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Research Article

STUDY OF STRUCTURAL VARIATION AT CATALYTIC SITE OF ADENYLATE DOMAIN OF ITURIN SYNTHETASE ENZYME WITH RESPECT TO AMINO ACID CHANGE

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A B S T R A C T

The geometrical and energetic variation at catalytic site of enzyme with respect to amino acid change is helpful to optimize the rate of catalysis of enzyme. In the present study, 22 iturin synthetase enzyme sequences comprised of 5 copies of iturin A, 8 copies of iturin B, 8 copies of iturin C and a single copy of iturin D of different strains of *Bacillus amyloliquefaciens* were retrieved and considered for structural variation study at catalytic site of AMP binding domain. A pattern derived from the Multiple sequence alignment was, [GAPH]-[LIPV]-X-[LIPV]-X-[IL]-[FTNR]-N-[EVH]-Y-G-P-T-E-[TN]-X-V-X[2,3]-[IVL] with a catalytic site pattern as, [N-E-Y-G-P-T-E]. A part of adenylate domain was separated from long iturin synthetase enzyme sequence and processed for 3D structure prediction by Homology modeling method on Phyre2 server. The obtained 3D protein structure were structurally aligned by MATRAS software. Structures were observed at catalytic site for structural variation with respect to amino acid change. Amino acid change at position 2 of pattern [N-E-Y-G-P-T-E], where Glu (E) is changed to either Val (V) or His (H) amino acid has increased the energy of adenylate domain of catalytic site of iturin synthetase turning it bit less stable, which was observed from the increase in energy of that amino acid and hence the total potential energy of enzyme. The variation in adjacent regions of conserved pattern was observed among the iturin synthetase adenylation domain of type A, B, C and D, indicating the variation of amino acids sequence in cyclic peptide, iturin molecule.

INTRODUCTION

Iturin is a large family of cyclic lipopeptides produced by *Bacillus spp*. The common structure of iturin is madeup of heptapeptide with a conserved chiral chain sequence LDDLLDL which is catalysed by amide bond formed between α-COO group of 7th amino acid and β-NH_2 group of β-amino fatty acid (βAA), which is peptide bonded through its α-COO group to N-terminal amino acid. The members of iturin family displays heterogeneity at 1, 4, 5,6 and 7 amino acid position in the peptide moiety as well as in the βAA length, which varies from 14 to 17 carbons. Depending on the variation of amino acids in peptide sequence, iturin have been classified as iturin A, iturin B, Iturin C, iturin D, iturin E, bacillomycin D, bacillomycin F, bacillomycin L, bacillomycin Lc and mycosubtilisin (Marion *et al.,* 1986). Amongst all iturins, iturin A has found to be predominantly secreted by most strains of *B.subtilis* and *B. amyloliquefaciens*.

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They have demonstrated the effectiveness of iturin A in prevention and treatment of fungal diseases caused by *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata, A. funigatus* and *C. newformans* (Tanaka *et al.,* 1997). The iturin Ahas ability to pass through the cell wall and interacts with lipid components of cell membrane and forms ion conducting pores (Maget and Peypoux, 1994). Bacillus group of bacteria are industrially and medically important hence isolation and characterization of potential species with human benefits challenge. The extent of variation in 16S rRNA copies and composition may mislead to incorrect identification of bacteria. Cyclic peptide synthesis enzyme complex comprised of different domains and variation in it is responsible for synthesis of different cyclic peptides. This study is aimed to structurally characterize the enzyme iturin synthetase produced by genus Bacillus. The study was performed with following objectives-

1. Diversity of iturin synthetase enzyme.
2. Domain analysis of iturin synthetase enzyme.
3. 3D structure prediction by Homology modeling techniques of iturin synthetase enzyme.
4. Structural co relationship in relevance to sequence mutation at catalytic site.

MATERIAL AND METHODOLOGY

Sequence screening and sorting

After retrieving the sequences, the protein sequences of *Bacillus* species which contain Iturin A synthetase enzymes was separated form coding DNA Sequences (CDS) file, " .faa" file and saved in fasta format in the notepad.

Fragmentation of the sequences

The sequences of iturin A synthetase separated from different *Bacillus amyloliquefaciens* strains were very long, so the sequences were cut into 1000 residues long fragments by the help of Bioedit tool, to facilitate domain analysis and structure modeling.

MSA (Multiple Sequence Alignment)

MSA of retrieved sequence was done so that, sequence homology can be inferred and phylogenetic analysis can be performed to get the sequence shared evolutionary origins. MSA is often used to check sequence conservation of protein domains, tertiary and secondary structure and also for individual amino acids or nucleotides (Wang *et al.,* 1994).

Phylogenetic analysis

Phylogenetic tree and Evolutionary tree is a branching diagram showing the evolutionary relationship among various biological species or other entities. Their phylogeny based upon similarities and difference in their physical and genetic characteristics. (Harrision and Langdale, 2005). Distance based methods: unweighted pair group method with arithmetic mean (UPGMA) method is simple, fast and has been extensively used in literature. In our work we used UPGMA method for the phylogenetic tree construction UPGMA employs a sequential clustering algorithm, in which local topological relationship are identified in order of similarity, and the phylogenetic tree is build in a appropriate manner. Bootstrap is a computer based method for testing the accuracy of almost any statistical estimate .The use of bootstrap in the estimation of phylogenetic tree has been widely used and it provides assessments of "confidence" for each clade of an observed tree, based on the proportion of bootstrap tree showing that same clade. Bootstrap confidence values obtained with 1000 resembling are given at the branch points. We input the 1000 value of bootstrap so we found the more replicates of our sequence.

Protein 3D structure Prediction

Homology modelling and threading are pioneer steps for protein 3Dstructure prediction. Homology modelling also known as comparative modelling of protein is the technique which allows constructing an unknown *atomic-resolution model* of the *"target"* protein. Homology modelling relies on the identification of an alignment that maps residue in the query sequence to residue in the template sequence. It has been shown that protein structures are more conserved than protein sequences amongst homologous but sequences falling below a 20% sequence identity can have very different structure (Chothia and Lesk, 1986)

3D Structural Alignment

Structural alignment attempts to establish homology between two or more polymer structure based on their shape and three dimensional conformations. Structural alignment tool is available for comparision of protein with low sequence similarities, where evolutionary relationships between proteins cannot be easily detected by standard sequence alignment techniques (Zemla, 2003).

RESULT AND DISCUSSION

A study titled as "Study of Structural Variation at catalytic site of Adenylate Domain of Iturin Syntheatse enzyme with respect to amino acid change" was performed with an objective to find out the diversity of iturin synthetase enzyme in genus *Bacillus*. Study was further extended for prediction of 3D structure and domain analysis of protein sequence of iturin synthetase from one of the *Bacillus* species.

During the current study the iturin synthetase gene sequences were observed only in *B. amyloliquefaciens* from the available genome sequences of *Bacillus* species. Total 22 iturin synthetase enzyme sequence comprised of 5 copies of iturin A, 8 copies of iturin B, 8 copies of iturin C and a single copy of iturin D were separated from coding DNA sequences (CDS) file, ".faa". Sequences were aligned by ClustalOmega software and phylogenetic analysis was done by MEGA6 software. Clustering was done by maximum evolution method, Kimura 2 parameter model and test of phylogeny was done by bootstrap method with 1000 replicates. Maximum similarity was observed with each iturin synthetase A of all strains of *B. amyloliquefaciens*.

Conserved Catalytic Patterns of Iturin Synthetase Enzyme

Pattern (fig 1) was generated to show the pattern based presentation of catalytic site of the sequences. According to the reference sequence with BioEdit position the pattern is spanning region between the amino acids 1081 – 1101, which is conserved catalytic site [NEYGPTE] for AMP binding. It is represented below.

Pattern

>1081[GAPH]-[LIPV]-X-[LIPV]-X-[IL]-[FTNR]-N-[EVH]-Y-G-P-T-E-[TN]-X-V-X[2,3]-[IVL][1101

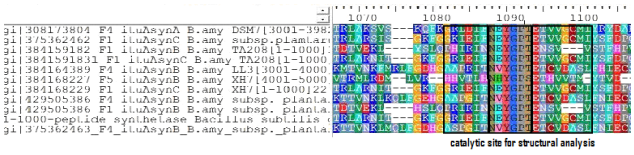

Fig 1 Conserved catalytic site of different *Bacillus amyloliquefaciens* strain containing enzyme iturin synthetase.

Multiple Sequence Alignment

Multiple Sequence Alignment of 32 iturin synthetase sequences of 22 different *Bacillus amyloliquefaciens* strains was carried out through ClustalOmega software for detection of conserved sites.

Fig 2 shows Multiple Sequence Alignment of Iturin synthetase sequence from *Bacillus amyloliquefaciens* strain. Star (*) shows highly conserved regions, double dots (:) shows partially conserved and single dot (.) shows weakly conserved regions.

Phylogenetic Analysis of Sequences of Iturin Synthetase

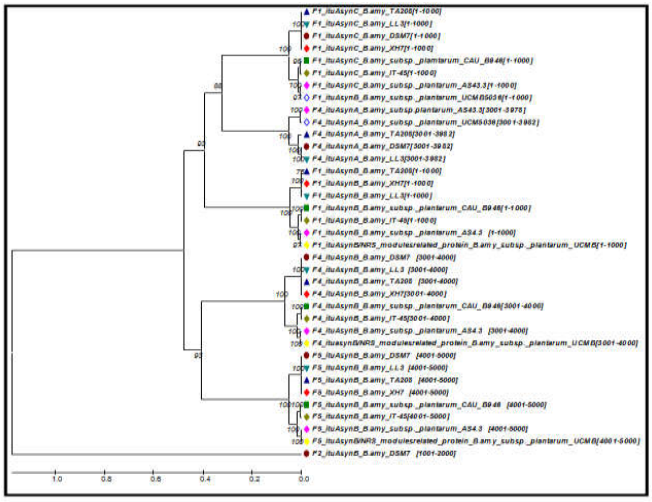

Fig. 3 Phylogenetic tree of adenylation domain of various iturin synthetase enzymes from different strains of *Bacillus amyloliquefaciens*

32 iturin synthetase sequences were processed for phylogenetic relationships by clustering method using the software MEGA6. Phylogenetic analysis was done by UPGMA method, Maximum Evolution model and Bootstrap with 1000 replicate as test of phylogeny. Iturin synthetase enzyme sequences clustered according to its specific class by slight variation 0.1 unit distance. Iturin-C formed a separate cluster and linked to cluster of iturin B by 0.4 unit distances. Iturin A class sequences are externally linked to cluster of Iturin C and iturin B by 0.5 unit distance. Iturin B of *Bacillus amyloliquefaciens* DSM 7 is externally linked to all by >0.5 unit distance (Fig. 4.3).

Domain and Catalytic site Analysis

Unit of iturin synthetase sequence from different *Bacillus amyloliquefaciens* strain are too long and is not acceptable for the domain analysis and 3D structure prediction through homology modeling and threading, so the sequences were fragmented into sub-regions of 1000 residues in length, with the help of BioEdit tool. Then each fragment was analyzed for the presence of adenylation domain by the online tool "InterProScan" (http://ebi.ac.uk). The sequence of iturin synthetase of different strains of Bacillus amyloliquefaciens domain for amino acid adenylation was separated and location is given in table-4.2 and table-2.5. Example of domain analysis by using the sequences of *Bacillus amyloliquefaciens* sub sp plantarum CAU B946. Domain analysis of fragment 1 of 1-1000 by InterProScan has indicated the presence of 8 domains in query protein.

Table 1 Domain analysis of fragment 1 of 1-1000 by InterProScan

Name of domain	Length of domain
AMP-dependent synthetase/ ligase	269-663 amino acid
Amino acid adenylation domain	290-687 amino acid
AMP-binding	403-423 amino acid
AMP-binding enzyme C-terminal domain	671-743 amino acid
Acyl carrier protein-like	761-834 amino acid
Condensation domain	847-1000 amino acid
AMP-binding, conserved site	408-419 amino acid
Phosphopantetheine attachment site	793-808 amino acid

In the current study the domain analysis extended to 3D structure prediction to understand the functional features of each domain.

3D structure Prediction of iturin Synthetase Enzyme

3D structure of iturin A was originally proposed by Garbay-Jaureguiberry *et al.,* (1992) and refined by Marion *et al.,* (1996). Cyclic peptide structure of iturin A is stabilized by two type II b-turns in the two tetrapeptide units: b-amino fatty acyl-Asn2-D-Tyr3-D-Asn4 (turn A) and L-Gln5-L-Pro6-D-Asn7-L-Ser8 (turn B). The variation of amino acids that will be added to the growing cyclo-peptide chain (Stachelhaus *et al.,* 1995; Roongsawang *et al.,* 2005). This study was extended to predict the 3Dstructure of Iturin A synthetase enzyme through various methods of 3D structure prediction mainly homology modeling and fold-recognition. Two online software tools were decided to be used for 3D structure prediction. SWISS-MODEL (Schwede *et al.,* 2003) and Phyre[2] (http://www.sbg.bio.ic.ac.uk/phyre2) (Kelly and Sternberg, 2009).

Homology modeling

3D structure model of iturin A synthetase protein was determined by SWISS-MODEL and Phyre2. Sequence of iturin synthetase in *B. amyloliquefaciens* strains containing adenylation domain were determined by SWISS-MODEL and Phyre2 (Kelley and Sternberg, 2009) through homology modeling and threading process. Role of adenylation domains of the free standing enzymes belonging to the Eukaryotic non-ribosomal peptide synthetase like family were studied through the *in-silico* domain structure prediction and analysis by Vincenzo and co-worker (2005).

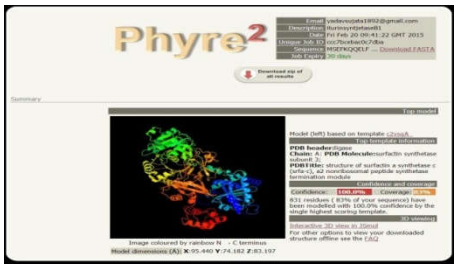

Fig 4 shows the modeled structure of enzyme iturin synthetase of *Bacillus amyloliquefaciens* CAU B946.

3D Ligand Site

Here we represent the figures of sequences of *B.amyloliquefaciens CAU B946* having amino acid adenylation domain and catalytic site similar to reference sequence. The ligand site prediction is mainly based on the homologous template structure from PDB bound to ligands or unbound to ligands. The prosthetic groups predicted that may bind to the iturin synthetase are Acetyl Co enzyme A, Phosphopantetheine, Ca &Mg ion, AMP and ATP molecule.

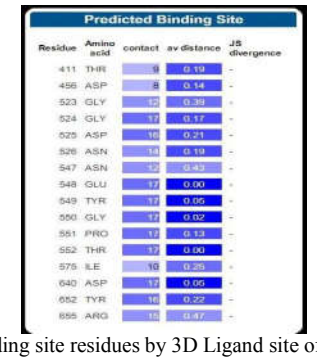

Fig 5 Predicted binding site residues by 3D Ligand site of iturin synthetase of *Bacillus amyloliquefaciens* CAU B946

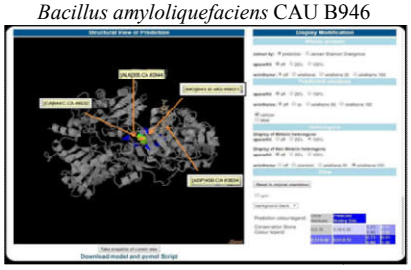

Fig 6 Modeled 3D structure of iturin synthetase C of *Bacillus amyloliquefaciens CAU B946* with Ligands (Aln), (Mg), (Asp), (Ca) and (Ala) at catalytic site

Structural Alignment Studies of Catalytic site of iturin synthetase

MATRAS structural alignment software was used for the structural comparison of 3D modeled adenylation domain of iturin synthetase catalytic site of different strains of *Bacillus amyloliquefaciens* with its reference sequence. MATRAS is program sets for protein 3D structure comparison; it stands for Markovian TRAnsition of protein Structure. It score using Markov transition model of structure evolution, which is supposed to be better for detecting homologous structure similarity.

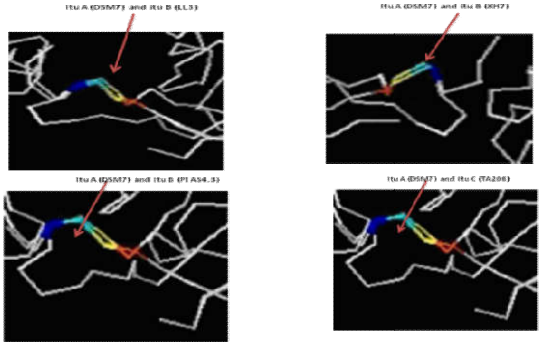

Fig 7 3D structural alignment of the catalytic site of some *Bacillus amyloliquefaciens* strain

The 3D structural alignment of some strains of *B. amyloliquefaciens* was observed through MATRAS online software tool. The clearly visible 3D alignments of the structures were obtained. The structures were the conserved at catalytic site [NEYGPT] for AMP binding region in iturin synthetase enzyme. The structural comparison of strain DSM 7 (iturin C) with other strains such as, (iturin B) LL3, (iturin B) XH7, iturin B plantarum AS4.3 and (iturin C) TA208 shows that the structure is similar in each comparison with slight structural variations.

Structures of Catalytic Site Through RasMol and their energy calculated by SPDBV software tool

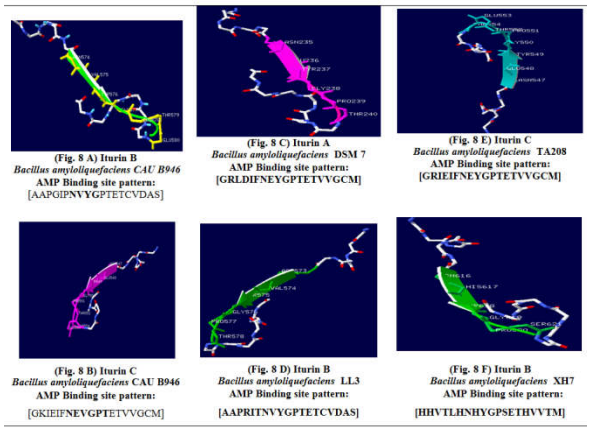

Fig 8 Structures of catalytic site through Rasmol

Energy optimization of catalytic site through SPDBV

Table 2 Energy of catalytic site of different strains of *B. amyloliquefaciens*

/ residue		bonds	angles	torsion	improper	nonBonded	electrostatic constraint //	TOTAL
*ASN	235	1.441	2.561	5.673	0.024	99999904.00	0.00	0.0000 // E=99999904.000
GLU	236	2.200	2.090	5.271	0.135	-36.49	117.27	0.0000 // E= 90.472
TYR	237	2.885	12.498	4.394	1.669	-35.24	0.00	0.0000 // E= -13.794
GLY	238	1.800	3.682	1.099	0.541	-13.24	0.00	0.0000 // E= -6.118
PRO	239	9.006	19.884	20.126	0.821	26.42	0.00	0.0000 // E= 76.254
THR	240	3.286	1.817	4.098	0.084	-5.34	0.00	0.0000 // E= 3.942

Iturin A *Bacillus amyloliquefaciens DSM 7*

Iturin C *Bacillus amyloliquefaciens CAU B946*

ASN	574	1.441	2.561	5.673	0.024	-42.00	-167.35	0.0000 // E= -199.653
VAL	575	1.733	3.893	3.335	1.141	-22.42	-9.81	0.0000 // E= -22.129
TYR	576	2.885	12.498	4.394	1.669	-38.51	-32.15	0.0000 // E= -49.216
GLY	577	1.800	3.682	1.099	0.541	-13.61	61.69	0.0000 // E= 55.140
PRO	578	9.006	19.884	20.126	0.821	26.60	-39.92	0.0000 // E= 36.519
THR	579	3.286	1.817	4.098	0.084	-5.33	-14.71	0.0000 // E= -10.759

Iturin B *Bacillus amyloliquefaciens XH7*

ASN	616	1.441	2.561	5.673	0.024	-39.93	-166.43	0.0000 // E= -196.659
*HISA	617	16.057	7.518	3.978	0.137	18631.48	1.31	0.0000 // E= 18660.490
TYR	618	2.885	12.498	4.394	1.669	-39.20	-31.92	0.0000 // E= -49.668
GLY	619	1.800	3.682	1.099	0.541	-15.41	61.29	0.0000 // E= 52.995
PRO	620	9.006	19.884	20.126	0.821	25.44	-39.15	0.0000 // E= 36.125
SER	621	3.266	1.478	3.706	0.076	-16.07	-15.61	0.0000 // E= -23.151

Iturin B *Bacillus amyloliquefaciens CAU B946*

ASN	573	1.441	2.561	5.673	0.024	-42.95	-169.28	0.0000 // E= -202.528
VAL	574	1.733	3.893	3.335	1.141	-23.19	-9.73	0.0000 // E= -22.816
TYR	575	2.885	12.498	4.394	1.669	-38.58	-32.67	0.0000 // E= -49.807
GLY	576	1.800	3.682	1.099	0.541	-13.62	61.54	0.0000 // E= 55.046
PRO	577	9.006	19.884	20.126	0.821	26.61	-39.63	0.0000 // E= 36.820
THR	578	3.286	1.817	4.098	0.084	-5.32	-14.14	0.0000 // E= -10.183

Iturin C *Bacillus amyloliquefaciens TA208*

The pattern observed at catalytic site of adenylation domain of iturin synthetase is-

[FTNR]-N-[EVH]-Y-G-P-T-E-[TN]-X-V-X[2,3]-[IVL]

And in reference sequence it is [N-E-Y-G-P-T-E].

The conserved catalytic site of AMP binding is [N-E-Y-G-P-T-E]. The study was mainly concentrated around this pattern and adjacent amino acids on either side of this pattern. Fig.7 shows the pairwise structural alignment at catalytic site. The alignment is of β-C-atom of protein backbone. The alignment

shows slight variation between each pair. As the alignment is just based on β-C-atom, variation cannot be judged properly without side chain confirmation analysis.

Structural variation with respect to sequence variation at catalytic site was further analysed by individual structural inspection in SPDBV software and energy of local catalytic site of protein. Greatly varying structure of *B. Amyloliquefacience* strains such as, CAUB946, CAUB946, DMS7, TA208 and XH7 were analyzed for stability of local structure (Fig. 8 A B C D E F). Pattern [NEYGPTE] is considered for energy evaluation (table-2). The deviation in energy was seen in two structures namely iturin A of *Bacillus amyloliquefaciens* DSM 7 and iturin B of Bacillus amyloliquefaciens XH7. In case of strain DSM7 deviation in energy at catalytic site occurs in Asn residue at position 235 and in case of XH7 deviation occurs at His residue at position 617. At both position the energy is higher as compared to other residues. In DSM7 a energy of Asn amino acid is increased due to variation at N-terminal region indicating unstable conformation. In CAUB946 structure is more stable due to presence of hydrophilic amino acid at N-terminal adjacent side. In case of IturinB of XH7 strain His is present inplace of Glu. It has high positive energy (18660 kacl/mol), indicating unstable local conformation. In TA208 more or less low local energy values indicating stable conformation, where Val is present inplace of Glu or His at second position in pattern [N-E/V/H-Y-G-P-T-E].

From this study the Glu or His at second position is more unstable and reactive as compared to Val at that position. Glu is acidic and His is basic amino acid, capable of changing the local conformation and re-activity of catalytic site.

CONCLUSION

The current study titled as "Study of Structural Variation at catalytic site of Adenylate Domain of Iturin Synthetase enzyme with respect to amino acid change" was carried out to observe the geometrical and energetic variation at catalytic site of enzyme with repsect to amino acid change. 22 iturin synthetase enzyme seqeunce comprised of 5 copies of iturin A, 8 copies of iturin B, 8 copies of iturin C and a single copy of iturin D of different starins of *Bacillus amyloliquefacience* were retrieved and considered for study of structural variation studies at catalytic site of AMP binding domain. A pattern derived from the Multiple sequence alignment was, [GAPH]-[LIPV]-X-[LIPV]-X-[IL]-[FTNR]-N-[EVH]-Y-G-P-T-E-[TN]-X-V-X[2,3]-[IVL] with a catalytic site pattern as, [N-E-Y-G-P-T-E].

A part of adenylate domain was separated from long iturin synthetase enzyme sequence and processed for 3D structure modeling by Homology modeling method on Phyre2 server. The obtained 3D protein structure were structurally aligned by MATRAS. Structures were observed at catalytic site for structural variation with respect to amino acid change. Amino acid change at position 2 as pattern [N-E-Y-G-P-T-E], where Glu(E) is changed to either Val(V) or His(H) amino acid has increased the energy of adenylate domain catalytic site of iturin synthetase turning it bit less stable, which was observed from the increase in energy of that amino acid and hence the total potential energy of enzyme.

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