Current Research in Microbiology and Biotechnology
Vol. 5, No. 2 (2017): 997-1001
Research Article
Open Access

ISSN: 2320-2246

Primer design for isolation of Phosphotransbutyrylase gene enabling its cloning studies in pHIS1522 shuttle vector

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Received: 19 January 2017 Accepted: 24 February 2017 Online: 02 March 2017

ABSTRACT

Phosphotransbutyrylase (Ptb) is one of the key enzymes responsible for Polyhydroxybutyrate (PHB) synthesis in *Bacillus megaterium*. This enzyme is encoded by Phosphotransbutyrylase (ptb) gene. To enhance the expression of Phosphotransbutyrylase gene in order to increase Polyhydroxybutyrate production, its isolation and cloning in suitable vector is required. *Escherichia coli–Bacillus megaterium* shuttle vector pHIS1522 has been used as a versatile expression vector in recent years. In the present study, we designed primers for ptb gene isolation from *Bacillus megaterium*. Restriction sites were added in primers considering cloning of isolated gene in pHIS1522 shuttle vector. NCBI primer blast tool used to design oligonucleotides sequence for amplification of ptb gene. Results demonstrate a high specificity of the three oligonucleotides for all soil isolates of the *Bacillus megaterium* group. The size of amplified gene was confirmed by PCR amplification and gel electrophoresis.

Keywords: Primer design, ptb gene, pHIS1522 shuttle vector, PCR amplification, Polyhydroxybutyrate.

1. INTRODUCTION

Polyhydroxybutyrate (PHB) and other biobased polymers are promising candidates for the development of environment-friendly and totally biodegradable plastics. These are synthesized by a range of microorganisms viz. *Bacillus* spp., *Clostridium* spp., *Azotobacter vinelandii, Ralstonia eutrophus, Methylobacterium rhodesianum* and *Azospirillum brasilense* etc as storage material inside the cell [1]. PHB extracted from bacterial cells exhibits the properties that are similar to synthetic polypropylene which make them natural, renewable and biocompatible for mankind use [2]. Several genes involved in PHB biosynthesis pathway have been studied so far. phosphotransbutyrylase enzyme plays an important role in the process of synthesizing PHB in bacterial species like *Bacillus* and *Clostridium*. Its role in PHB accumulation has been reported by many researchers [3-6]. Vazquez et al reported that increase in Ptb enzyme activity correlates with increase in PHB accumulation in *Bacillus megaterium* [4].

This organism has been gaining more and more importance in scientific as well as industrial applications because of its unique properties viz. Stable and high yield protein production, stable replication and maintenance of recombinant plasmids and tightly regulated and efficiently inducible xylA operon etc [7, 8]. XylA promoter has been successfully employed for increased gene expression in *Ecoli* and *Bacillus megaterium* as well [9]. Therefore XylA promoter containing pHIS1522 shuttle vector is selected for the study. This study aims to design nucleotide sequence for isolation of ptb gene that allows direct cloning of isolated gene in pHIS1522 vector.

2. MATERIALS AND METHODS

There are wide range of programs have been written to perform primer selection, varying significantly in selection criteria, comprehensiveness, interactive design, and user-friendliness [10-18]. We used NCBI primer blast tool to design target specific primers. The skeleton of basic PCR primer for molecular cloning consists of:

a) **Hybridization Sequence**: It is the region of the primer that binds to the sequence to be amplified (usually 18-21bp)

b) **Restriction Site:** It is sequence of restriction enzyme for cloning (usually 6-8bp

c) **Leader Sequence/5' extension site:** Extra base pairs on the 5' end of the primer assist with restriction enzyme digestion (usually 3-6bp)

Below procedure explain in detail to design such primers:

1) **Find out the target gene sequence-** Using accession no AJ278958.1 and NCBI 'nucleotide' blast tool, complete ptb gene sequence [4] obtained in fasta formate. The length of ptb gene is of 1048 base pair, its start and stop is at 134 and 1048 position respectively (Fig 1).
2) **Primer blast:** Blast search against accession no/ gene sequence, retrieved various primer pairs. Among these three primers were selected for further study based on melting temperature, self-complementarity and large product size (Table 1).
3) **Forward primer:** We have gene sequence from 5'→3' direction, so the sequence of forward primer would be same as 5'→3' sequence of gene. Some initial base pair of 5'→3' of gene selected. Forward primer is designed in such a way that the sequence of start codon 'atg' at 134 position is not changed in ptb gene.
4) **Reverse primer:** It was designed as the reverse complement of 5'→3' gene from the end of gene sequence. Similarly sequences of restriction site and 5' extension site were also added as reverse complement.
5) **Addition of cloning/restriction site:** For cloning of gene into a vector restriction sites must be added in a gene and that restriction site should be same or provide same sticky ends to the multiple cloning site of vector. By using two different restriction enzymes, we constrain our PCR product to a particular orientation in the plasmid. This process is known as *directional cloning*. [19,20]
   All restriction sites present in gene was identified with the help of NEB cutter V2.0 (New England Biolabs), two restriction enzymes BsrGI and SphI were selected that don't cut within the gene and also present in the plasmid. Hence, the sequence of BsrGI (TGTACA) and SphI (GCATGC) was added to forward and reverse primer respectively.
   Both enzymes cut the DNA well in the same conditions (buffer, temperature) and so they can be performed together, to save time and resources.
6) **Addition of 5' extension:** A 5' extension of the restriction site with 6 bases added to forward and reverse primer both to enhance the cleavage efficiency of enzymes[21]. Final sequence of designed primers showed in Table2. These nucleotide sequence of forward and reverse primers were got synthesized from Bio innovations laboratory, Mumbai.
7) **PCR reaction**: These primers were used in PCR reaction set up. To get template in this PCR amplification reaction, *Bacillus megaterium* isolated from various soil samples. Sodium hydroxide used for cell lysis and extraction of DNA.
   For twenty five micro liter reaction volume master mix contained 14.75ul Nuclease free water, 2.5ul Buffer, 4.0ul dNTPs, 0.25ul Taq polymerase (all components purchase from TAKARA) and 1.0ul of different combination of primers (primer 5,3 and 8) lastly template DNA added in master mix solution, mixed properly by tapping and briefly centrifuged. Applied Biosystems thermocycler programmed to run amplification reaction and the conditions included an initial denaturation step at 94°C for 7 min, 30 cycles of denaturation 94°C for 1min, annealing 60°C for 1min, extension 72°C for 2 min, and final extension at 72°C for 10 min.
8) **Observation of amplified gene:** The PCR amplicons were examined by agarose gel electrophoresis [22]. Amplified gene size was compared against 1kb SRL ladder. PCR products were examined by gel electrophoresis at 80V for 45mins in a 1% (w/v) agarose gel 1 x TAE buffer.
9) **Gel documentation:** To visualize bands, gel was observed and photographed using Gel doc (Vilber Gel Documentation System) with Megacapt software.

3. RESULTS AND DISCUSSION

The primer pairs with restriction site and 5' extension site as shown in Table 2, were again blast searched to get specifications and information about melting temperature as annealing temperature in PCR amplification needs to be set approximately 5°C below melting temperature, details shown in Table 3. The efficiency of the designed primers in amplifying DNA fragments of the corresponding ptb gene was analyzed by PCR amplification. Gel image shows that all three designed primers successfully amplified phosphotransbutyrylase gene with slight variations as shown in Fig 2. Among these three primers, Primer 5 was selected for further study based on the bands appearance on gel (brightness and thickness).

```
   1 agcagctata ttatgtaata tatgcagagg atggcacaaa tcttgcatgt attttaggtg
  61 actgcataaa cgcgcagtat gctctttaaa acggagcaaa cgaaagaatg aaaaaggaga
 121 ggtcgaacat tccatgactc ttgctaccat cttatctcgg gttgctaagc agtcttcttt
 181 gactgttgct gtagctgcag ctgaagactt agaaacaatt gaggcagtgt atgaagctgt
 241 gtcaagggaa acgtttctgg aatttatttt gtttggcaac gaaacgacaa ttaatgaatt
 301 attcaggcag taccgcccat ccttactcac tcataaggct gtgacggtcg tacatacgga
 361 gtccgtttta gaagcggctc aattggctgt gcgttcagtt cagcaaggtc aagctcatat
 421 cgtaatgaaa ggggatttat ccacatcgct tatcttaaaa gctgtgttaa ataaagagca
 481 tggtttgcgt acgggaaatg ttttatcaca tgtagctgtg tttgacgttc ctcactatga
 541 ccgtccgatt cttgtaacag acgcagctat gaatattacg ccatctctgg aagagaaagt
 601 tcaaatcatt caaaatgcgg tgaacgttgc ccactctatt ggggtagaga tgccaaaggt
 661 agctcctatt gcagcggttg aagttgtgaa ccctttgatg cctgcaacag tagaggctgc
 721 gcttcttaca cagatgaacc gcagaggaca aattaaaggc tgcgttattg atggaccttt
 781 agcactcgac aatgccatta acatagaagc agccaagcaa aaaggcattc aaagtgaagt
 841 tgccggtcaa gcagatatat tactcgttcc agcaattgaa acagggaacg tcttatataa
 901 atccctcatt tattttgcta aagcaaaagt tggagctatt ctagcaggag caaaagctcc
 961 ggtcgtctta acatccagag cagattcatc agaaagtaaa ttatactcac tggcgcttgc
1021 tatcagcgtc gcccaacaga attcttag
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Figure 1: Complete sequence of phosphotransbutyrylase gene of *Bacillus megaterium* (Accession number GenBank: AJ278958.1, start and stop codon shown in bold letters)

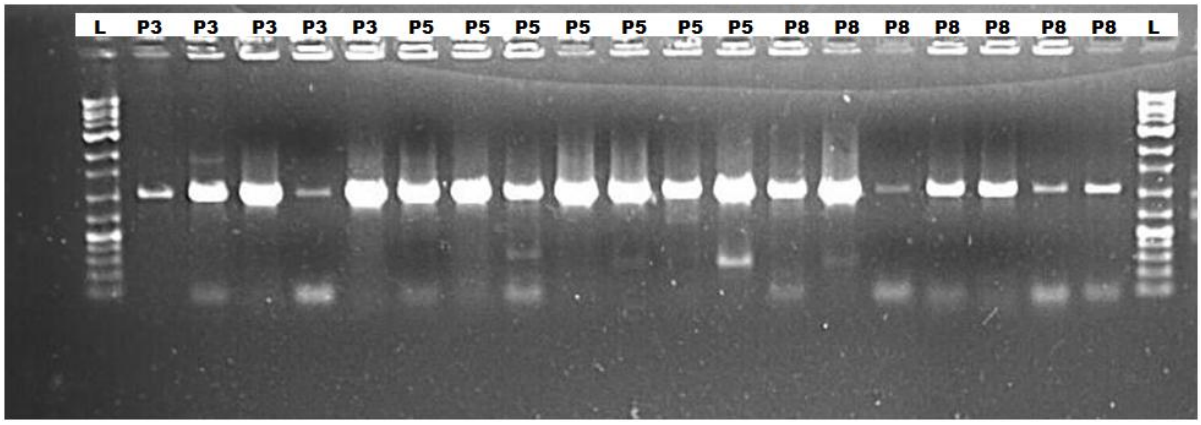

Figure 2: Lane L- 1kb SRL Ladder, Lane- P3, P5 & P8 represents the amplification of ptb gene with respective primers in 1% agarose gel.

It can be suggested from this study that primers designed with NCBI primer blast tool were efficient in amplifying desired ptb gene. NCBI Primer-BLAST helps to make primers that are specific to intended PCR target. It uses Primer3 to design PCR primers and then uses BLAST and global alignment algorithm to screen primers against user-selected database in order to avoid primer pairs (all combinations including forward-reverse primer pair, forward-forward as well as reverse-reverse pairs) that can cause non-specific amplifications. Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR.

Table 1: Primer pairs retrieved from NCBI primer blast.

Primers	Forward Sequence (5'->3')	Reverse Sequence (5'->3')
Primer 3	GCAGAGGATGGCACAAATCT	AATTCTGTTGGGCGACGCT
Primer 5	TGGCACAAATCTTGCATGT	TTCTGTTGGGCGACGCTG
Primer 8	AGAGGATGGCACAAATCT	CTAAGAATTCTGTTGGGC

Table 2: Final sequences of Forward and reverse primers with the addition of restriction sites and 5' extension sites.

Primer pair 3	NCBI blast primer sequence (5'->3')	Restriction Sites	5' extension sites	Final sequence
Forward primer	GCAGAGGATGGCACAAATCT	TGTACA	TCCAGC	TCCAGCTGTACAAGAGGATGGCACAAATCT
Reverse primer	AATTCTGTTGGGCGACGC	GCATGC	TTACT	AGTAAGCATGCAATTCTGTTGGGCGACGC
Reverse complement	GCGTCGCCCAACAGAATT			
	GCGTCGCCCAACAGAATT**GCATGCTTACT**			

Primer pair 5				
Forward primer	TGGCACAAATCTTGCATGT	TGTACA	GACTG	GACTGTGTACATGGCACAAATCTTGCATGT
Reverse primer	TTCTGTTGGGCGACGCTG	GCATGC	TATTA	TAATAGCATGCATTCTGTTGGGCGACGCTG
Reverse complement	CAGCGTCGCCCAACAGAA			
	CAGCGTCGCCCAACAGAAT**GCATGCTATTA**			
Primer pair 8				
Forward primer	AGAGGATGGCACAAATCT	TGTACA	CTAGCG	CTAGCGTGTACAAGAGGATGGCACAAATCT
Reverse primer	CTAAGAATTCTGTTGGGC	GCATGC	TCACTA	TAGTGAGCATGCCTAAGAATTCTGTTGGGC
Reverse complement	GCCCAACAGAATTCTTAG			
	GCCCAACAGAATTCTTAG**GCATGCTCACTA**			

Table 3: Specifications of all three finally designed primer pairs.

Primer 3	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCCAGCTGTACAAGAGGATGGCACAAATCT	30	67	46	6.00	2.00
Reverse primer	AGTAAGCATGCAATTCTGTTGGGCGACGC	29	69	51	6.00	5.00
Primer 5						
Forward primer	GACTGTGTACATGGCACAAATCTTGCATGT	30	66	43	8.00	8.00
Reverse primer	TAATAGCATGCATTCTGTTGGGCGACGCTG	30	69	50	10.00	1.00
Primer 8						
Forward primer	CTAGCGTGTACAAGAGGATGGCACAAATCT	30	66	46	6.00	2.00
Reverse primer	TAGTGAGCATGCCTAAGAATTCTGTTGGGC	30	66	46	8.00	6.00

4. CONCLUSION

Recombinant DNA technology (Molecular Cloning) has been successfully employed in various industries, food production, human and veterinary medicine, agriculture and bioengineering [23]. Advantage of choosing shuttle vector in cloning studies is that the construct (Vector + gene) can be easily propogated in *E.coli* and then used in a desired system which is comparatively difficult or slower to use. It is well known that xylA promoter of *Bacillus megaterium* can induce high level of intracellular expression of heterologous gene [6,24]. Hence, cloned construct of amplified gene in pHIS1522 vector can be used for over expression of ptb gene rendering increased PHB production in *Bacillus megaterium*.

Primers designed in this study can be used for ptb gene isolation to better elucidate the regulatory mechanisms in production of polyhydroxybutyrate, acetate, butyrate, acetone and butanol [25,26] etc. The methodology of primer design described in this paper can serve as a useful tool in various molecular cloning studies.

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