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# Role of Val289 Residue in the α-Amylase of *Bacillus amyloliquefaciens* MTCC 610: An Analysis by Site Directed Mutagenesis

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**The Val289 residue in the α-amylase of *Bacillus amyloliquefaciens*, which is equivalent to the Ala289 and Val286 residues in the α-amylases of *B. stearothermophilus* and *B. licheniformis*, respectively, was studied by site-directed mutagenesis. This residue was substituted with 10 different amino acids by random substitution of the Val codon. In these mutant α-amylases, Val289 was substituted with Ile, Tyr, Phe, Leu, Gly, Pro, Ser, Arg, Glu, and Asp. Compared with the wild-type α-amylase, the mutant α-amylase Val289Ile showed 20% more hydrolytic activity, whereas Val289Phe and Val289Leu showed 50% lesser activity. On the other hand, the mutant α-amylases Val289Gly, Val289Tyr, Val289Ser, and Val289Pro showed less than 15% activity. The substitution of Val289 with Arg, Asp, or Glu resulted in complete loss of the α-amylase activity. Interestingly, the mutant α-amylase Val289Tyr had acquired a transglycosylation activity, which resulted in the change of product profile of the reaction, giving a longer oligosaccharide.**

**Keywords:** *Bacillus amyloliquefaciens*, α-amylase, site-directed mutagenesis, starch hydrolysis, transglycosylation

The amylase family of enzymes is of great significance owing to its wide area of potential application. Amylases find application in a number of bioprocesses in the food, fermentation, textiles, and paper industries [11]. Liquefying α-amylases are used widely in bread-making, production of glucose and fructose syrup and fuel ethanol from starch material, and textile treatment [5]. α-Amylases (endo-1,4-α-D-glucan glucohydrolase; E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. These are endoenzymes that split the substrate in the interior of the molecules and are classified according to their action and properties. α-Amylases that produce free sugars are termed "saccharogenic" and those that liquefy starch without producing free sugars are known as "starch-liquefying" [11]. Saccharifying amylases are more efficient than that of the liquefying amylases with respect to the reducing sugar released after the hydrolytic reaction. Both types of α-amylases have been found in bacteria, but fungal amylases are mainly saccharifying [12].

The α-amylase family or family 13, the clan GH-H of glycoside hydrolases, is the largest family of starch-processing enzymes and is the largest in sequence entries that exceed 700 and enzyme specificities up to 30 [6, 15]. The most complex of the clan GH-H families is family 13, containing hydrolases, transferases, and isomerases, with over 22 known specificities, and include α-amylase, CGTase, amylosucrase, sucrose isomerase/isomaltulose synthase, sucrose hydrolase, sucrose phosphorylase, cyclodextrin-hydrolyzing enzyme, maltogenic α-amylase, maltogenic amylase, branching enzyme, maltohexose-producing amylase, and pullulanase. The product and reaction specificity vary widely, and many enzymes from this family have been well studied to identify key residues involved in the catalysis and reaction pattern [8].

The α-amylases are classified based on their amino acid sequence similarities, structural similarity, and common catalytic mechanisms [4]. The members of the α-amylase family are enzymes that (i) act on α-glucosidic linkages; (ii) hydrolyze or form by transglycosylation α-glucosidic linkages; (iii) contain four conserved regions; and (iv) contain Asp, Glu, and Asp residues corresponding to the Asp206, Glu230, and Asp297 of Taka-amylase A [15, 16].

α-Amylases may be derived from several bacteria, yeasts, and fungi. Bacterial α-amylase, however, is generally preferred over fungal α-amylase owing to several characteristic advantages. Strains of *Aspergillus* sp. and *Bacillus* sp., mainly *B. amyloliquefaciens* and *B. licheniformis*, are employed for commercial applications. *Bacillus* species

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are considered to be the most important sources of α-amylase and have been used for enzyme production [11]. The well-characterized and commercial amylases are produced by three different species of *Bacillus*, namely, *B. licheniformis* (BLA), *B. amyloliquefaciens* (BAA), and *B. stearothermophilus* (BStA). Despite the strong sequence similarity between these three enzymes, they exhibit significant differences in thermostability and activity. *Bacillus* α-amylases provide an interesting target for protein engineers, not only for improving its industrial performance but also for investigating the molecular basis of differences in their properties.

The amino acid sequence of α-amylase of *B. amyloliquefaciens* shows 77% identity (87% similarity) with α-amylases of *B. licheniformis* [19] and 64% identity (78% similarity) with *B. stearothermophilus* α-amylase [10]. It has been shown previously that replacing Ala289 to Tyr or Phe in BStA favored the production of a smaller oligosaccharide [13]. Similarly, the mutant α-amylase V286F of BLA favored the transglycosylation activity, whereas another mutant amylase V286Y improved the hydrolytic activity [12]. In liquefying amylase, the corresponding residue at position 286 is occupied mainly by smaller residues, whereas in saccharifying amylase, aromatic residues are predominant in that position.

The BAA α-amylase (AmyA) has at least 3-fold higher activity for starch hydrolysis than BLA α-amylase and targeting AmyA for generating mutant amylase, which provides altered product profile, and is of significant value for the food and enzyme industries. We report here random substitution of V289 (equivalent to V286 of BLA α-amylase and A289 of BStA α-amylase) with different amino acids by site-directed mutagenesis, and certain mutant amylases had improved hydrolytic activity over other mutant amylases and changed the product profile of another mutant α-amylase.

## Materials and Methods

### Materials

*Bacillus amyloliquefaciens* MTCC 610 (Microbial Type Culture Collection, Chandigarh, India) and *Escherichia coli* XL-1 Blue were used in this study. The plasmid pTZ59R (MBI Fermentas, Opelstrasse, Germany) was used in cloning and expression. Glucose, maltose, soluble potato starch, malto–oligosaccharides, maltotetrose (DP4), IPTG, and ampicillin were purchased from Sigma-Aldrich Fine Chemicals. Restriction and modification enzymes were obtained from MBI Fermentas (Opelstrasse, Germany). The oligonucleotides used were obtained from MWG Inc. (Chennai, India).

### Cloning and Expression of α-Amylase Gene

The *B. amyloliquefaciens* α-amylase gene (*amyA*) from the genomic DNA was PCR amplified with primers PBAF (5'- CCTGGTACCGA CTGATGATTTGGCTGAAGAAGTG-3') and BARXhoI (5'- GCCCT CGAGTTATTTCTGAACATAAATGGAGAC-3'). The PCR product was cloned into TA cloning vector pTZ57R (MBI Fermentas, Opelstrasse, Germany), and the ligated mixture was used to transform *E. coli* XL1 Blue. 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 0.2% (w/v) $I_2$ solution and 2% (w/v) KI and the formation of a clear halo around the colonies indicated the expression of amylase. The recombinant plasmid carrying the amylase gene (*amyA*) was designated as pPBA and sequenced (MWG, India, and Macrogen, South Korea).

### Site-Directed Mutagenesis

Plasmid pPBA was isolated using a mini-prep kit (Qiagen, Hilden, Germany) and 10–20 ng of template was used for reaction with mutagenic oligonucleotides BAA289VF (5'-CAATCCGTGTTTGATN NKCCGCTTCATTTC-3') and BAA289VR (5'-ATCAAACACGGAT TGATTAAAGCTTGTT-3') (N: A/C/G/T; K: G/T). Mutagenesis was carried out using a Quik-change mutagenesis kit (Stratagene, U.S.A.) as per the manufacturer's protocol. Multiple sequence alignment between different glycoside hydrolases was performed using the ClustalX sequence alignment tool [18]. The mutations in the α-amylase gene were confirmed by DNA sequencing (MWG, India, and Macrogen, South Korea) using the internal primer SeqAF (5'-TGGTATGCGAA TGAACTG-3') or SeqAR (5'-GGCGGAGCTGTCACCTTC-3').

### Production and Assay of α-Amylase

The *E. coli* XL-1 Blue cells harboring the recombinant plasmids were grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml) and 1% starch. After 16 h of growth, cells were harvested and washed with sterile distilled water and centrifuged at 4,000 rpm for 10 min at 4°C. The pellet was resuspended in Tris-acetate buffer (pH 7.0) and sonicated (Braun, Labsonic 2000, Germany) in the same buffer for 30 s twice, at 35 W, with a 60-s interval. The lysate was centrifuged at 13,000 rpm at 4°C for 30 min to remove the debris and the supernatant was used as the enzyme source.

The amylase activity of parent and mutant amylases was measured according to the DNS method [9] with a few modifications. The assay volume was 1 ml and it consisted of 0.5% (w/v) soluble starch in an appropriate buffer (pH 5, 100 mM) 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 protein that produced 1 μM of reducing glucose per minute under the reaction condition. All experiments were performed in duplicates from at least three independent assays. The protein concentration of the cell extract was determined by the method of Lowry with bovine serum albumin as the standard.

For certain reactions, maltotetraose (100 mM) was used as the substrate and the reaction was carried out at 50°C for 24 h. The product profile was analyzed by HPLC with a Luna amino column (25 cm Phenomenex with 4.6 mm diameter). Acetonitrile:water (75:25) was used as the mobile phase. The flow rate at 40°C was 1.0 ml/min and measured with a RI detector (Shimadzu RID-10A). The peaks were compared against standard oligosaccharides from G1 to G7.

### Bioinformatics Analysis

ClustalW multiple sequence alignment of various bacterial amylases was performed using Bioedit (Version 7.0.4.1) (http://www.mbio.ncsu.edu/BioEdit). The sequences included for alignment were retrieved from

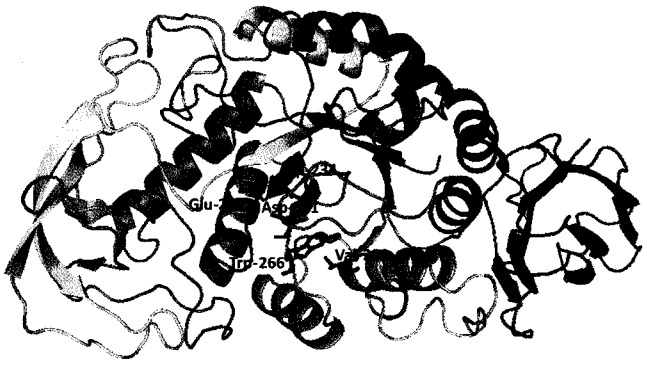

**Fig. 1.** Structure of α-amylase from *Bacillus amyloliquefaciens* showing the positions of V289, W266, and active site amino acids D234, E264, and D331.

the Protein Data Bank (PDB). The homology models of the wild-type AmyA and the mutant were generated using Swiss-Model [1, 3, 14] and edited using the PyMol editor [2] (Fig. 1 and 5). The template used was 1E3X, retrieved from the PDB. It is the fusion of the 1–300 amino acids of AmyA and 300–483 amino acids of BLA α-amylase and shows 93% homology with AmyA. This was the closest match for AmyA available in the PDB. The quality of the predicted structure of AmyA was found to be 94.947 using the SAVS–Structure Analysis and Validation Server (http://nihserver.mbi. ucla.edu/SAVES).

## RESULTS AND DISCUSSION

### Cloning and Expression of α-Amylase Gene

The *B. amyloliquefaciens* α-amylase gene (*amyA*) along with its promoter was cloned in pTZ59R and expressed in *E. coli* XL 1 Blue under the control of its own promoter and the resulting recombinant plasmid was designated as pPBA. The sequence analysis of the *amyA* gene revealed 99% identity with *B. amyloliquefaciens* 1H α-amylase gene [17]. The cloned α-amylase gene was constitutively expressed in *E. coli* XL1 Blue (pPBA) and the expression of amylase was enhanced in the presence of starch in the medium.

### Site-Directed Mutagenesis

The V289 residue (equivalent to V286 of BLA α-amylase and A289 of BStA α-amylase) is present in the semi-conserved loop 6 region of amylase (Fig. 2), and substitution of A289 in BStA with F or Y resulted in transglycosylation activity [14]. Similarly, mutant amylase V286F of BLA also showed transglycosylation activity, and V286Y showed a 3-fold increased hydrolytic activity [12].

In the present study, random substitution of V289 in AmyA was performed using the NNK degenerate codon in the mutagenic primer. By screening 45 mutant clones by sequencing, we obtained 10 different variants of α-amylase in which Val 289 was replaced by Ile, Tyr, Phe, Leu, Gly, Pro, Ser, Arg, Glu, and Asp.

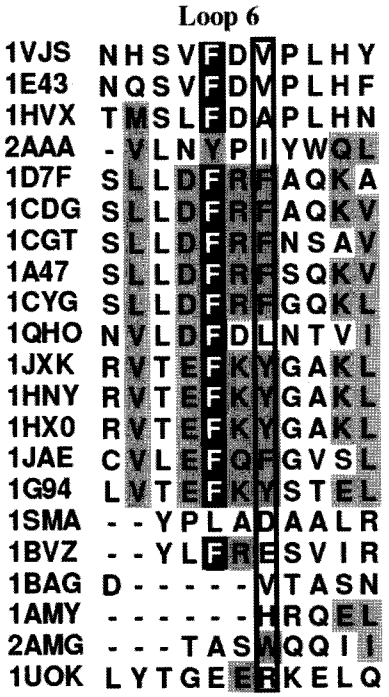


**Fig. 2.** Clustal W multiple sequence alignment of amino acid sequences of various α-amylases.

The PDB code for each sequence is indicated in the left. 1VJS, *B. licheniformis* α-amylase; 1E43, *B. amyloliquefaciens* chimera α-amylase with decacarbose bound; 1HVX, *B. stearothermophilus* α-amylase; 1BAG, *B. subtilis* 2633 α-amylase ; 2TAA, *Aspergillus oryzae* α-amylase; 2AAA, *A. niger* α-amylase; 1JXK, *Homo sapiens* salivary α-amylase; 1HNY, *H. sapiens* pancreatic α-amylase; 1HX0, *Sus scrofa* pancreatic α-amylase; 1JAE, *Tenebrio molitor* α-amylase; 1G94, *Pseudoalteromonas haloplanktis* α-amylase; 1AMY, *Hordeum vulgare* α-amylase; 1CDG, *B. circulans* 251 cyclodextrin glycosyltransferase (CGTase); 1CGT, *B. circulans* 8 CGTase; 1D7F, *Bacillus* sp. 1011 CGTase; 1CYG, *B. stearothermophilus* CGTase; 1A47, *Thermoanaerobacter thermosulfurogenes* CGTase; 1QHO, *B. stearothermophilus* maltogenic α-amylase; 1SMA, *Thermus* sp. maltogenic amylase; 1BVZ, *Thermoactinomyces vulgaris* neopullulanase; 2AMG, *Pseudomonas stutzeri* maltotetraose-forming amylase; 1UOK, *B. cereus* oligo-glucosidase; 1G5A, *Neisseria polysaccharea* amylosucrase. The V289 residue chosen for mutagenesis is boxed.

### Activity of Mutant α-Amylases

When V289 was replaced with all other amino acids by site-directed mutagenesis, the hydrolytic activities of the mutated enzymes were drastically altered (Table 1 and Fig. 3). The hydrolytic activities of the V289F, V289L, V289Y, V289G, V289D, V289E, V289P, and V289S mutant amylases showed decreased activity compared with that of the wild-type enzyme, whereas the V289I mutant α-amylase had improved activity (16.5 U/mg) over the wild-type amylase (13.8 U/mg).

The AmyA hydrolyzed oligosaccharide DP4 and produced the oligosaccharide G3 as the major hydrolytic product in 24 h. The hydrolytic activity of V289I amylase did not show any change, whereas V289L had decreased hydrolytic activity and produced G2 as major oligosaccharide products

**Table 1.** Starch hydrolysis activity of wild-type AmyA and mutant AmyA.

| Enzyme | Specific activity (U/mg) | Relative activity % |
|---|---|---|
| V289 (Wild type) | 13.8±0.45 | 100 |
| V289I | 16.57±1.3 | 120 |
| V289F | 6.75±0.25 | 48.9 |
| V289L | 5.02±0.20 | 36.4 |
| V289G | 2.01±0.52 | 14.5 |
| V289Y | 1.27±0.16 | 9.2 |
| V289S | 0.70±0.13 | 5 |
| V289P | 0.3±0.10 | 2.2 |
| V289R | n.d | 0 |
| V289E | n.d | 0 |
| V289D | n.d | 0 |

n.d, no detectable amylase activity.

and a small amount of G3. In the case of V289F amylase, there was a 50% decrement in hydrolytic activity resulting in G3. Upon hydrolysis of oligosaccharide DP4 the mutant V289G gave G2, and mutants V289P and V289S gave G2 and G3. The mutant V289Y amylase showed less than 15% activity of the wild-type amylase and produced G2 and G3 as major products. However, this mutant amylase had acquired transglycosylation property giving rise to higher oligosaccharides G5 and G6 (Fig. 4).

The changes in the hydrolytic property of α-amylase may be due to factors like the geometry and electrostatics of the environment around the active site [7, 11, 12]. The size and polarity of the side chain in the site results in the interaction with the Trp266, which is near to the catalytic E264. The network of interaction with Trp266 affects the geometry at the active site and influences the ionization state of the active site (Fig. 5). In wild-type α-amylase, 289$^{th}$ position is occupied by Val, which is nonpolar aliphatic, so the network of interaction surrounding the Val289 position is important for its hydrolytic activity,

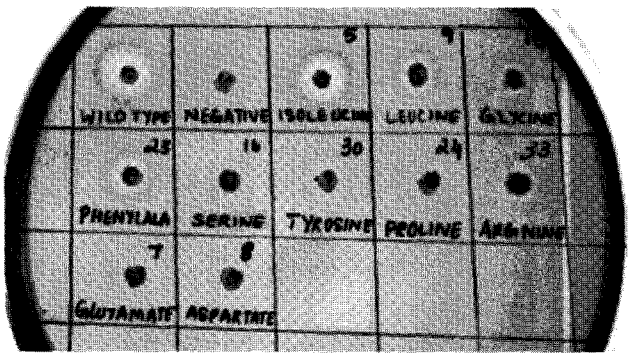


**Fig. 3.** Amylase activity of *E. coli* XL1 Blue transformants harboring different α-amylase genes.
The clear halos on 0.25% starch azure–ampicillin plate indicate the hydrolytic activity of α-amylase.

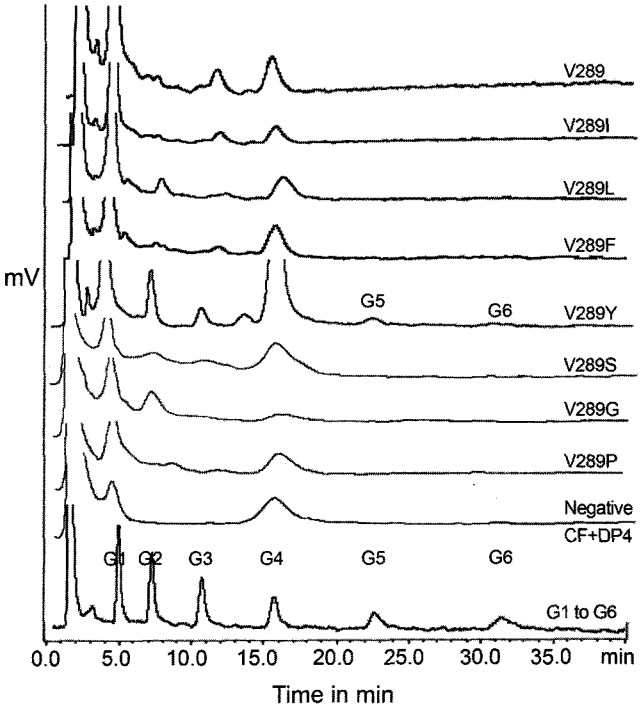


**Fig. 4.** Hydrolytic activities of the wild-type and mutant α-amylase enzymes determined by HPLC after 24 h of reaction at 50°C.
The negative control was substrate DP4 and total cellular fraction that was inactivated (CF).

where both the size and polarity are important. Replacing with a similar nonpolar aliphatic amino acid like Ile improved the activity. At the same time, replacing with Gly (smaller than Val) and replacing with a bulky aromatic amino acid like Phe and Tyr resulted in the decrease or loss of activity, thereby indicating the importance of size. Replacing with polar, basic, or acidic amino acids like Ser, Arg, or Glu, respectively, abolished the amylase activity indicating the importance of ionization and charge. Glu264 is the proton donor in the hydrolytic reaction. The activity of the alpha-amylase depends on proper positioning of the substrate (starch) in the cleft, which depends on the residues including Trp266. Proper orientation of the active site residues is important for its catalysis, which depends on the neighboring residues. In our present study, we have targeted Val289, which influences the active-site residue Glu264 indirectly by affecting the nearby residue Trp266. The size and polarity of the side chain in the site results in the interaction with the Trp266, which is near to the catalytic Glu264. Change of the size and polarity at Val289 affects the microenvironment around Trp266, which is near the catalytic residue Glu264.

The transglycosylation activity was seen in the mutant amylase V289Y. The mutant amylase V289F did not show transglycosylation activity while retaining 48% of its hydrolytic property. The substitution of V289 with Ile resulted in a 20% improved hydrolytic activity of amylase,

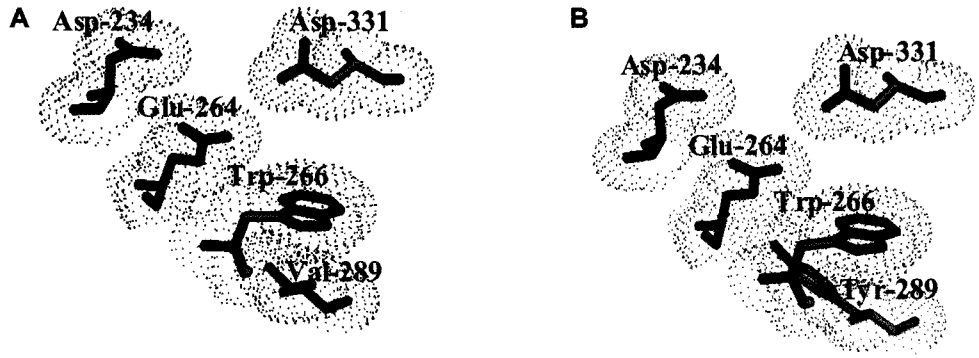


**Fig. 5.** Structural orientation of the modeled AmyA (**A**) and mutant V289Y (**B**) showing the active-site residues along with Trp-266 with Val-289 (**A**) and Trp-266 with Tyr-289 (**B**).
Trp-266 at the interface between Val-289 and the active-site Glu-264 influences the catalytic property.

but with Leu its activity decreased. Thus, the size and polarity of the amino acids in the mutants were the factors important for its retaining or decreasing the hydrolytic activity. On the other hand, substitution of V289 with a smaller residue, Gly or Ser or Pro, resulted in drastic decrease in the hydrolysis, indicating the importance of electrostatic interaction of the side chain present in the wild-type Val289 with the Trp266, which is present near the catalytic residue Glu264. The does not affect the electrostatic interaction with the Trp266, and therefore the mutant amylase V289I had retained its activity. In the case of V289E or V289Q, the change in charge might affect Trp266, which indirectly affects the nucleophilic acceptor Glu264.

In conclusion, changing residues near the active site resulted in an altered hydrolytic activity, and substution with a hydrophobic residue resulted in amylase acquiring transglycosylation activity but with a significant loss of hydrolytic activity. Thus, an increase in transglycosylation occurred at the expense of the hydrolytic activity, and the hydrophobicity and electrostatics nature at the catalytic site are important for this transglycosylation activity.

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