGCTGTAAACGTACGGGCGC
AAAAAA

Fig.2 Sequence similarity between the phbB gene of Ralstonia eutropha stain MTCC 1954 and Ralstonia eutropha stain H16 was 96%

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Query 11  CGCCGGCCGGATGCACAGCGAGGCCAGGCCCTTCTTCGCGTCACGGCGCTTCATCTCGTG 70
          |||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1186 CGCC-GCC-GATGCACAGCGAGGCCAGGCCCTTCTTCGCGTCACGGCGCTTCATCTCGTG 1129

Query 71  CAGCAGCCCCACCAGGATACGGCAGCCCGACGCGCCGATCGGGTGGCCGATGGCGATGGC 130
          |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1128 CAGCAGCGTCACCAGGATACGGCAGCCCGACGCGCCGATCGGGTGGCCGATGGCGATGGC 1069

Query 131 GCCGCCGTTCACATTGACCTTGGAGGTGTCCCAGCCCATCTGCTGGTGCACCGCCAGCGC 190
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1068 GCCGCCGTTCACATTGACCTTGGAGGTGTCCCAGCCCATCTGCTGGTGCACCGCCAGCGC 1009

Query 191 CTGCGCGGCAAAGGCCTCGTTGATCTCCATCAGGTCCAGGTCTTGCGGGGTCCACTCGGC 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1008 CTGCGCGGCAAAGGCCTCGTTGATCTCCATCAGGTCCAGGTCTTGCGGGGTCCACTCGGC 949

Query 251 GCGCGACAGGGCGCGCTTGGAGGCCGGCACCGGGCCCATGCCCATCACCTTGGGATCGAC 310
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 948  GCGCGACAGGGCGCGCTTGGAGGCCGGCACCGGGCCCATGCCCATCACCTTGGGATCGAC 889

Query 311 ACCGGCGTTGGCATAGCTCTTGATCGTGGCCAGCGGGGTCAGGCCCAGTTCCTTGGCCTT 370
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 888  ACCGGCGTTGGCATAGCTCTTGATCGTGGCCAGCGGGGTCAGGCCCAGTTCCTTGGCCTT 829

Query 371 GGCCGCCGACATCACCACCACCGCGGCGGCGCCGTCGTTCAGGCCCGAGGCGTTGGCCGC 430
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 828  GGCCGCCGACATCACCACCACCGCGGCGGCGCCGTCGTTCAGGCCCGAGGCGTTGGCCGC 769

Query 431 GGTCACCGTGCCGGCCTTGTCNAAGGCGGGCTTGAGGCCGGACATGCTGCCCAGCGTGGC 490
          |||||||||||||||||| |||||||||||||||||||||||| |||||||||
Sbjct 768  GGTCACCGTGCCGGCCTTGTCGAAGGCGGGCTTGAGGCCGGACATGCTGTCCAGCGTGGC 709

Query 491 GCCCTGGCGCACGAACTCNTCGGTCTTGAAGGCCACCGGNTCCCCCTTGCGCTGNGGGAT 550
          |||||||||||||||| |||||||||||||||| || |||||||||| |||||
Sbjct 708  GCCCTGGCGCACGAACTCGTCGGTCTTGAAGGCCACCGGGTCGCCCTTGCGCTGCGGGAT 649

Query 551 CAGCACCGGGACGATCTCTTCCTCAAACTTGCCGGCCTTCTGCGCGGCTTCGGCCTTGTT 610
          |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 648  CAGCACCGGGACGATCTCTTCGTCAAACTTGCCGGCCTTCTGCGCGGCTTCGGCCTTGTT 589

Query 611 CTGCGAGCCAACGGCGAACTCCTCCTGAACCTCGGGCGTGATGCCATACTCCTTGN--AC 668
          |||||||| |||||||||| ||||| ||||| | ||||||| || ||||||  ||
Sbjct 588  CTGCGAGCCGACGGCGAACTCATCCTGCGCCTCGCGTGTGATGCCGTATTCCTTGGCCAC 529

Query 669 TTTTCTCGGCGGTGATGTCT-TGCGGTACTTGCTGTAAACGT 709
          || |||||||||||| | || |||||| | |||| ||||
Sbjct 528  GTT-CTCGGCGGTGATGCCCATGTGGTACTGGTTGTACACGT 488
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Fig.3 1% Agarose gel showing PCR amplified profile of *Ralstonia eutropha strain genes* (MTCC 1954) well 1, DNA marker; well 2.*phbB* gene

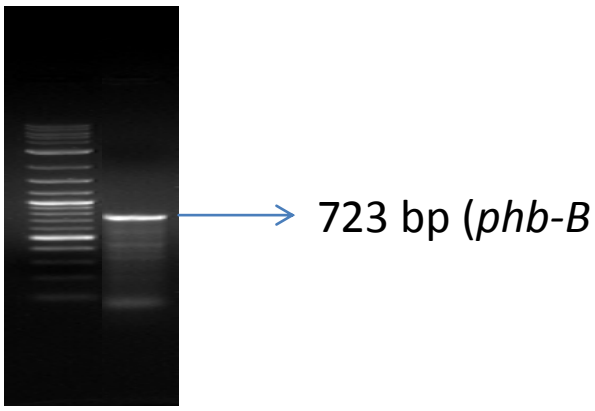

Table.1 Forward primer and reverse primers sets of *phbB* gene

PHBB-R: CTT CCT TAT TTG CGC TCG ACT	
LENGTH:	21
GC CONTENT:	60.87 %
MELT TEMP:	47.62 ºC
PHBC-F: GGA CGT GTA CAA CCA GTA CCA C	
LENGTH:	22
GC CONTENT:	59.31%
MELT TEMP:	54.55ºC

Results and Discussion

The gene specific primer pairs was designed for *phbB* gene (Table.1) from DNA isolated from *Ralstonia eutropha* was used as template to PCR amplify *phbB,* genes. The *phbB* gene was successfully isolated, cloned and The PCR amplicon 742 bp corresponding to *phbB* gene (Fig.1) was identified through the agrose gel and this fragments was further gel purified and sequenced and *phbB* gene yielded a 723 bp which matched with sequence of *phbB* gene from *Ralstonia eutropha* strain. The identity of the sequences was further validated by blasting the sequence (BLASTn) against all the reported nucleotide sequences in the NCBI GenBank and almost 96% identity was observed with both the forward and reverse run, thus confirming the *phbB* gene sequence (Fig-2 and 3). PHB biosynthetic pathway has been worked out in a bacterial species viz., *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and genes encoding the enzymes, β-ketothiolase (*phb A*), acetoacetyl-CoA reducatase (*phbB*), and polyhydroxybutyrate synthase (*phb*C) have been characterized (Peoples and Sinskey,1989a and b). Put of the three, the β –ketothiolase (*phbA*) is only one present in the plant cytosol while the reaming two enzymes *phbB* and *phbC* are absent in plants which limited PHB production in plant system. Scientific efforts are on to develop bacterial strains and transgenic plants that are efficient in PHB production (Poirier *et al.*, 1992 and 1995; Valentin *et al.*, 1999). The later would require cloning and over expression of *phb A, phb-B* and *phb*C genes in plants system. These genes are either

lacking or have limited expression in plants. It has been shown that PHB can be synthesized in transgenic plants (Poirier *et al.,* 1992 and 1995). Saruul *et al.,* (2002) reported that the synthesis of PHB in transgenic alfa alfa roots and leaves. In the present study we have attempted and successfully cloned the one (*phb-B*) gene from *Ralstonia eutropha* (MTCC 1954) strain out of three (*phb A, phb-B* and *phb*C) genes and preserved for future construction plant transformation vector cassette with all the three important genes *phbA, phb B and phbC* involved in PHB biosynthesis to develop the transgenic mean of producing PHB in higher amount in a plant system. Future line of work is need to be cloned remain *phbA, and phbC* from *Ralstonia eutropha* (MTCC 1954) strain.

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