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Repeated Random Mutagenesis of α-Amylase from *Bacillus licheniformis* for Improved pH Performance

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The α-amylases activity was improved by random mutagenesis and screening. A region comprising residues from the position 34–281 was randomly mutated in *B. licheniformis* α-amylase (AmyL), and the library with mutations ranging from low, medium, and high frequencies was generated. The library was screened using an effective liquid-phase screening method to isolate mutants with an altered pH profile. The sequencing of improved variants indicated 2–5 amino acid changes. Among them, mutant TP8H5 showed an altered pH profile as compared with that of wild type. The sequencing of variant TP8H5 indicated 2 amino acid changes, Ile157Ser and Trp193Arg, which were located in the solvent accessible flexible loop region in domain B.

Keywords: Directed evolution, high-throughput screening, error-prone PCR, *Bacillus* α-amylase, random mutagenesis

α-Amylases (1,4-α-D-glucan glucanohydrolase; E.C. 3.2.1.1) catalyze the hydrolysis of internal α-D-(1,4)-glucosidic linkages in starch, glycogen, and related oligo- and polysaccharides to produce maltodextrins, maltooligosaccharides, and glucose [30]. The industrial applications of α-amylase include (i) starch liquefaction and hydrolysis, (ii) manufacturing fructose and glucose syrups, (iii) improving flour in the baking industry, (iv) production of modified starches for the paper industry, (v) removal of starch from textiles (desizing), and (vi) additive to detergents in washing machines [11, 12, 28]. These industrial processes are operated under diverse physical and chemical conditions. In the industrial production of glucose, starch-liquefaction by α-amylase and saccharification by β-amylase are carried out sequentially [17]. The liquefaction of the starch is performed at a pH of 4.5 and a temperature of 105°C. Since α-amylase is unstable under these conditions, the pH of the reaction is increased to 5.7–6.0 and calcium is added. The second step in the process is saccharification of the liquefied product using a glucoamylase. Because pH 4.2–4.5 is the optimum for the glucoamylase activity, the pH is adjusted to 4.5 to facilitate the saccharification process. The processing of high fructose syrup involves glucoamylase for the conversion of saccharified glucose to high fructose corn syrup. The calcium added in the first step and the salts from the second step must be removed, using expensive processes. This could be avoided if the liquefaction step utilizes α-amylase capable of liquefying starch at pH 4.5 and 105°C without the addition of calcium [4]. In the detergent industry, α-amylases stable at alkaline pH are desired.

Both rational and random mutageneses have been used for the improvement of α-amylase in the food and detergent industries over the past. Directed evolution involves repeated cycles of random mutagenesis and/or gene recombination, followed by high-throughput screening or selection of the functionally improved mutants [26]. The other method is rational design, in which proteins are modified based on the understanding of the structural and mechanistic consequences of a particular change or set of changes. Our present knowledge of structure–function relationships in proteins is still insufficient to make a rational design, and directed evolution is used to explore the unknown sequence space. Using directed evolution, the thermostability of bacterial α-amylases was improved at low pH in the absence of added calcium [21]. *Bacillus amyloliquefaciens* α-amylase activity was improved at alkaline pH by DNA shuffling [3]. The thermostability of maltogenic α-amylase from *Thermus* sp. strain IM6501 was improved greatly by random mutagenesis and using DNA shuffling [16]. Employing error-prone PCR and DNA shuffling, an increase in the thermal stability at pH 4.5 was obtained in Novamyl from *Bacillus* sp. TS-25 [13].

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In the present study, repeated random mutagenesis of a selected domain in the *Bacillus licheniformis* α-amylase was performed, followed by screening to isolate mutants that are active at adverse pH conditions. One mutant α-amylase that had improved activity at both acidic and alkali pH conditions was obtained.

Materials and Methods

Materials

B. licheniformis MTCC 6598 (Microbial Type Culture Collection, Chandigarh, India) and *Escherichia coli* BL21(DE3) were used in this study. The plasmid pET20b (Novagen, Madison, WI, USA) was used in cloning and expression. Glucose, soluble potato starch, IPTG, and ampicillin were purchased from Sigma-Aldrich Fine Chemicals. Restriction and modification enzymes were obtained from MBI Fermentas (Germany). The oligonucleotides used were obtained from MWG Inc. (Chennai, India). The GeneMorph II EZClone domain mutagenesis kit (Stratagene, CA, USA) was used for random mutagenesis.

Cloning and Expression of α-Amylase Gene

The *B. licheniformis* α-amylase gene was PCR-amplified from genomic DNA with primers BLF 5'-TAGAAGCTTATGAAACAAC AAAAACGCGCT-3' and BLR 5'-TAGAAGCTTCTATCTTTGAAC ATAAATT-3' (*Hin*dIII restriction site is underlined). The PCR product was cloned at the *Hin*dIII site of pET20b and the ligated mixture was used to transform *E. coli* BL21 (DE3). The *E. coli* transformants carrying recombinant plasmid were screened on starch agar plates. After incubation for 12 h at 37°C, plates were flooded with iodine solution [0.2% (w/v) I_2 and 2% KI] and the formation of a clear halo around the colonies indicated the expression of amylase. The recombinant plasmid carrying the amylase gene (*amyL*) was designated as pBLA1 [20].

Random Mutagenesis and Mutant Library Construction

Polymerase chain reactions (PCRs) were carried out on an epigradient PCR machine (Eppendorf Mastercycler), using 5–20 ng of *amy*L, 10× mutazyme II reaction buffer (Stratagene, USA), 40 mM dNTP mix, 10 pmol primers (BLF34F, 5' -GGGACGCTGATGCAGTATTTTG-3' and BLR281R, 5'-CGTTTTTTCCCTGACATGATTAAC-3'), and 1.25 U of mutazyme II DNA polymerase (Stratagene, USA) in a 25-µl reaction with initial temperature of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, annealing temp. 55°C for 30 s, and extension at 72°C for 1 min, with a final extension of 72°C for 10 min. Initially, an error frequency of approximately 1–2 mutations per kilobase DNA was generated by adjusting the initial template concentration according to the manufacturer's instructions. This megaprimer was subsequently used as a template in varying concentrations to generate additional 2–7 mutations and 4–14 mutations as suggested by the manufacturer. The library generated using this megaprimer was called the second-generation library. This generated megaprimer was used as a primer to amplify the whole plasmid, as per the manufacturer's protocol. PCR was carried out in an epigradient PCR machine (Eppendorf Mastercycler), using 5–20 ng of *amy*L template, 250 ng of megaprimer, 2× EZClone enzyme mix, and EZClone solution in a 50-µl reaction with one cycle of 95°C for 2 min followed by 25 cycles of 95°C at 1 min, annealing temp. 60°C for 1 min, and extension of 2 min per kilobase, with a final extension of 68°C for 10 min. The samples were stored at 4°C. Then the PCR product was treated with *Dpn*I for 2 h to remove the wild-type plasmid.

Electrotransformation

Electrocompetent cells were prepared and transformed according to the standard protocol [22], with a few modifications. Plasmid DNA (50 ng) was diluted with 9 µl of sterile MilliQ water and mixed with electrocompetent cells (50 µl). The suspension was mixed well and then transferred to a 2-mm electroporation cuvette. The cells were given a pulse of 1.8 kV for 5 milliseconds, following which the SOC medium was added to recover the cells immediately. After resuspension in the medium, the cells were transferred to a 2-ml microfuge tube and allowed to express the antibiotic marker for a period of 45 to 60 min. The cells were then harvested by centrifugation and resuspended in 1 ml of fresh SOC medium, and 200-µl aliquots were plated on LB ampicillin plates. These plates were incubated overnight at 37°C and the transformants were screened on starch agar plates. The active clones were taken for further analysis.

Growth and Expression in Microplates

Active clones were grown in sterile 96-well microplates (200 µl LB-Amp per well) for 12 h at 37°C under agitation of 200 rpm. These microplates (referred to as starter microplates) were used for storage by adding 50% glycerol. Ten µl of the culture was transferred to sterile 96-well microplates (per well: 200 µl LB-Amp–IPTG) and grown for 12 h at 37°C under agitation at 200 rpm. These microplates (referred to as production microplates) were then used for the assay of α-amylase after freeze–thaw cycles (–70°C for 30 min followed by 37°C for 1 h twice). The enzyme preparation was taken for the microscale assay.

Microtiter Plate Assay and High-Throughput Screening of α-Amylase

Primary screening assays were performed in duplicates at pH 4.5 and pH 7. The reaction mixture contained 100 mM Tris-HCl, 1 mM $CaCl_2$, and 10 mM NaCl. Initially, 10 µl of the crude enzyme was transferred from the induction plate to the assay plate using an 8-channel micropipette. Forty µl of the appropriate buffer was added to all wells and 50 µl of 0.05% (w/v) starch was added. The contents were mixed well and incubated at 50°C for a period of 20 min, following which the reaction was stopped with the addition of 50 µl of 10 N HCl. Then 50 µl of iodine reagent was dispensed into all wells including necessary reaction controls. The activity of the enzyme in each well was calculated according to the method described earlier [25].

Assay for pH Activity of α-Amylase

The activity as a function of pH was measured over the pH range of 3.0 to 10 at 50°C. The buffers used were as follows: 100 mM sodium citrate buffer (pH 3), 100 mM sodium acetate buffer (pH 4 to 6), 100 mM sodium phosphate buffer (pH 6 to 8), and 100 mM sodium carbonate buffer (pH 9 to 10).

DNS Assay

The amylase activity was measured according to the dinitrosalicylic acid (DNS) method [18], with a few modifications. The assay mixture

(1 ml) consisted of 0.5% (w/v) soluble starch in an appropriate buffer and 100 µl of suitably diluted enzyme. The hydrolysis was allowed to proceed for 10 min at 50°C. The reaction was stopped by adding 1 ml of DNS to the reaction mixture, followed by placing the tubes in boiling water for 10 min. After cooling the tubes, the absorbance was measured at 570 nm. One unit of enzyme activity was defined as the amount of enzyme that is required to release 100 µmoles of reducing glucose per minute under the reaction conditions. All experiments were performed in duplicates from at least three independent assays.

Modeling of the Mutants

The homology models of the wild-type AmyL and the mutant TP8H5 were generated using Swiss-Model [2, 24] and viewed using the PyMol editor [7] (Fig. 2).

RESULTS

Expression of α-Amylase from *B. licheniformis* in *E. coli*

The wild-type α-amylase gene (*amy*L) from *B. licheniformis* MTCC 6598 was cloned in pET20b and expressed in *E. coli* BL21 under the control of the T7 promoter. The amylase activity of the recombinant clone was easily detected on LB agar plate supplemented with 1% (w/v) soluble starch (Fig. 1).

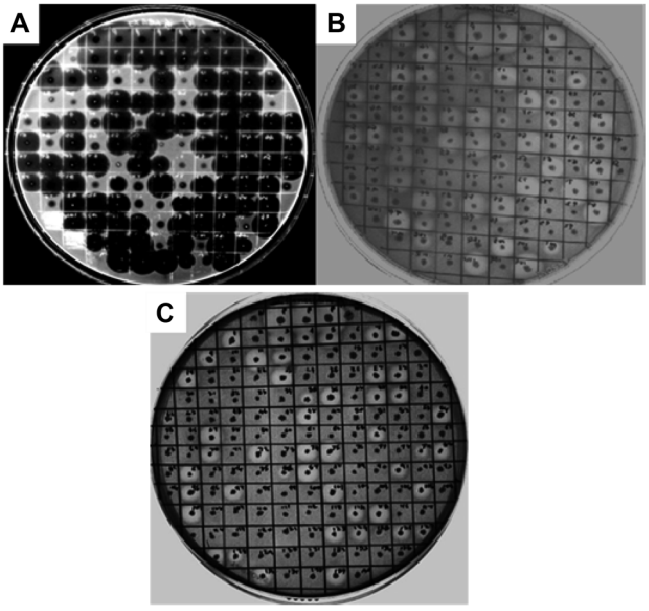

Fig. 1. Screening of mutant library on selective agar plate.
A. Colonies obtained from the first-generation library was replica-plated on starch agar with IPTG and flooded with KI-I solution after overnight incubation. **B**. Colonies obtained from the second-generation (2–8 mutations) library were replica-plated on starch azure with IPTG. Overnight incubation revealed zones of hydrolysis. **C**. Colonies obtained from the second-generation (4–14 mutations) library were replica-plated on starch azure with IPTG. Overnight incubation revealed zones of hydrolysis.

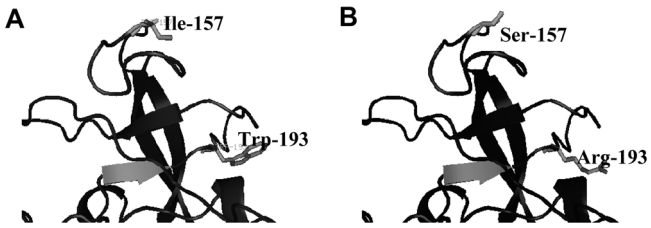

Fig. 2. Cartoon representation of the structures of AmyL (**A**) and mutant TP8H5 AmyL (**B**), showing the amino acid positions mutated by random mutagenesis.
The upmutant had Ser at position 157 (Ile in AmyL) and Arg at position 193 (Trp in AmyL). Both mutations are present in the flexible loop region of the α-amylase. The figure was prepared with the PyMol open source program version 0.98.

Generation and Screening of α-Amylase Expression Library

Random mutations were introduced in domain A and a part of domain B (residues 34–281) of *amy*L by error-prone PCR, followed by whole plasmid amplification. The mutation rate was adjusted by controlling the error rate (low, medium, to high) according to the inactivation rate.

Initially, an error frequency of approximately 1–2 mutations per kilobase DNA was generated in the first cycle by adjusting the initial template concentration. The library created using this megaprimer was called the first-generation library. This PCR product was subsequently used as a template in various concentrations to generate additional 2–8 mutations and 4–14 mutations. The library generated using this megaprimer was called the second-generation library. The first and second generation libraries (2.5×10^3 clones) were replica-plated on LB amp and LB amp starch/starch azure plate containing IPTG. After overnight incubation, clones showing no activity and much less activity were excluded from further screening.

Table 1. Effect of pH on the activity of wild-type AmyL and mutant TP8H5 AmyL.

pH	BLA	TP8H5
	Relative activity (%)	
3	00±0	4±0
4	37±1	43±1
4.5	50±2	56±1
5	95±3	98±1
6	100±0	100±0
7	88±3	97±1
8	81±1	93±0
9	39±1	49±1
10	10±0	19±1

The enzymatic activity was measured using soluble starch as a substrate in 100 mM buffers (pH 3 to 10). Relative activity was expressed as a percentage of the maximum activity of the enzyme at pH 6.

Screening of α-Amylase Library

A semi-automated high-throughput liquid-phase screening method was followed. Active α-amylase clones were grown for enzyme production in 96-well microplates and assayed for activity at pH 7 as well as pH 4.5. The assay plates were scanned and the images were used for digital image analysis. Six lead clones were picked out from different microplates based on improved activity at pH 4.5 or pH 7 or both. These were subjected to secondary assay by the DNS method.

To enable high-throughput screening of large libraries of α-amylase variants, simple freeze–thaw cycles were employed to release the enzyme. The success of directed evolution depends on the use of a rapid method in screening large libraries of variants for a targeted property. In this study, preliminary screening was done on starch agar plates to rapidly eliminate inactive or much less-active clones. The secondary screening involved a simple yet sensitive liquid assay with soluble starch as substrate in a microtiter plate format.

For an effective screening method, several factors such as cell growth, gene expression, cell lysis, and assay of activity have to be considered. This variability in the data is linked to the fidelity of the screening method to identify significantly improved variants but not false positives [9]. The coefficient of variance (CV) is a useful statistical parameter to estimate the reproducibility of the data. In the present study, the activity data collected from 96-well microplates containing clones expressing wild-type amylase had a CV of approximately 18%.

The comparison of activities of the mutants and wild type at pH 4.5 and 7 eliminated the selection of wild-type-like clones. The estimation of total activity by measuring the value obtained at one particular pH is often unreliable [3]. Hence, variants were assayed at pH 4.5 and pH 7. A MATLAB-based image analysis script (VMR) was used to quantify the enzyme activity from scanned images of the assay microplates [25].

Of the six leads obtained, one mutant (TP8H5) had an altered pH profile compared with wild-type enzyme. An average of 2–5 amino acid changes were observed in the sequenced active α-amylase clones that had several amino acid substitutions, such as Q to H, S to G, S to D, W to R, S to I, and I to S. The mutant TP8H5 had two mutations, I157S and W193R, in the loop region of domain B accessible to solvent (Fig. 2).

DISCUSSION

The mutations that modify the thermostability of α-amylase are generally concentrated in domain B and a nearby region of domain A [6, 13, 27]. Moreover, the primary substrate binding site and the active site are located in the same region; that is, in the cleft resulting from the folding of domain B over the top of the $(\alpha/\beta)_8$ barrel forming domain A. The domain B and its interface with domain A is particularly sensitive to any structural perturbations [14].

Electrostatistics of the active site residues play a major role in the α-amylase activity in adverse pH [19]. Single amino acid substutions in and around the active site of the amylase would alter the electrostatistics of the active site [19]. Analysis of the phenotypic effects of the mutations in α-amylase revealed that even neutral-to-neutral amino acid substutions have made significant changes in the pH profile of the α-amylase, as much as the neutral-to-charged amino acid substutions. This finding suggested that the other factors in addition to the electrostatistics of the active site of the α-amylase may contribute to the altered pH activity profile [19]. Thus, they suggest the use of more random strategies to point out the actual determinants of the pH activity profile of BLA. Before choosing sites in rational design, the dynamic properties of the protein have to be considered. Thus, a random mutagenesis approach will give us a comprehensive view of the factors affecting the activity at adverse pHs.

Taq DNA polymerase has the highest error rate among the known thermostable DNA polymerases (0.1×10^{-4} to 2×10^{-4} per nucleotide) [1,5]. This error rate is however not sufficient to produce a library of variants. Moreover, these errors introduced by the *Taq* DNA polymerase are heavily biased towards AT→GC changes [15]. The domain mutagenesis system used provides an easier method to circumvent the problems associated with the the synthesis of mutants using megaprimers and subsequently synthesis of whole plasmids. Proteins with improved or novel function are isolated from high-error-rate generated mutant libraries. High mutation rates unlock regions of sequence space that are enriched in combined beneficial mutations. Typically, 2 to 5 amino acid changes appear in a library of clones with 30% to 40% inactivation rates. Therefore, a library with amylase inactivation rates of 40% to 60% was taken for screening mutants.

Using this strategy, screening of a mutant library of 2.5×10^3 clones at pH 4.5 resulted in identification of six clones with improved activity at pH 4.5, pH 7, or both. One mutant amylase, TP8H5, showed improved activity at extreme acidic and alkali pH values compared with the wild type. This mutant was found to have two amino acid substitutions: I157S and W193R. Mutation W193R should have occurred during the first round of directed evolution since it appeared in all of the mutants, whereas the mutation I157S is exclusive to TP8H5. The change I157S is from the nonpolar hydrophobic residue to a polar hydrophilic residue. The residue W193 is highly conserved in amylases of various *Bacillus* sp. The substitutions seen in the mutant enzyme are located on the surface and are not in the

vicinity of the active-site residues. The mutation W194R in combination with a few other changes was also seen in the alkaline-pH-tolerant mutant BAA 42 of *B. amyloliquefaciens* alpha amylase [3]. In many directed evolution studies, the beneficial mutations favor structurally tolerant sites, which are located on the protein surface [29]. The amino acid side chain of arginine consists of a 4-carbon aliphatic straight chain, the distal end of which is capped by a complex guanidinium group. The guanidinium group is positively charged, with a pKa of 12.48 in neutral, acidic, and even most basic environments, and thus imparts basic chemical properties to arginine. Changes in the amino acid content on the solvent-accessible surface were detected in proteins adapted to extreme temperatures and high salinity [8] and acidic pH [10, 23]. The presence of charged arginine on protein surfaces where interactions with the environment are possible is greatly favored. Thus, this replacement of a nonpolar, hydrophobic, neutral tryptophan with a polar, hydrophilic, basic arginine could have possibly occurred for want of a positive charge. Thus, the paper demonstrates using a random mutation approach to obtain enzyme active at extreme pH values.

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