Structural analysis of 4-α-Glucanotransferase from *Pyrococcus furiosus*

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Certificate of Examination

This is to certify that the dissertation titled **"Structural analysis of 4-α-Glucanotransferase from *Pyrococcus furiosus*"** submitted by **Mr. Gurkaran Singh Mehta** (Reg. No. MP14001) for the partial fulfillment of MS degree programme of the Institute, has been examined by the thesis committee duly appointed by the institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Prof. A. K. Bachhawat	Prof. P. Guptasarma	Dr. Shashi B. Pandit
(Head of the Department)	(Supervisor)	(Co-supervisor)

Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. P. Guptasarma and Dr. Shashi B. Pandit at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Gurkaran Singh Mehta
(Candidate)

Dated: April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. P. Guptasarma
(Supervisor)

Dr. Shashi B. Pandit
(Co-supervisor)

Acknowledgment

First of all, I express my sincere gratitude towards my mentor **Prof. P. Guptasarma** for allowing me to work in his laboratory with full freedom. I wonder if I could've been more fascinated by proteins in my lifetime if I hadn't joined under his supervision. I doubt whether I would have completed this work successfully without his moral support and valuable advises during my downtime. The real life examples and suggestions given by him is more than enough for any person to make the right decision in life. I thank the fortune or **God**, whatever it maybe, who gave me chance to start my research career under the hegemony of such an avant-garde mentor.

I would also like to express my heartiest regards and gratitude to **Dr. Shashi B. Pandit,** who have been my co-supervisor, for giving constant guidance throughout the course of the project which enabled this project to be a fruitful one. We had many arguments and discussions which turned to be for my betterment. The scientific discussions done with him have never failed to surprise me how wonderful structural biology is.

I express my sincere thank to our Head of Department **Prof. A. K. Bachhawat**, for his friendly approach towards me which made me work comfortably.

I would like to thank all the **Research Scholars** of Protein Biochemistry and Biophysics lab and Structural Biology lab, especially **Ms. Pallavi Kaila** and **Ms. Preeti Choudhary**, for their constant guidance and support during my stay in the lab. I could successfully complete this work because of the extensive help by the lab scholars.

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Gurkaran Singh Mehta

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Abstract

4-α-Glucanotransferase, an enzyme from *Pyrococcus furiosus*, catalyzes the hydrolysis of a glucose unit from a donor molecule, and transfers it to an acceptor molecule. The sequence of this enzyme is very much similar to 4-α-Glucanotransferase from *Thermococcus litoralis*. The donor and acceptor molecules are carbohydrates of varying length. The donor and acceptor sites are present within the enzyme only. The donor site is present in domain-I having catalytic residues Glutamate-124 and Aspartate-215, working in a acid-base catalysis kind of mechanism, similar in case of the enzyme from *Thermococcus litoralis*. The acceptor site is present in domain-II having interacting residues Histidine-369 and Arginine-372. The function of domain-III is not yet known.

Chapter-1

INTRODUCTION

1.1 An overview of *Pyrococcus furiosus*.

1.2 Glucanotransferases: A structural perspective.

1.3 Three Dimensional structures and domain organization of Glucanotransferases.

1.4 4-α-Glucanotransferase from *Pyrococcus furiosus*.

1.5 Nature of study.

1.6 Purpose of study.

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1. Introduction

1.1 An Overview of *Pyrococcus furiosus*:

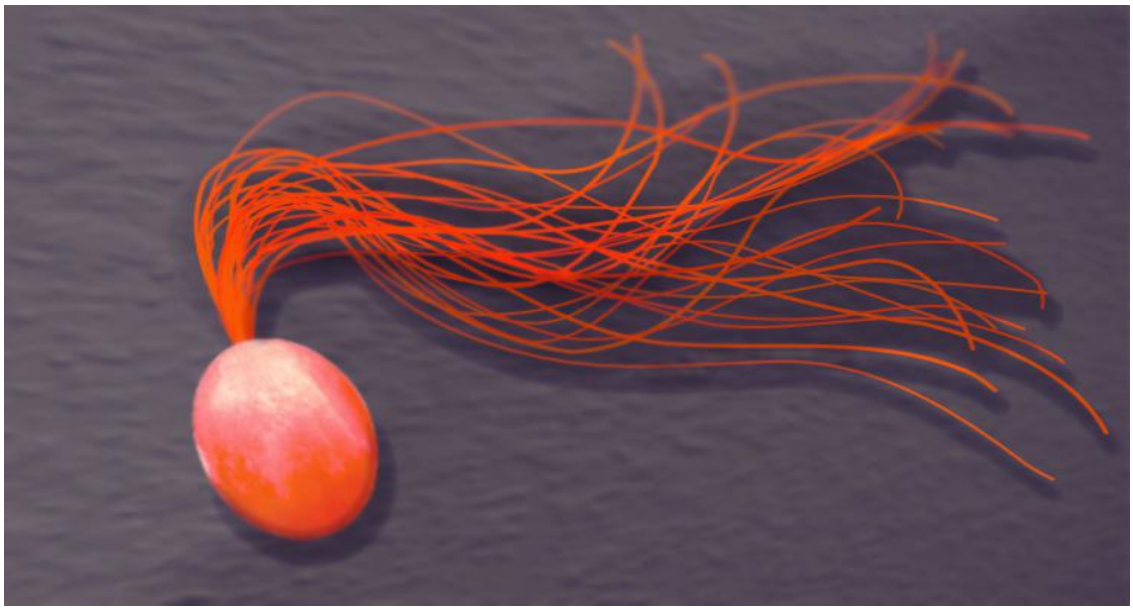
Pyrococcus furiosus is an aquatic, anaerobic, hyperthermophilic archaeon. It's optimal growth temperature is 100°C, so it's enzymes are extremely thermostable. It is one of the first hyperthermophiles to be studied extensively by scientists, and it was found that it's enzymes and proteins are highly resistant to heat shock and radiation. It is unique among it's kind because it can use a wide range of compounds as a carbon source, such as peptides and carbohydrates **(Source = microbe-wiki)**.

Domain	Archea
Kingdom	Euryarchaeota
Phylum	Euryarchaeota
Class	Thermococci
Order	Thermococcales
Family	Thermococcaceae
Genus	*Pyrococcus*
Species	*P. furiosus*

Table-1 Scientific classification of *Pyrococcus furiosus*

1.1.1 Cell Structure:

Pyrococcus furiosus has flagella that are attached to one pole of the cell. It is composed of mainly one type of glycoprotein similar to bacterial flagellin, but differs in other aspects from bacterial flagella (Photograph-1). While bacterial flagella are hollow tubes of a single flagellin growing from the tip, archael flagella are formed from many flagellin. It also allows *P. furiosus* cells to attach themselves to a solid surface, along with connection to other cells. *P. furiosus* can live in a community that's similar to that of a bio-film of bacteria **(Source = microbe-wiki)**.



Photograph.-1 Structural overview of *Pyrococcus furiosus* **(Source = Wikipedia)**

1.1.2 Mechanism:

Living in such an extreme environment, *P. furiosus* has other remarkable mechanisms to protect and proliferate itself. In a more general study of hyperthermophiles, it has been found that their enzymes are generally more rigid in structure to prevent from environmental adverse effect.

P. furiosus is very unique among its genus, for it can use both peptides and carbohydrates as its carbon source. It can also use a wide range of carbohydrates, main is the metabolism of beta linkage glucose polymers (such as cellubiose, chitin, etc). It also has alpha glucosidase activity to degrade alpha linkage sugars like, maltose, malto-triose, malto-tetraose, etc **(Source = microbe-wiki)**.

1.2 Glucanotransferases:- A Structural Perspective:

Glucanotransferase, the enzymes that catalyze glycosidic bond, creates a diverse range of mono- and oligo-saccharides in nature.

Glucanotransferase has been classified by sequence homology into 96 families in the Carbohydrate Active enZyme database (CAZy).

Earlier, three main categories of glucanotransferases were known, namely, cyclodextrin glucanotransferase (CGTase), disproportionationg enzyme (D-enzyme) and glycogen debranching enzyme. Takaha and Smith (1999) proposed the three glucanotransferase be referred as type I, type II and type III. Based on the primary structure and reaction specifities, enzymes from other thermophiles and hyperthermophiles were characterized under type IV and type V category.

The system of classification was based on the analysis of smallest acceptor, smallest donor and the smallest transferred unit.

Types of GTase	Name	Reaction catalyzed	Smallest Donor	Smallest acceptor	Smallest ransferred unit
Type I	Cyclodextrin transferase	coverts starch into a mixture of cyclic α-1,4 glucans	Glucose	Maltose, Maltotriose, Maltotetraose	Glucose
Type II	Disproportionating enzyme	Amylomaltase like function	Maltotriose	Glucose	Maltose
Type III	Glycogen debranching enzyme	Bifunctional-amylo-1,6-glucosidase + glucanotransferase function	Maltotriose	Maltotetraose	Maltose
Type IV	Consists of enzymes from hyperthermophilic bacteria *Thermotoga maritima*		Maltotetraose	Maltose	Maltose
Type V	Consists of enzymes from hyperthermophiles like *Thermococcus litoralis* and *Pyrococcus furiosus*		Maltose	Glucose	Glucose

Table-2. Types of Glucanotransferases

1.3 Three Dimensional structures and domain organization of Glucanotransferases:

Three dimensional structures of several glucanotransferases belonging to families GH 13, 57 and 77 have been solved using X-ray crystallography. It revealed structural similarities in the domain-I of GH-13 and GH-77 family members, but they differ in other domains. GH-57 family members, however, differ even the structural similarity of domain-I from the other families of glucanotransferases (Fig.-1).

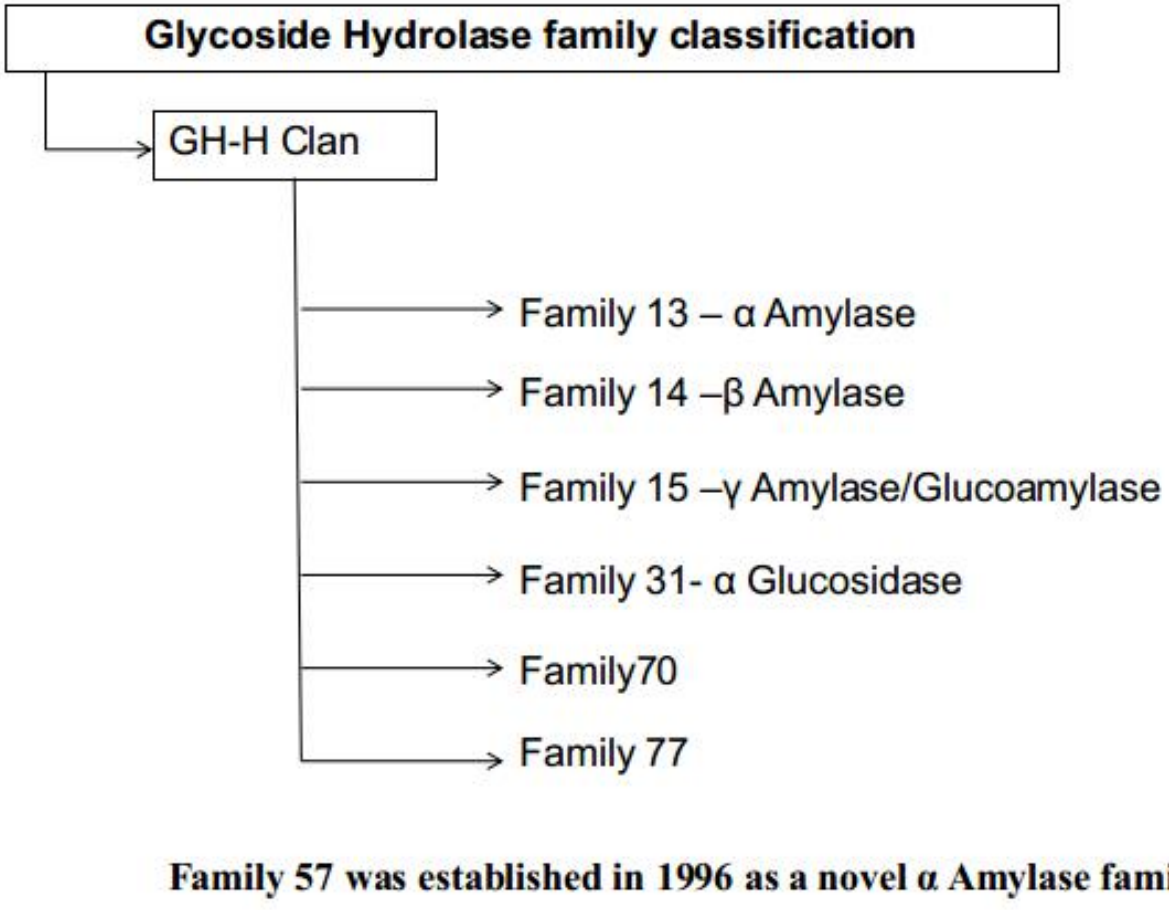


Fig.-1 Glycoside hydrolase family classification

1.3.1 GH-13 family enzymes:

Among the family of GH-13 family enzymes, crystal structures of α- amylase (Matsuura et al. 1984), CGTase (Klein and Schulz 1991), 4-α-glucanotransferase (Roujeinikova et al. 2002) and isoamylases (Katsuya et al. 1998) has been solved. The analysis revealed that they are multi-domain proteins consisting of three domains (A, B and C).
Domain-A is the core domain having a $(\beta/\alpha)_8$ - barrel (TIM-barrel) like structure.
Domain-B varies in length and sequence depending upon the type of enzyme and its function. Domain-C is a domain of unknown function (DUF domain) and is in the form of two sandwiched β-sheets at C-terminus (Fig.-2).

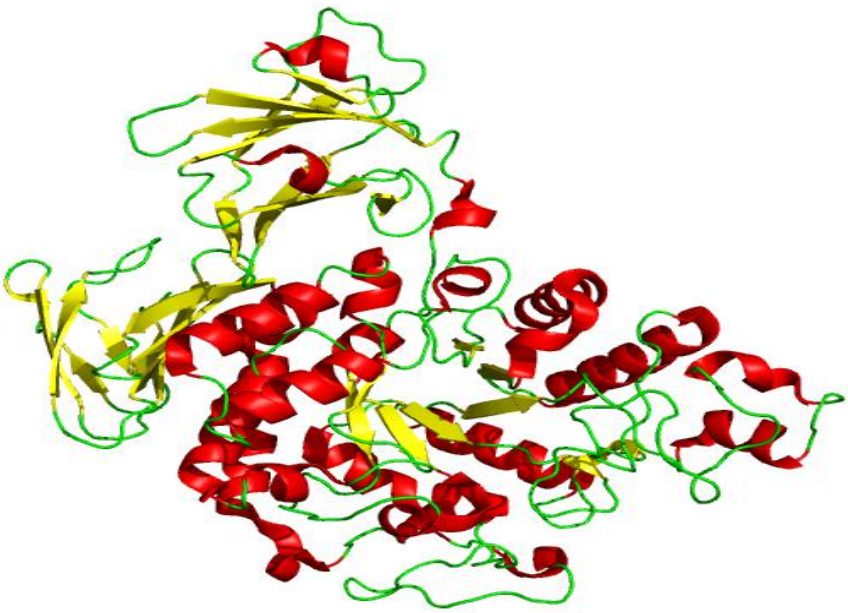


Fig.-2 Crystal structure of alpha amylase II from *Thermoactinomyces vulgaris* (PDB ID - 1g1y)

1.3.2 GH-77 family enzymes :

It is a mono-specific family containing only amylomaltases either from bacterial or archael origin. Among GH-77 family enzymes, the enzymes from *Thermus aquaticus* and *Thermus thermophilus* are the most studied (Przylas et al., 2000). The crystal structure of amylomaltases revealed that domain-A, which is the core domain resembles with GH-13 family enzymes as both have a $(\beta/\alpha)_8$ - barrel like structure. Domain-B of GH-77 family enzymes is split into three sub-domains, namely, B1, B2 and B3 (Przylas et al., 2000). β strands of domain-B are embedded as an insertion in $(\beta/\alpha)_8$ - barrel structure of domain-A. Amylomaltases does not possess a C-domain, which is one of the most striking difference between GH-13 and GH-77 family enzymes (Fig.-3).

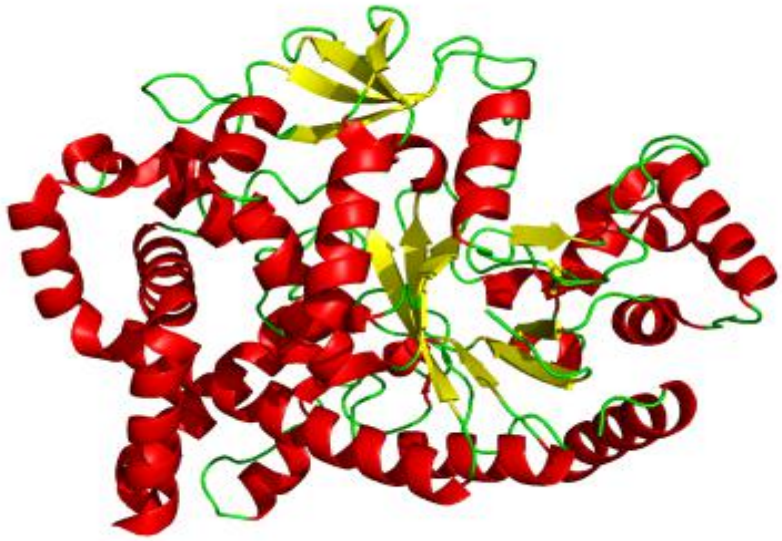


Fig.-3 Crystal structure of amylomaltase from *Thermus aquaticus* (PBD ID - 1cwy)

1.3.3 GH-57 family enzymes:

The sequence of GH-57 family enzymes is very diverse and lack similarities to the members of α-amylase superfamily. 4-α-Glucanotransferase from *Thermococcus litoralis* (Imamura et al. 2003), alpha-amylase C from *Thermotoga maritima* (Dickmanns et al. 2006), and some branching enzymes from *Thermococcus kodakaraensis* (Santos et al. 2011) are some of the most widely studied enzymes from GH-57 family. The core structure of these enzymes looks like a distorted TIM-barrel like structure comprising of seven β-strands and α-helices [$(\beta/\alpha)_7$]. This core domain constitutes the catalytic domain of the enzyme (Ahmad et al. 2015) at the N-terminus. It also has two other domains whose function is not yet known. The C-terminal domain is comprised mainly of β stands and a few α helices (Fig.-4). Janeček and Kuchtová (2012) recently demonstrated that the structural domains and catalytic machinery are somewhat closely related to the members of GH-119 family enzymes.

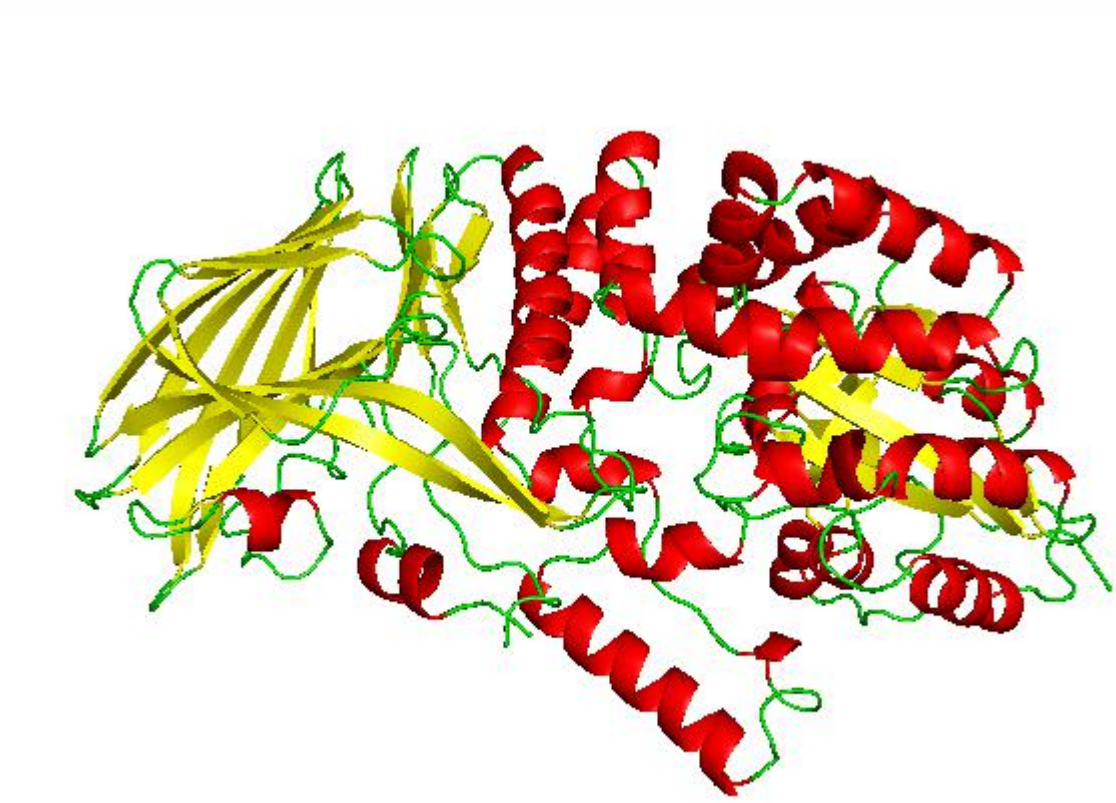


Fig.-4 Crystal structure of 4-α-Glucanotransferase from *Thermococcus litoralis* (PDB ID- 1k1x)

1.4 4-α-Glucanotransferase from *P. furiosus:*

4-α-Glucanotransferase (EC 2.4.1.25) is an enzyme that catalyzes a chemical reaction that transfers a glucose unit of a 1,4-alpha-D-glucan, $(C_6H_{10}O_5)nH_2O$, to a new position in an acceptor carbohydrate, which may be a glucose or a 1,4-alpha-D-glucan.

This enzyme belongs to the family of glycoside hydrolase. It consists of 649 amino acids. The enzyme is further bifurcated into 3 domains, namely, the N-terminal domain belonging to GH-57 family, a middle DUF-1925 domain and a C-terminal DUF-1926 domain (Fig.-5).

Source	Domain	Start	End
Pfam	Glyco_hydro_57	8	292
Pfam	DUF 1925	312	385
Pfam	DUF 1926	393	644

Fig.-5 Pfam classification of 4-α-Glucanotransferase from *Pyrococcus furiosus*

Among the members of GH-57 family, many conserved regions among the sequences have been identified between different organisms (Fig.-6).

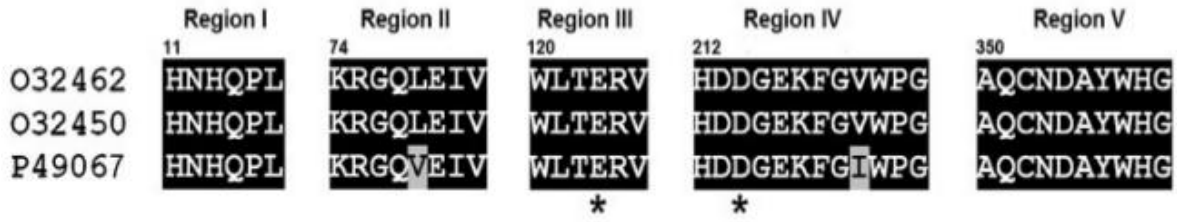


Fig.-6 Regions conserved among sequences of selected members of family GH-57. Catalytic residues are indicated by asterisks. Uniprot accession numbers of 4-α-Glucanotransferases : O32462-*Thermococcus litoralis*, O32450- *Thermococcus kodakaraensis*, P49067- *Pyrococcus furiosus*. (Ahmad et al. 2015)

1.5 Nature of study:

In this study, the focus was on examining the structural aspects of 4-α-Glucanotransferase from *Pyrococcus furiosus* like catalytic residues, catalytic sites (if more than one), number of residues involved in catalysis, mechanism of action, affinity towards various substrates, secondary structure analysis like presence of tunnel or grove at the binding sites, etc.

1.6 Purpose of study:

There was need to analyze this through a computational approach because:-

1. Definite donor and acceptor sites are not known for glucanotransferases.
2. Few crystal structures (from Archae) are known.
3. No information about the mechanism of action of 4-α-Glucanotransferase from *Pyrococcus furiosus* is known.

4. Not enough background information about this enzyme for mutational analysis.

Chapter-2

MATERIALS AND METHODS

2.1 Databases employed

2.2 Servers used

2.3 Bioinformatics Tools used

2.4 Methodology

2. Materials and Methods

2.1 Databases employed:

2.1.1 Databases of NCBI (National Center for Biotechnology Information):

A) GenBank:

GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis.

B) PubMed:

PubMed is a database developed by NCBI National Library of Medicine (NLM), it works as a part of the NCBI Entrez retrieval system. PubMed provides links that allow to access the full-text journal articles at Web sites of particular publishers. MEDLINE database is the primary data source for PubMed, which includes the fields of medicine, dentistry, nursing, health care system, veterinary and the preclinical sciences. PMC provides the permanent access to all of its content and is managed by NLM.

2.1.2 UniProt database:

UniProt is a comprehensive, high-quality and freely accessible database of protein sequence and functional information, many entries being derived from genome sequencing projects. It contains a large amount of information about the biological function of proteins derived from the research literature. The UniProt consortium comprises the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB), and the Protein Information Resource (PIR).

2.1.3 Protein Data Bank:

The Protein Data bank (PDB) is a crystallographic database for the three dimensional structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography, NMR spectroscopy, or, increasingly, cryo-electron microscopy, and submitted to biologists and biochemists from around the world, are freely accessible on the internet via the websites of its member organizations. The PDB is overseen by an organization called the Worldwide Protein Data Bank, wwPDB. The PDB is a key resource in the area of structural biology.

2.1.4 Carbohydrate Active enZYmes database:

The Carbohydrate Active enZYmes database (CAZy), describes the families of structurally related catalytic and carbohydrate binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds. Online since 1998, CAZy is a specialist database dedicated to the display and analysis of genomic, structural and biochemical information Carbohydrate Active Enzymes (CAZymes). CAZy data are accessible either by browsing sequence based families or by browsing the content of the genomes in carbohydrate active enzymes. New genomes are added regularly shortly after they appear in the daily releases of GenBank. New families are created based on published evidence for the activity of at least one member of the family and all families are regularly updated, both in content and in description.

2.1.5 PDBsum:

PDBsum is a database that provides an overview of the contents of each 3D macro-molecular structure deposited in the Protein Data Bank. Each structure in the PDBsum database includes an image of structure, molecular components contained in the structure, enzyme reaction diagram if appropriate, gene ontology functional assignments, description of bound molecules and graphic showing interaction between protein and secondary structure, schematic diagrams of protein-protein interactions, analysis of clefts contained within the structure and links to external databases.

2.1.6 Pfam database:

Pfam is a database of protein families that includes their annotations and multiple sequence alignments generated using Hidden Markov Models. The general purpose of the Pfam database is to provide a complete and accurate classification of protein families and domains. It is used by experimental biologists researching specific proteins, by structural biologists to identify new targets for structure determination, by computational biologists to organize sequence and by evolutionary biologists tracing the origins of proteins.

2.2 Servers used:

2.2.1 I-TASSER:

I-TASSER (Iterative Threading ASSEmbly Refinement) is a bioinformatics method for predicting three dimensional structure of protein molecules from amino acid sequences. It detects structure templates from Protein Data Bank by a technique called fold recognition (or threading). The full length structure models are constructed by reassembling dtructural fragments from threading templates using replica exchange Monte Carlo simulations. I-TASSER is one of the most successful protein structure prediction methods in the community wide CASP experiments.

2.2.2 EMBOSS Needle Pairwise sequence alignment tool:

EMBOSS Needle reads two input sequences and writes their optimal global sequence alignment to file. It uses the Needleman-Wunsch alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length. This tool can be used in the following contexts:

1. Protein alignment
2. Nucleotide alignment

2.2.3 TM-align:

TM-align is an algorithm for sequence-order independent protein structure comparisons. For two protein structures of unknown equivalence, TM-align first generates optimized residue to residue alignment based on structural similarity using dynamic programming iterations. An optimal superposition of the two structures, as well as the TM-score value which scales the structural similarity, will be returned. TM_score has the value in (0, 1), where 1 indicates the perfect match between two structures. Following strict statistics of structures in the PDB, scores below 0.2 corresponds to randomly chosen unrelated proteins whereas score higher than 0.5 assume generally the same fold in SCOP/CATH.

2.3 Bioinformatics Tools used:

2.3.1 PyMOL:

PyMOL is a computer software, a molecular visualization system created by Warren Lyford DeLano. It is user sponsored, open source software, released under the Python License. PyMOL can produce high quality 3D images of small molecules and biological macro-molecules, such as proteins. The *Py* part of the software's name refers to that it extends, and is extensible by, the programming language Python. PyMOL uses Open GL Extension Wrangler Library (GLEW) and Freeglut, and can solve Poisson-Boltzmann equations using Adaptive Poisson Boltzmann solver.

2.3.2 AutoDock:

AutoDock is a molecular modeling simulation software. It is especially effective for protein-ligand docking. It is one of the most cited docking software in the research community. AutoDock consists of two main programs:

1. AutoDock for docking of the ligand to a set of grids describing the target protein.
2. AutoGrid for pre-calculating these grids.

Usage of AutoDock has contributed to discovery of several drugs, including HIV-1 integrase inhibitors.

2.4 Methodology:

2.4.1 Sequence analysis of 4-α-Glucanotransferase from *T. litoralis* and *P. furiosus:*

1. Sequence of 4-α-glucanotransferase from *T. litoralis* and *P. furiosus* were obtained from UniProt in FASTA format.
2. Both the sequences were submitted to EMBOSS Needle for pairwise sequence alignment.
3. A significant amount of identical and similar regions were observed.

2.4.2 Domain analysis of 4-α-Glucanotransferase from *T. litoralis* and *P. furiosus:*

1. Domain characterization of 4-α-glucanotransferase from *T. litoralis* and *P. furiosus* was done using Pfam database.
2. Strikingly similar domains were seen in the enzyme of both the organisms.

2.4.3 Structural analysis of 4-α-glucanotransferase from *T. litoralis*:

1. Structure of 4-α-glucanotransferase from *T. litoralis* was derived from Protein Data Bank as its crystal structure is known in *Thermococcus litoralis.*
2. Structural analysis was done using PyMOL.

2.4.4 Homology model structure of 4-Glucanotransferase from *Pyrococcus furiosus* through I-TASSER:

1. The sequence derived from UniProt was submitted to I-TASSER for modeling the structure of the enzyme.
2. The server used 4-α-glucanotransferase from *T. litoralis* as a template for modeling the structure of the enzyme.
3. The efficiency of the modeled structure was evaluated on the bases of C-score.

2.4.5 Structural alignment using TM-align:

1. The structures of 4-α-glucanotransferase from *T. litoralis* as well as the modeled structure from *P. furiosus* were submitted to TM-align.
2. The alignment was evaluated on the bases of TM-score and RMSD value.

2.4.6 Prediction of residues involved in interaction:

1. The interactive residues were predicted using PDBsum.
2. The LigPlot function in PDBsum allows to identify specific atoms of the residues involved in interaction.
3. It can be used for those proteins only whose crystal structure is known (4-α-glucanotransferase from *T. litoralis*).

4. Similar set of residues observed in 4-α-glucanotransferase from *P. furiosus.*

2.4.7 Protein-ligand Docking studies for 4-α-Glucanotransferase using AutoDock:

1. The enzyme has specificity for a wide range of carbohydrate molecules which can be used as substrates.
2. The docking studies were done using the following molecules as substrates:
   A) Glucose
   B) Fructose
   C) Maltose
   D) Malto-triose
   E) Malto-tetraose

Chapter-3

RESULTS AND DISCUSSIONS

3.1 Sequence analysis of 4-α-glucanotransferase from *Thermococcus litoralis* and *Pyrococcus furiosus*.

3.2 Domain analysis of 4-α-glucanotransferase from *Thermococcus litoralis.*

3.3 Structural analysis of 4-α-glucanotransferase from *Thermococcus litoralis.*

3.4 Homology based structural modeling of 4-α-glucanotransferase from *Pyrococcus furiosus* using I-TASSER.

3.5 Structural alignment of 4-α-glucanotransferase.

3.6 Determination of interacting residues of 4-α-glucanotransferase from *Thermococcus litoralis* and *Pyrococcus furiosus*.

3.7 Molecular Docking analysis of 4-α-glucanotransferase from *Pyrococcus furiosus*.

3.8 Discussion.

3.9 Proposed mechanism of action.

3. Results and Discussions

3.1 Sequence analysis of 4-α-glucanotransferase from *Thermococcus litoralis* and *Pyrococcus furiosus*:

According to CAZy database, 4-α-glucanotransferase from *Thermococcus litoralis* is very well studied in the Archael family. Also, its crystal structure is known (PDB ID:- 1k1x, 1k1y, 1k1z). Therefore, the sequences of 4-α-glucanotransferases was obtained in FASTA format for both *Thermococcus litoralis* and *Pyrococcus furiosus* through UniProt and were subjected to pairwise sequence alignment using EMBOSS Needle (Fig.-7)

On performing pairwise sequence alignment of 4-α-glucanotransferase from *Pyrococcus furiosus* with 4-α-glucanotransferase from *Thermococcus litoralis*, following results were obtained:-

1. Domain-1 (length = 291 amino acids) showed similarity of 90.4% and identity of 78% with each other.
2. Domain-2 (length = 74 amino acids), also known as Domain of Unknown Function-1925 (DUF-1925) showed 100% similarity as well 100% identity with each other.
3. Domain-3 (length = 265 amino acids), also known as Domain of Unknown Function-1926 (DUF-1926) showed 64.2% similarity and 45.3% identity with each other.

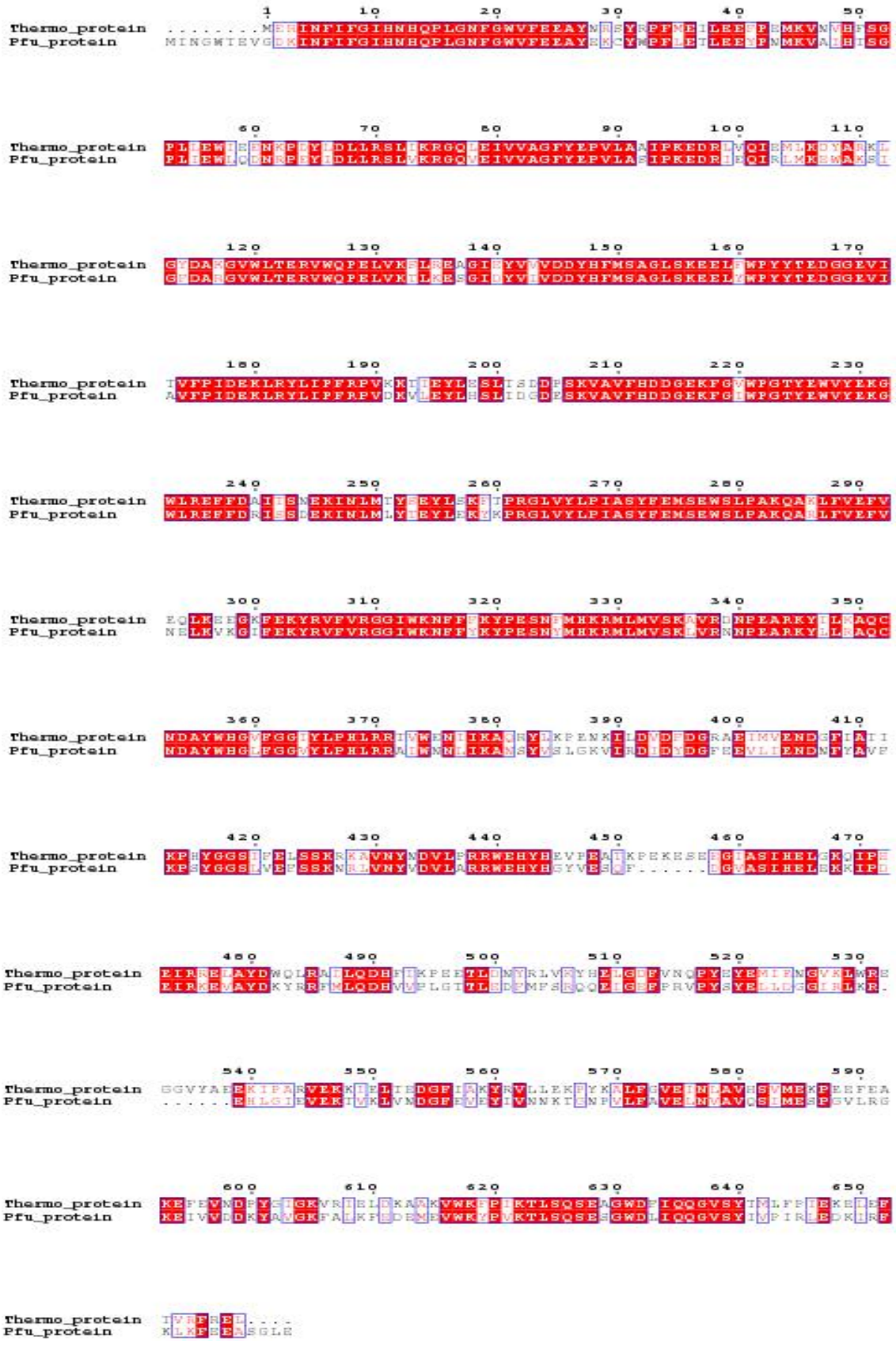


Fig.-7 Pairwise sequence alignment of 4-α-glucanotransferase from *Thermococcus litoralis* and *Pyrococcus furiosus* using EMOBOSS Needle and data represented using ESpript.

3.2 Domain analysis of 4-α-glucanotransferase from *Thermococcus litoralis*:

4-α-glucanotransferase (UniProt ID = O32462) consists of three domains according to Pfam classification (Fig.-8).

The N-terminal domain belongs to GH-57 family, consisting of 284 amino acids. The middle domain is a domain of unknown function, consisting of 73 amino acids. Members of this family adopt an immunoglobulin/albumin binding domain like fold (Imamura et.al. 2003). The C-terminal domain is also a domain of unknown function, consisting of 264 amino acids. Members of this family adopt a β-sandwich fold, in which two layers of anti-parallel β-sheets are arranged in a parallel fashion. It has been proposed that they may play a role in tranglycosylation reactions (Imamura et al. 2003).

Source	Domain	Start	End
Pfam	GH-57	7	291
Pfam	DUF-1925	311	384
Pfam	DUF-1926	392	656

Fig.-8 Arrangement of domains in 4-α-glucanotransferase from *Thermococcus litoralis* (UniProt ID = O32462). **Source - Pfam.**

3.3 Structural Analysis of 4-α-glucanotransferase from *Thermococcus litoralis*:

3.3.1 Crystal structure of 4-α-glucanotransferase:

The crystal structure of 4-α-glucanotransferase from *Thermococcus litoralis* is known (Imamura et al. 2003), therefore, it was derived from Protein Data Bank (PDB ID = 1k1y). Structural analysis using PyMOL revealed that it is a homo-dimeric protein and the two sub-units (Chain-A and Chain-B) were joined in a head to head kind of fashion, i.e., domain-1 of chain-A was attached to domain-1 of chain-B, but in opposite orientation (Fig.-9). The crystal structure was solved by Imamura et al. by using an inhibitor, the acarbose, which is a structural analog of malto-tetraose. The authors were unable to explain the presence of maltose in the crystal structure (Imamura et al. 2003). Although it has been mentioned by Imamura et al. that as the maltose binding site on Chain-A was involved in crystal contact, therefore, it is possible that maltose was found on Chain-B rather than Chain-A. It is also unclear that why acarbose was not found on Chain-B. Presence of maltose in the crystal structure led us to formulate a hypothesis that maybe the acceptor site in also present within the enzyme only.

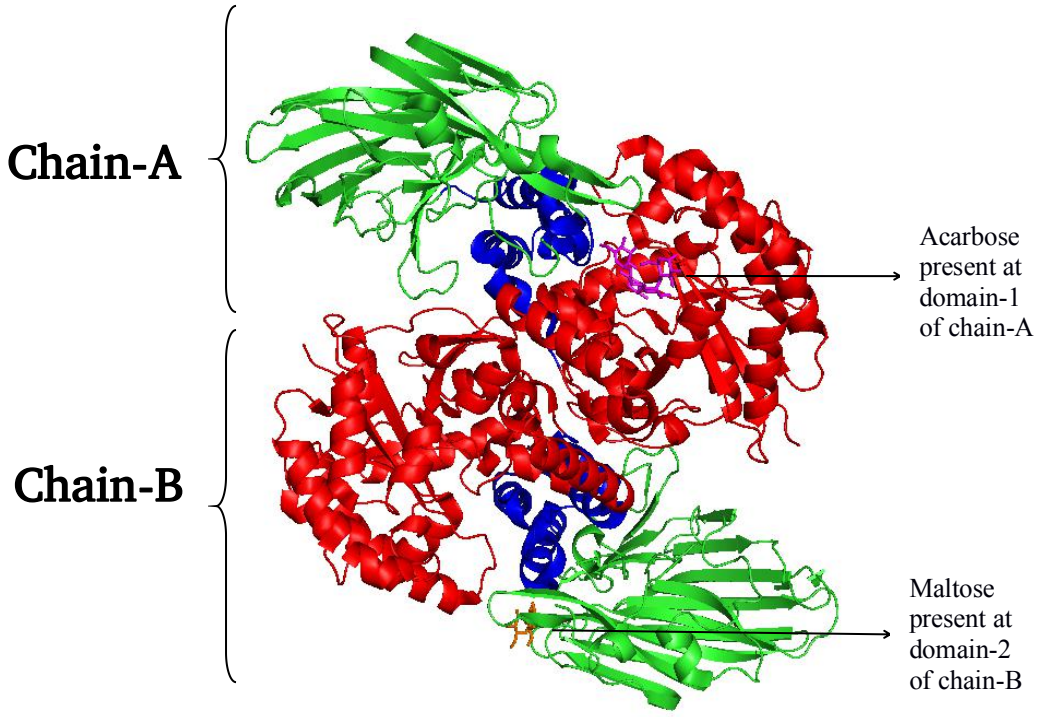


Fig.-9 Crystal structure of 4-α-glucanotransferase from *Thermococcus litoralis* complexed with an inhibitor, acarbose (PDB ID = 1k1y).

3.3.2 Domain Annotation study:

Using SCOP classification (Structural Classification Of Proteins), individual domain study was also done for both the chains of 4-α-glucanotransferase from *Thermococcus litoralis* (Table-3).

Chains	Domain info	Class	Fold	Super-family	Family	Domain
A 1-310	d1k1ya3	Alpha and Beta proteins	7- stranded beta/alpha barrel	Glycoside hydrolase/de-acetylase	4-alpha-glucanotransferase, N-terminal Domain	4-alpha-glucanotransferase, N-terminal Domain
A 311-384	d1k1ya1	All alpha Proteins	Immunoglobulin/albumin binding like domain	Families 57/38 glycoside transferase middle domain	4-alpha-glucanotransferase, Domain-2	4-alpha-glucanotransferase, Domain-2
A 385-659	d1k1ya2	All Beta proteins	Supersandwich	Galactose-mutarotase like	4-alpha-glucanotransferase, C-terminal Domain	4-alpha-glucanotransferase, C-terminal Domain
B 2-310	d1k1yb3	Alpha and Beta proteins	7- stranded beta/alpha barrel	Glycoside hydrolase/de-acetylase	4-alpha-glucanotransferase, N-terminal Domain	4-alpha-glucanotransferase, N-terminal Domain
B 311-384	d1k1yb1	All Alpha proteins	Immunoglobulin/albumin binding like domain	Families 57/38 glycoside transferase middle domain	4-alpha-glucanotransferase, Domain-2	4-alpha-glucanotransferase, Domain-2
B 385-659	d1k1yb2	All Beta proteins	Supersandwich	Galactose-mutarotase like	4-alpha-glucanotransferase, C-terminal Domain	4-alpha-glucanotransferase, C-terminal Domain

Table-3 Domain annotation of 4-α-glucanotransferase from *Thermococcus litoralis* (PDB ID - 1k1y)

Source = RCSB PDB

3.4 Homology based structure modeling of 4-α-glucanotransferase from *Pyrococcus furiosus* using I-TASSER:

Since the sequence was already derived from UniProt (ID = P49067), it was submitted to I-TASSER for homology based modeling of 4-α-glucanotransferase (Fig.-10).
The efficiency of the model was evaluated on the bases of C-score. It is a confidence score for estimating the quality of predicted models by I-TASSER. C-score is typically in the range of -5 to 2, where a C-score of higher value signifies a model with a high confidence.

The server used the structure of 4-α-glucanotransferase from *Thermococcus litoralis* as a template to model the structure.

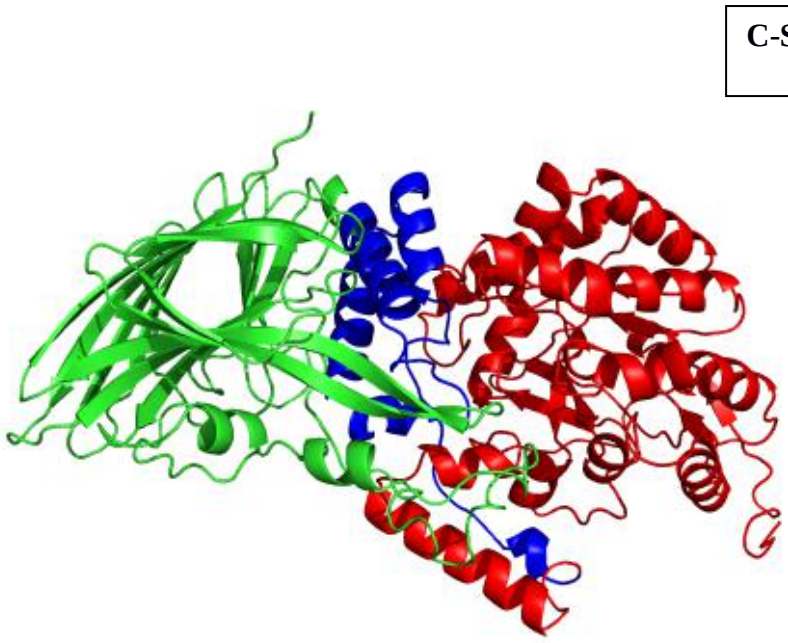


Fig.- 10 Modeled structure of 4-α-glucanotransferase from *Pyrococcus furiosus* using I-TASSER.

3.5 Structural alignment of 4-α-glucanotransferase:-

Since, the sequence alignment showed a very significant similarity and identity among the enzyme from two different organisms, the modeled structure of 4-α-glucanotransferase from *Pyrococcus furiosus* and the known structure from *Thermococcus litoralis* were submitted to TM-align server for structural alignment with both chains of glucanotransferase from *Thermococcus litoralis*.

The efficiency of the alignment was evaluated on the bases of TM-score and RMSD value provided by the server itself after the alignment(Fig.-11). TM-score is a recently proposed scale for measuring the structural similarity between two structures. The purpose of proposing TM-score is to solve the problem of RMSD which is sensitive to local error. Because RMSD is an average distance of all residue pairs in the structure, a local error will araise a big RMSD difference. In TM-score, however, the small distance is weighted stronger than the big distance which makes the score insensitive to local modeling error. 0.0<TM-score<0.30, indicates random structural similarity, whereas, 0.5<TM-score<1.00, indicates about the same fold in both the proteins.

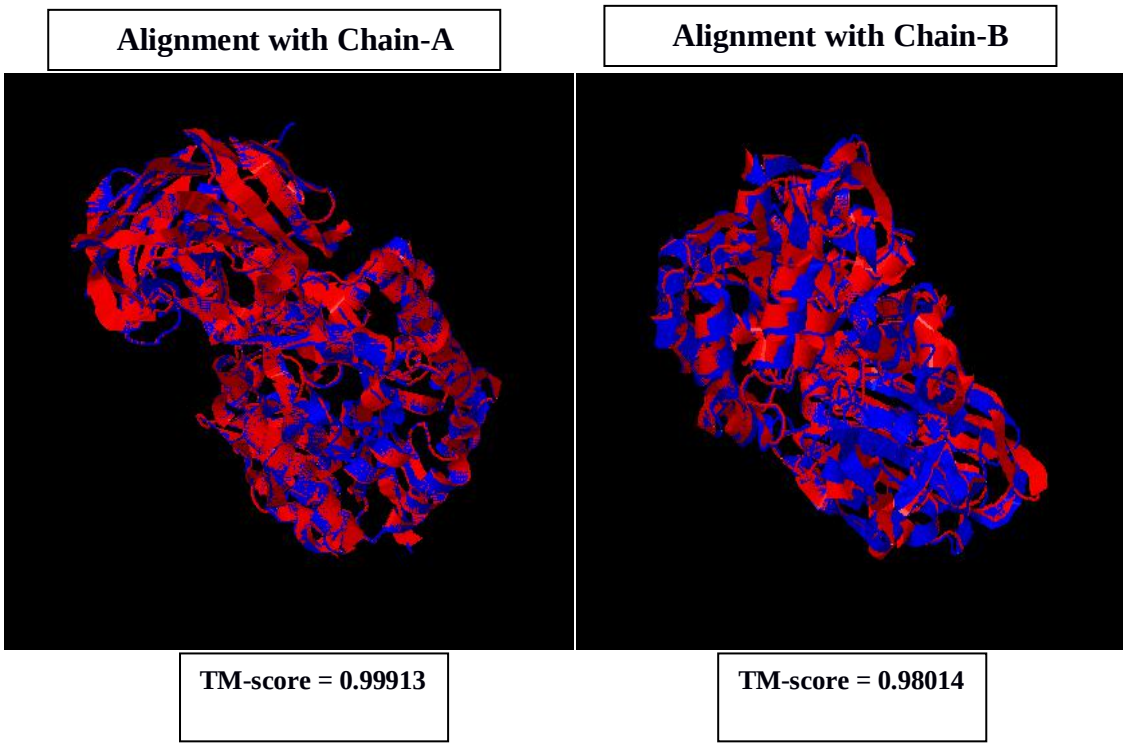


Fig.-11 Structural alignment of glucanotransferase using TM-align. Structure in blue is the glucanotransferase from *Thermococcus litoralis* and structure in red is the glucanotransferase from *Pyrococcus furiosus*.

3.6 Determination of interacting residues of 4-α-glucanotransferase from *Thermococcus litoralis* and *Pyrococcus furiosus*:

Since the structural alignment was very efficient (based on TM-score), determination of interacting residues for glucanotransferase in *Pyrococcus furiosus* was easy as similar residues would be observed in glucanotransferase from *Thermococcus litoralis* as its crystal structure is known (PDB ID = 1k1y). 4-α-glucanotransferase uses a wide variety of carbohydrate molecules as substrates/donor for its activity. One of the substrates in malto-tetraose whose structural analog, acarbose, was used as an inhibitor for determining the crystal structure. The residues interacting with acarbose would be the interacting residues involved in the hydrolysis of substrates for glucanotransferases (Fig.-12). To identify the interacting residues, the PDB ID was entered in the PDBsum server for determination of LigPlot (ligand binding plot). Similarly the interacting residues for maltose was also determined (Fig.-13).

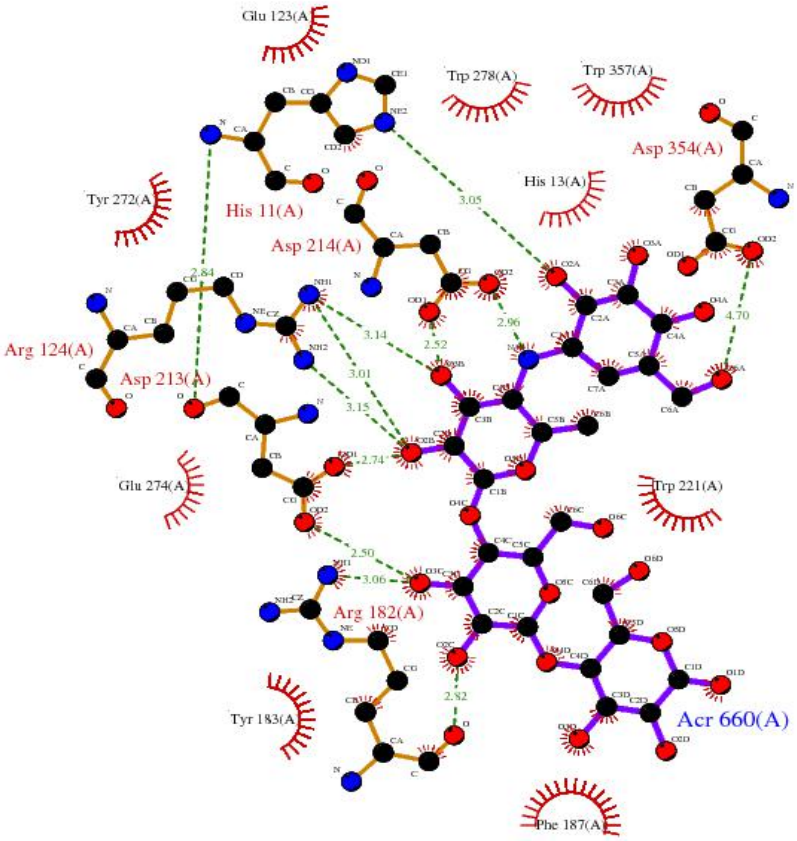


Fig.-12 LigPlot of interactions involving ligand Acarbose. **Source = PDBsum**

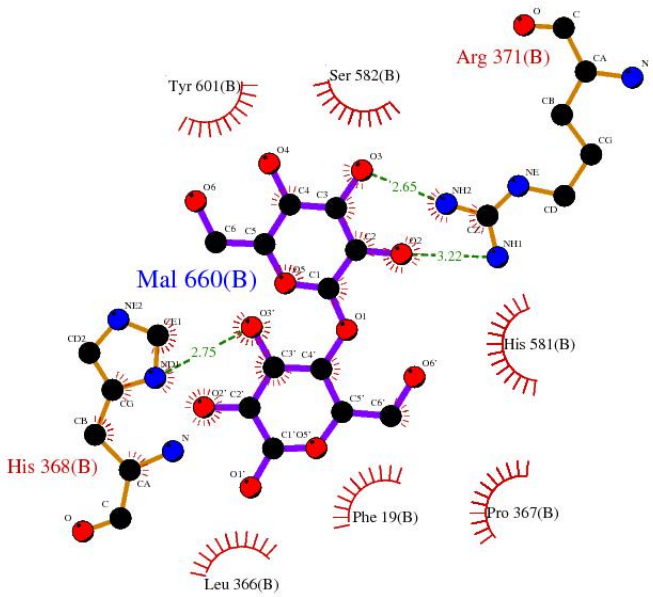
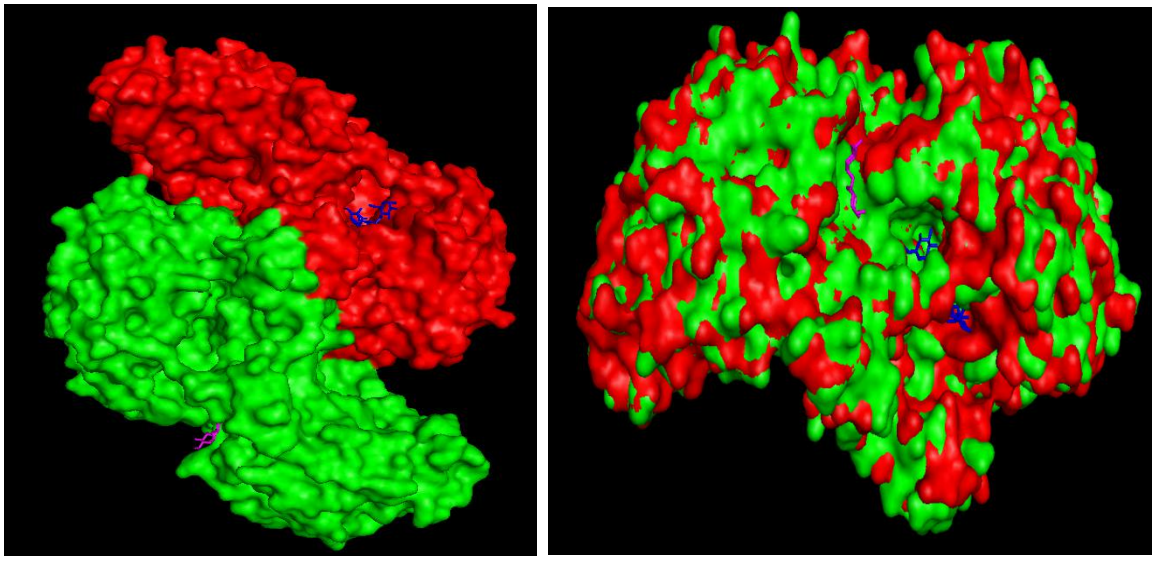


Fig.-13 LigPlot of interactions involving ligand maltose. **Source = PDBsum**

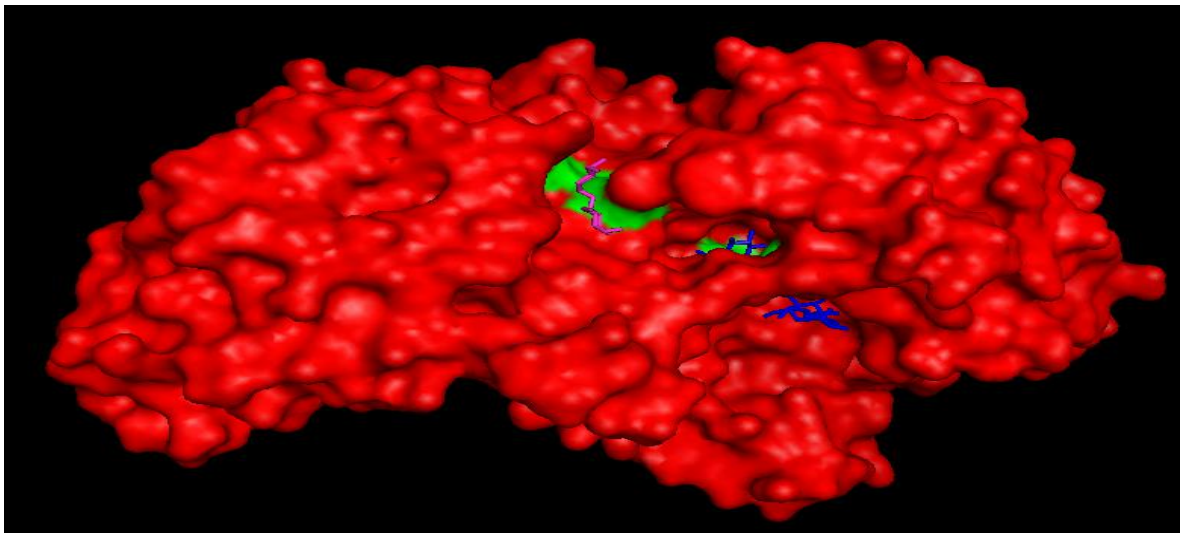
The two structures were also superimposed in PyMOL to check whether the interacting residues in both the enzymes acquire same position or not. For this, the two chains of glucanotransferase from *Thermococcus litoralis* (PDB ID = 1k1y) were first superimposed to each other to confirm that the interacting residues in both the chains are similar (Fig.-14).



a) **b)**

Fig.-14 Structure of glucanotransferase from *Thermococcus litoralis* in PyMOL. Chain-A is shown in red color, Chain-B is shown in green color, acarbose is blue in color and maltose is magenta in color. a) shows the crystal structure of the enzyme and b) shows the superimposition of the two chains.

Superimposition of the two chains revealed that the interacting residues for acarbose as well as maltose are same in both the chains (Fig.-15).



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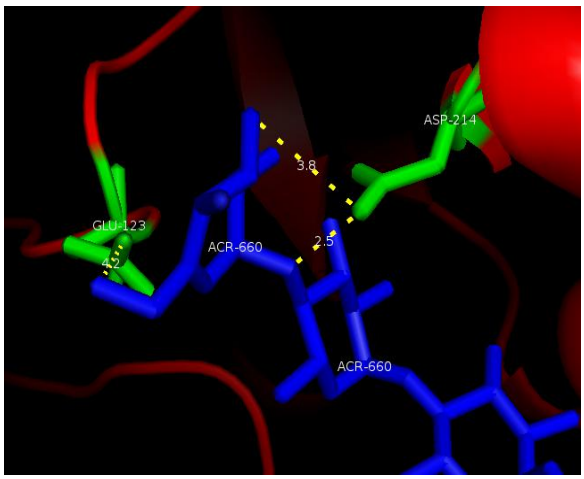
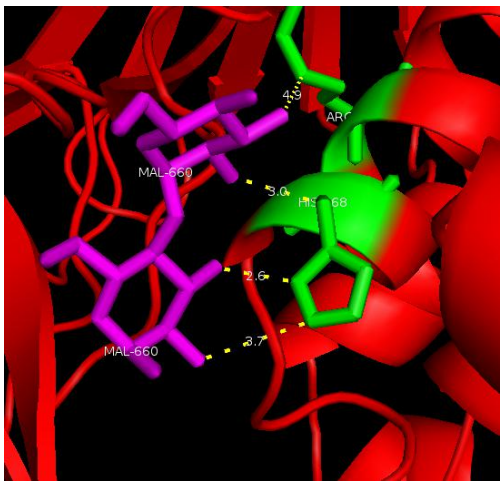
a)



b)



c)

Fig.-15 a) shows position of acarbose and maltose in the crystal structure of glucanotransferase from *Thermococcus litoralis*. b) shows the interacting residues at the donor/active site and their distance from acarbose. c) shows the interacting residues at the putative acceptor site and their distance from maltose.

Table 4 summarizes the list of interacting residues at the donor/active as well as the putative acceptor site:

Ligand Name	Chain	Interacting Residues
Acarbose	Chain-A	Glutamate-123 Aspartate-214
Maltose	Chain-B	Histidine-368 Arginine-371

Table-4 List of interacting residues of 4-α-glucanotransferase from *Thermococcus litoralis.*

To check whether the modeled structure of glucanotransferase from *Pyrococcus furiosus* also had same interacting residues as like the glucanotransferase from *Thermococcus litoralis*, superimposition of the modeled structure was done with one of the chains of glucanotransferase from *Thermococcus litoralis* and checked for the position of acarbose and maltose and the residues around them (Fig-16).

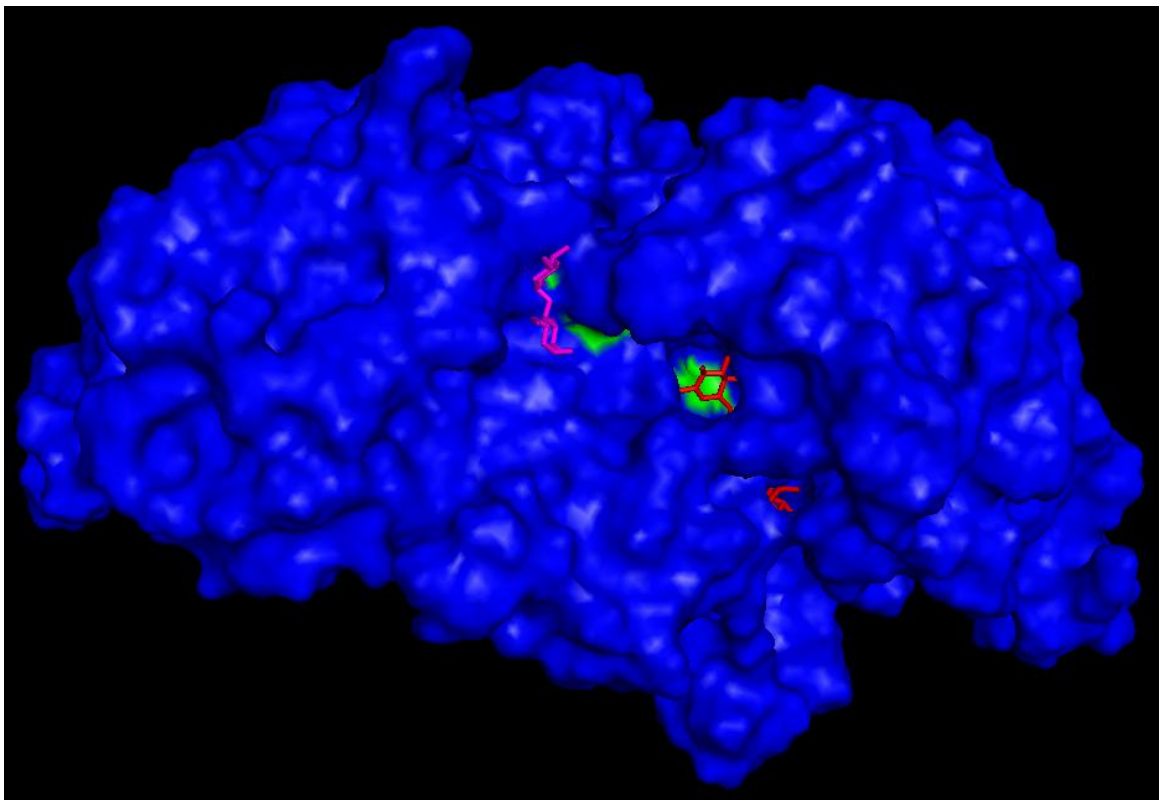
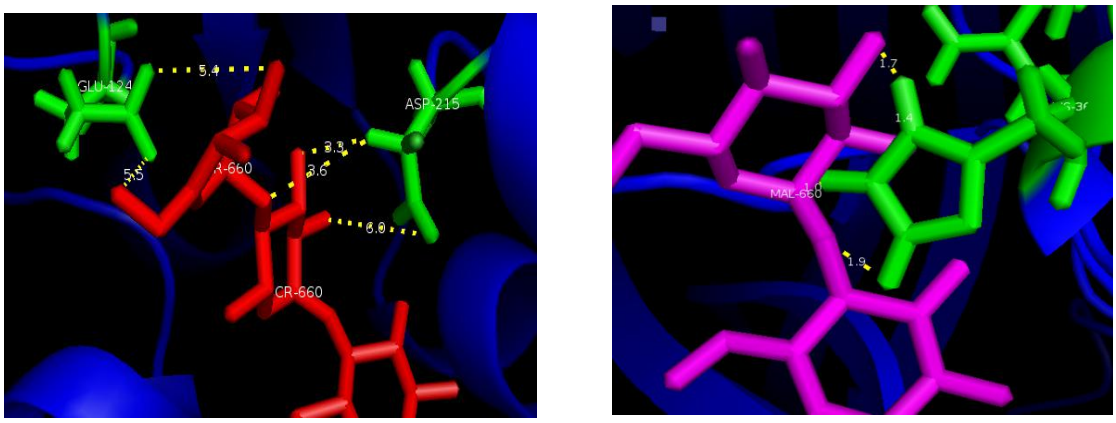


Fig.-16 Modeled structure of glucanotransferase from *Pyrococcus furiosus* bearing the same donor/active and putative acceptor sites as glucanotransferase from *Thermococcus litoralis*. Donor/ substrate (acarbose) is shown in red color and maltose is shown in magenta color. Highlighted green color shows position of interacting residues inside the tunnel.

Detailed analysis of this structure showed that same residues are involved in interaction with acarbose and maltose (Fig.-17).



a) **b)**

Fig.-17 Shows the distance between the ligands and the interacting residues. Figure a) shows acarbose (red) in proximity with the donor/active site residues. Figure b) shows maltose (magenta) in close proximity with putative acceptor site residues. The enzyme, glucanotransferase from *Pyrococcus furiosus* is shown in blue color.

It was also observed that the donor/active site comprises of a tunnel and the putative acceptor site is like a groove.

Table-5 summarizes the list of interacting residues at the donor/active site as well as putative acceptor site for glucanotransferase from *Pyrococcus furiosus*:

Ligand Name	**Chain**	**Interacting Residues**
Acarbose	Chain-A	Glutamate-124 Aspartate-215
Maltose	Chain-A	Histidine-369 Arginine-372

Table-5 List of interacting residues of 4-α-glucanotransferase from *Pyrococcus furiosus*.

3.7 Molecular Docking analysis of 4-α-Glucanotransferase from *Pyrococcus furiosus*:

Since the interacting residues were identified for glucanotransferase from *Pyrococcus furiosus*, molecular docking was performed using AutoDock Tools using various carbohydrates as substrates to compare their binding affinities, to predict the conformation of ligand when it interacts and to predict minimum glucan unit required to act as donor as well as acceptor. The substrates used were fructose, glucose, maltose, malto-triose, malto-tetraose and acarbose. Docking was performed for both donor as well as putative acceptor sites. Molecular docking was done for 1000 runs.

The .pdb file of the modeled structure was studied in text format and the coordinates of all the interacting residues for donor and putative acceptor sites were obtained. The mean was taken and a grid around the molecular area to be docked by the ligand was formed using those coordinates. For docking at the donor site, a grid having the coordinates X = 2.065, Y = 54.265 and Z = 90.228 and of dimension of 60 was formed. Similarly, for docking at the putative acceptor site, a grid having the coordinates X = -4.300, Y = 74.739 and Z = 100.197 and of dimension 60 was formed.

3.7.1. Docking of Fructose at the donor site:

Fig.-18 shows the formation of grid around the donor/active site of glucanotransferase from *Pyrococcus furiosus*. Formation of grid ensures that the ligand will try to dock in the area under the grid only.

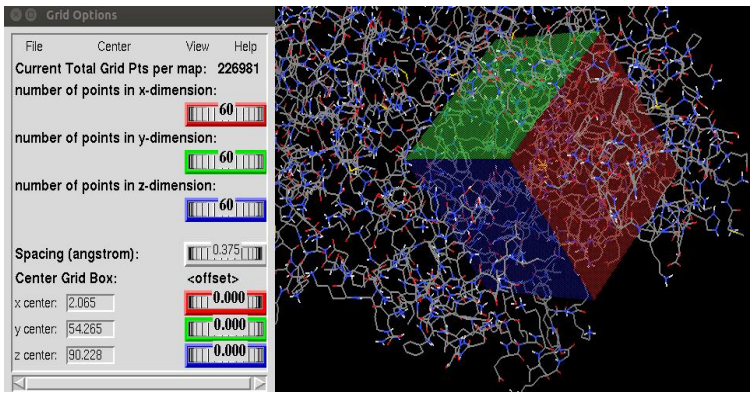


Fig.-18 Grid formation around the active site for docking of fructose to the enzyme.

Once the docking was completed, results were analyzed in the AutoDock software itself. The results appear in the form of cluster of ranks and can be arranged in decreasing order of binding energy so as to make analysis simpler. Fig.-19 shows the most suitable conformation of fructose docked with the enzyme having the binding energy of -6.83 kJ/mol. Negative sign indicates that the reaction is thermodynamically favorable.

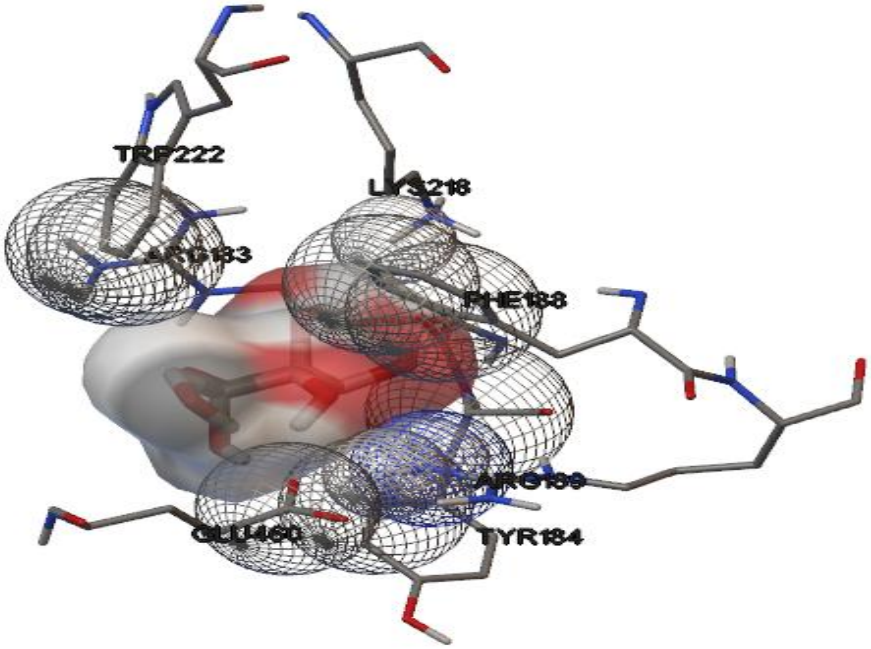


Fig.-19 Image of fructose with glucanotransferase from *Pyrococcus furiosus*.

No significant interaction was observed between fructose and the catalytic residues of the enzyme.

3.7.2 Docking of Glucose at the donor site:

Similar kind of grid was formed for docking of glucose to the enzyme. Fig.-20 shows the possible conformation adopted by glucose while binding at the active site of the enzyme. The binding energy obtained was -7.53 kJ/mol.

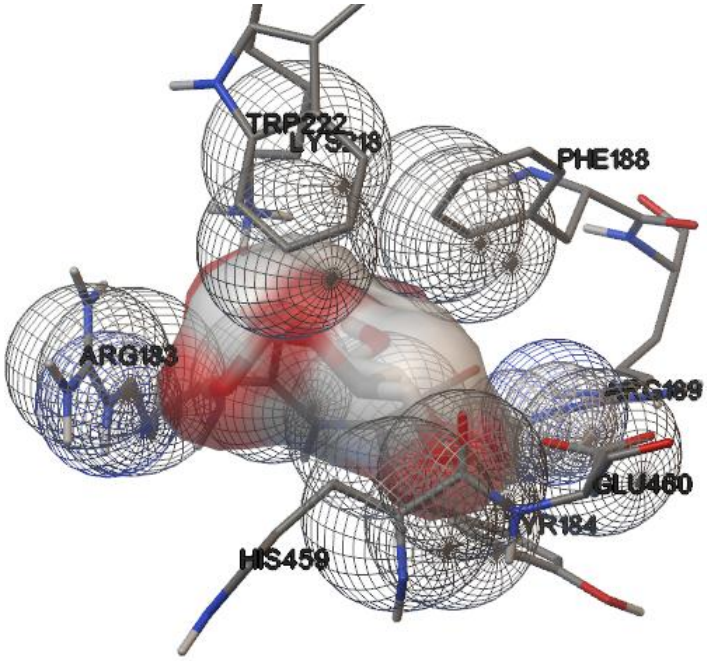


Fig.-20 Image of glucose with glucanotransferase from *Pyrococcus furiosus*.

Here also, no significant binding was observed between glucose and the catalytic residues of the enzyme.

3.7.3 Docking of Maltose at the donor site:

Fig.-21 shows the most favorable conformation adopted by maltose to bind at the donor/active site of the enzyme. The binding energy was found to be -7.74 kJ/mol.

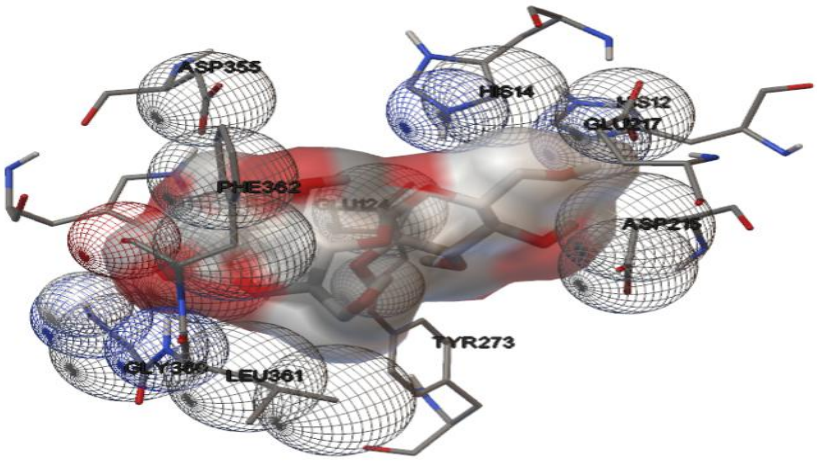


Fig.-21 Image of maltose with glucanotransferase from *Pyrococcus furiosus*.

Fig.-21 shows that maltose was able to interact with the active site residues of the enzyme along with some other residues which were in close proximity with maltose. It could lead to another conclusion that may be maltose is able to enter the the tunnel at the donor/active site where the catalytic residues are present.

3.7.4 Docking of Malto-triose at the donor site:

Malto-triose is three glucose unit molecule linked to each other by α-1,4-glycosidic linkage. Fig.-22 shows the Image of malto-triose at the donor/active site of the enzyme in its most favorable conformation. The binding energy was found to be -10.00 kJ/mol.

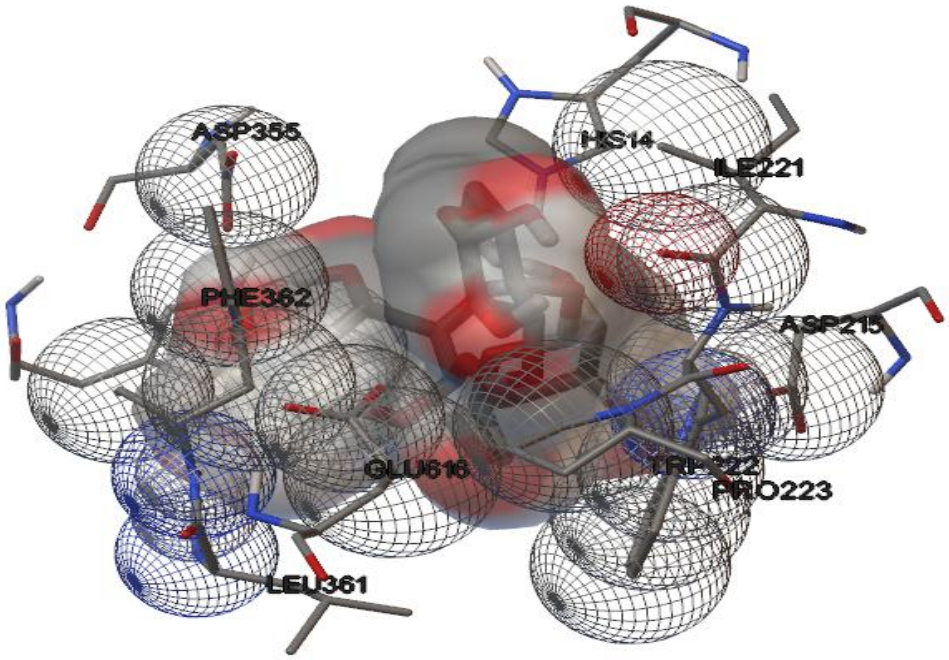


Fig.-22 Image of malto-triose with glucanotransferase from *Pyrococcus furiosus*.

A sudden rise in the binding energy indicates that may be malto-triose is one of the favored substrates for this enzyme and it can easily interact with the catalytic residues as well as other residues in close proximity as they are all part of the tunnel too.

3.7.5 Docking of Malto-tetraose to the donor site:

Fig.-23 shows the Image of malto-tetraose at the donor/active site if the enzyme in its most thermodynamically favored conformation. The binding energy was observed to be -10.03 kJ/mol.

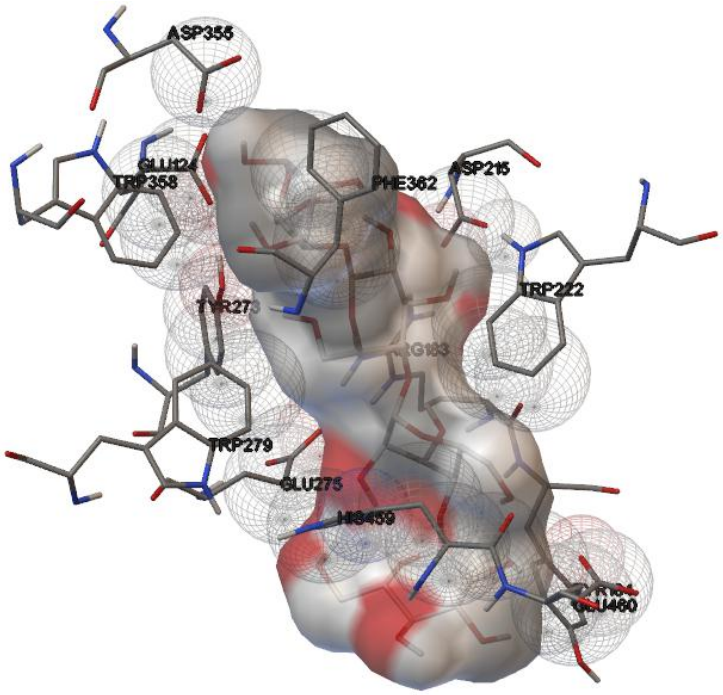


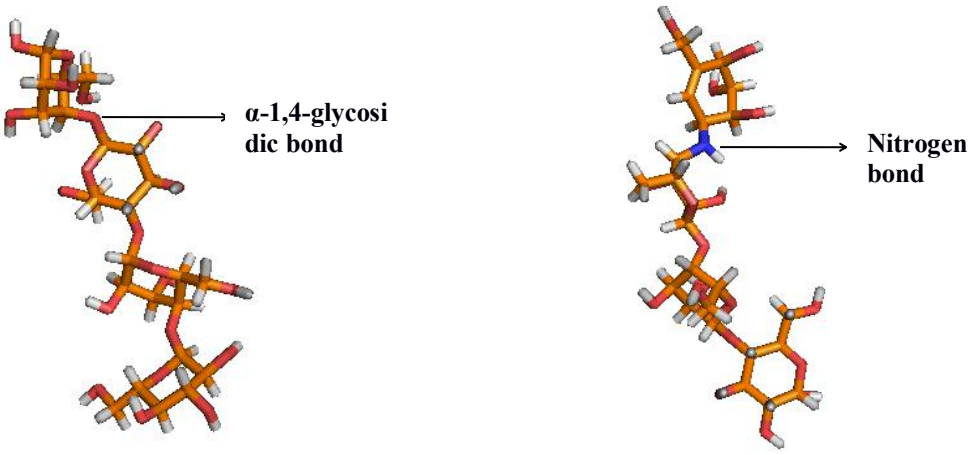
Fig.-23 Image of malto-tetraose with glucanotransferase from *Pyrococcus furiosus*.

Here also it is seen that apart from the catalytic residues, many other residues present in the tunnel, which are in close proximity with the ligand, also seem to have affinity towards the ligand.

Affinity of other residues in the tunnel towards ligand also suggests that may be these residues are temporarily binding the ligand and assisting it to enter the tunnel properly in the correct orientation.

3.7.6 Docking of Acarbose to the donor site:

Acarbose is a structural analog of malto-tetraose having a nitrogen bond instead of a glycosidic bond between the first and second glucose unit (Fig.- 24).



Fig,-24 Structural difference between malto-tetraose and its analog acarbose. **Tool used = PyMOL**

Since glucanotransferase cannot catalyze nitrogen bond, therefore, acarbose was used as an inhibitor of the reaction.

Docking was also performed using acarbose as the ligand to compare the results. Fig.-25 shows the docking of acarbose to the donor/active site of the enzyme having a binding energy of -10.18 kJ/mol.

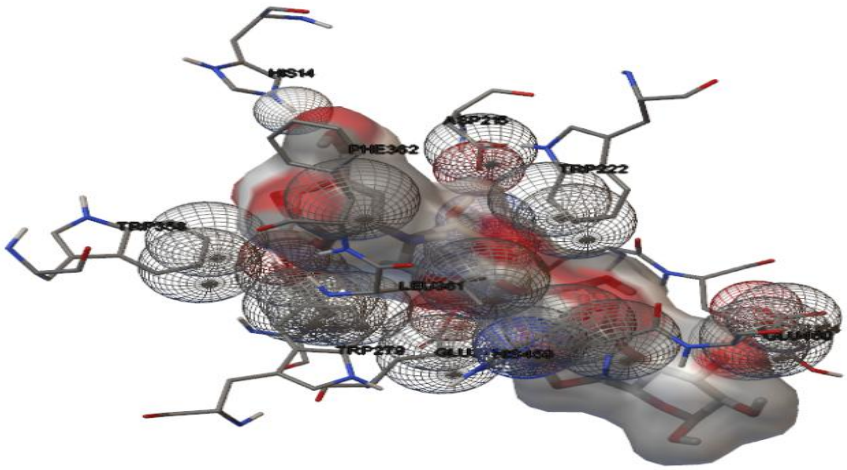


Fig. -25 Image of acarbose docked with glucanotransferase from *Pyrococcus furiosus*

Acarbose seems to be interacting with Aspartate-215 more efficiently as compared with Glutamate-124 as the interaction with Glutamate-124 was not seen to be thermodynamically favorable in the molecular docking approach.

The summarized result of molecular docking at the donor/active site is represented as a graph in Fig.-26.

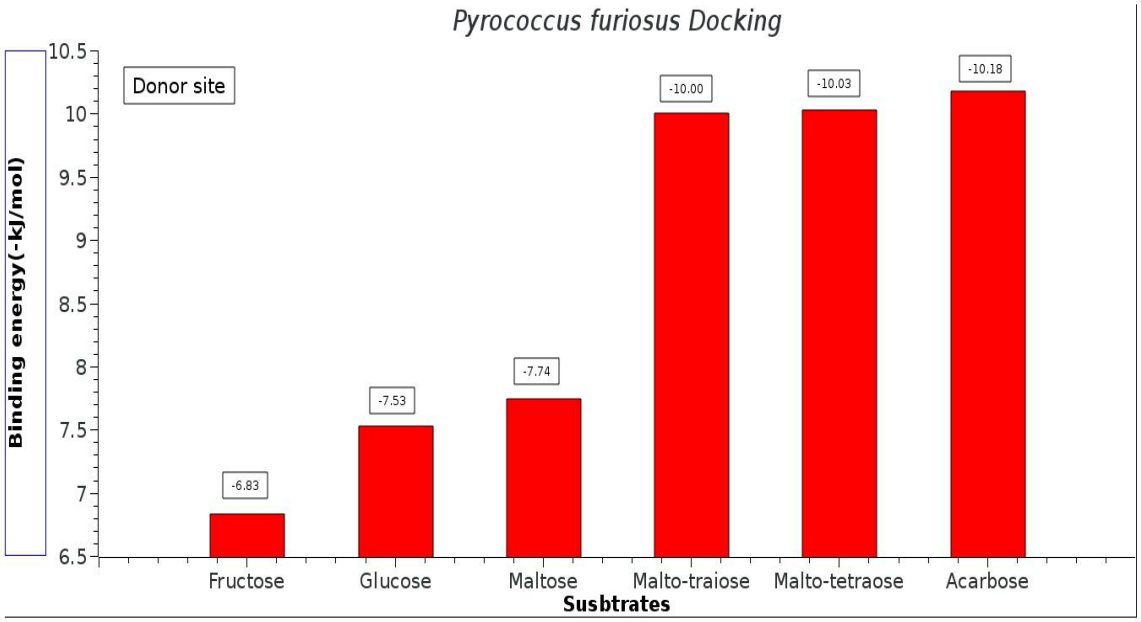


Fig.-26 Molecular Docking analysis of various substrates at the donor/active site of glucanotransferase from *Pyrococcus furiosus*.

Similar molecular docking studies was performed at the putative acceptor site of glucanotransferase from *Pyrococcus furiosus*.

3.7.7 Docking of Fructose at the putative acceptor site:

The residues found to be a part of putative acceptor site are Histidine-369 and Arginine-372. Same protocol was followed for docking at putative acceptor site as it was done with the donor/active site.

Fig.-27 shows docking of fructose at the putative acceptor site. The binding energy was found to be -2.87 kJ/mol.

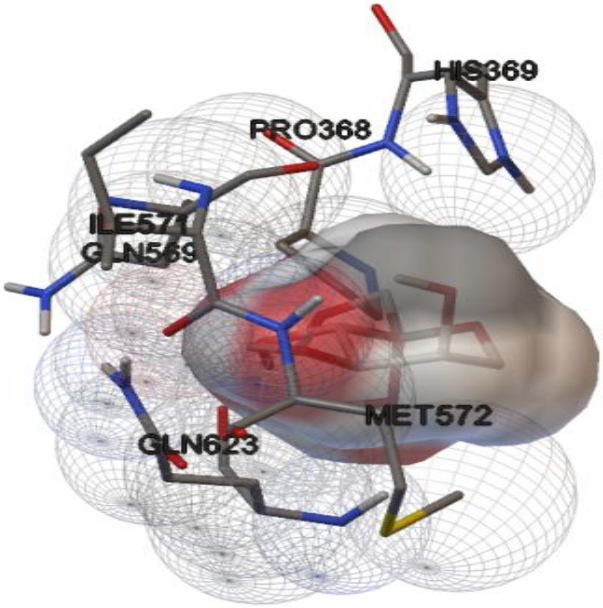


Fig.-27 Image of fructose docked at the putative acceptor site of glucanotransferase from *Pyrococcus furiosus*.

Fructose, although being in close proximity with Histidine-369, was seen to interact with other residues (Glutamine-623). This could be possible because:-

1. Molecular docking is affinity based reactions rather that specificity based reactions.
2. Fructose, being a small mono-meric carbohydrate unit, can adopt many conformations for binding which was not yet seen in molecular docking reactions.
3. It is also possible that other residues are also involved in binding of carbohydrate molecule at the putative acceptor site which are yet to be found.

3.7.8 Docking of Glucose at the putative acceptor site:

Fig.-28 demonstrates binding of glucose at the putative acceptor site having a binding energy of -3.33 kJ/mol. Glucose is also seen interacting here with Glutamine-623, suggesting that may be Glutamine-623 is involved in accepting the carbohydrate monomer from donor site to the putative acceptor site.

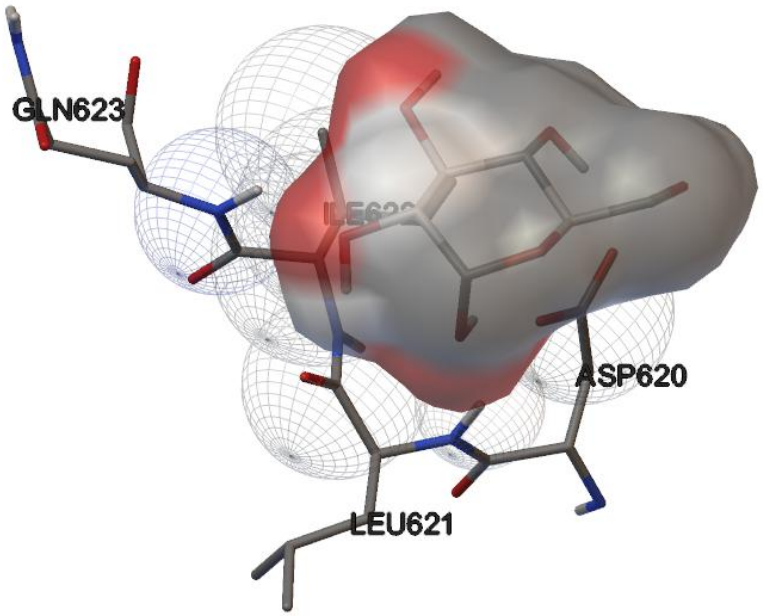


Fig.-28 Image of glucose docked at the putative acceptor site of glucanotransferase from *Pyrococcus furiosus*.

3.7.9 Docking of Maltose at the putative acceptor site:

Maltose, being a di-saccharide, can provide more atoms to form bond at the putative acceptor site as compared to glucose and fructose. Fig.-29 shows the image of maltose docked at the putative acceptor site having binding energy of -4.32 kJ/mol.
Maltose was seen binding with Histidine-369 and Arginine-372, which are thought to be the interacting residues at the putative acceptor site.

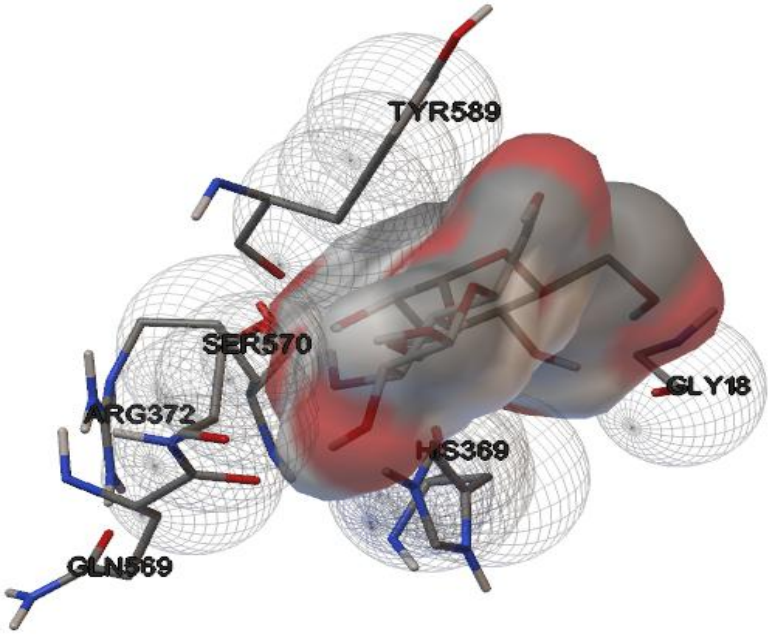


Fig.-29 Image of maltose docked with glucanotransferase from *Pyrococcus furiosus*.

3.7.10 Docking of Malto-triose at the putative acceptor site:

Fig.-30 shows docking of malto-triose at the putative acceptor site. The estimated binding energy was observed to be -5.24 kJ/mol.

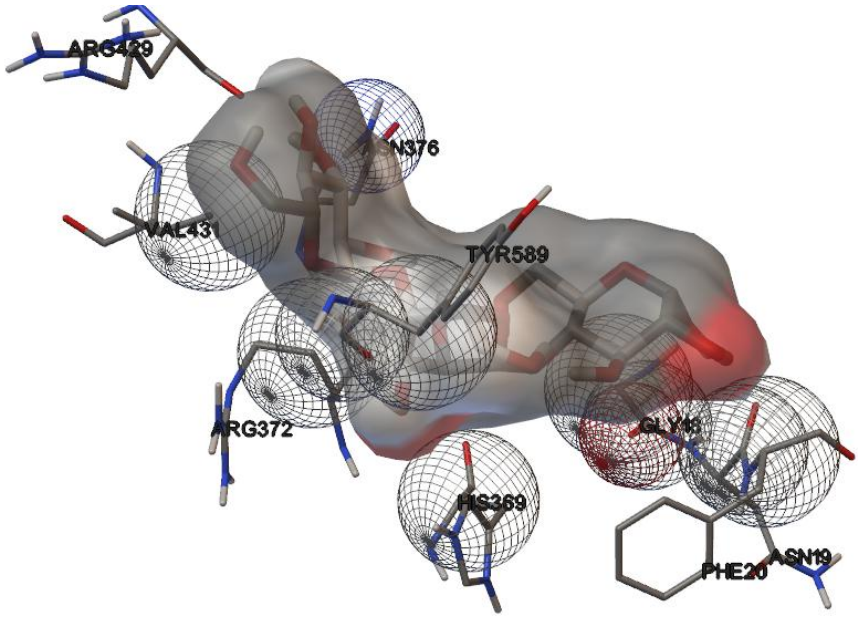


Fig.-30 Image of malto-triose interaction with glucanotransferase from *Pyrococcus furiosus*.

Malto-triose is also able to bind to Histidine-369 and Arginine-372, suggesting that these two are in-fact the residues, may be along with other residues, involved for accepting the carbohydrate molecule coming from the donor molecule.

3.7.11 Docking of Malto-tetraose at the putative acceptor site:

Fig.-31 shows the docking of malto-tetraose at the putative acceptor site of glucanotransferase. The estimated binding energy was found to be -5.56 kJ/mol. Malto-tetraose was also seen binding to binding to Histidine-369 and Arginine-372 along with some other residues.

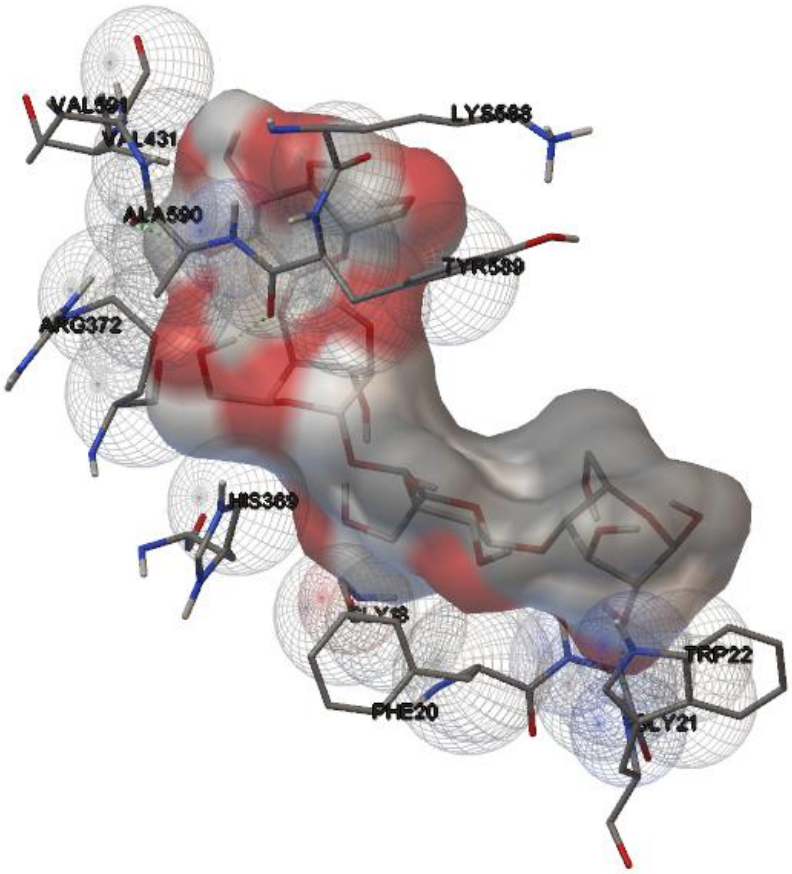


Fig.-31 Image of malto-tetraose docked with glucanotransferase from *Pyrococcus furiosus*.

3.7.12 Docking of Acarbose at the putative acceptor site:

Since acarbose is a structural analog of malto-tetraose, therefore, the binding energy at the putative acceptor site should almost be same as of malto-tetraose. The binding energy was found to be -5.60 kJ/mol. Fig.-32 shows docking of acarbose at the putative acceptor site of glucanotransferase.

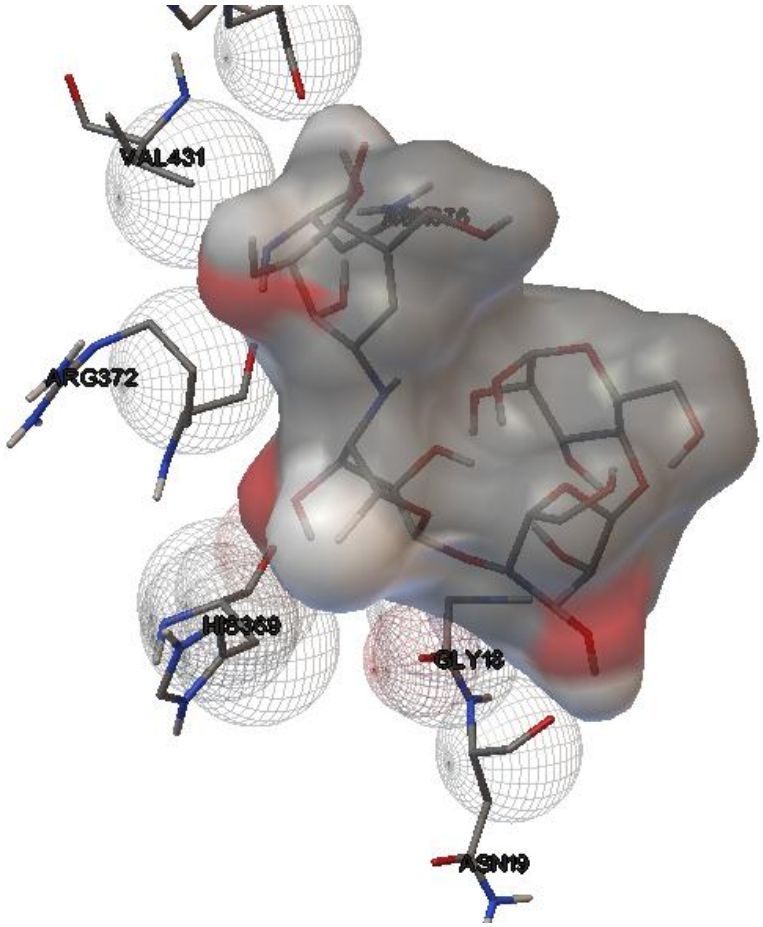


Fig.-32 Image of acarbose docked with glucanotransferase from *Pyrococcus furiosus*.

Acarbose is also seen interacting with Histidine-369 and Arginine-372.

The summarized results of molecular docking at the putative acceptor site are shown in Fig.-33.

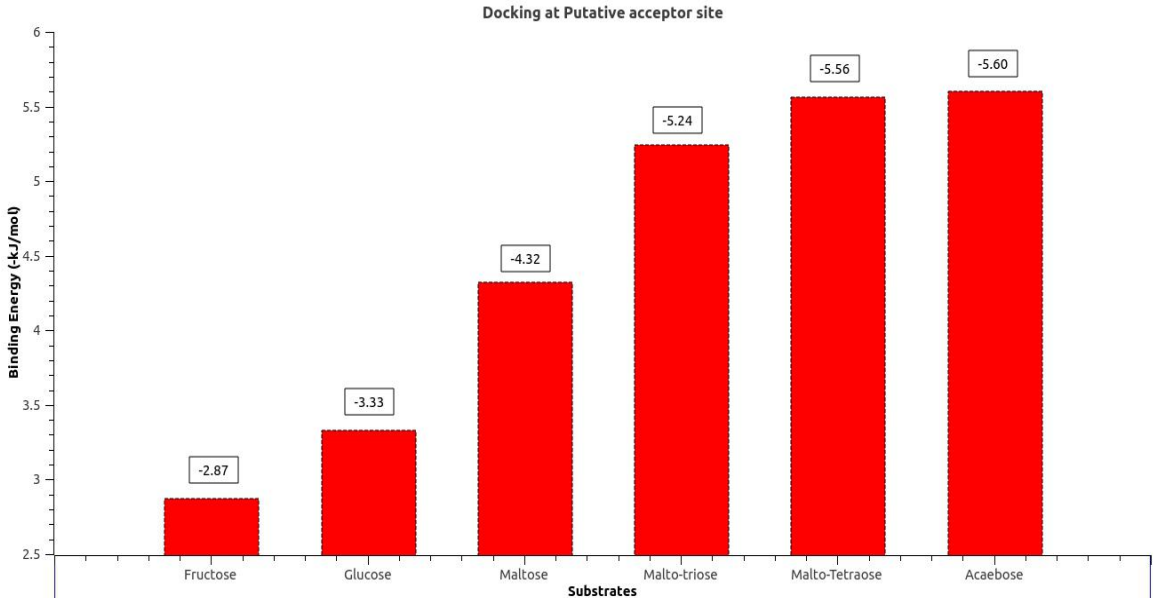


Fig.-33 Molecular Docking analysis of various substrates at the putative acceptor site of glucanotransferase from *Pyrococcus furiosus*.

3.8 Discussion:

Results from molecular docking at the donor/active site indicates that may be malto-triose and higher forms of carbohydrates are preferred substrates for glucanotransferase as their binding energy was much lower as compared to fructose, glucose and maltose. On the other hand, not much difference was seen from docking at the putative acceptor site among various substrates leading to a possibility that may be all the carbohydrates can be used as an acceptor for the the carbohydrate unfit coming from the donor molecule, resulting in the formation of higher carbohydrate species. It can also be reflected from the fact that the binding pockets at the donor and the putative acceptor site are different.

At the donor site, the binding pocket looks like a tunnel, in which the catalytic residues specifies their position. Presence of a tunnel reflects the specificity of substrates at the donor site as well as to the fact that once the substrate has entered the tunnel, it cannot leave easily, leading to the efficiency of the enzyme.

At the putative acceptor site, instead of a tunnel, there is a groove, indicating that the diversity in binding of acceptor molecules is much higher here. One possible reason could be that since the acceptor site is more exposed to solvent, the affinity of substrates would be less as compared to the affinity of the substrates at the donor site. Also, it suggests that when more than one acceptor molecule is present, each of them has equal probability to bind at the putative acceptor site and accept the carbohydrate unit coming from the donor molecule.

3.9 Proposed mechanism of action:

There are two sites in the enzyme, a donor site and an acceptor site. The donor site is like a tunnel and the acceptor site is in the shape of a groove (Fig.-34).

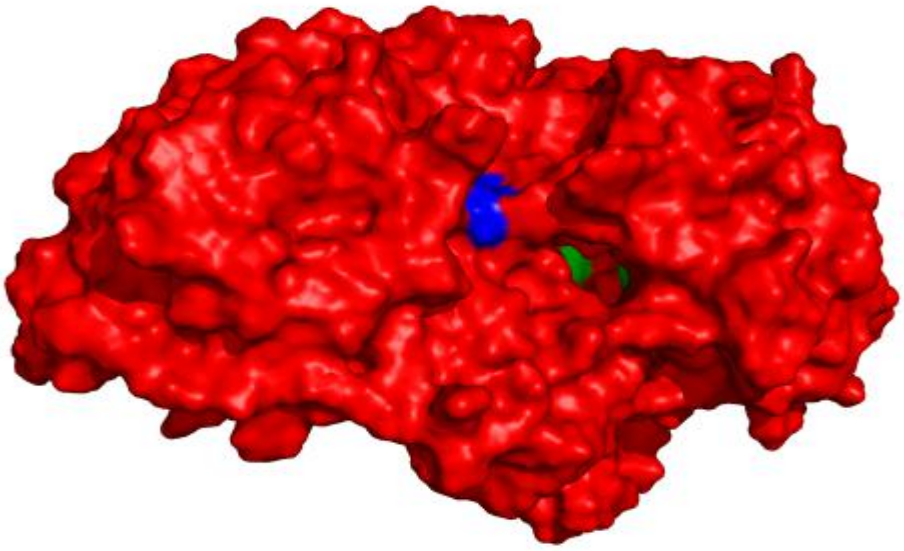


Fig.-34 Glucanotransferase from *Pyrococcus furiosus*. The residues shown in green color are the catalytic residues at the donor site, embedded in a tunnel and the residues shown in blue color are the residues at the acceptor site, at the groove.

The substrate molecule/donor molecule enters the tunnel and binds to the catalytic residues Glutamate-124 and Aspartate-215. The activity of these residues results in the hydrolysis of one glucose unit from the donor, to be accepted by an acceptor carbohydrate molecule present at the acceptor site. The acceptor molecule, having *n* number of glucose units, now becomes *n+1* in number of glucose units. This process is continued till the donor molecule is completely hydrolyzed or becomes too small to be fit into the tunnel.

Between the donor and acceptor site, there happens to be a loop which could be assisting in the transfer of glucose unit from the donor to the acceptor molecule (Fig.-35) as has been demonstrated by Qasba PK et al. in 2005 in glycosyltransferases family enzymes. Similar kind of loop and residues have been identified in glucanotransferases from *Pyrococcus furiosus* (Fig.-36). Mutational studies needs to be carried out to confirm the donor and the putative acceptor sites as well as the transfer of glucose by the loop as predicted by this study.

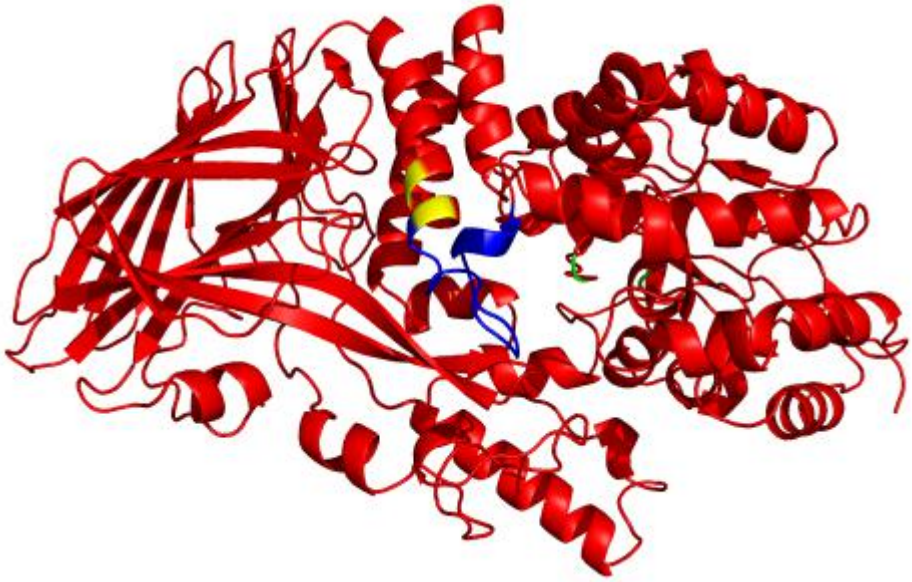


Fig.-35 Glucanotransferase from *Pyrococcus furiosus*. The catalytic residues at donor site are shown in green color, residues at acceptor site are shown in yellow color and the loop is shown in blue color.

```
                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Pfu                                                                GIWKNFFYKYPESNYMHKRMLMYSKLYRNNPEARKYLLRAQCNDAYWHG----LFGGVYLP------HLRRAIWNNLIKANSYV
sp|P04547|GSTB_BPT4  MKIAIINMGNNVINFKTVPSSETIYLFKVISEMGLNVDIISLKNGVYTKSFDEVDVNDY-DRLIVVNSSINFFGGKPNLAILSAQKFMAKYKSKIYYLFTDIRLPFSQSWPNVKNRPWAYLYTEEELLIK
Consensus       ..........................................G!wknfFdeyden#Y.drrilmnSkinrnngearlaiLrAQcndAkwhg....LFgd!rLP......nlrraiWanLika#ell..

                131     140       150       160       170       180       190       200       210       220       230       240       250       260
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Pfu
sp|P04547|GSTB_BPT4  SPIKVISQGINLDIAKAAHKKVDNVIEFEYFPIEQYKIHMNDFQLSKPTKKTLDVIYGGSFRSGQRESKMVEFLFDTGLNIEFFGNAREKQFKNPKYPWTKAPVFTGKIPMNMVSEKNSQAIAALIIGDK
Consensus       ..........................................................................................................................................

                261     270       280       290       300       310       320       330       340       35052
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+-|
Pfu
sp|P04547|GSTB_BPT4  NYNDNFITLRVWETMASDAYMLIDEEFDTKHRIINDARFYVNNRAELIDRYNELKHSDVLRKEMLSIQHDILNKTRAKKAEWQDAFKKAIDL
Consensus       ..........................................................................................
```

Fig.-36 Sequence alignment between *Pyrococcus furiosus* glucanotransferase and T4 β-glycosyltransferase.

Chapter-4

CONCLUSION

4. Conclusion

4-α-Glucanotransferase (EC 2.4.1.25) from *Pyrococcus furiosus*, is an enzyme that catalyzes the transfer of a glucose unit from a donor to an acceptor molecule. We have predicted that the sites for donor and acceptor molecules are within the enzyme only. We have also proposed a mechanism of transfer of glucose from the donor site to the acceptor site involving a loop based on the studies carried out by Qasba PK et al. and our sequence alignment results. The present study has opened possibilities for many experimental analysis to be done to the enzyme which will be useful for all Glucanotransferases as well.

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