Carbohydrates constitute a large share of all the macromolecules found in nature. The study of their structure, function, and synthesis has increased in importance greatly in recent times, in the fields of both food and health. In the food industry, modified carbohydrates are in the limelight. In the health sector, the purpose, function, and patterns of glycosylation of proteins are gaining importance. Modifications of carbohydrates are mainly carried out by carbohydrate-modifying enzymes. Such enzymes have recently been classified and categorized on the basis of their similarities of sequence, in the CAZy database, and on the basis of their functions in glycosidic bond cleavage, bond formation, debranching functions, isomerization functions etc. The product of each of these enzyme functions is essential at some stage of carbohydrate metabolism and, consequently, in the industry related to carbohydrates. This had led to the search for enzymes with new specificities, and features, involving the formation of unique products, or versatile enzymes featuring multiple specificities or catalytic functions. In particular, there is a focus on enzymatic functions that include both the breaking as well as synthesizing of glycosidic bonds, as glycosyl hydrolases and as glycosyltransferases, respectively. Enzymes that perform these functions take part in various metabolic pathways, and are useful in the industry: in the generation of sweeteners, novel dietary carbohydrates, etc., cosmetic industry: as thickening agents, detergent industry, textile industry and with the recent emphasis on generation and use of clean energy such enzymes find a huge role in production of biofuels.

Glycosyl Hydrolases are enzymes that hydrolyze/break a glycosidic bond to generate products smaller in size than the initial substrate. They include broadly endo-acting enzymes as well as exo-acting enzymes to create a variety of oligosaccharide species. Glycosyltransferases are enzymes that transfer sugars/glucans from donor to acceptor molecules to produce oligosaccharides of varying lengths. The choice of donor and acceptor molecules and the degree of polymerization of the products formed depends upon the source of the enzyme i.e. the organism from which it is derived and the role evolution has played in its maturation.

The glycosyltransferases of higher organisms are broadly classified into Leloir and non-Leloir enzymes. The Leloir enzymes use glycosyl esters of nucleoside mono or diphosphates as donor molecules. Whereas, non-Leloir enzymes use glycosyl phosphates as donors molecules. Glycosyltransferases from lower order organisms are however classified on the basis of the smallest donor and acceptor molecules they utilize (since they mainly utilize glucose based saccharides).

The Archaea was categorized as the third domain of life in the late 1970s. The use of archaeal enzymes in different processes has many advantages when used in large-scale industrial processes. Since most archaea are found in habitats characterized by harsh physical and chemical conditions, enzymes isolated from them are found to be able to function at high temperatures and highly acidic/alkaline pH conditions, and they are found to contain stable secondary, tertiary and quaternary structures. These properties make archaeal enzymes highly suitable candidates for various industrial processes. Most enzymes of archaeal origin are also promiscuous in nature, i.e., they are less specific and can work with a wider range of chemically-similar substrates. This property can be utilized by incorporating protein engineering techniques and creating modifications in order to exploit their full potential.

Of the carbohydrate-modifying enzymes, glucanotransferases are enzymes which cleave a glycosidic bond from a donor molecule to release a small sugar (which can either be a monosaccharide or a disaccharide) which is then transferred to another carbohydrate or non-carbohydrate entity, to yield products of varying lengths and chemical structures.

The present thesis deals with an enzyme from Pyrococcus furiosus which was characterized as an α-amylase in 1993, but which was re-classified as a 4-α-Glucanotransferase in 2005. Since then, the enzyme has been referred to in the literature as a 4-α-Glucanotransferase, and there is no discussion of its possible amylase function. We refer to this enzyme as PfuAmyGT, keeping in mind its organism of origin (Pfu), and its possible functions as an amylase (Amy) and a glucanotransferase (GT). To better understand the action of hyperthermophilic 4-α-Glucanotransferases, we also cloned a related enzyme from another organism Thermococcus onnurineus, which we refer to as TonAmyGT. Both of these enzymes were studied biophysically to assess their: (1) Secondary structure, using Circular Dichroism (CD) Spectroscopy, (2) Tertiary structure, using Fluorescence Spectroscopy, (3) Quaternary structure, using Gel filtration chromatography and Dynamic Light Scattering, (4) Thermal stability, using CD Spectroscopy and Differential Scanning Calorimetry, and (5) Chemical stability, using CD Spectroscopy. They were also studied biochemically and in terms of their activity as an amylase and as a glucanotransferase by (6) the Starch-iodide method to establish amylase activity, (7) Zymography to study amylase activity, and (8) Thin Layer Chromatography (TLC) to study both amylase and transferase activity. In addition, these proteins were also studied: (9) Bioinformatically, for comparison with 4-α-Glucanotransferases whose crystal structures are available, and to build reasonable hypotheses regarding their mechanisms of function, in keeping with all the experimental data from the biochemical and other studies.

With PfuAmyGT, we found it to possess an exo-amylase activity when starch was used as a substrate which appeared to be enhanced in the presence of maltose. The exo-amylase activity which is observed at 90 °C with starch, could be observed at ambient temperatures too when maltose was present along with starch. These results were further confirmed by zymography. In TLC experiments, we observed the formation of varying lengths of oligosaccharides by the action of PfuAmyGT on starch. The intensities of these oligosaccharides increased when maltose was added to the reaction mixture. The formation of oligosaccharides can only be Explained when PfuAmyGT is considered to be an amylase-cum-glucanotransferase, which uses maltose as a sink for the addition of glucose units derived through exo-amylase action upon starch. Thus we established that PfuAmyGT contains both exo-amylase and transferase activities. As a glucanotransferase, we established the smallest donor to be maltotriose, the smallest acceptor to be glucose, and the smallest transferred unit to be glucose. These features cause us to categorize PfuAmyGT as a new type of glucanotransferase, rather than as a known type of glucanotransferase.

Bioinformatic analyses based on a sequence-homology-based prediction of the structure revealed that the structure of PfuAmyGT contains three domains:

Domain 1 (D1): a (β/α) 7 barrel domain housing the catalytic site for glycosidic bond cleavage.

Domain 2 (D2): a small segment made of α helix and loops belonging to the Domain of Unknown Function (DUF) 1925.

Domain 3 (D3): a β-sandwich fold belonging to the Domain of Unknown

Function (DUF) 1925.
In order to understand the function of each domain in PfuAmyGT and also gain insights into its mode of action, we individually cloned each domain as well as in combinations, but could not observe any activity. On comparison of the predicted structure of PfuAmyGT with a 4-α-Glucanotransferase from Thermococcus litoralis (whose crystal structure is available), we identified the putative donor and acceptor sites in PfuAmyGT and also a tryptophan bearing loop which could possibly act to transfer the excised glucose unit from donor to acceptor molecule. To test this hypothesis, we created three point mutations in the loop region, one residue is likely to participate in a catalytic action upon the donor glucan, and other two are likely to be involved in the transfer of the excised glucan. We observed loss of transferase activity on TLC in case of the mutation (to alanine) of a loop residue suspected to participate in glucoside bond hydrolysis (involving an aspartate residue), and also in one case of mutation of a residue suspected to transfer the glucan (a tryptophan). Therefore, we established that the tryptophan is an essential residue taking part in the transferase action of PfuAmyGT.
With TonAmyGT, we cloned, expressed and purified the protein to determine its structure, oligomeric status, thermal and chemical stability. Its activity was also examined using TLC. Briefly, in comparison with PfuAmyGT, we found that it uses maltose as the smallest donor as well as the smallest acceptor glucan, although this enzyme also uses glucose as the smallest transferred glucan.
We believe that these studies of two amylases-cum-glucanotransferases provide new mechanistic insights, and applications, to the field of carbohydrate modifying enzymes.