SAGE-Hindawi Access to Research
Biotechnology Research International
Volume 2011, Article ID 305956, 5 pages
doi:10.4061/2011/305956

Research Article

Cloning and Expression of Randomly Mutated *Bacillus subtilis* α-Amylase Genes in HB101

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Received 12 April 2011; Accepted 26 May 2011

Academic Editor: Triantafyllos Roukas

The aim of this study was to isolate and express the randomly mutated *α-amylase* gene from *B. subtilis* strain 168. BS168F: 5′-gtgtcaagaatgtttgc-3′ and BS168R: 3′-gttttgttaaaagatga-5′ primers were used to amplify the amylase gene using the following cycle in error-prone PCR method: 94°C for 30 s, 40°C for 2 min, and 72°C for 2 min in 30 cycles that were followed with 72°C for 2 min as a post cycle. *E. coli* XL1 blue was used as host for plasmid construction. Amylase enzyme activity assay was performed using continuous spectrophotometric procedures. Results of sequencing showed that sequence was cloned from the first ATG and with the correct open reading frame. Having confirmed the integrity of the insert, the gene was ligated into expression vector pET-15b and then further confirmed using digestion analysis. Amylase activity showed 3 clones with higher enzymatic activity compared with the wild type. Error-prone PCR method produced a mutated gene that provides amylase activity much higher than that of wild type. Sequencing the mutated genes should shed light on the important region of the genes that could be manipulated in future studies.

1. Introduction

α-Amylases are extracellular enzymes which randomly cleave the α-1,4 linkages between adjacent glucose units in the linear amylase chain, generating glucose, maltose, and maltotriose units [1]. According to the type of bonds they hydrolyze and their products, they are classified as α, β, and γ amylases [2]. They are among the most important commercial enzymes, with wide applications in starch processing, brewing, alcohol production, textile, paper, detergent, clinical, medicinal, and analytical chemistry [1]. Amylase enzymes make up approximately 25% of the enzyme market. Although α-amylases can be derived from plants and animals, they are the enzymes from microbial sources (typically *Bacillus* spp.) that are generally used to meet the expanding industrial demands [3]. Mesophilic and thermophilic fungi are also good source of amylolytic enzymes due to their working conditions and reaction specificity properties [4].

The Gram-positive bacterium *B. subtilis* naturally thrives in the soil and the plant rhizosphere. Due to its life in unfavorable conditions, *Bacillus* produces and secretes a wide variety of enzymes with industrial applications [5]. *B. subtilis* is preferred organism, as it transports proteins across the cytoplasmic membrane into the growth medium [6]. Being an excellent model for investigating the mechanisms of gene regulation, differentiation, and metabolism, *B. subtilis* has been extensively studied in hundreds of laboratories worldwide for more than half a century using a variety of laboratory strains. The laboratory strain 168 is the only

B. subtilis strain with known genomic sequence, obtained through an extensive collaboration more than ten years ago [7]. *B. subtilis* 168 was constructed by mutagenic X-rays and UV treatment of the wild-type *B. subtilis* (Marburg) strain [8].

Considering the widespread application of α-amylases in different industrial processes, any improvement in the enzyme production, thermostability, and activity will have a direct impact on the process performance, economics, and feasibility. The requirements for an optimally performing enzyme are quite different for different industries, especially the optimal pH and temperature required. Protein engineering techniques have been applied to improve the different characteristics of a targeted enzymes by introducing mutations in the α-amylases-encoding DNA sequence. The methods for protein engineering can be roughly categorized into two: site-directed mutagenesis and random mutagenesis. Site-directed mutagenesis is to introduce additions/deletions/substitutions of specific amino acids, and structural information of the targeted enzyme is essential to make those alternations. Random mutagenesis is to introduce mutations at random along the entire length of a gene using error-prone PCR [9]. Error-prone PCR method is widely used for constructing a randomly mutagenized DNA library [10]. This method has been successfully applied for various purposes such as substrate specificity, improve thermal stability, and to identify critical residues for protein-ligand interaction [10]. Considering the importance of amylase in various industries, the aim of the current project was to randomly mutagenise the bacillus subtilis 168 α-amylase gene using error-prone PCR technique. The mutated genes were later expressed in suitable expression vector and their enzymatic activities were compared with the wild-type enzyme.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, Enzymes, and Chemicals. *Escherichia coli* strains XL1Blue and *B. subtilis 168* were purchased from PTCC (Iran) and DSMZ (Germany), respectively. All bacterial strains were cultured in Luria-Bertani (LB) liquid or on LB plates, supplied with appropriate antibiotics, at 37°C. InsT/A clone PCR product cloning kit was purchased from Fermentas (Hungary). Restriction enzymes and reagents for gene manipulation were obtained from Fermentas and were used according to the manufacturer's recommendations. Primers were synthesized by Fazapazo (Iran). Genemorph II random mutagenesis kit was purchased from Stratagene (Switzerland). QIA quick gel extraction kit and high pure PCR template preparation kit were purchased from Roche (Germany). Electrophoresis equipment for agarose gel electrophoresis was from Bio-Rad. All other chemicals and reagents were of analytical grade and were purchased from Sigma or Merck unless otherwise stated. All buffers and solutions were prepared with Milli Q water (Millipore).

2.2. Media and Growth Conditions. *E. coli* and *B. subtilis 168* were grown in Luria-Bertani (LB) medium, adjusted to pH 7.2 with NaOH on a gyratory shaker with 220 rpm at 37°C. Ampicillin 50 μg/mL was used as the identical final concentrations, in plates and in liquid media.

2.3. Genomic DNA Extraction. Genomic DNA was isolated from *B. subtilis* cells according to Roche "high pure PCR template preparation kit" instruction manual. Briefly, cells were harvested in midexponential growth phase by centrifugation (3000 ×g, 5 min) and suspended in PBS buffer (4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 137 mM NaCl, 1.4 mM KH_2PO_4, and 2.7 mM KCl (pH 7.2)). Cells were treated with lysozyme (10 mg/mL in 10 mM Tris-HCl, pH 8.0) for 30 min at 37°C and subsequently with proteinase K (100 mg/mL) at 72°C for 10 min. DNA was precipitated with 0.6 volumes of isopropanol and harvested by centrifugation (10,000 ×g, 15 min at 4°C).

2.4. Random Mutagenesis of α-Amylase Gene. The α-amylase gene of *BS*168 was amplified by Genemorph II random mutagenesis kit using purified genomic DNA as a tem plate. Based on the deduced ORF of the amylase gene, oligonucleotide primers were synthesized to amplify the intact region of α-amylase. The forward primer, *BS*168F 5′-CTAATTCCATGGGG*ATG*TTTGCAAAACGATTCA-3′, introduced *Nco*I site (underlined) just prior to the initiation site of amylase (italics). The reverse primer, *BS*168R 5′-GAA CGCGGATCCTCAATGGGGAAGAGAA-3′, was complementary to the nucleotides positioned before the termination codon and introduced *BamH*I site (underlined) just downstream of the termination codon (italics). These primers correspond to the boundaries of the coding sequence of the gene from translation initiation to the termination codon (underlined), thus the final PCR product was 1983 Kb.

The PCR mixture consisted of 5 μL of 10X Mutazyme II reaction buffer, 1 μL of 40 mM mutagenic dNTP mix (200 μM each final), 25 μL each of forward and reverse primers (2.5 μM each primer), 1 μL of Mutazyme II DNA polymerase (2.5 U/μL), and 400 ng of template in a total volume of 50 μL.

The PCR was performed with the following cycling profile: initial denaturation at 94°C for 5 min, followed by 35 cycles of 1 min denaturation at 94°C, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The time for the final extension step was increased to 20 min.

The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and cloned into pTZ57R vector. Ligation stage was achieved by adding T4 DNA ligase to solution containing PCR product and pTZ57R vector at 22°C overnight. The recombinant plasmids were transformed into *E. coli* XL1Blue and HB101, using the Hanahan protocol [11], for amplification. Plasmid DNA from the resulting clones was purified using the QIAprep spin miniprep kit and screened through restriction digestion for the insert of the PCR product. Plasmids identified as carrying the insert were digested with *Nco*I/*BamH*I, and after isolation through gel electrophoresis and purification, the insert was ligated into the expression vector pET-15b predigested with *Nco*I and *BamH*I. The

ligation mixture was transformed into *E. coli* HB101 for plasmid propagation. Recombinant plasmids were screened through *Nco*I/*BamH*I digestion, and clones containing the insertion of the intact coding region for α-amylase were identified. The presence of α-amylase gene was determined at 92°C using the 12/KI method as described previously [12].

2.5. Enzyme Assay. Amylase activity was measured by the dinitrosalicylic acid (DNS) method (Rick, 1974). Soluble starch was prepared by heating a suspension of starch at concentrations ranging from 0 to 2 mg/mL in 100 mM phosphate buffer (77 mM Na_2HPO_4, 23 mM NaH_2PO_4) until a homogeneous, viscous solution was obtained. The reaction mixture containing 1% soluble starch and 1 mL of enzyme solution was kept for 12 min at 25°C. To the above reaction, 0.1 mL of DNS reagent was added, and the mixture was boiled for 15 min. After cooling, the reaction mixture was diluted by distilled water, and the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme causing the release of 1 μmole of reducing sugars in one min under the assay conditions.

2.6. Statistics. The statistical analysis of the results was carried out using SigmaStat 3.10 program and based on analysis of variance (ANOVA) followed by the Tukey post-hoc test. $P < 0.05$ was considered significant. All data are expressed as mean ± S.E.M.

3. Results

3.1. Construction of the Expression Vector pet-15b. PCR amplification of the α-amylase gene of *BS*168 under normal or random mutagenesis conditions resulted in production of a band in the expected region of 1983 bp as is shown in Figures 1(a) and 1(b), respectively. *BS*168F and *BS*168R primers correspond to the boundaries of the coding sequence of the α-amylase gene from translation initiation to the termination codon, thus the final PCR product of 1983 bp should be observed.

Using Qia quick gel extraction kit, PCR products were purified and used for ligation into the pTZ57R plasmid with the aid of T/A cloning kit. The constructs were then used to transform *E. coli* XL1-Blue and *E. coli* HB101. From the library of pTZ57R bacterial plasmids, several clones were shown to contain the α-amylase gene in the correct orientation. The cloned DNA fragment in pTZ57R was digested from upstream and downstream of the gene by *Bam*HI and *Nco*I restriction enzymes, and the 1983 bp DNA fragment was inserted into the same endonuclease sites of the pET-15b expression vector. The orientation of the insert in the vector was confirmed using *Eco*RI restriction enzyme as depicted in Figure 2. Digestion of the recombinant pET-15b with *Eco*RI should produce two bands in the region of 1764 and 5857 bp.

The amylase activity of the recombinant vector was assessed by transforming the vector in *E. coli* HB101. The cells harbouring the plasmid were grown on ampicillin plate parallel with negative controls cells containing pET-15b. All

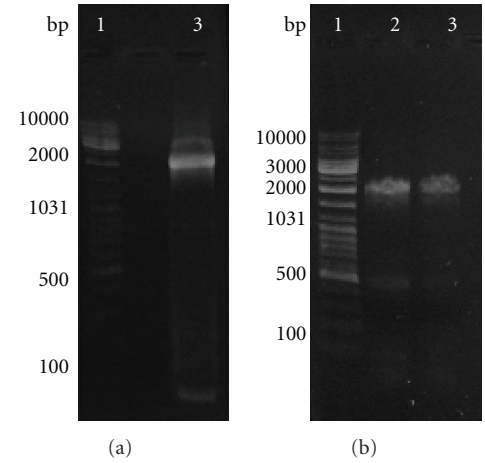

Figure 1: PCR amplification of α-amylase gene of *BS*168. (a) amplification of α-amylase gene. Lanes 1: DNA molecular size marker, 3: PCR product. (b) Error-prone PCR product of α-amylase gene. Lanes 1: DNA molecular size marker, 2 and 3: error-prone PCR products. 10 μL of samples were applied to a 0.7% agarose gel for electrophoretic separation.

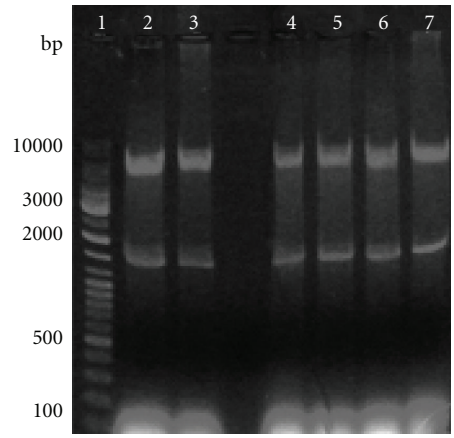

Figure 2: Digestion of the pET-15b containing α-amylase plasmid with *Eco*RI restriction enzyme. Lane 1: molecular weight marker. Lanes: 2, 3, 5, 6, and 7: digested pET-15b plasmid harbouring α-amylase with *Eco*RI restriction enzyme.

the colonies of *E. coli* carrying PET plasmid tested by the KI-I2 method showed haloes in starch-containing plates indicating the presence of α-amylase gene.

3.2. Nucleotide Sequences of B. subtilis 168 α-Amylase Gene. The complete nucleotide sequence of the DNA fragment encompassing the α-amylase gene was determined with both strands using T7 primers. Sequence analysis with BLAST program revealed 100% homology between this gene and standard α-amylase gene that outlined in GenBank with NC_000964 accession number.

3.3. Enzymatic Properties of Randomly Mutated Amylase Genes. A comparison of the catalytic activity of the clones

Table 1: The α-amylase activity of wild, error-prone gene and empty vector.

Samples	Mean absorbance at 540 nm	α-amylase activity (U/mL)
Sample 3	0.306	605.39 ± 0.0416
Sample 27	0.278	553.92 ± 0.0134
Sample 6	0.377	735.29 ± 0.0124
Wild-type 1	0.283	561.27 ± 0.0015
Wild-type 2	0.271	541.67 ± 0.0032
B. subtilis	0.0217	95.10 ± 0.0107
Plasmid pET15-b	0.0233	97.79 ± 0.0153

produced by error-prone PCR with those of the wild-type enzyme was carried out using soluble starch as a substrate, measuring the release of reducing sugars. Amylase activity was first measured under different IPTG concentrations (0.25, 0.5, 1, 1.5 mM) in both supernatant and intracellular fractions of cells during different times (4, 17, 24, and 48). *E. coli* HB101 cells harbouring pET-15b were used as a negative control and represented no amylase activity. IPTG concentration required for optimal amylase starch hydrolysis activity was 0.5 mM. The time for optimum expression cultivation condition for amylase activity was found to be 4 h. From the total of 97 clones that were screened, 3 samples showed the maximum enzymatic activities (Table 1). The activities of the three samples were significantly higher than that of the wild types ($P < 0.05$).

4. Discussion

The aim of the current project was to isolate amylase genes from *B. subtilis* 168 and then using error-prone PCR technique to mutate the PCR product. *Bacillus* species are among bacteria that are widely used for production of commercial enzymes. At present, about 60% of the commercially available enzymes are produced by *Bacillus* species, mostly being homologous proteins that are naturally secreted in the growth medium such as alkaline proteases as washing agent or amylases for the starch industry [13, 14]. The first step in the process of obtaining recombinant protein was to select a suitable microorganism that carries the gene of the request protein or enzyme. In this study, *B. subtilis* 168 was used as a source of DNA for amylase gene. *B. subtilis* 168, which is second only to *E. coli* in the extent to which tools of genetic and protein engineering have been developed, has contributed to the extensive use for the industrial production of bioengineered materials. The advantage of *B. subtilis* 168 over other bacterial strains has been increased even more by the availability of the complete sequence of the *B. subtilis* 168 genome [15].

Using the designed pair of primers, the PCR reaction was carried out under a standard condition that was stated in Section 2. The expected band of approximately 2000 bp was observed after several repeats and changes in annealing temperature. Transformation of recombinant pTZ57R plasmid in two *E. coli* strains, XL1-Blue, and HB101 produced several colonies which harboured the recombinant plasmid. *Eco*RI digestion of the recombinant plasmid resulted in production of several bands on agarose gel. *Eco*RI cuts amylase gene at 538 bp region and the pTZ57R cloning vector at 615 bp region. Depending on the orientations of α-amylase gene in pTZ57R plasmid, two different size bands would be expected. If the orientation of α-amylase gene is such that its 5′ end is near T7 promoter, then, following the digestion with *Eco*RI enzyme, two bands of 537 and 4296 bp should be produced, otherwise, two bands at about 1480 and 3389 bp regions are resulted. Double digestion of the recombinant plasmid with *Nco*I and *Bam*HI restriction enzymes provided the final endorsement for the correct orientation of the insert. As explained previously, when the double digestion was carried out only two bands of approximately, 2000 bp and 2900 bp are observed which corresponded to the insert and the pTZ57R, respectively.

Having confirmed the integrity of the insert in the plasmid, the next step was to determine the sequence of the insert. T7 primers were used for nucleotide sequencing of DNA fragment encompassing the α-amylase gene. The sequence of the fragment was analyzed using BLAST program and showed a 100% homology with the standard α-amylase gene that outlined in GenBank with NC_000964 accession number.

A large number of colonies containing the recombinant plasmids were selected and analyzed for the existence of the newly inserted wild-type or error-prone amylase genes in them. After confirmation of the clones at this stage, amylase activity was measured according to the method that was stated in previous sections. From the total of 97 clones that were analyzed, 3 clones proved to have an enzymatic activity greater than those of the wild-type genes. The activity of recombinant amylases is relatively poor compared to those of most bacterial α-amylases. Comparison of this activity to closely related amylases is difficult as most have been partially or completely purified. These 3 clones are now required to be taken for further detailed studies. The fact that only 3 clones had activity greater than those of wild type requires further adjustment in the process. Alteration in the process of mutation by using another error-prone kit or manually altering the gene could be an alternative pursuit that due to lack of fund could not be seriously carried out in this study. Confirmation of the amylase activity by other methods and the sequencing of the gene could pave the way for production in the lab scale. Testing of the enzyme for activity in industrial process is another point that is worth perusing in future studies.

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