Frontiers in Life Science, 2013
Vol. 7, Nos. 3–4, 140–147, http://dx.doi.org/10.1080/21553769.2013.852993

Taylor & Francis
Taylor & Francis Group

Molecular modelling and docking studies of an α-1,4-amylase from endophytic *Bacillus amyloliquefaciens*

Amith Abraham[a], Sunilkumar Puthenpurackal Narayanan[b], Shaji Philip[c], Divya Gopalakrishnan Nair[d] and Jayachandran Kochupurackal[a]*

[a]*School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India;* [b]*Institute of Biophysics and Physical Biochemistry, Centre of Magnetic Resonance in Chemistry and Biomedicine (CMRCB), University of Regensburg, D-93040 Regensburg, Germany;* [c]*Rubber Research Institute of India, Kottayam, Kerala, India;* [d]*Department of Biotechnology and Microbiology, School of Lifesciences, Kannur University, Thalassery Campus, Kannur, Kerala, India;*

(*Received 1 July 2013; accepted 4 October 2013*)

α-1,4-Amylase is one of the most important industrial enzymes and there is enormous interest in isolating α-1,4-amylase with better properties. The α-1,4-amylase producing endophytic *Bacillus amyloliquefaciens* was isolated and characterized from *Hevea brasiliensis*. The α-1,4-amylase gene after cloning and sequencing contained 1542 base pairs. A homology model of the α-1,4-amylase enzyme was built from the deduced amino acid sequence. The modelled and template α-1,4-amylase enzyme (PDB ID:3bh4) showed 97.7% sequence identity with similar secondary and tertiary structures. Computer aided docking studies of the substrate (maltotetraose) with the modelled as well as the template enzymes showed that although the binding energies were almost the same in both the complexes, the number of hydrogen bonds and van der Waals interactions in the active sites of the two enzymes were different. These variations might be due to the change in the amino acid residues of the active site regions of two enzymes. The mutated polar amino acids in the active site of modelled α-1,4-amylase favoured more hydrogen bond formation with the substrate. The difference in the active site interactions may improve the specificity of the enzyme and affect the catalytic potential of α-1,4-amylase.

Keywords: α-1,4-amylase; *Bacillus amyloliquefaciens*; docking; endophyte; homology modelling

Introduction

α-Amylases (α-1,4-*D*-glucan-glucanohydrolase, E.C.3.2.1.1) catalyse the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and related polysaccharides. α-Amylase comprises about 30% of the world's enzyme production (Gomes & Steiner 2004). This enzyme has widespread applications in starch processing, brewing, alcohol production, textiles, pharmaceuticals and several other industries. Microbial production of α-amylase is viable and bacteria have been extensively screened for the production. Better production of α-amylase has been reported from *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. sterothermophilis* (Mamo & Gessesse 1999).

The suitability of any α-amylase to a meticulous process will depend on its characteristics, e.g. specificity, stability, pH and temperature dependence (Pandey et al. 2000). The characteristics and catalytic potential of an enzyme depend on its three-dimensional structures, which are uniquely determined by the amino acid sequence. The amino acids at the enzyme active site act as acid/base, nucleophiles or electrophiles and also make hydrogen bonds either with the substrate or with other residues of an enzyme to stabilize the transition state (Zvelebil & Sternberg 1988). Structural level information, at atomic level, can help us to understand the features of new enzymes. The search for novel microorganisms capable of producing enzymes with unique features is a continuous process and there are microorganisms useful for biotechnological applications still surviving under unexplored environments (Staley et al. 1997). The endophytic microorganisms dwell in a relatively unexplored site and may represent a new source of enzymes with different potentialities and unique characteristics. To understand the functions of proteins at a molecular level, it is often necessary to determine their three-dimensional structure. The availability of sequence information from newly isolated microbial genes and the comparison with homologous enzymes having similar folding allow us to identify the molecular determinants and amino acid residues involved in desired features.

In this paper, we report the analysis of the complete nucleotide sequence of the α-1,4 amylase gene from a novel endophytic *B. amyloliquefaciens*. A homology model of the new α-1,4 amylase enzyme was constructed and was used to explore the interaction features of this enzyme with the

*Corresponding author. Email: jayansbs@yahoo.in

substrate. Homology modelling was done using the online SWISS-MODEL work space (SIB-Biozentrum Basel) and the docking studies were done using the Autodock program (https://www.scripps.edu/).

Materials and methods

Isolation of α-1,4-amylase producing bacterial endophytes

Root, petiole and leaf tissues of *H. brasiliensis* were used for the isolation of bacterial endophytes by surface sterilization and trituration method (Caetano-Anolles et al. 1990). The isolates were screened for starch hydrolysis on 1% starch agar plate and the enzyme production of the selected isolates was carried out in mineral salt starch medium (MSSM) (Bose & Das 1996) inoculated with 1% bacterial inocula. The cell free supernatant was collected after incubation at 37°C for 48 h and was taken as crude enzyme extract of α-1,4-amylase. The enzyme assay was done by the 3,5-Dinitrosalicylic acid method (Bernfeld 1955) and protein estimation by Lowry's method. One unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol glucose equivalent per minute under the assay conditions. The isolate producing the highest amount of enzyme (IU/ml and specific activity) in the given conditions was selected for further study.

Molecular characterization of the bacterial endophyte

Genomic DNA was prepared (Pitcher et al. 1989) and the conserved eubacterial primers used for the amplification of 16S ribosomal DNA were as follows: (1) pA – 5′-AGAGTTTGATCCTGG CTCAG-3′; and (2) pH – 5′-AAGGAGGTGATC CAGCCGCA-3′ (Edwards et al. 1989). Each reaction mixture contained Taq DNA polymerase (Fermentas, Vilnius, Lithuania), magnesium chloride at a concentration of 1 mM, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 100 pmol and 50 ng of DNA per 20 μl reaction mixture. The PCR reaction was carried out in an Eppendorf AG22331 Thermal Cycler (Eppendorf AG, Hamburg, Germany) with the following PCR cycle: one cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by final 2 min incubation at 72°C. The PCR products were size fractionated on 1% agarose gel and the bands were excised from the gel and purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich, Steinheim, Germany). Purified 16S rDNA sequences were cloned in pGEMT Easy vector (Promega, Madison, WI, USA), transformed in JM 109 cells (Promega) and sequenced at Macrogen, Seoul, South Korea. The sequence similarity was analysed by sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST analysis (Altschul et al. 1990) and isolates were identified on the basis of the best match in the database. Sequences of antagonistic bacterial endophytes and reference sequences from NCBI GenBank were aligned using the multiple sequence alignment program ClustalW2. Using the alignment file generated by ClustalW2, phylogenetic analysis was performed in MEGA4 (Tamura et al. 2007).

Cloning and sequencing of the α-1,4-amylase encoding gene

The genomic DNA with following primers was used for the amplification of amylase gene: (1) forward primer: 5′-GGAAACATGATTCAAAAACG-3′; (2) reverse primer: 5′-ATTACCTTATT TCTGAACAT-3′. The PCR reaction was carried out in an Eppendorf AG22331 Thermal Cycler with following PCR cycle: one cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by final 2 min incubation at 72°C. The PCR products were size fractionated on 1% agarose gel and the bands were excised from the gel and purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich). The purified gene sequence was cloned in pGEMT Easy vector (Promega), transformed in JM 109 cells (Promega) and sequenced at Macrogen. The sequence similarity was analysed by sequences available in the NCBI database using BLAST analysis (Altschul et al. 1990).

In silico *studies of α-1,4-amylase*

Amino acid sequence and physico-chemical properties

The amino acid sequence of α-1,4-amylase enzyme was obtained from nucleotide sequence of the α-1,4-amylase gene by translation using the EXPASY tool (http://web.expasy.org/translate/). Secondary structure elements of the α-1,4-amylase enzyme were predicted using the PredictProtein Server (https://www.predictprotein.org/. Amino acid sequences of α-1,4-amylase from related strains of *Bacillus* sp. were aligned with our own sequence using the multiple sequence alignment program ClustalW2. Using the alignment file generated by ClustalW2, phylogenetic analysis was performed in MEGA4 (Tamura et al. 2007). Extracellular nature of the enzyme was checked by Signal-P programme (Petersen et al. 2011).

Homology modelling

Homology modelling using the SWISS-MODEL workspace (Arnold et al. 2006) was used to build three-dimensional protein structure models using experimentally determined structures of related family members as templates. Alpha-amylase from *B. amyloliquefaciens* (PDB ID: 3bh4) was used as template for modelling. Energy minimization

of the built model was done using the GROMOS96 implemented in the Swiss PDB viewer programme (Guex & Peitsch 1997). Quality of the predicted model was analysed by PROCHECK (Laskowski et al. 1993). Visualization and analysis of the model were done using the Swiss PDB viewer and PyMOL (DeLano 2002) programmes. The active sites of both the enzymes were identified using the online Q-site finder tool (Laurie & Jackson 2005) to predict possible binding sites.

Docking studies

Computer aided molecular docking of the substrate (maltotetraose) to the active sites of the modelled and template enzymes was done using the Autodock 4.2 program (Morris et al. 2009). Autodock allows consistent computational docking of flexible ligands and receptor with a maximum of 32 torsional degrees of freedom. For the best placement of the ligand, Lamarckian genetic algorithm was used in the program. Initial co-ordinates of the substrate were obtained from the Chemspider database. The modelled structure of the α-1,4-amylase was taken as receptor proteins. Docking parameters modified from the defaults were: number of individuals in the population (set to 300), maximum number of energy evaluations (set to 2,500,000), maximum number of generations (set to 27,000) and number of hybrid GALS runs (set to 100). For each of the substrate–receptor complexes, 100 independent docking runs were calculated and the lowest energy pose with acceptable geometry was selected, for further analysis. Hydrogen bond interactions were determined using the following criteria: (i) the distance between proton donor (A) and acceptor (B) atoms $\leq$3.2 Å; and (ii) the A-H- - -B angle=120°. Van der Waals contacts between the heavy atoms were determined within a distance limit of $\leq$4.0 Å.

Results

Isolation of α-1,4-amylase producing bacterial endophytes

A total of 35 morphologically different bacterial endophytes were isolated from surface disinfected tissues of *H. brasiliensis*. Among 35 isolates, six isolates hydrolysed the starch on starch agar and were selected for further study of amylase production. Secondary screening resulted in the selection of the strain REB20 which could give 150 units of enzyme in 100 ml mineral salt starch medium (MSSM) with a specific activity of 75 (Table 1).

Table 1. α-1,4-Amylase from bacterial endophytes. Enzymatic and specific activities of six isolates are given.

Isolates	Enzyme activity (IU/ml)	Specific activity
REB3	84^{e}	56^{c}
REB4	115^{b}	65^{b}
REB 16	95^{d}	43^{e}
REB 20	150^{a}	75^{a}
REB 24	111^{c}	52^{b}
REB 29	76^{f}	48^{b}

Means within columns followed by the same letter are not significantly different at $p < 0.01$ according to Duncan's multiple range test.

Molecular characterization of the bacterial endophyte

Molecular and phylogenetic analyses were used to characterize the α-1,4-amylase producing bacterial endophyte from *H. brasiliensis*. PCR amplification of the 16S rDNA generated a fragment of approximately 1.6 Kb and sequence of the isolate was compared to the sequences of organisms represented in the GenBank database. The isolate showed 99% identity to *Bacillus amyloliquefaciens*. Phylogenetic analysis was conducted in MEGA4 based on UPGMA (unweighted pair group method with arithmetic mean) method. The highest score sequences were recovered from the database as reference sequences and aligned with the 16S rDNA sequence of the endophytic isolate from *H. brasiliensis*. Evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. A phylogenetic tree constructed using 16S rDNA sequences demonstrated that the α-1,4-amylase producing endophytic *B. amyloliqufaciens* was distinctly unique from the reference strains (Figure S1 in supplementary material).

Cloning and sequencing of the α-1,4-amylase encoding gene

DNA was isolated from *B. amyloliquefaciens* and selectively amplified with the primers designed for α-1,4-amylase. The PCR amplification yielded a $\sim$1.6 Kb band in agarose gel. The BLAST analysis of the amplified gene sequence showed identity with other α-1,4-amylase genes. The open reading frame (ORF) of the α-1,4-amylase gene was found to be 1542 bp. The gene was deposited in NCBI gen bank with an accession no JQ710747.

In silico *studies of α-1,4-amylase*

Amino acid sequence and physico-chemical properties

The amino acid sequence obtained by translation of the nucleotide sequence of α-1,4-amylase gene has 513 amino acids. The secondary structure prediction showed 25.15% α-helix, 18.91% β-sheets and the remaining percentage of random coil conformation (55.95%). A phylogenetic tree constructed on the basis of amino acid sequence alignment revealed the similarity and uniqueness of the sequence with the other bacterial α-1,4-amylase sequences (Figure 1). Signal P-4.0 prediction showed that the α-1,4-amylase enzyme was secretory in nature with a cleavage site existing between

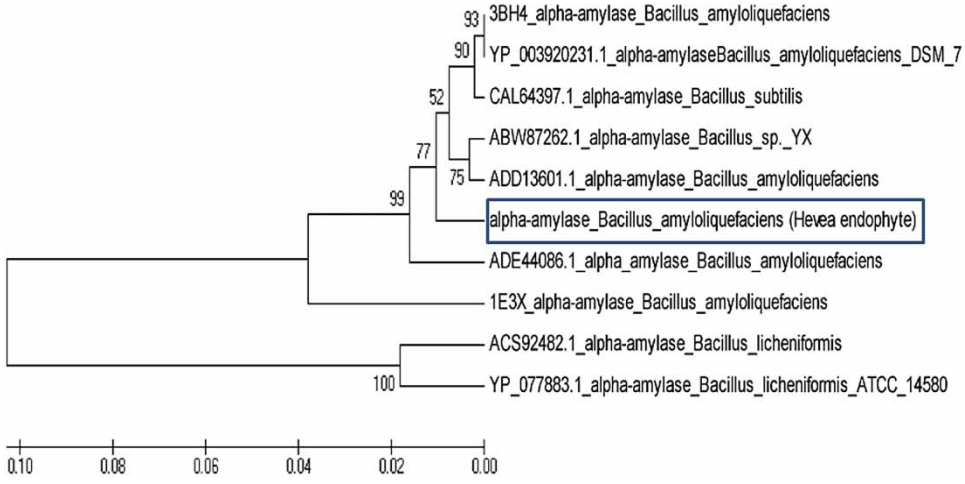

Figure 1. Dendrogram representing the amino acid sequence homology of α-1,4-amylase from endophytic *B. amyloliquefaciens* with other *Bacillus* sp. The amino acid sequence of α-1,4-amylase from endophytic *B. amyloliquefaciens* showed divergence from other sequences. Bootstrap values are given to the left of the respective nodes.

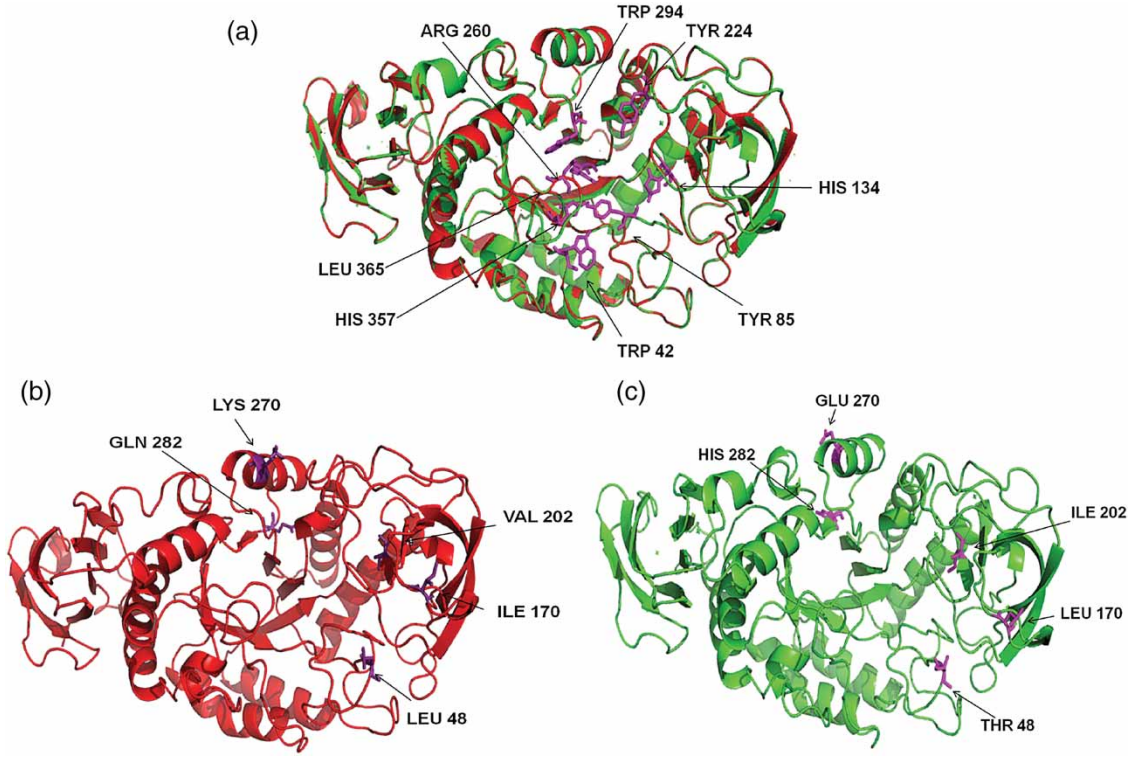

Figure 2. (a) Super imposition of homology modelled structure of the new α-1,4-amylase (green) and crystal structure of template α-1,4-amylase enzyme (red, PDB ID 3bh4). Similar active site residues, identified by Q-site finder, are labelled. (b) Amino acid residues in the active site region of the template α-1,4-amylase showing difference with modelled α-1,4-amylase. (c) Amino acid residues in the active site region of the modelled α-1,4-amylase showing difference with template α-1,4-amylase.

amino acid positions 31 and 32. The functional enzyme without signal peptide has 482 amino acids.

Homology modelling

The three dimensional structure of α-1,4-amylase enzyme, from *B. amyloliquefaciens*, is given in Figure 2. The template α-amylase enzyme from (PDB ID: 3bh4) *B. amyloliquefaciens* has 97.7% sequence similarity with the target enzyme (α-1,4-amylase) and also an E-value of zero. A Q MEAN Z-score value of -0.984 showed the reliability of the model. The stereochemistry of the modelled structure was checked by the Ramachandran plot in which 85% of the total amino acids were present in most favoured regions and 15% in allowed regions (Figure S2 in supplementary materials). The residues showing difference

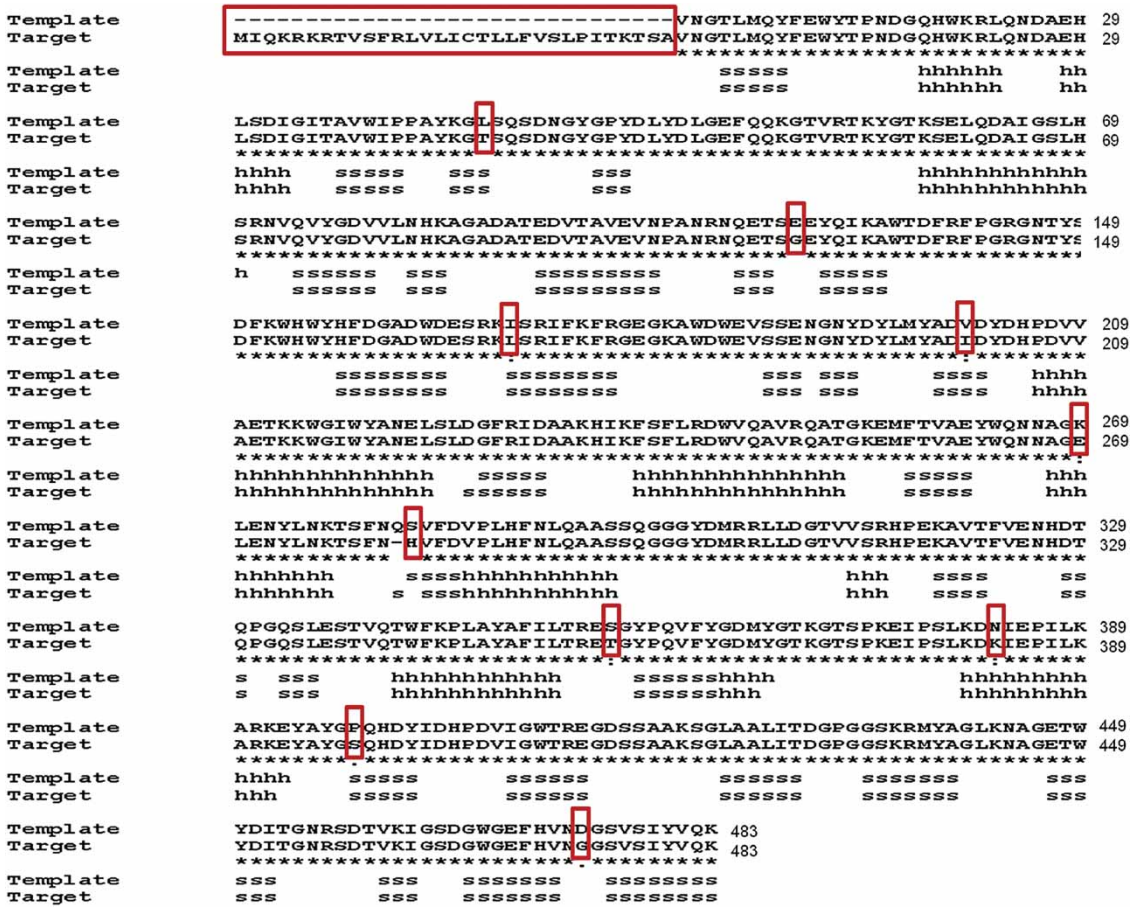

Figure 3. Amino acid sequence alignment between the new α-1,4-amylase enzyme (target) and the template α-1,4-amylase enzyme. The mismatched amino acids are marked by rectangles. The secondary structural elements in both the enzymes are also given. 'S' represents β-sheet structure and 'h' represents helical structure. The numbering of amino acids in template enzyme started after the signal peptide. Numbering of amino acids in the target enzyme excluded the signal peptide.

Table 2. Docking features of modelled and template α-1,4-amylase enzymes with substrate.

Properties	Modelled α-1,4-amylase	Template α-1,4-amylase (PDB ID 3bh4)
Binding energy (kcal/mol)	−25.35	−24.05
Hydrogen bond forming residues	Trp12, Asp53, Asp163, Leu197, Ser335	Asp163, Leu197,Ser335
No. of van der Waals interactions (heavy atoms only)	66	256

between the target and template enzymes are marked in Figure 3. The position and length of secondary structure elements in the modelled structure are exactly matching with that of the template enzyme (Figure 3). The active site residues in the modelled structure obtained by Q-site finder tool are Trp42, Tyr85, His134, Tyr224, Arg260, Trp294, His357 and Leu365, which are shown in Figure 2a. Comparison of the 3D structure and active site residues showed good matching with the template enzyme. The five residues Leu 48, Ile 170, Val 202, Lys 270 and Gln 282 in the active site region of the template enzyme (Figure 2b) are replaced by Thr 48, Leu 170, Ile 202, Glu 270 and His 282 respectively in the corresponding positions of modelled α-1,4-amylase (Figure 2c).

Docking studies

Docking studies showed that the substrate (maltotetraose) has almost comparable binding energies with the modelled (−25.35 kcal/mol) and template (−24.05 kcal/mol) enzymes. The list of hydrogen bonding interactions between the substrate and the receptor proteins are given in Table 2. The modelled α-1,4-amylase–substrate complex is stabilized by six hydrogen bonds formed by the amino acid residues Trp 12, Asp 53, Asp 163, Leu 197 and Ser 335. The template–substrate complex is stabilized by four hydrogen bonds formed by the amino acid residues Asp 163, Leu 197 and Ser 335. The hydrogen bonding interactions between the substrate and the enzymes (modelled and template) are

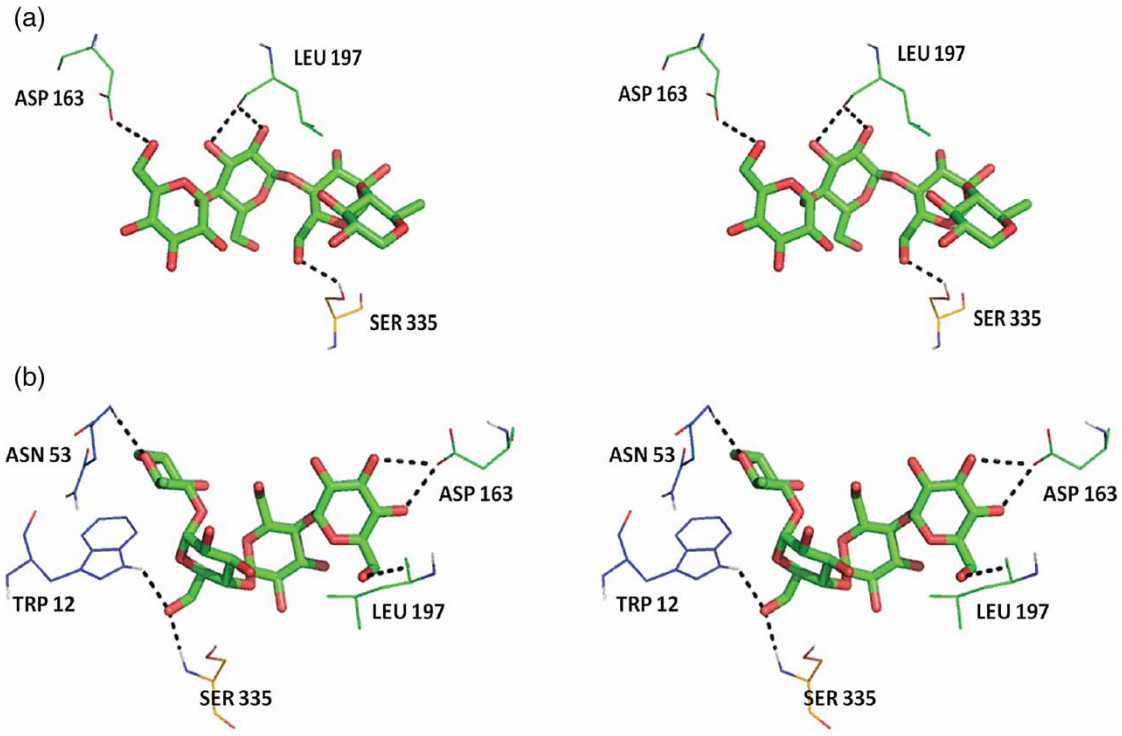

Figure 4. Hydrogen bond interactions of maltotetraose with catalytic residues in the active site of (a) template α-1,4-amylase and (b) modelled α-1,4-amylase (stereo view).

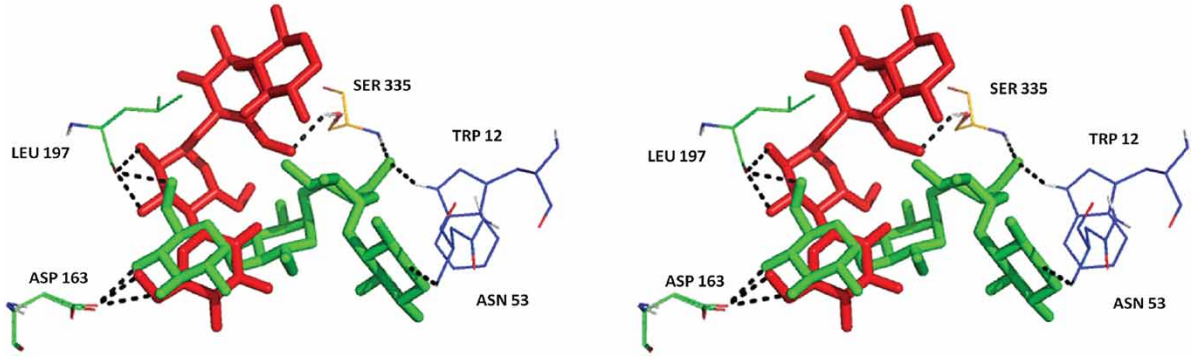

Figure 5. Superposition of the substrate-modelled enzyme (green) and substrate–template enzyme (red) complexes (stereo view).

shown in Figure 4a and 4b. The numbers of van der Waals contacts (heavy atoms only) in the enzyme–substrate complexes for modelled α-1,4-amylase and template enzymes were 66 and 256 respectively. The hydrogen bonding pattern also altered the orientation of the substrate in the binding pocket of the enzyme (Figure 5).

Discussion

Enzymes have evolved over years to operate most effectively under physiological conditions, on a narrow range of natural substrates, and usually at concentrations in the low mM range. An efficient industrial process requires a biocatalytic enzyme operating on non-natural substrates, under extreme conditions of temperature, pH, pressure, and in the presence of organic solvents in which enzyme becomes unstable or inactive (Sylvestre et al. 2006). The search for enzymes with potential features to overcome the hurdles in the industrial process is an important area in enzyme research. Screening of α-1,4-amylase producing microorganisms in a different environment could facilitate the discovery of novel amylases suitable for new applications (Gupta et al. 2003). The endophytic microorganisms occupy a relatively unexplored site for microorganism isolation and represent a new source in obtaining more enzymes with different potentialities. Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as pear (Whitesides & Spotts 1991), oak (Brooks et al. 1994), citrus plant and Scots pine (Pirttila et al. 2005), to herbaceous crop plants, such as sugar beets (Jacobs et al. 1985) and maize (McInroy & Kloepper 1995). In this study a novel

α-1,4 amylase producing endophyte was isolated from a tree crop, *H. brasiliensis*. The production of α-1,4 amylase was evaluated and an efficient isolate was characterized as *B. amyloliquefaciens*. The α-1,4-amylase from various *Bacillus*species is reported and *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are the most common species (Mielenz 1983; Syu & Chen 1997; Mendu et al. 2005; Mishra et al. 2005). This is the first report of the isolation of an α-1,4-amylase producing endophytic microorganism from the tissues of *H. brasiliensis*.

The unique sequence of an enzyme is so important for its function that the mutation of any single amino acid can disrupt the entire fold, and thereby function of a protein, by upsetting the balance of interacting forces within the enzyme (Ishida 2010). Prediction of structure for new sequences via homology modelling offers an alternative way to obtain structural information and is of great importance especially in unlocking the full potential of an enzyme. In the present study, the α-1,4-amylase gene from *B. amyloliquefaciens* was cloned and sequenced. The sequence was translated and novel molecular and structural properties of α-1,4-amylase were analysed using bioinformatics tools and compared with the template used in homology modelling. The functional region of the new α-1,4-amylase has 482 amino acids and its primary structure and secondary structure elements are given in Figure 3. Phylogenetic tree analysis of the α-1,4-amylase from the endophytic *B. amyloliquefaciens* against most similar protein sequences from the NCBI database indicated the uniqueness of the sequence. Secondary structure prediction showed a higher α-helix, 25.15%, over other regular secondary structures, which is a common feature of α-1,4-amylase. The three-dimensional structure of the α-1,4-amylase from the endophytic *B. amyloliquefaciens* was built by homology modelling using SWISS PDB Workspace. The secondary as well as tertiary structures of the modelled α-1,4-amylase are similar to that of the template enzyme. The stochiometric and thermodynamic properties of the modelled α-1,4-amylase were estimated by Q-MEAN Z Score and according to Ramachandran plot 85% of residues in most favoured regions. These results indicated that the model was geometrically reliable. Comparison of predicted active site regions of the modelled and template enzymes revealed the five point mutations as shown in Figure 2b and 2c.

Molecular docking has been used to compare the binding modes of the substrate with the modelled α-1,4-amylase and the template enzymes. The substrate showed almost equal binding energies with the modelled α-1,4-amylase and the template proteins. The substrate–template enzyme complex is stabilized by four hydrogen bonds (Table 2) and 256 van der Waals interactions. The substrate-modelled α-1,4-amylase complex is stabilized by six hydrogen bonds and 66 van der Waals interactions. The three residues forming the hydrogen bonds in the modelled enzyme are conserved in the template enzyme. Interactions between the binding partners not only depended on the conserved residues but also on the nature of the active site regions. Orientations of the substrate in the binding pockets of the two enzymes are different, as shown in Figure 5. Comparison of the active site regions showed the point mutations of Leu 48, Ile 170, Val 202, Lys 270 and Gln 282 residues in the template enzyme by Thr 48, Leu 170, Ile 270, Glu 270 and His 282, respectively, in the modelled enzyme. The increased polar nature of the replaced amino acid residues made the active site region of the modelled enzyme more hydrophilic than the template enzyme, which favours a greater extent of hydrogen bonding and other polar interactions with the substrate molecule. The critical role of hydrogen bonding contacts between polar active-site residues and the substrate molecule in enzyme catalysis has been established by various studies (Kraut et al. 2003; Warshel et al. 2006). Unlike other chemical interactions, hydrogen bonds require directionality between the hydrogen-bond acceptor and donor. This directionality provides the enzyme's specificity for its substrate (Kortemme et al. 2003). The Gln102Arg mutant of lactate dehydrogenase showed an extreme effect of a single ionic hydrogen bond. The wild-type enzyme selectivity was 1000:1 for lactate as compared to malate, but it is converted into a malate dehydrogenase on introduction of Arg102. Due to the new ionic hydrogen bond between the malate g-carboxylate group and the arginine side chain, the reaction rates are now 10,000 times faster for malate than for lactate, resulting in a selectivity change of seven orders of magnitude (Wilks et al. 1988).

Conclusion

The demand for enzymes in the chemical industry has expanded rapidly. α-1,4-Amylases are some of the most versatile enzymes in the industrial enzyme sector and account for approximately 30% of the enzyme market. The suitability of α-1,4-amylase to a particular process will depend on its specificity and characteristics. α-1,4-Amylases can catalyse reactions at room temperature and have great substrate, stereo- and chemo selectivity based on their three-dimensional structure, as defined by their unique sequence. Enzymes of different origin have distinct preferences and the present study could explain differences in electrostatic nature at the active site regions of the modelled α-1,4-amylase and the template enzymes. The high polar nature might endorse more hydrogen bond interactions in the active site region of the new α-1,4-amylase, which might result in increased specificity in its catalytic activity. Considering the outstanding importance of novel enzymes for diverse applications, computational methods allow easy and fast predictions of structural and substrate binding features of enzymes from diverse environments.

Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/21553769.2013.852993

Acknowledgements

The authors are grateful for the facilities provided at Rubber Research Institute of India, School of Biosciences, Mahatma Gandhi University, Kottayam and Department of Biotechnology and Microbiology, Kannur University, Kannur.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215:403–410.

Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics. 22:195–201.

Bernfeld P. 1955. Amylase α and β. Methods Enzymol. 1: 149–155.

Bose K, Das D. 1996. Thermostable α-amylase production using *B. licheniformis* NRRL B1438. Indian J Exp Biol. 34: 1279–1282.

Brooks DS, Gonzalez CF, Appe DN, Filer TH. 1994. Evaluation of endophytic bacteria as potential biological control agents for oak wilt. Biol Control. 4:373–381.

Caetano-Anolles G, Favelukes G, Baeur WD. 1990. Optimizations of surface sterilization for legume seed. Crop Sci. 30:708–712.

DeLano WL. 2002. The PyMOL Molecular Graphics System. San Carlos, CA, USA: DeLano Scientific.

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. 1989. Isolation and direct complete nucleotide determination of entire gene. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17:7843–7851.

Gomes J, Steiner W. 2004. The biocatalytic potential of extremophiles and extremozymes. Food Technol. 42: 223–239.

Guex N, Peitsch MC. 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling. Electrophoresis. 18:2714–2723.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. 2003. Microbial α-amylases: a biotechnological perspective. Process Biochem. 38:1599–1616.

Ishida T. 2010. Effects of point mutation on enzymatic activity: correlation between protein electronic structure and motion in chorismate mutase reaction. J Am Chem Soc. 132: 7104–7118.

Jacobs MJ, Bugbee WM, Gabrielson DA. 1985. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. Can J Bot. 63:1262–1265.

Kortemme T, Morozov AV, Baker D. 2003. An orientation-dependent hydrogen bonding potential improves prediction of specificity and structure for proteins and protein-protein complexes. J Mol Biol. 326:1239–1259.

Kraut DA, Carroll KS, Herschlag D. 2003. Challenges in enzyme mechanism and energetics. Annu Rev Biochem. 72: 517–571.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst. 26:283–291.

Laurie AT, Jackson RM. 2005. Q-Site Finder: an energy based method for the prediction of protein-ligand binding sites. Bioinformatics. 21:1908–1916.

Mamo G, Gessesse A. 1999. Effect of cultivation conditions on growth and alpha–amylase production by a thermophilic *Bacillus* sp. Lett App Microbiol. 29:61–65.

Mclnroy JA, Kloepper JW. 1995. Population dynamics of endophytic bacteria in field grown sweet corn and cotton. Can J Microbiol. 41:895–901.

Mendu DR, Ratnam BVV, Purnima A, Ayyanna C. 2005. Affinity chromatography of α-amylase from *Bacillus licheniformis*. Enzyme Microb Technol. 37:712–717.

Mielenz JR. 1983. *Bacillus stearothermophilus* contains a plasmid- borne gene for α-amylase. Proc Natl Acad Sci. 80:5975–5979.

Mishra S, Noronha SB, Suraishkumar GK. 2005. Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. Process Biochem. 40: 1863–1870.

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. 2009. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem. 30:2785–2791.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. 2000. Advances in microbial amylases. Biotechnol Appl Biochem. 31:135–152.

Petersen TN, Brunak S, Von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 8:785–786.

Pirttila AM, Pospiech H, Lakkanen H, Myllyala R, Hohtola A. 2005. Seasonal variations in location and population structure of endophytes in buds of Scots pine. Tree Physiol. 25: 289–297.

Pitcher DG, Nauders NA, Owen RJ. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol. 8:151–156.

Staley JT, Castenholz RW, Colwell RR, Holt JG, Kane MD, Pace NR, Salyers AA, Tiedje JM. 1997. The microbial world: foundation of biosphere. Washington, DC, USA: American Society for Microbiology.

Sylvestre J, Chautard H, Cedrone F, Delcourt M. 2006. Directed evolution of biocatalysts. Org Process Res Dev. 10:562–571.

Syu MJ, Chen YH. 1997. A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*. Chem Eng J. 65:237–247.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24:1596–1599.

Warshel A, Sharma PK, Kato M, Xiang Y, Liu H, Olsson MH. 2006. Electrostatic basis for enzyme catalysis. Chem Rev. 106:3210–3235.

Whitesides SK, Spotts RA. 1991. Frequency, distribution, and characteristics of endophytic *Pseudomanas syringae* in pear trees. Phytopatholgoy. 81:453–457.

Wilks HM, Hart KW, Feeney R, Dunn CR, Muirhead H, Chia WN, Barstow DA, Atkinson T, Clarke AR, Holbrook JJ. 1988. A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework. Science. 242: 1541–1544.

Zvelebil M, Sternberg M. 1988. Analysis and prediction of the cellular localization of catalytic residues in enzymes. Prot Engg. 2:127–138.