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ARTICLE

Enhancement of thermostability of *Bacillus subtilis* endoglucanase by error-prone PCR and DNA shuffling

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Abstract The *Bacillus subtilis* endo-β-1,4-glucanase gene (*beg*), which encodes the enzyme BEG that comprises 499 amino acid residues, was mutated by error-prone polymerase chain reaction and DNA shuffling to make variants with improved functionalities. The mutated DNAs were transformed into *Escherichia coli* DH5α, and among the 1370 transformants, a positive clone 8-20 was obtained finally based on a halo assay and thermostability and alkaline tolerance analyses. The mutated enzyme $BEG_{8\text{-}20}$ of clone 8-20 was changed at seven amino acid residues compared to the wild-type enzyme BEG_{wt}: K45E, I102Y, M112V, D226Y, D295E, L423S, and D460G. The optimum temperature and pH of $BEG_{8\text{-}20}$ were nearly the same as those of BEG_{wt}. However, the thermostability of $BEG_{8\text{-}20}$ was increased 1.53-fold (21.4 min vs. 14.0 min) based on the half-life of the residual activity at 70 °C. This increase in enzyme thermostability is advantageous for the hydrolysis of lignocellulosic materials to produce fermentable sugars. In the activity staining experiment, only a truncated smaller enzyme was observed for $BEG_{8\text{-}20}$, whereas two forms, a matured (52 kDa) and a truncated smaller (34.5 kDa) enzyme, were observed for BEG_{wt} owing to proteolytic internal cleavage at the linker region between the 297th and 356th amino acid residues. These results indicate that amino acid substitutions in the mutant enzyme have rendered the protein prone to cleavage at the C-terminal region.

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Introduction

Proteins with new or improved functionality can be made by in vitro mutations such as error-prone polymerase chain reaction (EP-PCR) or DNA shuffling (Cadwell and Joyce 1992; Stemmer 1994). DNA shuffling involves random fragmentation of several mutant DNAs followed by their reassembly using PCR under mutagenic conditions. Many proteins have been made by EP-PCR and DNA shuffling, including β-1,4-glucanases from cultured or uncultured microbial sources (Ni et al. 2005; Lin et al. 2009; Liang et al. 2011; Anbar et al. 2012; Telke et al. 2013). In these mutated and evolved enzymes, enzymatic properties such as catalytic efficiency, substrate specificity, heat stability, and organic solvent resistance have been improved to a moderate or great extent. Very recently, a mutant of endoglucanase and a mutant of β-1,3-1,4-glucanase mutated by EP-PCR showed 1.61- and 1.49-fold increment in catalytic activity, respectively (Lin et al. 2016; Mao et al. 2016).

Endoglucanases (e.g., 1,4-β-D-glucan cellobiohydrolase, endo-β-1,4-glucanase, EC 3.2.1.4) hydrolyze cellulose homopolymers consisting of glucose units linked by β-1,4-glucosidic bonds. These enzymes are used as keys or additive components and as synergistic proteins in the hydrolysis of lignocellulosic materials (Kim et al. 2014, 2015). Many endo-β-1,4-glucanases (also called carboxymethyl cellulase, CMCase) from *Bacillus* species have been reported (Cantwell and McConnell 1983; MacKay et al. 1986; Robson and Chambliss 1987; Lo et al. 1988; Park et al. 1991; Akita et al. 2005). Among

them, an endo-β-1,4-glucanase gene (*beg*) from *Bacillus subtilis* BSE616 was cloned, and its biochemical properties were characterized (Kim and Pack 1988; Park et al. 1991).

In this study, we performed EP-PCR and DNA shuffling to make enzyme variants for obtaining improved enzymes using the *B. subtilis* BSE616 *beg*, and we analyzed the biochemical properties and proteolytic internal cleavage of the enzymes.

Materials and methods

Bacterial strains and culture medium

Escherichia coli DH5α and transformants were grown at 37 °C in Luria–Bertani (LB) broth medium with shaking at 200 rpm. LB supplemented with 0.5% carboxymethyl cellulose (CMC) (LBC) was used for detecting BEG production. When necessary, ampicillin (Amp, 50 μg/mL) was added to the media. Plasmid pUC19 was used as a cloning vector.

Chemicals

Restriction enzymes, T4 DNA ligase, and DNase I were purchased from Promega (Madison, WI, USA). Oligonucleotide primers were synthesized by Bioneer Co. (Daejeon, Korea). CMC, *p*-nitrophenol (PNP), *p*-nitrophenyl-β-D-cellobioside (PNPC), 4-methylumbelliferyl cellobioside (MUC), 3,5-dinitrosalicylic acid (DNS), and Congo red were purchased from Sigma (St. Louis, MO, USA).

EP-PCR and DNA shuffling

DNA shuffling was performed as described by Stemmer (1994) with a modification. Plasmid pBS1 (Park et al. 1991) carrying *beg* was digested with *Pst*I and then ligated to *Pst*I-digested pUC19. This mixture was transformed into *E. coli* DH5α, and a positive colony was selected. The plasmid isolated from the positive clone was named pBSH62.

pBSH62 was amplified by standard or EP-PCR using universal primers (Table 1). The standard PCR conditions were as follows: 93 °C for 5 min (pre-melting), 30 cycles of template DNA denaturation at 93 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. EP-PCR was performed under modified conditions. The reaction mixture contained PCR buffer (10 mM Tris–HCl, pH 9.0, 40 mM KCl), 3 mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.25 mM dATP, 0.75 mM dTTP, 0.75 mM dCTP, 0.75 mM dGTP, 50 pmol of each primer, and 5 units of *Taq* polymerase in a 100-μL reaction.

The PCR product was extracted from an agarose gel, and the DNA was randomly digested by DNase I (0.05 U/μL) at 15 °C for 15 min. The DNase I digestion buffer contained 40 mM Tris–HCl, pH 8.0, 10 mM $MgSO_4$, and 1 mM $CaCl_2$. The digested 100- to 300-bp DNA fragments were purified using a Gel Extraction Kit. The 100- to 300-bp DNA fragments for *beg* were reassembled by PCR under the following conditions: 93 °C for 5 min, 90 cycles of 93 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The reaction mixture contained PCR buffer (10 mM Tris–HCl, pH 9.0, 40 mM KCl, and 1.5 mM $MgCl_2$), 0.25 mM of each dNTP, 10 pmol of each primer, and 1 unit of *Taq* polymerase in a 20-μL reaction volume. The reassembled DNA fragments were amplified by conventional PCR (30 cycles) with 1 μL of reassembled DNA fragments in a 20-μL reaction using inner primers (Table 1). The forward and reverse inner primers contain *BamH*I and *Pst*I sites, respectively, at each 5′ end. The amplified product was cut from the gel, and the DNA was extracted.

Screening for mutants

The product of the EP-PCR and DNA shuffling was digested with *BamH*I-*Pst*I and then ligated into the pUC19 vector digested with the same enzymes. The ligation mixture was introduced into *E. coli* DH5α competent cells prepared by Hanahan's method (1985) using a conventional transformation method or by electroporation. Electroporation was performed with a pulse of 2.5 kV, 25 μF, and 200 Ω in a 0.2-cm electroporation cuvette (Bio-Rad, Hercules, CA, USA) per 500 ng of DNA. The transformants were picked using sterile toothpicks, replicated on LBC plates containing ampicillin, incubated for 24 h at 37 °C, and selected by a halo assay using staining with 0.2% Congo red and destaining with 1 M NaCl. The mutants producing thermostable endoglucanase were

Table 1 Primers used in EP-PCR and DNA shuffling for amplifying the endoglucanase gene from *Bacillus subtilis*

Primer	Sequence
Outer gene forward primer (23-mer)	AGCGGATAACAATTTCACACAGG
Reverse primer (23-mer)	CCCAGTCACGACGTTGTAAAACG
Inner gene forward primer (27-mer)	ACTC**GGATCC**TGTTCTATATGTATCAT
Reverse primer (27-mer)	TGTAA**CTGCAG**GATGCTGGATGTCTTT

screened by heating for 2 h at 70 °C in a dry oven before the halo assay. After incubation for 1 h at 37 °C, the agar plates were subjected to a halo assay. Those mutants that produced alkaline-stable endoglucanase were screened by LBC plates adjusted to pH 9.5 and selected by the halo assay.

Enzyme assay

CMCase activity was determined by measuring the amount of released reducing sugar by the DNS method (Miller 1959) after 30 min of reaction at 55 °C using 0.5% CMC in 50 mM sodium citrate, pH 5.5, as the substrate. One unit of enzymatic activity was defined as the amount of enzyme that released reducing sugar equivalent to 1.0 µmol of glucose per minute under the above conditions. The PNPC-hydrolyzing activity was estimated by measuring the release of PNP from PNPC according to Deshpande et al.'s method (1984). Assay mixtures contained 0.9 mL of 2.2 mM PNPC in 50 mM sodium citrate buffer, pH 5.5, and 0.1 mL of the enzyme sample. After 30 min at 40 °C, 3 mL of 1 M Na_2CO_3 solution was added to the reaction mixture, and then, the absorbance was measured at 400 nm. One unit of PNPC-hydrolyzing activity was defined as the amount of enzyme liberating 1 µmol of PNP in 1 min. The protein concentration was determined by Lowry et al.'s method (Lowry et al. 1951) with bovine serum albumin as the standard.

Effect of temperature and pH of mutants

The optimum temperature was analyzed by measuring the enzyme activity at 40–70 °C. The optimum pH was determined using 50 mM sodium citrate (pH 4.0–6.0) or Tris–HCl (pH 7.0–9.0). The heat stability of the mutants was determined by measuring the remaining activity after treating the enzyme in the absence of the substrate at 55, 65, and 70 °C for designated time periods in 50 mM sodium citrate buffer (pH 5.5).

Activity staining (Zymogram)

Activity staining was performed as described previously (Lee et al. 1999) with a slight modification. The crude enzyme was electrophoresed on an 11.5% SDS-polyacrylamide gel. After electrophoresis, the gel was washed in 20% isopropanol (3 × 15 min) and then in 50 mM sodium citrate buffer (pH 5.5) (3 × 15 min). The washed gel was kept in contact with 2 mM MUC for 15 min and incubated for 1 h at 55 °C. The fluorescent band of the active protein was photographed under a UV lamp. The protein was stained with Coomassie Brilliant Blue R-250.

Results and discussion

Screening of mutants after EP-PCR and DNA shuffling

In this study, the recombinant plasmid pBSH62 (6.2 kb) was constructed using the plasmid pBS1 carrying *beg* from *B. subtilis* and a vector pUC19 by the ligation of *Pst*I-cut fragments. Then, pBSH62 was amplified using universal primers by standard PCR or EP-PCR. The resulting 3.5-kb PCR product was isolated and randomly digested by DNase I, and then, DNA fragments of 100–300 bp were reassembled without primers. Finally, the reassembled DNA fragments were amplified using inner primers by conventional PCR. The 1.8-kb product was extracted and digested with *Bam*HI-*Pst*I and then ligated into the pUC19 vector digested by the same enzymes. The ligation mixture was introduced into *E. coli* DH5α by heat-shock transformation or electroporation. In the first screening, 59 positive clones for BEG were identified among 1370 transformants based on the halo assay at 37 °C. After repeated transfer, nine transformants were selected from the positive clones and tested for heat stability and alkaline tolerance (data not shown). Based on the results of heat treatment and alkaline tolerance of the enzymes, three positive clones (7-215, 8-20, and 8-85) were finally selected and tested repeatedly (Fig. 1).

Amino acid change and thermostability of enzyme

From the DNA sequences of the plasmid isolated from the clones, it was realized that the mutated enzyme $BEG_{8\text{-}20}$ of clone 8-20 was changed at seven amino acid residues: K45E, I102Y, M112V, D226Y, D295E, L423S, and D460G (Table 2). No changes were observed in two other enzymes of clones 7-215 and 8-85. This halo screening method is powerful and convenient, but has limitation about precise quantitation of the enzyme production depending on the degree of enzyme secretion, other physiological conditions, and/or other unknown reason.

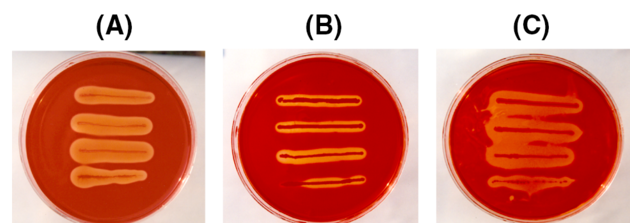


Fig. 1 Detection of endoglucanase positive clones by Congo red staining after no heat treatment (**A**), preheat treatment for 2 h at 70 °C (**B**), and growth in alkaline medium (pH 9.5) (**C**). The four positive clones were wild type, 7-215, 8-20, and 8-85 from the top of each plate

Table 2 Amino acid substitutions in the mutant enzyme $BEG_{8\text{-}20}$

Residue number	BEG_{wt}	$BEG_{8\text{-}20}$
45th	Lys	Glu
102nd	Ile	Tyr
112th	Met	Val
226th	Asp	Tyr
295th	Asp	Glu
423rd	Leu	Ser
460th	Asp	Gly

Sometimes transformants with no change in amino acid of the structural gene were selected. Therefore, we have to perform repeated culture and determine the sequence of inserted DNA. The optimum temperature and pH of the mutated enzyme $BEG_{8\text{-}20}$ were 55 °C and 5.5, respectively; these are similar to those of the wild-type enzyme BEG_{wt} (Fig. 2). $BEG_{8\text{-}20}$, however, was more tolerant to the acidic pH region; the residual enzyme activity of $BEG_{8\text{-}20}$ at pH 4.0 was 97% of the optimal activity at pH 5.5, though the value of BEG_{wt} was 79% (Fig. 2B). In an experiment on heat stability with preincubation for 15 min at 70 °C, $BEG_{8\text{-}20}$ retained 67% of its activity, whereas BEG_{wt} retained 43% of its activity (Fig. 3). It was observed that the half-life of $BEG_{8\text{-}20}$ at 70 °C was 21.4 min, whereas that of BEG_{wt} was 14.0 min. These results indicate that the mutation in $BEG_{8\text{-}20}$ made by EP-PCR and DNA shuffling increased the thermostability of the enzyme 1.53-fold. This increase in enzyme thermostability can be advantageous for the hydrolysis of lignocellulosic materials to produce fermentable sugars.

Proteolytic internal cleavage of enzyme

In activity staining after SDS-PAGE and renaturation of the enzymes, it was found that the molecular mass of the active form of the mutated enzyme $BEG_{8\text{-}20}$ (34.5 kDa) was smaller than that of the intact form of the wild type (52 kDa) (Fig. 4). The internal cleavages of this enzyme were reported previously in *Bacillus megaterium* or *E. coli* transformants (Ahn et al. 1993; Kim et al. 1995); a matured and intact form of the enzyme (52 kDa) was cleaved at the C-terminal regions and truncated to smaller forms (33 or 34.5 kDa, respectively). In the BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), BEG comprises a signal peptide (29 amino acid residues), a catalytic domain (CD) belonging to the glycosyl hydrolase family 5 (50–296 amino acid residues), and a carbohydrate-binding module (CBM, 357–436 amino acid residues). The proteolytic cleavage was occurred around the connecting region

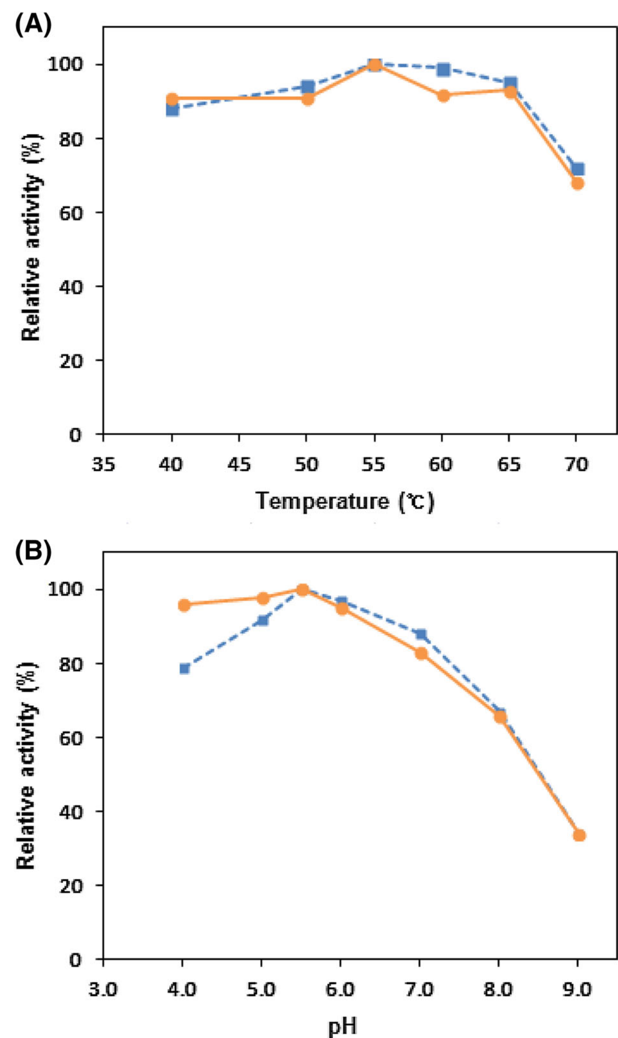
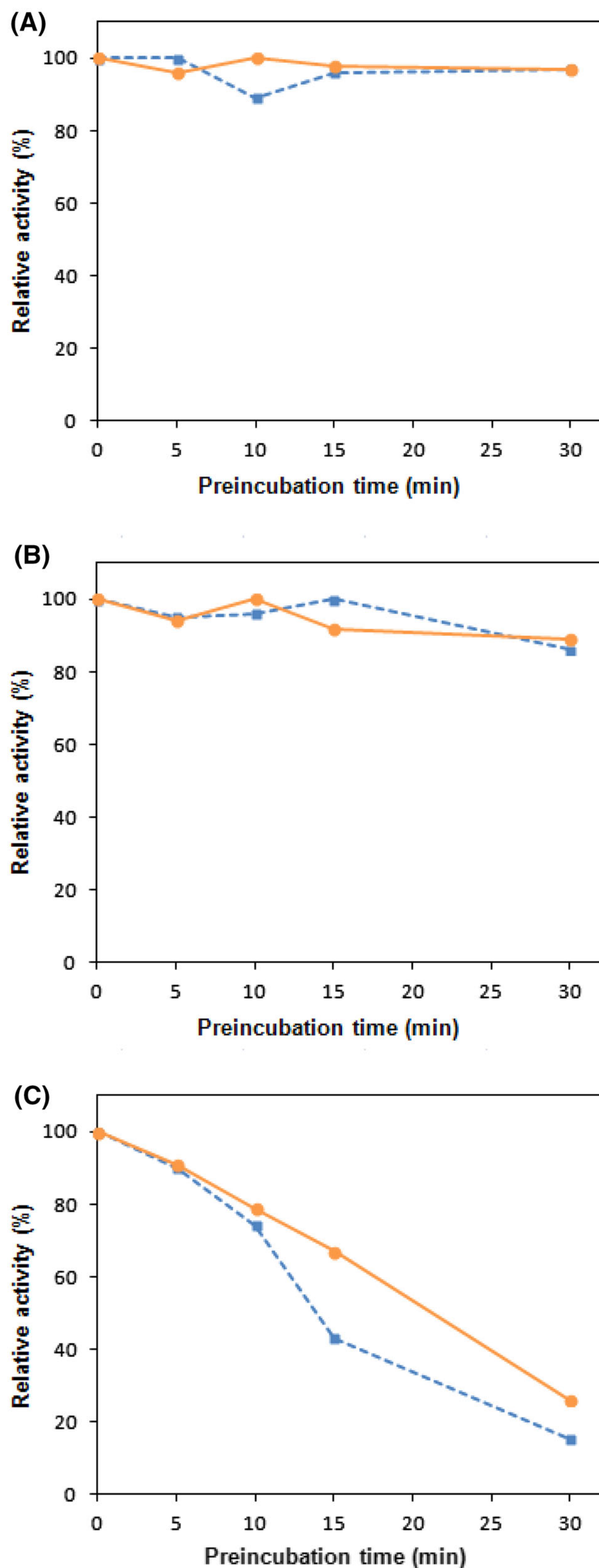


Fig. 2 Temperature (**A**) and pH dependency (**B**) of wild-type enzyme (*filled square*) and mutant enzyme (*filled circle*) of clone 8-20

of CD and CBM (Kim et al. 1995). It is likely that internal cleavages occur between 297th and 356th amino acid residues of the enzyme. In this study, the cleaved form (34.5 kDa) of the wild-type enzyme was well observed, and the band corresponded to the band of the mutated $BEG_{8\text{-}20}$ enzyme; however, the intact large form was not observed (Fig. 4). These results may suggest that the substitutions in Asp to Glu at the 295th residue, locating at the end of CD, and/or Leu to Ser at the 423rd residue, positioning near the end of CBM, play an important role in the elevated level of cleavage.

Catalytic properties

The specific activity of enzymes for a large or small polymeric molecule is shown in Table 3. The $BEG_{8\text{-}20}$ values for CMC or PNPC were, respectively, slightly



◀**Fig. 3** Influence of temperature on enzyme activities of wild-type enzyme (*filled square*) and mutant enzyme of clone 8-20 (*filled circle*). Thermostability was determined by the preincubation of the enzyme without the substrate at 55 °C (**A**), 65 °C (**B**), and 70 °C (**C**) for designated time periods

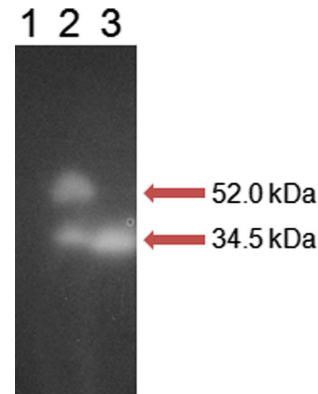


Fig. 4 Activity staining of wild-type enzyme BEG_{wt} and mutant enzyme $BEG_{8\text{-}20}$ after SDS-PAGE using crude extracts. *Lane 1*: *E. coli* DH5α (pUC19) as a control, *lane 2*: wild type, *lane 3*: mutant enzyme of clone 8-20

Table 3 Specific activities of wild-type and mutant enzymes

Enzyme	Specific activity (U/mg protein)	
	CMC	PNPC
BEG_{wt}	0.64	0.041
$BEG_{8\text{-}20}$	0.78	0.046

CMC Carboxymethyl cellulose, *PNPC p*-nitrophenyl-β-D-cellobioside

greater than or almost the same as the BEG_{wt} values. The kinetic parameters of the enzymes were determined using the Lineweaver–Burk plot. The K_m and V_{max} values of $BEG_{8\text{-}20}$ were similar to those of BEG_{wt} (Table 4). These results indicate that the catalytic properties of the mutated enzyme were nearly unchanged.

Since the DNA shuffling strategy was proposed as a directed evolution method, the properties of many enzymes have been improved artificially (Stemmer 1994; Lin et al.

Table 4 Kinetic parameters for *p*-nitrophenyl cellobioside (PNPC) of wild-type and mutant enzymes

Enzyme	K_m (mM)	V_{max} (ΔA/min)
BEG_{wt}	3.2	0.10
$BEG_{8\text{-}20}$	3.1	0.11

2009, 2016; Labrou2010; Tiwari 2016). In this study, a mutant endo-β-1,4-glucanase $BEG_{8\text{-}20}$ that was substituted at seven amino acid residues was obtained by EP-PCR and DNA shuffling. The optimum temperature and pH, specific activity, and kinetic parameters of the mutant enzyme were nearly the same as those of the wild type. The fact that the molecular mass of $BEG_{8\text{-}20}$ was smaller than that of the wild type suggests that amino acid substitutions in the mutant enzyme have rendered the protein prone to proteolytic cleavage at the C-terminal region. Compared to the wild type, the thermostability of $BEG_{8\text{-}20}$ was increased 1.53-fold based on the half-life at 70 °C, suggesting that the mutation in $BEG_{8\text{-}20}$ produced by EP-PCR and DNA shuffling increased the thermostability.

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