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Optimization of Gene Design, PCR Assembly and Site-Directed Mutagenesis for the Synthesis of *Beauveria Bassiana* Protease Gene

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Abstract

Synthetic gene construction is one of the components of synthetic biology. It can be used for various purposes such as to optimize gene expression. In this study, we proposed six predetermined criteria for designing oligos for the synthesis of the *Beauveria* bassiana protease gene. These criteria were set up to optimize the cost and to accommodate the oligos assembly. A total of 44 overlapping oligos were designed and synthesized 0.5 µM of oligos mixture was used in assembly PCR together with high fidelity DNA polymerase to produce 1.1 kbp fragment. The gene was visualized by agarose gel electrophoresis before subcloned into pCR™2.1-TOPO. The sequence of the gene was verified by DNA sequencing. Site-directed mutagenesis was performed to repair errors resulted from the gene synthesis. A sharp and distinguished band of the expected size of the protease gene was observed in agarose gel electrophoresis. Errors in the sequence which was detected by DNA sequencing was successfully repaired using our simplified site-directed mutagenesis protocol. The result indicated long DNA sequences (>1 kbp) can be synthesized with less error by using our method. Additionally, this method was easy to perform because it would require minimum optimization to synthesize other genes by following our guidelines.

Keywords: Gene synthesis; PCR assembly; Protease gene; Site-directed mutagenesis.

Introduction

Beauveria bassiana is an entomopathogenic fungus and commercially produced for biological control [1]. Protease is one of the enzymes secreted by *Beauveria bassiana* to degrade insect cuticle during infection [2].

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Protease gene can increase the pathogenicity of microbial pesticides such as baculovirus through genetic modification [3]. Nowadays, synthetic gene design and synthesis are much simpler compared to earlier days with the advancement in molecular biology and web-based application for DNA and protein analysis which are available on the net for free.

There are numerous PCR-based gene syntheses have been developed such as polymerase chain assembly (PCA) or also known as assembly PCR [4], sequential overlap extension PCR (OE-PCR) [5] and thermodynamically-balance inside-out (TBIO) [6]. As for PCA, the overlapping oligos are assembled in the first reaction, and then the gene assembly is amplified by PCR using the first and the last oligos as primers. In the sequential OE-PCR method, oligo pairs axre used to produce overlapping fragments, extended in the subsequent PCR steps until a full gene is obtained. An attempt to reduce the cost of gene synthesis by OE-PCR is made by decreasing the length of the oligos for gene synthesis and leaved gaps between them through a combination of dual asymmetric PCR and OE-PCR [7]. TBIO approach involves designing the oligos in such a way that the fragments start to extend from the centre to gradually form a full gene.

PCA method has been modified to synthesize longer genes which range from 1.0 to 5.4 kb with less mutation [8]. Using this method, the DNA fragments which constitute the full gene, 500 bp in length, were first synthesized from the oligos and then joined in the second PCR by the outermost oligos. Another approach includes joining the DNA fragments into a full sequence using ligation reaction by the addition of restriction sites [9]. This method can also be used to enable the synthesis of genes up to 32 kbp in length by insertion of the fragments into vectors using ligation-independent cloning (LIC) method and then fragments were assembled into full gene by digestion/ligation reaction [10]. Besides that, unmodified PCA has been simplified by combining the gene assembly and PCR reaction into one reaction [11–13]. Moreover, a combination of PCA and TBIO techniques has been shown to enable the synthesis of genes up to 1550 bp [14].

In this study, we proposed an optimized method for the construction of the protease synthetic gene of *B. bassiana* starting from designing oligos according to our predetermined criteria until its final product. This 1.1 kbp gene was successfully synthesized with a low error rate. In addition, we also described simplified site-directed mutagenesis (SDM) for the correction of the gene sequence.

Materials and Methods

Gene design

The synthetic *Beauveria bassiana* serine protease gene was constructed according to the sequence published in the GenBank (*Accession number*: GUI166155). The oligos for the gene construction were designed based on the following criteria:

1. The maximum length of the oligos was set to 50 nucleotides to optimize the cost.
2. The minimum overlapping region was set to 20 nucleotides.
3. The GC contents range from 40-60%.
4. Melting temperature differences between each oligonucleotide must not more than 5°C.
5. Tm of potential hairpin structures lower than annealing temperature. Delta G values are higher than -9.0 kcal/mole. The parameters were determined using OligoAnalyzer 3.1 (available at http://sg.idtdna.com/calc/analyzer).

A total of 44 oligos were designed and synthesized (IDT, Singapore), the locations of the oligos in the gene were shown (Supplementary Table 1 and Supplementary Figure 1).

Gene synthesis

The gene was synthesized by assembling the 44 oligos in the first step of PCR, and then the gene was amplified in the second step PCR (Fig. 1). The oligos mixture containing all the 44 oligos was prepared by mixing 10 µl of each oligo in a 2 ml centrifuge tube. The mixture was diluted to a final concentration of 10 µM. The oligos mixture was used at the concentration of 0.5 - 4 µM for optimization in PCR mix containing 1.5 mM MgCl2, 200 µM of each dNTP and 0.4 U Phusion enzyme (Thermo Scientific, US). Assembly PCR was carried out using the following condition: 98°C for 30 s, 55 cycles of 94°C for 60 s, 62°C for 120 s, 72°C for 60 s and a final extension of 72°C for 10 minutes.

The assembled oligo was further amplified with the addition of the BamHI (SP1.2) and NotI (SP44.2) restriction sites (Table 1). The assembled oligo was diluted 5-fold in a 20 µl PCR mix containing the same component as the first PCR. The second PCR was carried out using the following condition: 98°C for 30 s, 25 cycles of 94°C for 60 s, 62°C for 60 s, 72°C for 60 s and a final extension of 72°C for 10 minutes. The PCR product was visualized, and the size estimated by agarose gel electrophoresis. The amplicon was purified from the gel using Nucleospin Gel and PCR Clean-Up (Machery Nagel, Germany) for subcloning.

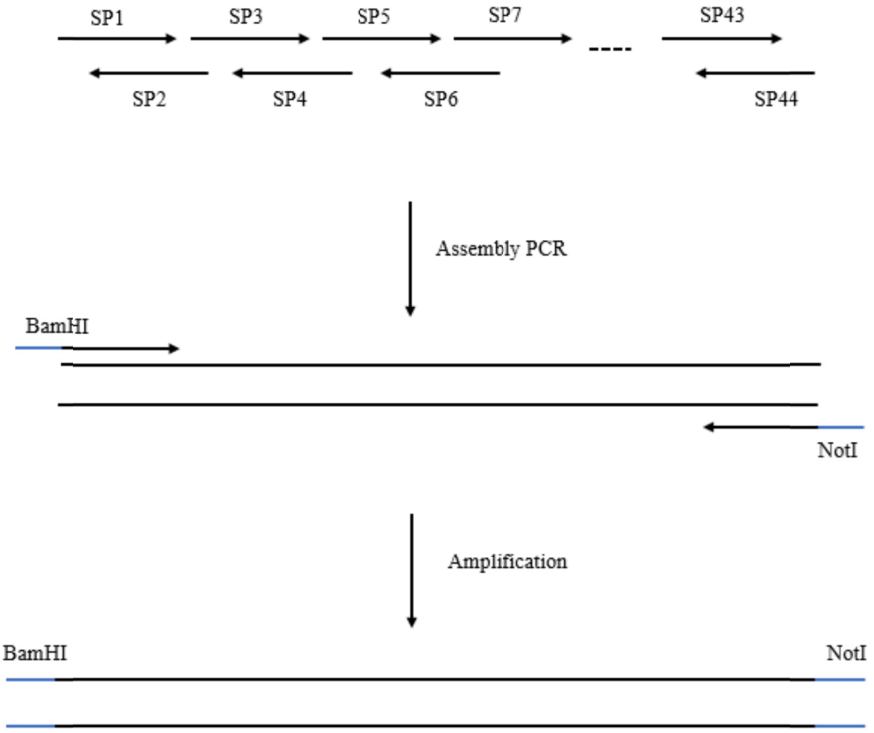

Figure 1. Schematic diagram of gene synthesis using assembly PCR. SP1 to SP 44 are the oligos covering both strands of the gene. These oligos were assembled in the first PCR and amplified in the second PCR with the addition of BamHI and NotI restriction sites forming a synthetic gene.

Table 1. Primers used in gene synthesis and SOE. Restriction sites are underlined.

Primers	Sequence (5'-3')
SP1.2	GCGGATCCCGACATAAATATGCGTTTGTCAATAATTGCAGCGGCTTTGCC
SP44.2	TCAAGCGGCCGCAAATCTTATATGCCGCCGTTAAATGCCAGGTAGTTGAC
SPF2	CCGCTTACGCCATGATCCAGATGTTGAAACAATAGAGC
SPR2	GCTCTATTGTTTCAACATCTGGATCATGGCGTAAGCGG
SPF3	CCATTTTCGGCGTCAAGGTACTCGAAGATAGTGGTTCGGGT
SPR3	ACCCGAACCACTATCTTCGAGTACCTTGACGCCGAAAATGG
SPF4	GAAAAGCTGTTGATATTTTTGCACCTGGCACTGGCAT
SPR4	ATGCCAGTGCCAGGTGCAAAAATATCAACAGCTTTTC

Subcloning and sequencing

The synthetic gene was digested with BamHI and NotI before ligated into pCR™2.1-TOPO vector (Thermo Scientific, USA). The vector was transformed into Top10 *Erischeria coli* competent cell which was grown on Luria Bertani (LB) agar containing 50ug/ml ampicillin. Transformant colonies were screened for the presence of the gene insert in forward sequence orientation using SP1 and backbone-specific primer (M13 Forward (-20)). Plasmid from the successful transformants was extracted using NucleoSpin Plasmid (Machery Nagel, Germany) according to the manufacturer's protocol and sequenced using backbone-specific primers (M13 Reverse and M13 Forward (-20)). The DNA sequences were analyzed using Bioedit version 7.0.5.3 [15].

Site-Directed Mutagenesis

Site-directed mutagenesis by overlap extension (SOE) using PCR was performed to replace the errors in the DNA sequence as described previously [16]. Initially, the forward and reverse primers containing the targeted nucleotides (Table 1) were designed to overlap with the region containing the nucleotides to be replaced or inserted, as shown in Figure 2. The primers were then combined with SP1.2 forward and SP44.2 reverse primers to produce two overlapping fragments. PCR was carried out using the following condition: 98°C for 30 s, 25 cycles of 94°C for 60 s, 62°C for 60 s, 72°C for 60 s and a final extension of 72°C for 10 min. The PCR

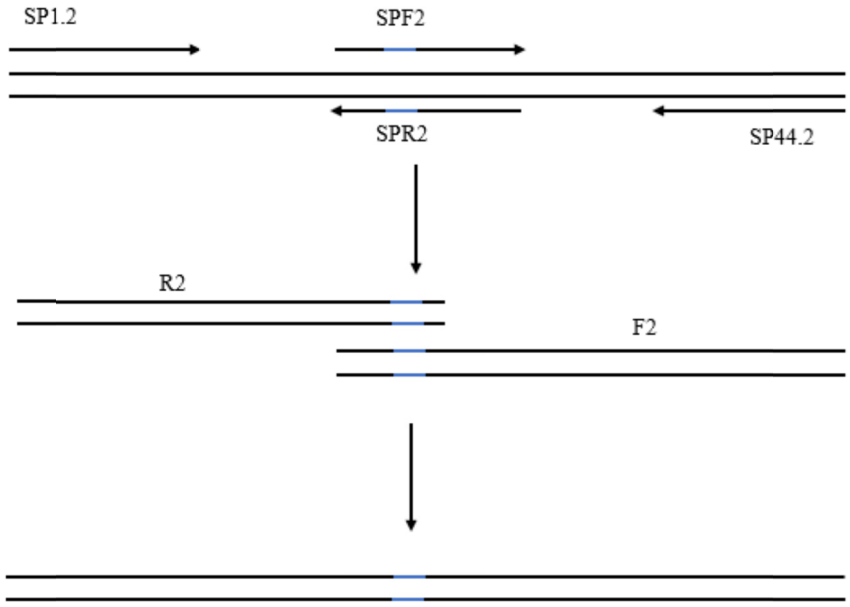

Figure 2. Schematic diagram of SOE. Two overlapping fragments covering the regions need to be corrected were generated in the first steps of SOE. These overlapping fragments were joined into full length gene by PCR using the outermost primers. The full-length gene was used as template to produce the next fragments.

products were then purified. The overlapping fragments were then joined to produce single fragments by PCR. The fragments were purified from the gel using NucleoSpin Gel and PCR Clean-Up (Machery Nagel, Germany) and used as the template for the next SOE. These techniques were repeated in each location to which nucleotides needed to be repaired. Next, the final fragments were subcloned and re-sequenced.

Results

The sequence of *B. bassiana* proteinase gene in this study was modified for the optimization of gene synthesis according to a previous study [17]. The melting temperature for all 44 synthesized oligos ranges from 66.1 to 68.9°C. The minimum length was 40 nucleotides to accommodate 20 nucleotides overlaps with the adjacent oligos. Not more than 50 nucleotides in length were synthesized to minimize the cost and error for oligos synthesis. Randomly spaced gaps were introduced between the oligos to reduce the cost and accommodate the melting temperature and GC content ranges.

Restriction sites were added to the outermost oligos to facilitate cloning into the vector instead of using TOPO TA cloning. TOPO TA cloning resulted in the gene cloned in the reverse direction as indicated by negative PCR results and confirmed by DNA sequencing. The plasmid of the successful transformants was sequenced using backbone specific forward and reverse primer to cover to whole gene sequence. The forward and reverse sequences were aligned using in BioEdit software. A total of four mutated regions were detected in the sequences (Supplementary Figure 2) and this represents a total of 6 mutations with a mutation rate of 0.5%.

In order to correct the errors in the sequence, two overlapping fragments containing the correct sequences were produced using PCR containing the correct nucleotides (Fig. 3). These fragments were purified and combined in the subsequent PCR into the full-length genes. These processes were repeated for other sites where mutations occurred. Joining of the overlapping fragments also yield background amplification (Fig. 4). The successfully joined fragments, as inferred by the amplicon size, were excised, purified and used as a template for generating the next fragments. After all of the errors have been repaired, the gene was cloned and re-sequenced for verification.

Discussion

A number of previous studies described simpler and rapid methods, but the cost for oligos synthesis was higher [5,14,18]. These long oligos synthesis (>50 bases) are prone to error, and higher quality and more

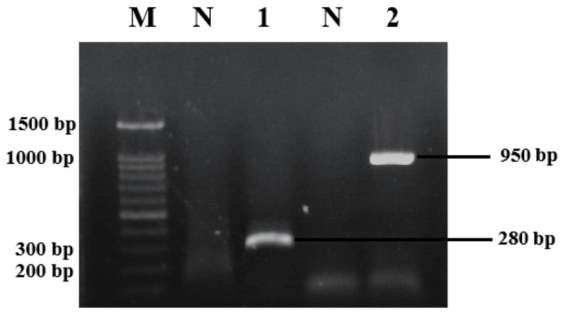

Figure 3. Two overlapping fragments produced from the first step of SOE. Lane 1 and 3: negative control; lane 2 and 4: Overlapping fragments of expected sizes.

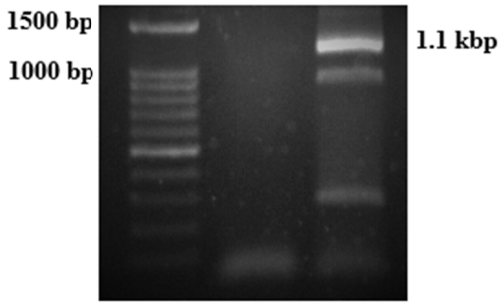

Figure 4. Full length gene produced from the amplification of the two overlapping fragments in SOE. Lane 1: negative control; lane 2: full length protease gene.

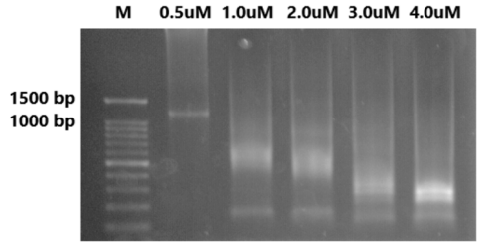

Figure 5. Oligos were assembled into a single fragment at the concentration of 0.5 µM. M,100 bp DNA marker; lane 1-5, products of assembly PCR at the oligos concentrations of 0.5 µM, 1.0 µM, 2.0 µM, 3.0 µM and 4.0 µM, respectively.

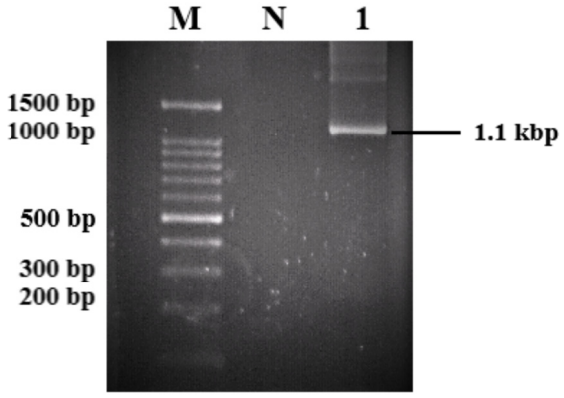

Figure 6. Assembled oligos was enriched and added with BamHI and NotI restriction sites. M is 100 bp DNA marker. Lane 1: negative control; lane 2: full length protease gene amplified from the assembled oligos.

expensive oligos are required [7].

Potential hairpin formation for each oligo was eliminated by designing oligos with annealing temperatures higher than the melting temperature of the hairpin. Delta value of each oligo was kept higher than -9.0 to minimize the primer dimer formation. These oligos were assembled in the first PCR, and a 1.1 kbp fragment at the concentration of 0.5 µM oligos mixture was visualized (Fig. 5). Smaller fragments were formed at higher concentrations of the oligos mixture. The assembled oligo was successfully amplified in the second PCR forming a 1.1 kbp band (Fig. 6).

Gene length was one of the limitations in gene synthesis methods, previously which is associated with mutations and the unsuccessfulness of gene assembly [6]. Mutation rate increases with the gene length. The oligos might not be assembled at all because of the presence of a high number of unspecific mismatches between oligos creating nonspecific annealing which leads to the formation of truncated sequences and hence premature termination of PCR [9]. In order to overcome their limitation, an extra gene fragments synthesis step was added. With this additional step, the length of the synthesized gene can be increased up to 32 kbp though mutations still cannot be eliminated [10]. Since this method involves an extra step, we would like to suggest that this method should only be performed for a long gene for when our method might not be successful.

In our study, we were able to synthesize the 1.1 kbp gene with a lesser number of errors. Gene synthesis has an advantage over long-range PCR and direct cloning in a such way that it requires no template. Besides, a high number of mutations that could arise especially when amplifying long template can be completely avoided. Longer genes have not been tested in this study. However, by following our criteria, it is still possible to synthesize longer genes given that the other methods require complicated optimization including PCR conditions, oligos concentration and design [14].

Besides, the additional gene fragments synthesis step also requires additional works to be performed such as digestion/ligation reaction, DNA purifications, sequencing and analysis, resulting in more laborious works [7,9,10,18]. Nevertheless, our method may not be suitable for a large gene because the number of mutations might increase with the length of the gene though high fidelity polymerase is used. In such cases, mutations may be accumulated to the point of beyond repair.

It was reported that the fragments produced in the first step of SOE need to be further purified before fused to avoid the formation of the secondary products [16]. In this study, secondary products were still formed even though purification has been done indicating that other factors contributed to their formations. However, the band of the desired product was more dominant as it was brighter than the others. The corrected gene was purified and used directly as a template for SOE of another site without the need to subclone. However, when this method was performed on a longer gene, the desired fragments could not be produced (data not shown). The fragment could only be produced when the longer gene was subcloned into a vector. The complexity of DNA structure inherited by longer gene sequences might hinder the direct amplification of this template.

Our method requires less effort and highly reproducible by following our criteria for designing good oligos. In addition, our method also serves as a cheaper alternative to the commercial synthetic gene. This method is also ideal for sequences equal to or less than 1 kbp in length. Furthermore, this method also allows customization of the DNA sequence for the purpose of mutation study or codon modification for optimization of gene expression. Moreover, synthesizing the gene using this method can prevent direct contact with dangerous organisms such as poisonous animals and pathogenic microorganisms. Nonetheless, in a study where even a single nucleotide change must be avoided, this method might not be suitable because in our method, nucleotide change in DNA sequence not only alters the protein it codes, it is required to adjust the properties of the oligonucleotides such as the GC contents and the melting temperatures to accommodate the oligonucleotides assembly. Additionally, this method might also need extra works such as the optimization of the PCR condition and DNA purification step.

Conclusions

Gene synthesis from oligos could be challenging especially for beginners without proper planning and design. In this study, we have listed five criteria for designing a gene as a guide towards more successful gene synthesis. By following these criteria, we successfully produced synthetic *B. bassiana* protease gene using assembly PCR. Thus, the criteria would provide useful guidance for PCR-based gene synthesis. Besides, we have also described the application of SOE for repairing the errors that occurred in the synthesized gene. Even though the technique might have several disadvantages, generally it is acceptable for small genes, simple study and low scale synthesis.

Acknowledgement

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References

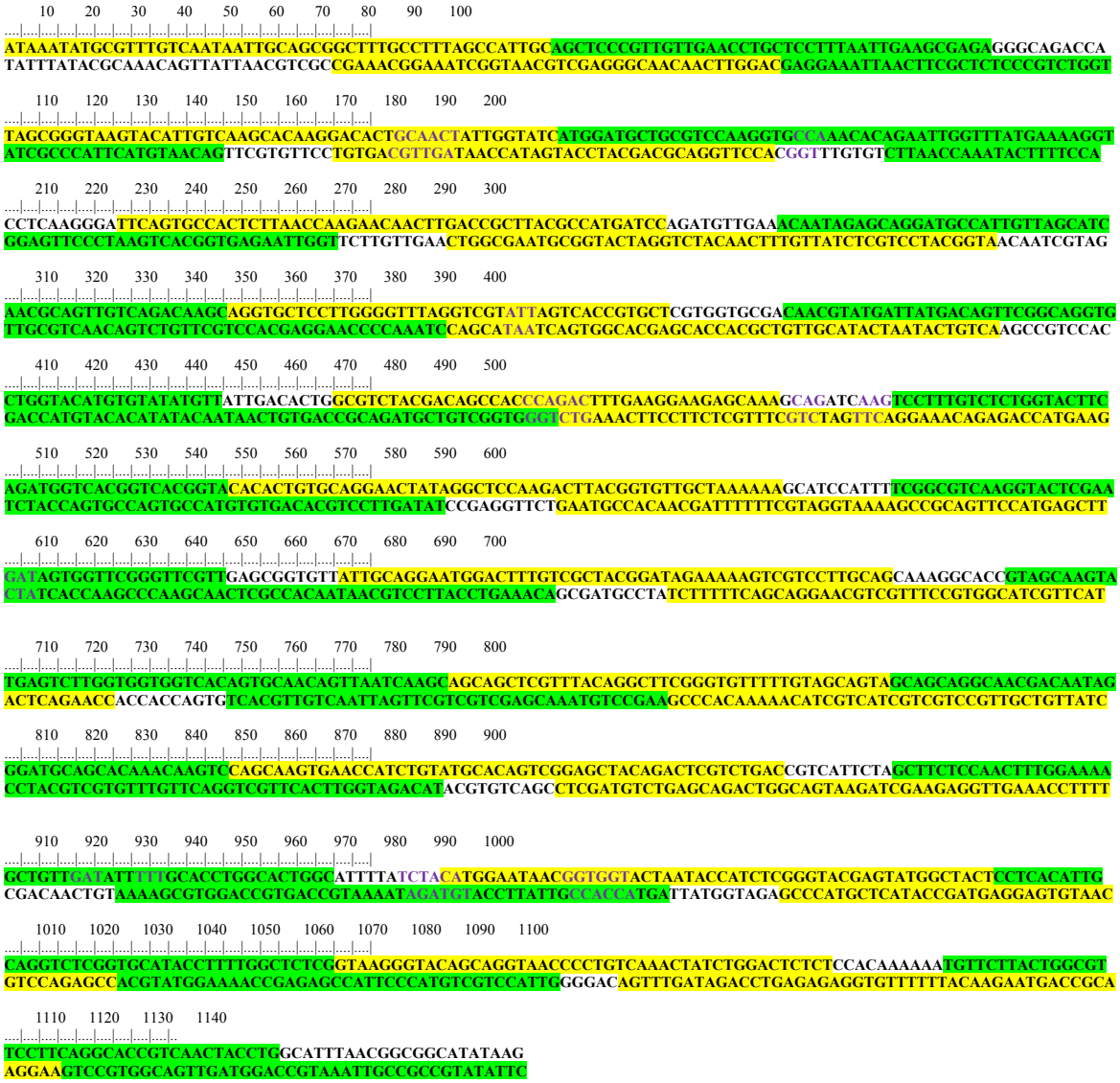
1. Xiao G, Ying SH, Zheng P, Wang ZL, Zhang S, Xie XQ, et al. Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana. Sci Rep. 2012;2.
2. Dhawan M, Joshi N. Enzymatic comparison and mortality of Beauveria bassiana against cabbage caterpillar Pieris brassicae LINN. Braz J Microbiol. 2017;48(3):522–9.
3. Gramkow AW, Perecmanis S, Sousa RLB, Noronha EF, Felix CR, Nagata T, et al. Insecticidal activity of two proteases against Spodoptera frugiperda larvae infected with recombinant baculoviruses. Virol J. 2010;7(1):143.
4. Stemmer WPC, Crameri A, Ha KD, Brennan TM, Heyneker HL. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene. 1995;164(1):49–53.
5. Zhang P, Ding Y, Liao W, Chen Q, Zhang H, Qi P, et al. A simple, universal, efficient PCR-based gene synthesis method: Sequential OE-PCR gene synthesis. Gene. 2013;524(2):347–54.
6. Gao X, Yo P, Keith A, Ragan TJ, Harris TK. Thermodynamically balanced inside-out (TBIO) PCR-based gene synthesis: a novel method of primer design for high-fidelity assembly of longer gene sequences. Nucleic Acids Res. 2003;31(22):e143–e143.
7. Young L, Dong Q. Two step total gene synthesis method. Nucleic Acids Res. 2004;32(7):e59–e59.
8. Xiong AS, Yao QH, Peng RH, Li X, Fan HQ, Cheng ZM, et al. A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequences. Nucleic Acids Res. 2004;32(12):e98–e98.
9. Yang J, Liu L. Codon optimization through a two-step gene synthesis leads to a high-level expression of Aspergillus niger lip2 gene in Pichia pastoris. J Mol Catal B Enzym. 2010;63(3):164–9.
10. Reisinger SJ, Patel KG, Santi D V. Total synthesis of

multi-kilobase DNA sequences from oligonucleotides. Nat Protoc. 2006;1(6):2596–603.
11. Wu G, Wolf JB, Ibrahim AF, Vadasz S, Gunasinghe M, Freeland SJ. Simplified gene synthesis: A one-step approach to PCR-based gene construction. J Biotechnol. 2006;124(3):496–503.
12. Ye H, Huang MC, Li M-H, Ying JY. Experimental analysis of gene assembly with TopDown one-step real-time gene synthesis. Nucleic Acids Res. 2009/03/05. 2009;37(7):e51–e51.
13. Sequeira AF, Brás JLA, Guerreiro CIPD, Vincentelli R, Fontes CMGA. Development of a gene synthesis platform for the efficient large scale production of small genes encoding animal toxins. BMC Biotechnol. 2016;16(1):86.
14. Shchur V V, Burankova YP, Shapira MA, Klevzhits D V, Usanov SA, Yantsevich A V. Programmed assembly of long DNA synthons: design, mechanism, and online monitoring. Appl Microbiol Biotechnol. 2019;103(21):9103–17.
15. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic acids symposium series. [London]: Information Retrieval Ltd., c1979-c2000.; 1999. p. 95–8.
16. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 1989;77(1):51–9.
17. Jiang Y, Deng F, Wang H, Hu Z. An extensive analysis on the global codon usage pattern of baculoviruses. Arch Virol. 2008/11/26. 2008;153(12):2273–82.
18. Gordeeva TL, Borschevskaya LN, Sineoky SP. Improved PCR-based gene synthesis method and its application to the Citrobacter freundii phytase gene codon modification. J Microbiol Methods. 2010;81(2):147–52.

Supplmemtary Table 1. Oligos used for the synthesis of the protease gene in this study.

Name	Sequence (5'-3')	Length	GC(%)	Tm(°C)
SP1	ATAAATATGCGTTTGTCAATAATTGCAGCGGCTTTGCCTTTAGCCATTGC	50	40.0	67.0
SP2	CAGGTTCAACAACGGGAGCTGCAATGGCTAAAGGCAAAGC	40	52.5	68.7
SP3	AGCTCCCGTTGTTGAACCTGCTCCTTTAATTGAAGCGAGA	40	47.5	67.2
SP4	GACAATGTACTTACCCGCTATGGTCTGCCCTCTCGCTTCAATTAAAGGAG	50	48.0	67.6
SP5	TAGCGGGTAAGTACATTGTCAAGCACAAGGACACTGCGACCATTGGTATC	50	44.0	66.9
SP6	ACCTTGGACGCAGCATCCATGATACCAATAGTTGCAGTGT	40	47.5	67.3
SP7	ATGGATGCTGCGTCCAAGGTGCCAAACACAGAATTGGTTTATGAAAAGGT	50	44.0	68.8
SP8	TGGTTAAGAGTGGCACTGAATCCCTTGAGGACCTTTTCATAAACCAATTC	50	42.0	66.5
SP9	TTCAGTGCCACTCTTAACCAAGAACAACTTGACCGCTTACGCCATGATCC	50	48.0	68.9
SP10	ATGGCATCCTGCTCTATTGTTTCAACATCTGGATCATGGCGTAAGCGGTC	50	48.0	68.9
SP11	ACAATAGAGCAGGATGCCATTGTTAGCATCAACGCAGTTGTCAGACAAGC	50	46.0	68.4
SP12	CTAAACCCCAAGGAGCACCTGCTTGTCTGACAACTGCGTT	40	52.5	68.7
SP13	AGGTGCTCCTTGGGGTTTAGGTCGTATTAGTCACCGTGCT	40	52.5	68.7
SP14	ACTGTCATAATCATACGTTGTCGCACCACGAGCACGGTGACTAATACGAC	50	48.0	68.1
SP15	CAACGTATGATTATGACAGTTCGGCAGGTGCTGGTACATGTGTATATGTT	50	42.0	66.1
SP16	TGGGTGGCTGTCGTAGACGCCAGTGTCAATAACATATACACATGTACCAG	50	48.0	68.1
SP17	GCGTCTACGACAGCCACCCAGACTTTGAAGGAAGAGCAAA	40	52.5	68.3
SP18	GAAGTACCAGAGACAAAGGACTTGATCTGCTTTGCTCTTCCTTCAAAGTC	50	44.0	66.2
SP19	TCCTTTGTCTCTGGTACTTCAGATGGTCACGGTCACGGTA	40	50.0	67.1
SP20	TATAGTTCCTGCACAGTGTGTACCGTGACCGTGACCATCT	40	50.0	67.3
SP21	CACACTGTGCAGGAACTATAGGCTCCAAGACTTACGGTGTTGCTAAAAAA	50	44.0	67.1
SP22	TTCGAGTACCTTGACGCCGAAAATGGATGCTTTTTTAGCAACACCGTAAG	50	44.0	67.5
SP23	TCGGCGTCAAGGTACTCGAAGATAGTGGTTCGGGTTCGTT	40	52.5	68.5
SP24	ACAAAGTCCATTCCTGCAATAACACCGCTCAACGAACCCGAACCACTATC	50	48.0	68.9
SP25	ATTGCAGGAATGGACTTTGTCGCTACGGATAGAAAAAGTCGTCCTTGCAG	50	46.0	68.0
SP26	CCAAGACTCATACTTGCTACGGTGCCTTTGCTGCAAGGACGACTTTTTCT	50	48.0	68.9
SP27	GTAGCAAGTATGAGTCTTGGTGGTGGTCACAGTGCAACAGTTAATCAAGC	50	46.0	67.3
SP28	AAGCCTGTAAACGAGCTGCTGCTTGATTAACTGTTGCACT	40	45.0	66.7
SP29	AGCAGCTCGTTTACAGGCTTCGGGTGTTTTTGTAGCAGTA	40	47.5	67.2
SP30	CTATTGTCGTTGCCTGCTGCTACTGCTACAAAAACACCCG	40	50.0	66.8
SP31	GCAGCAGGCAACGACAATAGGGATGCAGCACAAACAAGTC	40	52.5	68.4
SP32	TACAGATGGTTCACTTGCTGGACTTGTTTGTGCTGCATCC	40	47.5	66.7
SP33	CAGCAAGTGAACCATCTGTATGCACAGTCGGAGCTACAGACTCGTCTGAC	50	52.0	68.9
SP34	TTTTCCAAAGTTGGAGAAGCTAGAATGACGGTCAGACGAGTCTGTAGCTC	50	46.0	67.3
SP35	GCTTCTCCAACTTTGGAAAAGCTGTTGATATTTTTGCACCTGGCACTGGC	50	46.0	68.4
SP36	AGTACCACCGTTATTCCATGTAGATAAAATGCCAGTGCCAGGTGCGAAAA	50	44.0	68.0
SP37	CATGGAATAACGGTGGTACTAATACCATCTCGGGTACGAGTATGGCTACT	50	46.0	66.6
SP38	CCGAGACCTGCAATGTGAGGAGTAGCCATACTCGTACCCG	40	57.5	68.6
SP39	CCTCACATTGCAGGTCTCGGTGCATACCTTTTGGCTCTCG	40	55.0	68.6
SP40	GTTACCTGCTGTACCCTTACCGAGAGCCAAAAGGTATGCA	40	50.0	67.0
SP41	GTAAGGGTACAGCAGGTAACCCCTGTCAAACTATCTGGACTCTCT	45	48.9	66.9
SP42	AAGGAACGCCAGTAAGAACATTTTTTGTGGAGAGAGTCCAGATAGTTTGA	50	40.0	66.1
SP43	TGTTCTTACTGGCGTTCCTTCAGGCACCGTCAACTACCTG	40	52.5	68.1
SP44	CTTATATGCCGCCGTTAAATGCCAGGTAGTTGACGGTGCCTG	42	52.4	68.2

Supplementary Figure 1. The location of the oligos in the gene.



Supplementary Figure 2. The alignment of the expected sequence with the DNA sequence of the synthesised gene. The primer target sites were highlighted with yellow while the mutated sequences were highlighted with blue.