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Evolution and Adaptation of Enzymes' Active Sites: Enzymes Use Functionally Similar Active Site for Same Type of Reactions with Structurally Similar Substrates Irrespective of the Organism

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Abstract

Enzymes have played a crucial role in the evolution of life on the planet Earth, as they could accelerate thermodynamically favourable reactions approximately a million times faster. The evolution of enzymes with their unique 3D structures and specific active sites for each reaction is still a mystery. Interestingly, now it is found that two different transferases from two different metabolic pathways use the same active site amino acids, suggesting that active sites of enzymes are designed based on the specific molecular interactions between the active site amino acids and their substrate molecules. The present data further reveal that not only the active site is designed for a particular biological reaction during evolution, but also adapted and conserved in all life forms, from viruses to humans. This is based on the fact that just two-point mutations, viz. S^{356} and Q^{363}, instead of the conserved Phe^{356} and a positively charged residue K^{363}, respectively, rendered the enzyme, fructosyl transferase from *Geobacillus stearothermophilus* inactive. The importance of these two amino acids in fructosyl transferases was further confirmed from analysis of hundreds of fructosyl transferase sequences from levan- and inulosucrases, and other glycoside hydrolase GH68 family of enzymes from a large number of organisms. The fructosyl transferases are bifunctional enzymes and function both as a hydrolase and as a transferase. The hydrolase uses a catalytic triad in its active site, viz. 2 Asp residues where one acts as the nucleophile and the other acts as the transition-state stabilizer and a Glu residue that acts as a general acid-base catalyst. Similarly, the transferase also uses a catalytic triad, viz. a positively charged amino acid residue, K/R/H, as the proton abstractor and an R at -3 from the proton abstractor as the donor selection amino acid and an Y or a branched chain amino acid with a G as the template-binding pair. It is interesting to note that the same catalytic triad is observed in the active sites of nucleotidyl transferases like DNA/RNA polymerases [1-3]. For example, the nucleotidyl transferase (*E. coli* DNA polymerase I) and the fructosyl transferase (*Bacillus subtilis* levansucrase) possess more or less the same active site structure, (-**R**$^{-4}$RSA**K**^{758}AINFGLI**Y**^{766}**G**M- and -D^{358}S**R**$^{-3}$GS**K**^{363}MTIDGITSNDIYM**L**^{377}**G**Y-, respectively) where K^{758} in the *E. coli* enzyme is shown to be the proton abstractor, the R at -4 as the nucleotide selection amino acid and the –YG- as the template–binding pair. Furthermore, extensive analysis of different DNA/RNA polymerases and fructosyl transferases from a large number organisms, from viruses to humans, revealed the same active site structure [1-3]. These data confirm that during evolution, not only similar active site is designed and used for the same types of reactions, but also conserved and adapted to all forms of life, from viruses to humans.

Keywords: Evolution of Life; Evolution of Enzyme Active Sites; Fructans; Fructosyl Transferases; Hydrolase Active Site; Fructosyl Transferase Active Site; Nucleotidyl Transferases; Nucleotidyl Transferase Active Site; Mechanisms of the Fructosyl and Nucleotidyl Transferases

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1. Introduction

Understanding the origin of life is still a complex and a completely unsolved problem in biology. However, laboratory experiments suggest that life might have evolved in the early Earth by a series of progressive and spontaneous chemical reactions under the prevailing environmental conditions of the prebiotic era. The first phase of evolution, viz. the chemical evolution, actually set the very early stage for the development of life by the formation of simple organic molecules such as amino acids, simple sugars, fatty acids, nucleic acid bases, etc. from inorganic molecules. Such spontaneous formation of organic molecules from inorganic molecules is proved by groundbreaking experiments conducted by Urey [4] and Miller [5] in the 1950s where they simulated the conditions of early Earth's atmosphere and studied the formation of organic molecules. Miller and Urey [6] demonstrated that organic molecules such as amino acids could be spontaneously formed from inorganic compounds when an electrical arc (simulating lightning) was passed through a mixture of gases. Their seminal experiments suggested that electrical discharges might have played a significant role in the formation of organic compounds from a mixture of gases present in the primitive atmosphere.

Their findings of spontaneous formation of life's molecules in the primitive atmosphere were further confirmed by Criado-Reyes *et al*. [7]. They recently repeated the familiar Urey-Miller experiment, conducted about 70 years ago, but in a borosilicate vessel. Interestingly, they found a large number of additional compounds such as dipeptides, multi-carbon dicarboxylic acids, polycyclic aromatic hydrocarbon compounds (PAHs) and a complete panel of biological nucleobases in their reaction mixture. Furthermore, the natural formation of small peptides and oligonucleotides in the presence of inorganic catalysts like hydroxyapatite, clay, zeolites, etc. further advanced our knowledge on the evolution of life [8]. These experiments strongly support a widely accepted theory of "spontaneous generation," suggesting that chemical reactions could just start spontaneously when the reacting substances are kept in close proximity to each other. This theory of 'spontaneous formation' of life's molecules is further supported by a recent work by Mohajer *et al*. [9]. They have shown the spontaneous formation of urea, the simplest organic molecule and a key compound that connects chemistry and life and is also considered as a key molecule in the search for the origin of life. Its formation from ammonia and carbon dioxide requires either high pressures and temperatures, or, under milder conditions with catalysts or additional reagents. In contrast, Mohajer *et al*. [9] have demonstrated that the spontaneous formation of urea under ambient conditions (i.e., non-energetic conditions and without additional catalysts) from ammonia and carbon dioxide in the surface layer of aqueous droplets. Their experiment further corroborates that the evolutionary processes are spontaneous.

Further, additional advanced experiments on evolution have demonstrated that replicating autocatalytic RNA molecules could undergo spontaneous changes and that the variants of RNA molecules with the greatest autocatalytic activities prevailed in the prebiotic life [10]. Their results support the basis of the "RNA world" hypothesis. In support of this hypothesis, a recent study has shown that RNAs are not only autocatalytic, but also could help amino acids to assemble into peptides without a pre-existing protein machinery during evolution [10]. However, the "Metabolism First" camp argues that self-sustaining chemical networks should have evolved first for the self-replicating genetic molecules like RNA to use them. The 'Metabolism First' model suggests that life emerged as a result of simple chemical reactions, governed by the laws of chemistry and thermodynamics, rather than through improbable chance events.

The next crucial phase in the origin of life is the formation of 'protocells' that are enveloped with primitive type membrane structures, where the organic compounds that are formed are compartmentalized and the compartmentalized organic compounds with self-organization potential led to the formation of lower molecular weight compounds like simple oligopeptides, oligonucleotides, etc. Thus, they bridge chemistry and biology by establishing primitive metabolic activities inside them. The protocells later evolved into 'primitive cell' types. The primitive cells may be considered as the first living cells capable of basic metabolism and reproductive capabilities. Interestingly, small peptides, oligonucleotides that are assembled on inorganic matrixes like clay, silica, zeolites, etc., and other small molecules, nutrients, ions, etc. could also have diffused freely into the primitive cells expanding their metabolic capabilities [11]. It is also likely that at different prevailing environmental conditions on the planet Earth different types of protocells and primitive cells would have formed, giving rise to diversities. In the next phase, the glycan-based polymers would have formed a protective layer on the fragile cell membranes, leading to the formation of complete primitive cell types. The two crucial components of basic life processes, viz. enzymes (which are responsible for all the cellular reactions) and genetic materials (which carry the genetic information to the next generation) might have evolved much later.

Primitive types of catalytic activities in the primitive cells would have started with metal ions and minerals like clay, zeolites, etc. As each enzyme's active site is specific for its substrate, it would have formed after the binding of active site amino acids on to their respective existing substrates, followed by filling of the gaps between the active site amino acids with other amino acids and likely not *vice versa*. This is evidenced by the distance conservation between the active

site amino acids in enzymes. Then the enzymes could have formed their respective RNA molecules where three nucleobases (as a codon) recognize each of the amino acid and such codons are connected in the same order into a short RNA molecule representing that particular enzyme, which would later code for that enzyme. An intuitive addition of a Met codon at the start and a stop codon at the end make the translation events unidirectional. And, many such protein coding RNA molecules could have further assembled into a larger RNA molecule forming an RNA genome. The RNA genome, thus, carries all the molecular instructions of the enzymes in a cell. And, when the deoxyribonucleosides were available, the molecular instructions on the RNA molecule could have transferred to the more stable DNA molecule by complementary base-pairing. In the reverse direction, such DNA can give rise to RNA molecules (transcription) and the RNA molecules can give rise to enzymes (translation) which function in the cells as originally designed by nature. Therefore, it is tempting to speculate that the life's molecules could have evolved spontaneously, self-assembled and organized into primitive cell types, initiating all cellular reactions. The mutation and selection, addition(s) and deletion(s), are natural and progressive steps in the evolutionary process. As discussed elsewhere, enzymes have evolved over billions of years through natural selection, which act primarily at the level of biological function, and diverse types of enzymes are constantly evolved from mutation and selection, divergence of existing genes and recombination of domains. Further, adaptation to different new environments could also play a key role in further evolution of new enzymes with new biological functions. Besides, changes in the available chemicals, ions, minerals and environmental conditions could also favour further evolution of enzymes with new functions that are beneficial to the organisms.

In this communication, evidences are presented to show how two different unconnected enzymatic reactions, one in the carbohydrate metabolism and the other in the nucleic acid metabolism, use the same active site amino acids for polymerization reactions, which strongly supports the chemical basis of evolution enzymes and their active sites. Both the enzymes are classified under the Main class, 'Transferases', but one is a fructofuranosyl transferase and the other is a nucleotidyl transferase. The nucleotidyl transferases play the key role in DNA/RNA polymerases to polymerize the nucleobases into a DNA or an RNA. It is interesting to note that the requirement for such specialized nucleotidyl transferases to accurately replicate the genetic material is universal in all kingdoms of life and hence, they are also known as crucial 'life catalysts'. The properties, multiple sequence alignment (MSA) analysis and the mechanism of actions of DNA and RNA polymerases from viruses, plants to humans have been extensively analyzed and reported by the author [1-3]. Therefore, in this communication evidences are provided to support how these two different groups of enzymes use and have adapted the same active site amino acid structure to perform similar functions in all organisms.

1.1. Fructans and their Applications

Inulins, levans and fructooligosaccharides are collectively known as fructans. They mainly differ in their linkages and degree of polymerization (DP). The inulins and levans exhibit chain lengths of >20 DPs and the fructooligosaccharides are of only <20 DPs. Demand and interest for fructans have increased over the years, due to their versatile applications and with increased interest in healthy lifestyles as they exhibit many potential health benefits. In recent times, the global market for fructans has steadily increased, with an annual growth rate of >5%, and is expected to reach approximately 2.7 billion US dollars by 2026 [12]. The fructans are produced by a large number of organisms like bacteria, yeasts [13], higher fungi [14] and plants [15]. Bacterial levans (produced as exopolysaccharides, EPS) are much larger than those produced by plants and fungi with multiple branches and molecular weights ranging from 2 to 100 million Daltons [16]. Inulin-type fructans are mostly found in plants. The dicots accumulate them as long-term reserve carbohydrates, in underground storage organs such as roots and tubers and are mainly extracted from the plant sources like chicory, dahlia, yacon and Jerusalem artichoke as they accumulate up to 80% polysaccharides in their tubers [17-19]. In grasses, graminan (branched type fructans), levan, and neokestose-derived fructans mainly act as short-term storage compounds in stems, tiller bases, leaf sheaths, elongating leaf bases, and to a lesser extent in leaf blades and roots [17]. They find versatile applications in various industries and also in medicine. In industries, they are used as food thickeners, in cosmetics, as prebiotics, dietary fibres, mineral absorbing agents, anticancer agents, diabetic control agents, etc. Fructans exhibit many potential health benefits. As these fructans are non-digestible in our digestive system, they are considered as health-promoting 'prebiotics', i.e., they act as a food source for the beneficial gut bacteria. Therefore, they are gaining considerable attention the health industries as effective prebiotics to support the growth of beneficial gut bacteria, which play important roles in promoting digestive health, boosting the immune system, reducing the risk of chronic diseases, etc. [20-22]. Many bacteria, fungi and ~ 40,000 plant species use fructans as the primary carbohydrate reserves. Furthermore, it is found that in plants these fructans function as stabilizers of cell membranes and thus, confer tolerance to drought and frost. In plants, the fructan molecules are bound to the polar head groups of the lipid bilayer of the membrane to block water leakage during abiotic stress such as frost and drought [15, 23]. In addition to the above roles in industrial applications and as prebiotics, levan exhibits a wide range of specialized properties like film-forming ability, biodegradability, non-toxicity, self-aggregation, encapsulation, controlled release capacity, water retention, immunomodulatory activity, antimicrobial and anticancer activities, high biocompatibility

etc. With the strongest bio-adhesive properties and film-forming ability, levan could be used for healing wounds, burned tissues and for the development of bioresorbable electronic implants [24]. Furthermore, high DP fructans from bacteria are ideal substrates for the production of high fructose syrups (HFS), with very low glucose content. As levan is more soluble than inulin, it produces viscous solutions in water. In the industry, this property makes levan especially attractive as an emulsifier or encapsulating agent in a wide range of products, including biodegradable plastics, cosmetics, glues, textile coatings and detergents. These exceptional properties position levan as an attractive candidate for nature-based materials in food production, modern cosmetology, medicine, and pharmaceutical industries. Thus, with excellent medicinal properties and ease of production, microbial levans appear as a valuable and versatile biopolymer of the future.

1.2. Fructofuranosyl Transferases

The commonly used sugar, sucrose [α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] is a non-reducing disaccharide, where its two monosaccharide units, viz. glucose and fructose are linked by a glycosidic bond. Unlike most of the other disaccharides, as both the reducing ends of the monosaccharide units are involved in the glycosidic bond formation, it becomes non-reducing. Furthermore, in sucrose, the glucose is in its pyranose form and the fructose is in its furanose form. It is metabolized mainly by at least four types of enzymes, i.e., i) invertases (EC 3.2.1.26), which hydrolyze the sucrose to produce 1 mole each of glucose and fructose, ii) inulosucrases (E.C 2.4.1.9), which hydrolyze the sucrose to produce inulin (with a molecular mass of about 10 Da) and glucose units, iii) levansucrases (E.C 2.4.1.10), which hydrolyze the sucrose to produce levan (with a molecular mass up to $1x10^6$ Da) and glucose units and iv) dextransucrases (EC 2.4.1.5), which hydrolyze sucrose and produce dextran (with a molecular mass of about 1kDa to 2MDa) and fructose units. The chains of levan, inulin and dextran, like other biopolymers like starch, cellulose, and chitin grow stepwise by the repeated transfer of a hexosyl group (donor) to a growing acceptor molecule. These fructan-forming enzymes are commonly reported from bacteria, fungi and plants. Bacteria use the fructans and dextran as reserve EPS and utilize them by producing corresponding degradative enzymes like levanase, inulinase and dextranase when needed [25]. Among them, fructans are acid-labile, water-soluble, and polydisperse polymers of fructose built upon a sucrose starter unit. Between the two commonly occurring fructans, viz. inulin and levan, the inulosucrases polymerize fructosyl units into inulin which is mainly β-(2→1) linked, whereas the levansucrases, form levan from fructosyl units which is mainly β-(2→6) linked. Both belong to the Glycoside Hydrolase family GH68 (http://afmb.cnrs-mrs.fr/CAZY)) and specifically belong to the hexosyltransferases [25]. Levansucrases, in general, display a five-bladed β-propeller architecture, where the catalytic residues which are responsible for sucrose hydrolysis are located in a negatively charged cavity [20, 26]. The microbial fructan synthesis is more efficient than the plant systems because it is synthesized from sucrose by a single enzyme reaction and exhibits homogeneous structures with occasional branches. In contrast, plant fructans are shorter and exhibit diverse structures, which result from a combination of catalytic actions of various enzymes, including sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT), fructan:fructan 6G-fructosyltransferase (6G-FFT), and sucrose:fructan 6-fructosyltransferase (6-SFT). Fructosyltransferases (Fts) are also reported from fungi such as *Aspergillus*, *Penicillium*, *Aureobasidium*, *Kluyveromyces*, etc. Fungal Fts act on sucrose by cleaving the β-(2-1) linkage, releasing glucose, and then transferring the fructosyl units to an acceptor molecule. The fungal Ft from *Aspergillus* possesses both hydrolytic and transfructosylating activities as in other organisms. Interestingly, at sucrose concentrations >100 mM, the Fts exclusively exhibit a transfructosylation activity [27].

2. Materials and Methods

The protein sequence data of levansucrases, inulosucrases and other GH68 family of enzymes and DNA and RNA polymerases were obtained from PUBMED and SWISS-PROT databases. The advanced version of Clustal Omega was used for protein sequence analysis. Along with the conserved motifs identified by the bioinformatics analysis, and with the data already available from biochemical, site-directed mutagenesis (SDM) experiments and X-ray crystallographic analysis on these enzymes are used to confirm the possible amino acids at the active sites of these two different transferases, viz. fructofuranosyl and nucleotidyl transferases.

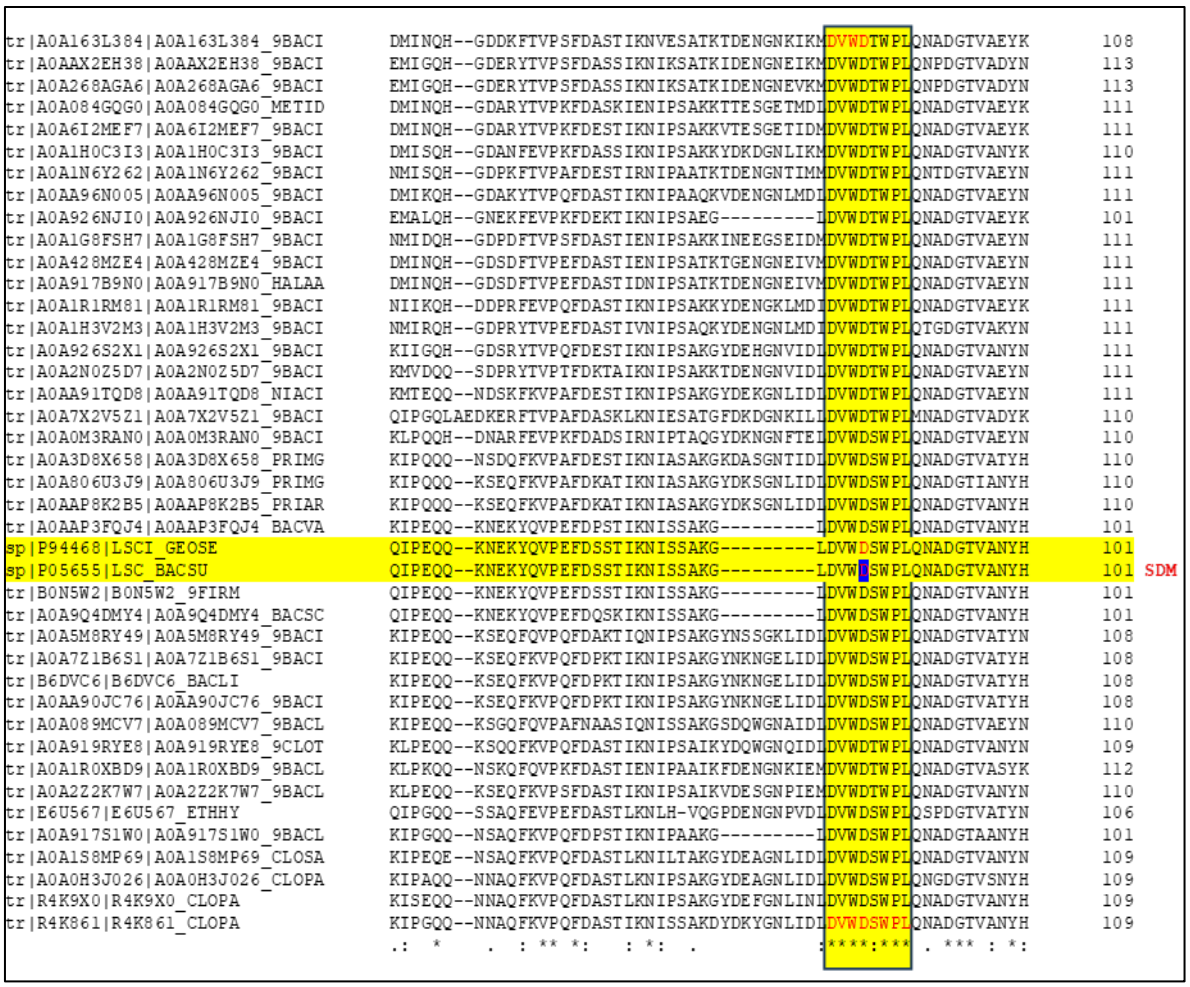
3. Results and Discussion

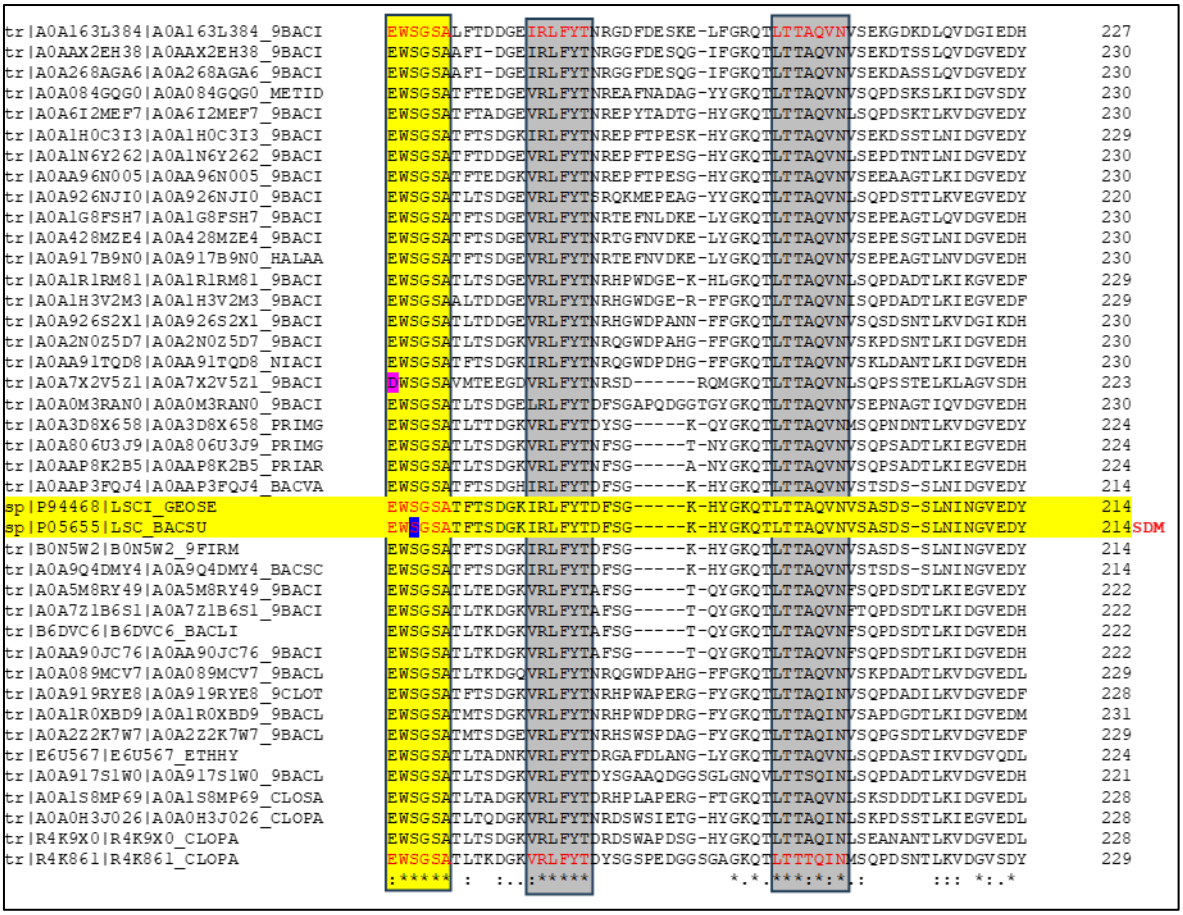
3.1. MSA analysis of levansucrases and other GH68 family glycoside hydrolases fructosyl transferases

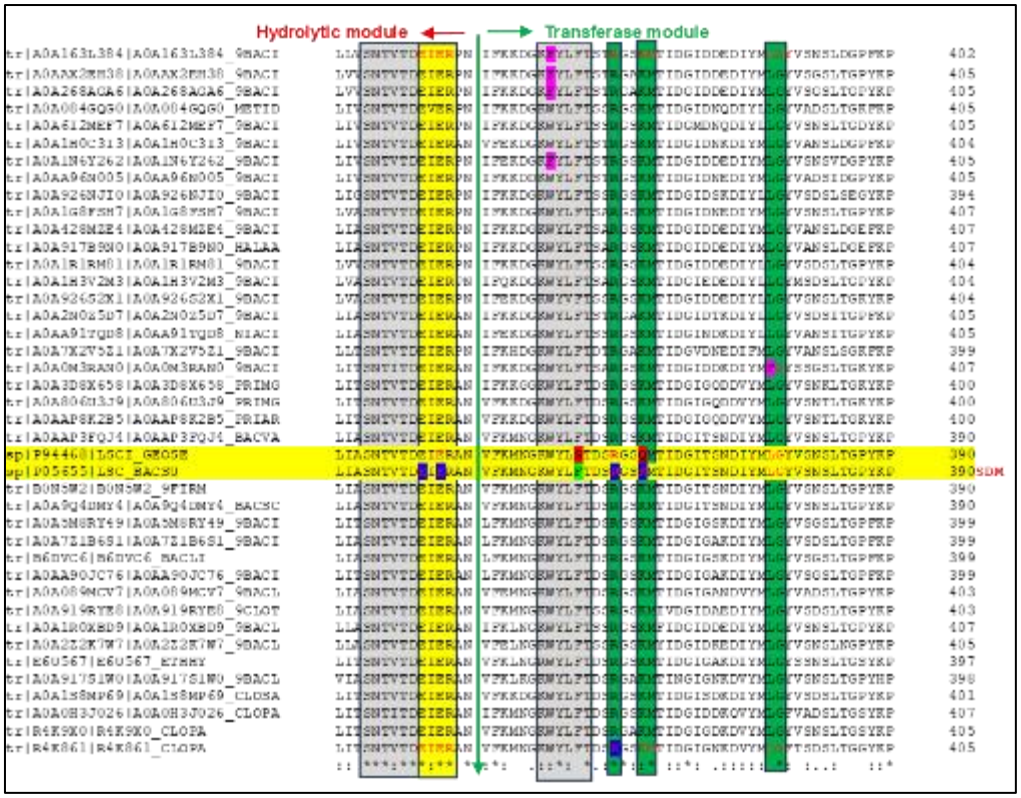
Figure 1 shows the MSA of levansucrases and GH68 family of fructosyl transferases from different bacteria, (only the required regions for the discussions are shown). The sources of active and inactive fructosyl transferase enzymes are highlighted in yellow. The green arrow mark tentatively separates the hydrolytic and transferase domains. As the fructosyl transferases are bifunctional enzymes, they exhibit both hydrolase and transferase activities and each activity

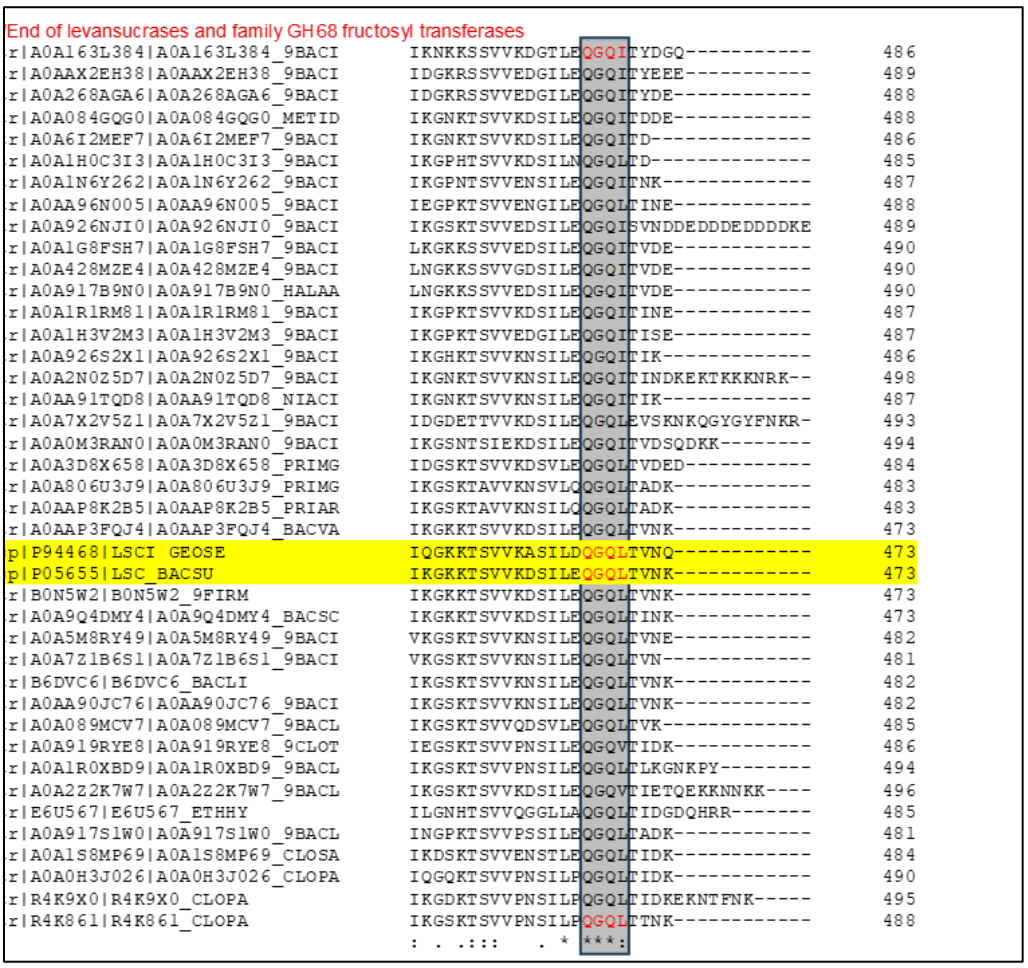
is composed of a catalytic triad of three invariant or highly conserved amino acids. Furthermore, they also possess two highly conserved sucrose-binding sites. The N-terminal region is not conserved among them except for a few amino acids. However, an octapeptide -DVW**D**S/TWPL-is completely conserved in these enzymes where the second D is shown to be the catalytic nucleophile [18]. The second conserved peptide -EW**S**GSA- is found around 160 amino acids from the N-terminal. The first S is known to play an important role in binding to the Glc unit of sucrose. By SDM, the levansucrase of *Bacillus subtilis* SacB (-EW**S**^{164}), where the conserved **S**^{164}→A, showed that this is an important residue in maintaining the nucleophile position in the active site, suggesting that the motif is possibly involved in sucrose-binding [28]. The third highly conserved region in these enzymes is a large peptide -DNHTL/M/FRDPHYVED- located around 250 amino acids from the N-terminal. The -RDP- triad is conserved in all and is shown to bind the Fru unit of sucrose. The D in this triad is also shown to play a role in stabilizing the reaction intermediate during catalysis [18]. The acid-base catalytic **E** is found around 340 amino acids from the N-terminal and located in the highly conserved tetrapeptide -EI**E**R- where the catalytic E is shown to act as the proton donor in catalysis [18]. The transferase module starts immediately after the hydrolytic module (indicated by a green arrow mark). The active site amino acids in the transferase module are highlighted in green. The catalytic site of transferases is also composed of a catalytic triad with an invariant basic amino acid K (proton abstractor), a template-binding pair -LG- (usually exists as a conserved triad –LGY/F) and an invariant amino acid R at -3, which is proposed in selecting incoming donor fructose units for polymerization. It is interesting to note that in the inactive levansucrase from *G. stearothermophilus*, the proton abstractor K is replaced by a Q (marked in red). Thus, this module is conserved in both fructosyl transferases and in nucleotidyl transferases. In the conserved pentapeptide -KW**Y**L*F*- the invariant Y is also implicated in the interaction with the fructose donor and the *F* is replaced by an *S* in the inactive form of the levansucrase from *G. stearothermophilus* (marked in red).

CLUSTAL O (1.2.4) MSA of levansucrases and other GH68 family glycoside hydrolase fructosyl transferases from bacteria






Hydrolytic module
Transferase module


End of levansucrases and family GH68 fructosyl transferases

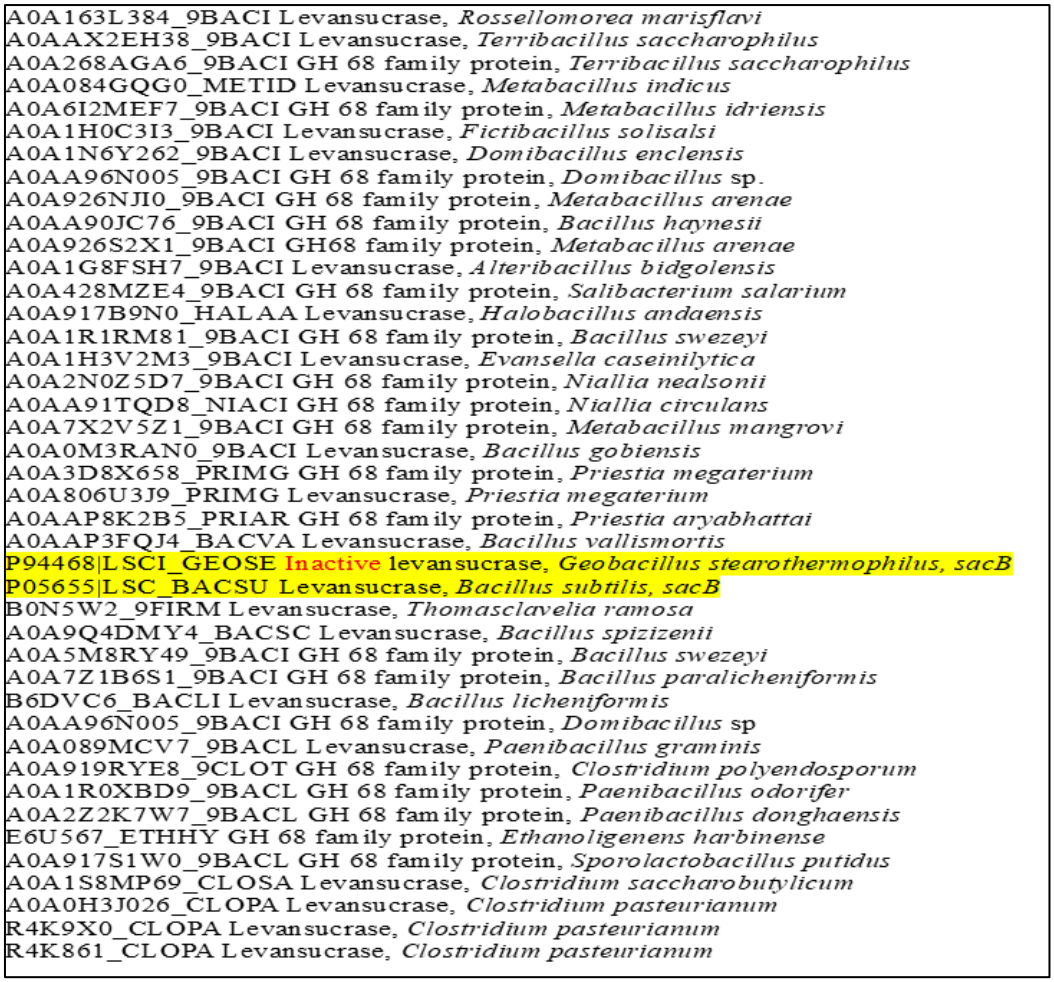
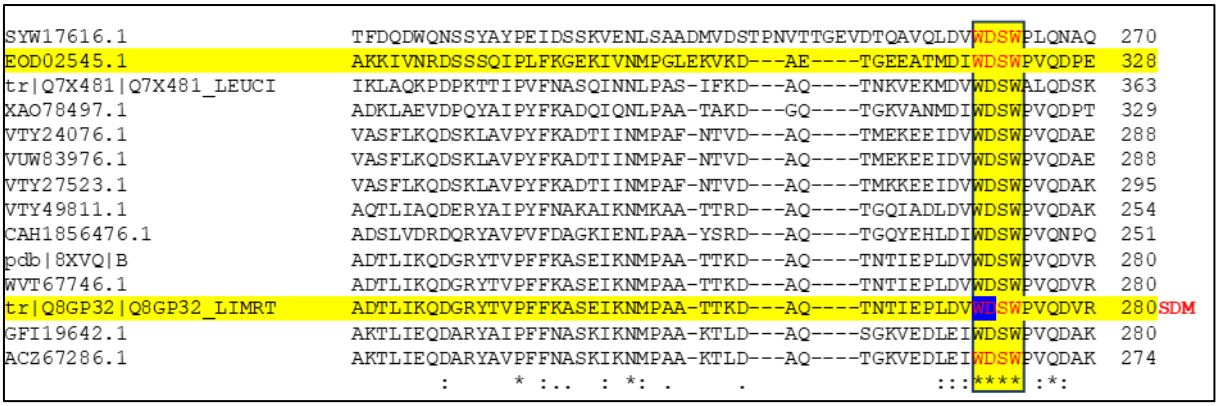
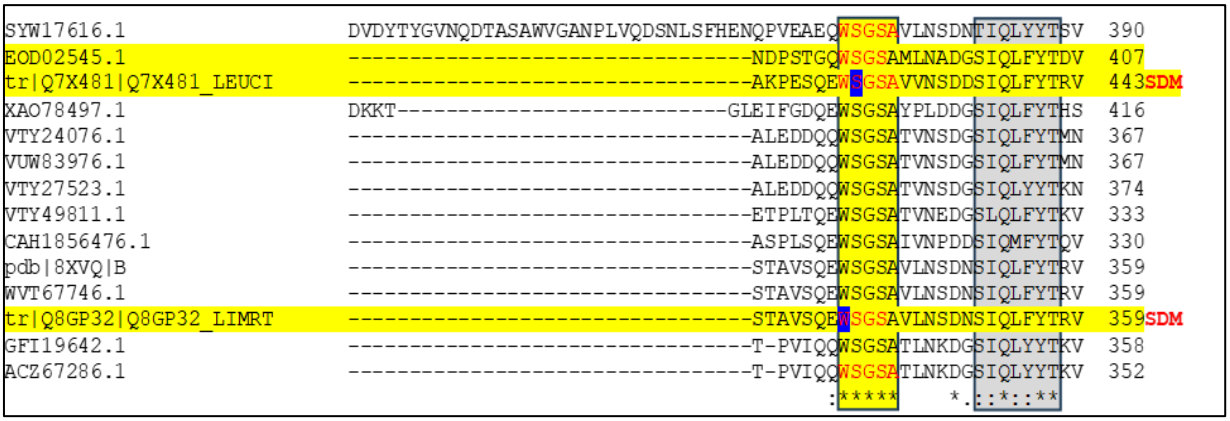


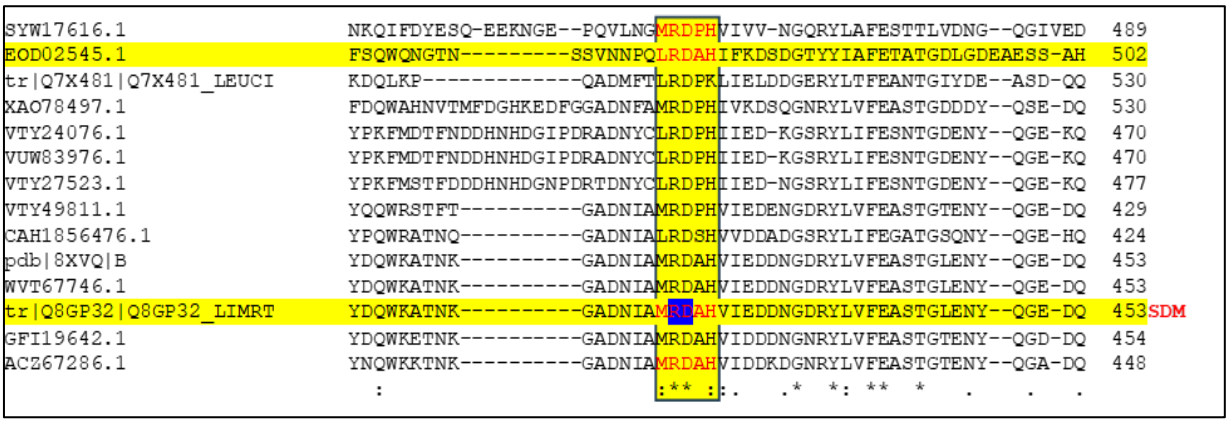
Figure 1 MSA of levansucrases and GH68 family glycoside hydrolase fructosyl transferases

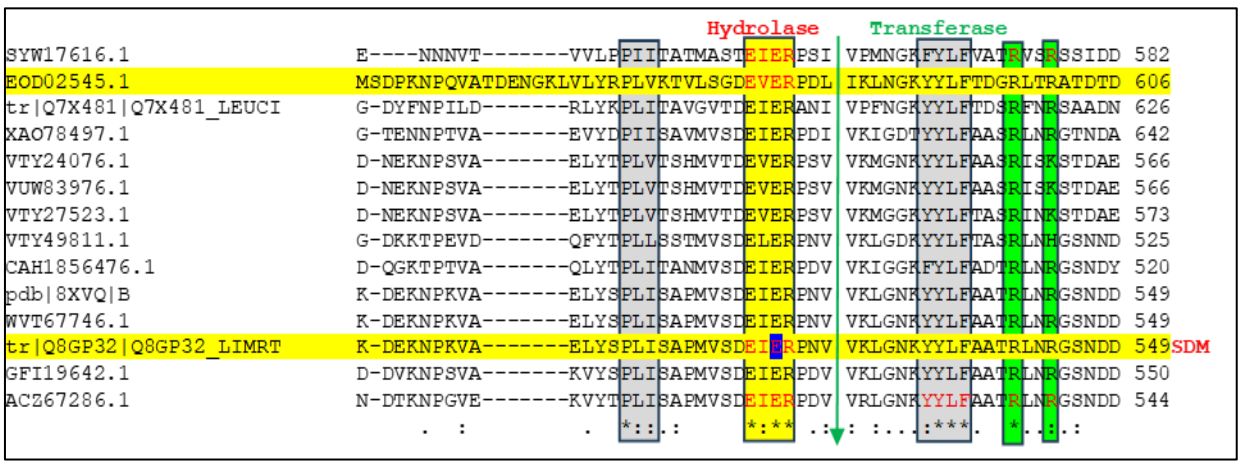
3.2. MSA analysis of inulosucrases

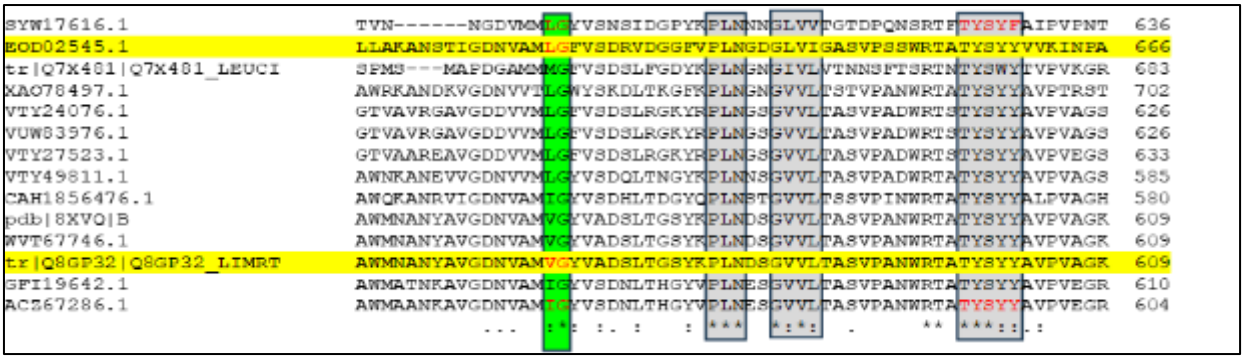
Figure 2 shows the MSA of inulosucrases from different bacteria, (only the required regions for the discussions are shown). These are also bifunctional enzymes like the levansucrases and exhibit two different activities, viz. hydrolytic and transferase activities. The active site amino acids of hydrolytic and transferase domains are highlighted in yellow and green, respectively. Like the levansucrases, the inulosucrases are also composed of the same catalytic triad. The N-terminal is not conserved among them until it reaches the first catalytic site amino acid, D (nucleophile). The catalytic triad is found in the completely conserved motifs, the nucleophilic amino acid D is found in -WDSW-. The proposed Glc-binding motif is the conserved, -WSGSA- motif. In the other two highly conserved motifs, one harbours the acid-base catalytic amino acid E, -EI/VER- and the other is the proposed Fru-binding motif, -M/LRDP/AH-, where the –RD- pair is found invariant in all and shown to involve in catalysis by SDM. As found in the levansucrases, the catalytic triad in the second transferase domain of inulosucrases is also highly conserved, viz. the basic amino acid, K/R for proton abstraction, and a completely conserved fructose donor selection amino acid, R at -3, and a template-binding pair, -MI//VLG- at -22 from the proton abstractor and usually exists as a conserved triad. Interestingly, the active site amino acids in the transferase domain are the same as the nucleotidyl transferase domain found in the DNA/ RNA polymerases [1-3]. Most of the inulosucrases' active site amino acids are also confirmed by SDM experiments and X-ray crystallographic data. For example, in *Lactobacillus reuteri* the catalytic triad is composed of D^{272}, E^{523} and D^{424} (highlighted) and also confirmed by SDM analysis. The other three completely conserved residues, viz. W^{271}, W^{340} and R^{423} (highlighted) are located at the -1 subsite. Inulosucrase mutants W^{340}→N and R^{423}→H were virtually inactive, confirming the essential role played by these residues in the inulosucrase active site [29, 30].








Hydrolase
Transferase



SYW17616.1 putative Inulosucrase, *Oenococcus oeni*
EOD02545.1 Inulosucrase InuJ, *Lactobacillus delbrueckii*
Q7X481_LEUCI Inulosucrase, *Leuconostoc citreum*
XAO78497.1 Inulosucrase, *Lactiplantibacillus plantarum*
VTY24076.1 Inulosucrase, *Streptococcus salivarius*
VUW83976.1 Inulosucrase, *Streptococcus thermophilus*
VTY27523.1 Inulosucrase, *Streptococcus salivarius*
VTY49811.1 Inulosucrase, *Streptococcus mutans*
CAH1856476.1 Inulosucrase, *Convivina intestini*
8XVQ|B Chain B, Inulosucrase
WVT67746.1 Sequence 14 from patent US 11859216
Q8GP32_LIMRT Inulosucrase, *Limosilactobacillus reuteri*
GFI19642.1 Inulosucrase, *Lactobacillus johnsonii*
ACZ67286.1 Inulosucrase, *Lactobacillus gasseri*

Figure 2 MSA of inulosucrases from different bacteria

3.3. 'Mix and Match' MSA analysis of levansucrases, inulosucrases and other Family GH68 fructosyl transferases

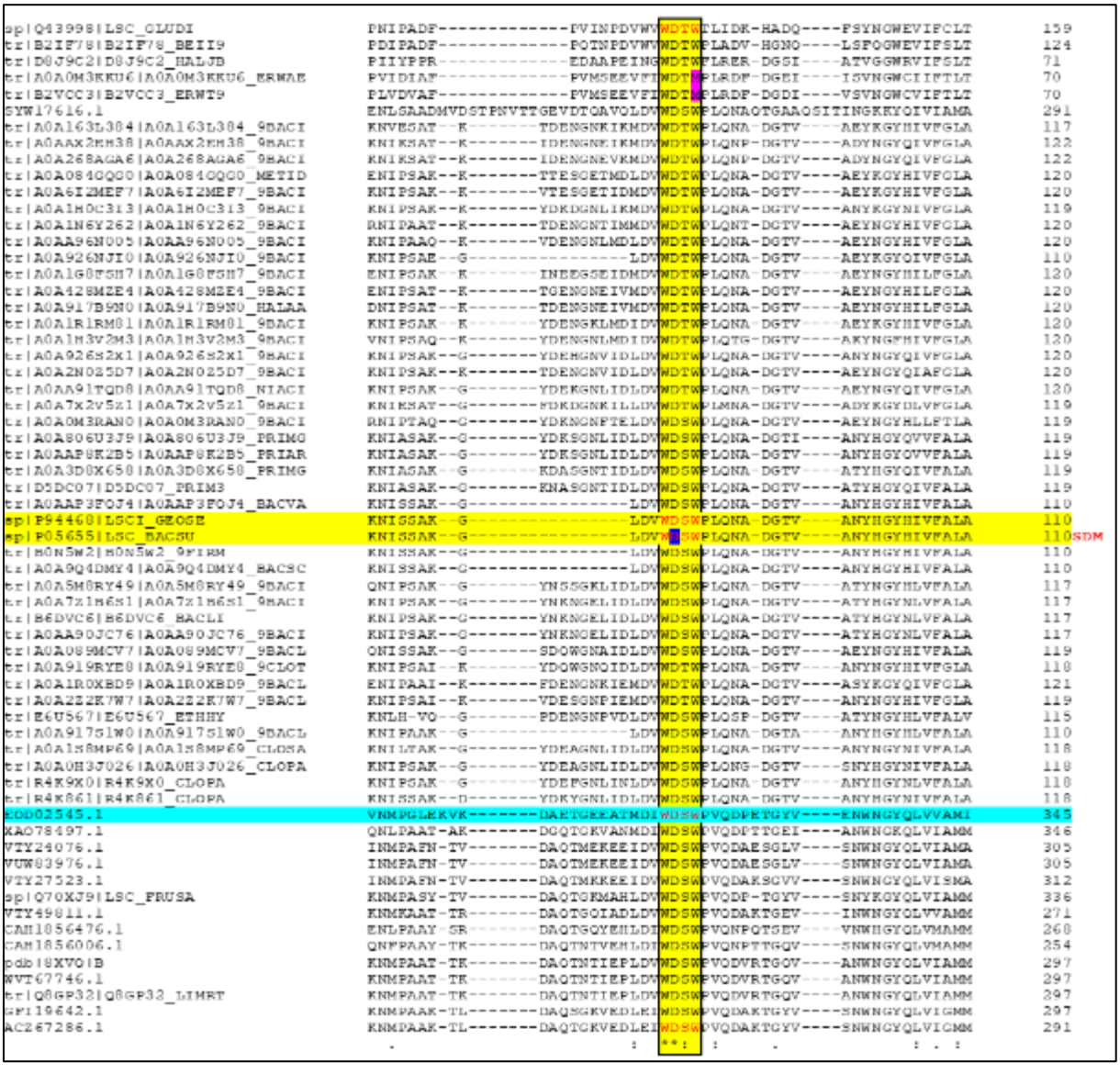
Figure 3 shows the 'Mix and Match' MSA of all the enzymes belonging to fructosyl transferases, viz. levansucrases, inulosucrases and other GH68 family glycoside hydrolases, (only the required regions for the discussions are shown). The active site amino acids of the hydrolytic and transferase domains from all three groups are highlighted in yellow and green, respectively. The active and inactive transferase enzyme from *B. subtilis* and *G. stereothermophilus* is highlighted in green and red, respectively. Strikingly, all three groups revealed high sequence similarities mostly at their catalytic sites only. Furthermore, it is interesting to note that the enzyme from *S. mutans*, the organism which causes dental caries in humans, synthesizes a β-(1→2)-linked fructan (inulin type), whereas the *B. subtilis* (a nonpathogenic organism) produces a β-(2→6)-linked polymer (levan type). As the hydrolytic site amino acids are the same, the structural difference of the fructans may be correlated with the sequence difference(s) around the transferase catalytic regions. For example, the comparison of the active site regions in levan and inulin synthetic enzymes show distinct differences in their distance motifs between the proton donor and the proposed template-binding pair. In fact, a longer distance motif between the proton abstractor and the template-binding pair is observed in inulosucrases of *S. mutans*, (e.g.), *B. subtilis* use, -SR-^{3}GSK^{1}MTIDGITSNDIYML^{15}GY- and *S. mutans* use, -SR-^{3}LNH^{1}GSNNDAWNKANEVVGDNVVML^{21}GY-). It is interesting to note that in *S. mutans*, the EPS of dental plaque contains approximately 30% inulin, which protects the cells from antimicrobial materials present in saliva by forming biofilm [31].

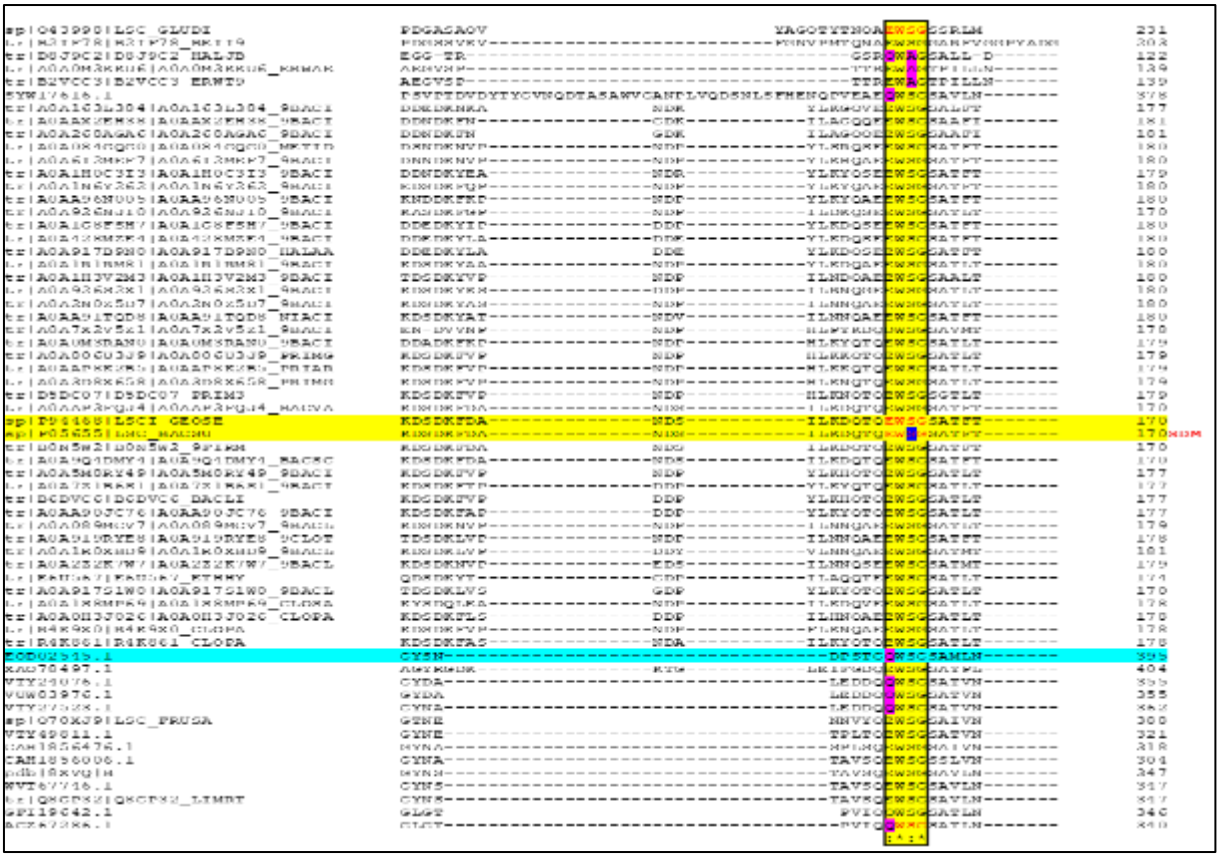
The inulosucrase sequences start from the light blue highlighted sequence. Interestingly, they are larger proteins, and are almost double the size of the other two groups of transferases. There are big gaps in the N-terminal region and more or less no conserved amino acids or regions (data not shown) are observed until it reaches the first highly conserved tetrapeptide, -WDS/T/W/M-. This tetrapeptide harbours the invariant D, which acts as the catalytic nucleophile in the hydrolytic reaction. Again, there are gaps in the alignment until it reaches the second highly conserved tetrapeptide, -Q/D/EWSG/A- and is suggested to interact with the Glu unit of the sucrose molecule. This is followed by the next conserved tetrapeptide is -MF/LRDP/A/S- which is highly conserved in all three groups suggesting its importance. This tetrapeptide is suggested to interact with the Fru unit of the sucrose molecule. Substitution of Asp^{309} by Asn in the –FRD^{309}P- motif of *A. diazotrophicus* levansucrase reduced the k_{cat} of the mutant enzyme 75-fold, indicating that Asp^{309} plays a major role in catalysis affecting the overall efficiency of sucrose hydrolysis. It is interesting to note that this tetrapetide motif is conserved not only in the fructosyltransferases, but also in invertases, levanases, inulinases and sucrose-6-phosphate hydrolases, suggesting a common functional role for this motif in these enzymes [32]. Yanase *et al*. [33] found that the substitution of Asp^{194}, located in the –FRD^{194}P- motif of *Z. mobilis* levansucrase abolished sucrose

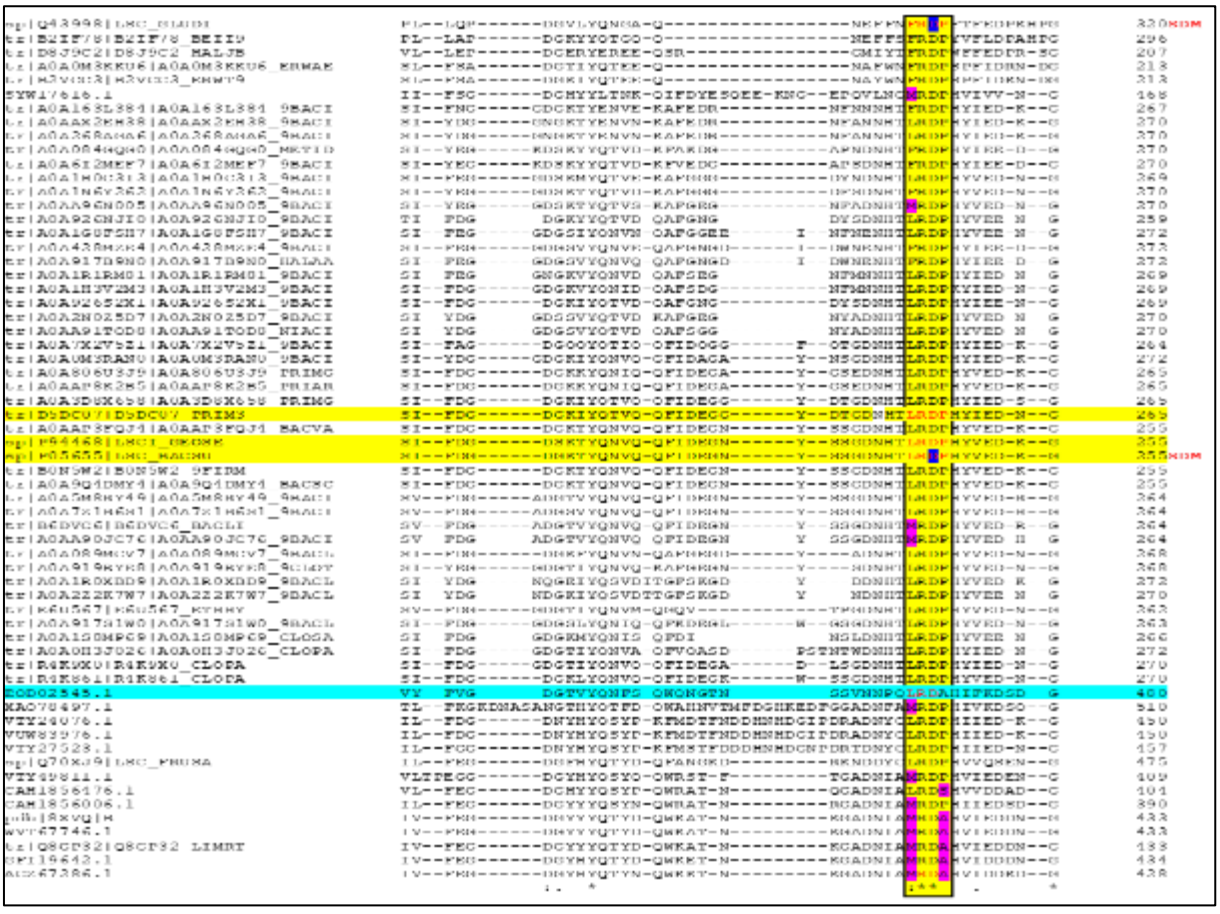
hydrolysis, suggesting its importance in catalysis. Furthermore, they found that the His[296], in the transferase domain is crucial for catalysis of the transfructosylation reaction –FT[-3]ISH[296]HSTYADGLSGPDGVY[16]GF-. Not much conservation is observed until it reaches the next highly conserved tetrapeptide – QT/EI/VER-. The –ER- pair is completely conserved in all and the invariant -E- is known to act as the acid/base catalyst in the hydrolytic reaction.

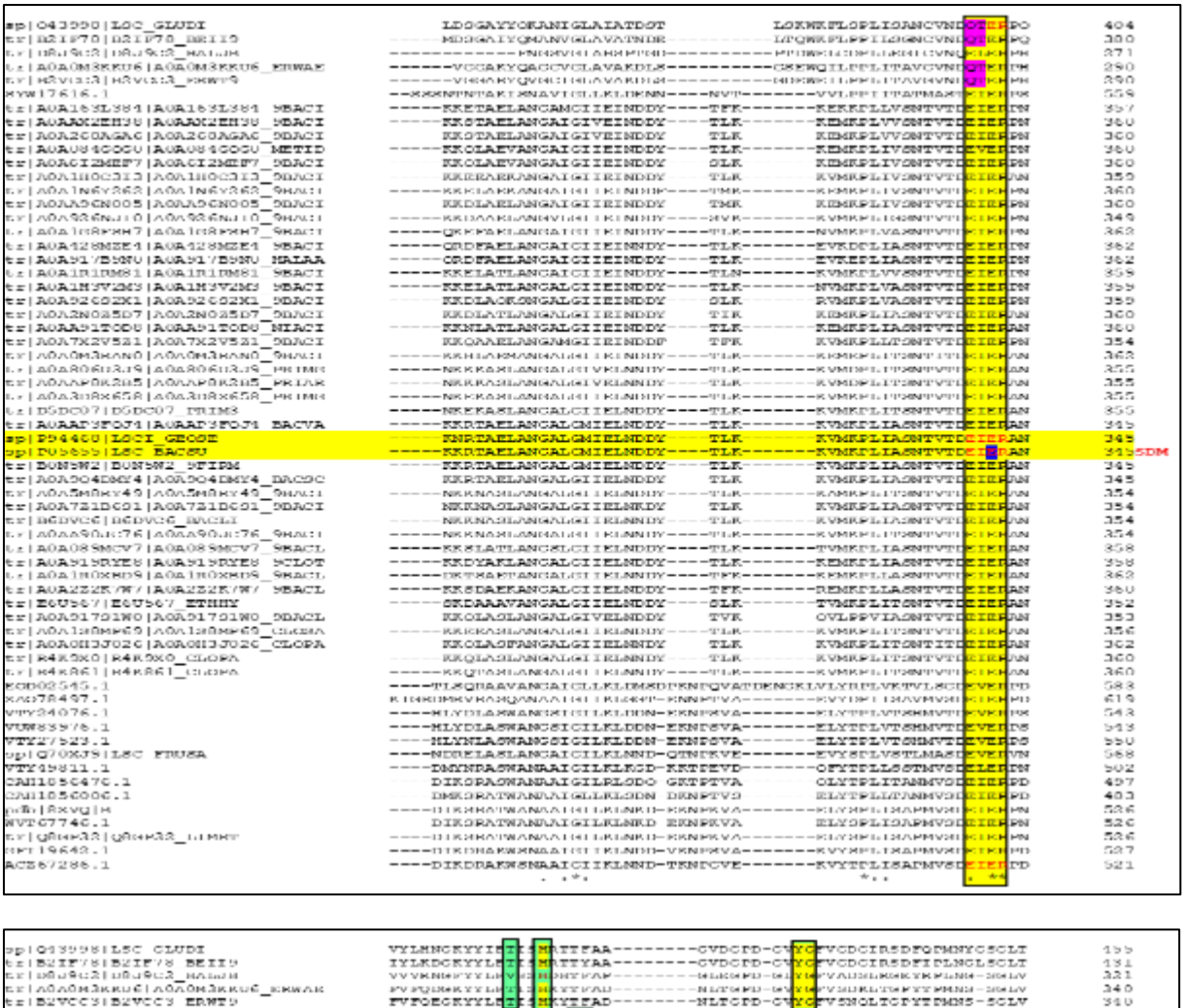
Immediately after the hydrolytic module, the transferase active site domain is placed (highlighted in green). It is interesting to note that the transferase module is very similar to the nucleotidyl transferases module found in the DNA/RNA polymerases and composed of a similar catalytic triad, viz. a proton abstractor (usually a basic amino acid in all DNA/RNA polymerases and is conserved in the fructosyl transferases also) and a donor selection amino acid R at -3 to -4 from the proton abstractor and a template-binding pair -L/I/GY or –YG- (a highly conserved -YG- pair is shown as the template-binding pair in most of the DNA/RNA polymerases (Table 2). Interestingly, in all these group of fructosyl transferases a highly conserved template-binding triad, rather than a pair, is observed. The levansucrase from *G. stearothermophilus*, is reported to be inactive. The main difference between the active (from *B. subtilis*) and the inactive (from *G. stearothermophilus*) levansucrases is the proton abstractor, where the K is replaced by Q, respectively (marked in red). As the Q does not act as a proton abstractor in transferases, the enzyme could be inactive which is further confirmed by MSA analysis and SDM data. Interestingly, the same highly conserved amino acids at the catalytic sites of the two different transferases, viz. the fructosyl and nucleotidyl transferases suggest common evolutionary principles in designing active sites for same type of reactions in living cells.

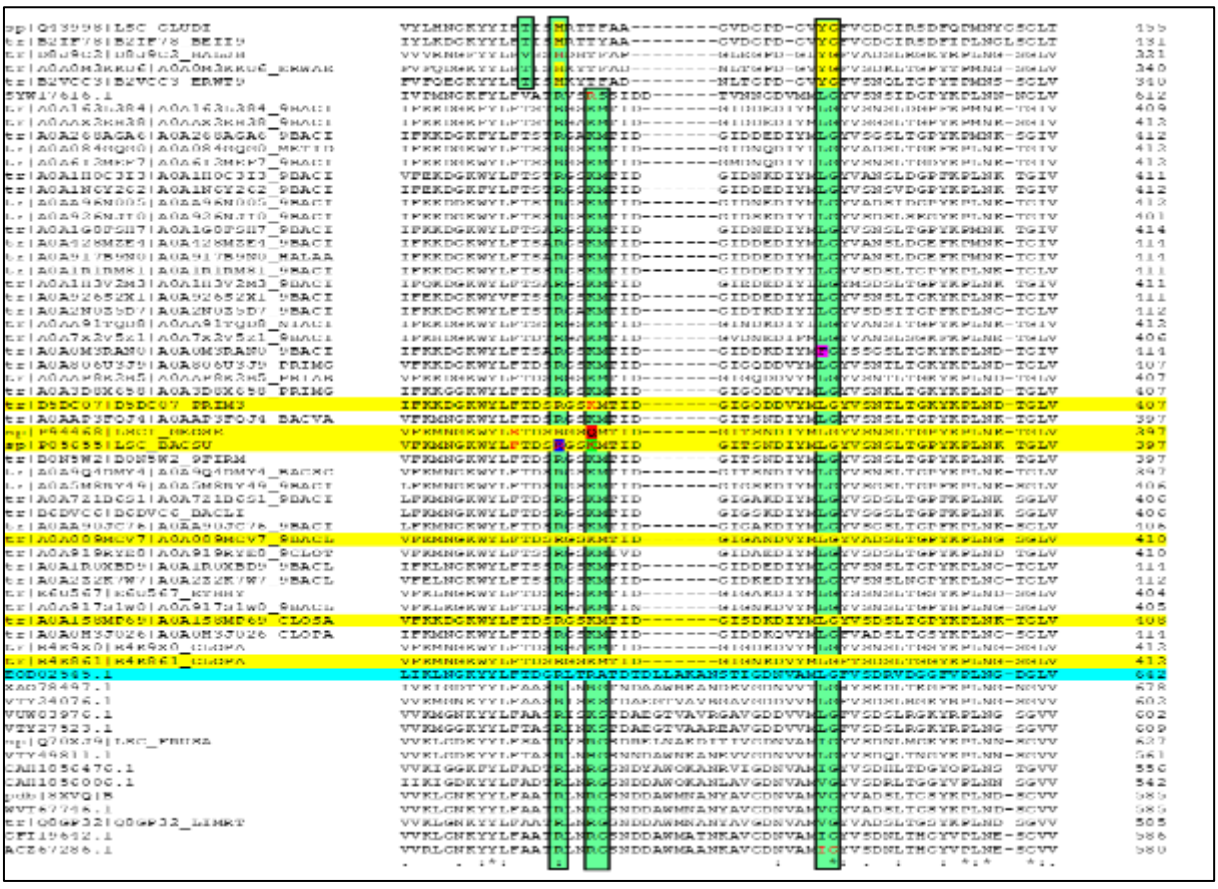
CLUSTAL O (1.2.4) 'Mix and Match' MSA of levan- and inulosucrases and other GH68 family glycoside hydrolase fructosyl transferases.

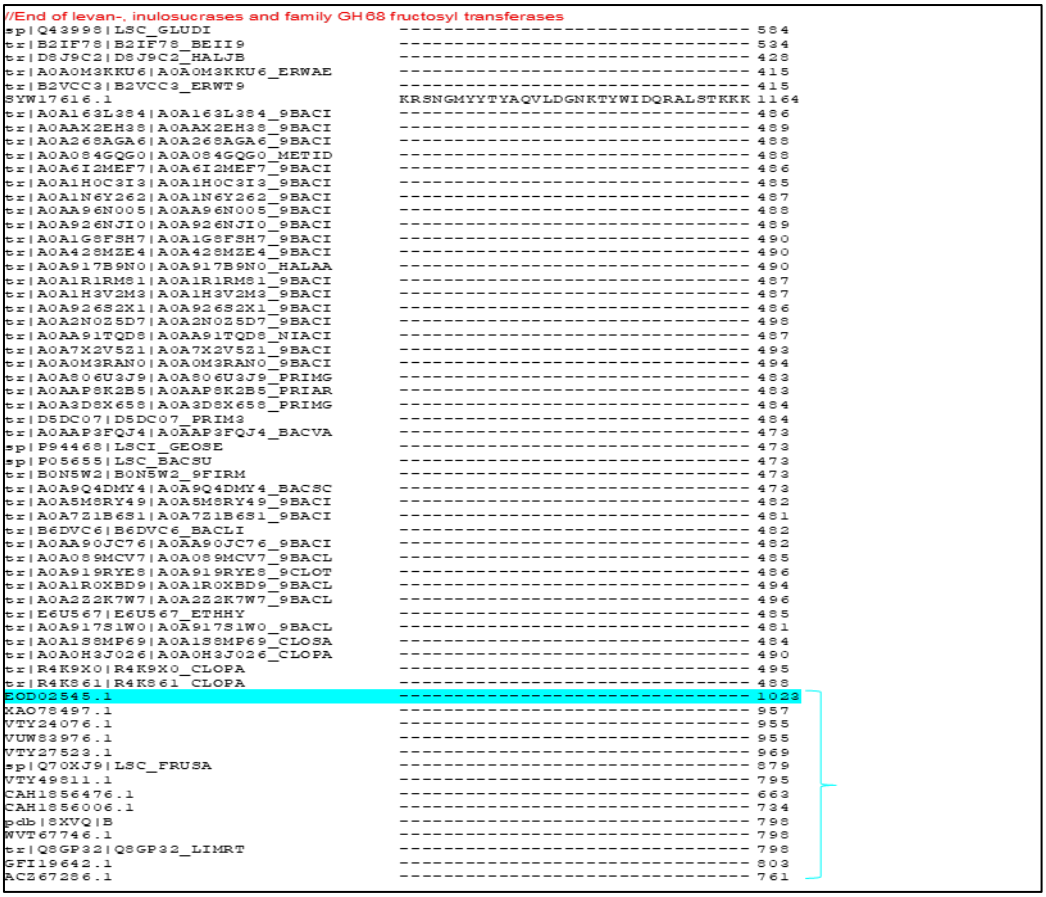












NB: The SDM and X-ray crystallographic data are highlighted in dark blue and light blue in bold, respectively.

B2IF78_BEII9 Levansucrase, *Beijerinckia indica*
D8J9C2_HALJB Levansucrase, *Halalkalicoccus jeotgali*
A0A0M3KKU6_ERWAE levansucrase, *Erwinia amylovora*
B2VCC3_ERWT9 levansucrase, *Erwinia tasmaniensis*
SYW17616.1 putative Inulosucrase, *Oenococcus oeni*
A0A163L384_9BACI Levansucrase, *Rossellomorea marisflavi*
A0AAX2EH38_9BACI Levansucrase, *Terribacillus saccharophilus*
A0A268AGA6_9BACI GH68 family protein, *Terribacillus saccharophilus*
A0A084GQG0_METID Levansucrase, *Metabacillus indicus*
A0A6I2MEF7_9BACI GH68 family protein, *Metabacillus idriensis*
A0A1H0C3I3_9BACI Levansucrase, *Fictibacillus solisalsi*
A0A1N6Y262_9BACI Levansucrase, *Domibacillus enclensis*
A0AA96N005_9BACI GH68 family protein, *Domibacillus* sp.
A0A926NJI0_9BACI GH68 family protein, *Metabacillus arenae*
A0A1G8FSH7_9BACI Levansucrase, *Alteribacillus bidgolensis*
A0A428MZE4_9BACI GH68 family protein, *Salibacterium salarium*
A0A917B9N0_HALAA Levansucrase, *Halobacillus andaensis*
A0A1R1RM81_9BACI GH68 family protein, *Bacillus swezeyi*
A0A1H3V2M3_9BACI Levansucrase, *Evansella caseinilytica*
A0A926S2X1_9BACI GH68 family protein, *Metabacillus arenae*
A0A2N0Z5D7_9BACI GH68 family protein, *Niallia nealsonii*
A0AA91TQD8_NIACI GH68 family protein, *Niallia circulans*
A0A7X2V5Z1_9BACI GH68 family protein, *Metabacillus mangrovi*
A0A0M3RAN0_9BACI Levansucrase, *Bacillus gobiensis*
A0A806U3J9_PRIMG Levansucrase, *Priestia megaterium*
A0AAP8K2B5_PRIAR GH68 family protein, *Priestia aryabhattai*
A0A3D8X658_PRIMG GH68 family protein, *Priestia (Bacillus) megaterium*
D5DC07_PRIM3 Levansucrase, *Priestia (Bacillus) megaterium*
A0AAP3FQJ4_BACVA Levansucrase, *Bacillus vallismortis*
P94468LSCI_GEOSE Inactive levansucrase, *Geobacillus stearothermophilus*, sacB
P05655LSC_BACSU Levansucrase, *Bacillus subtilis*, sacB

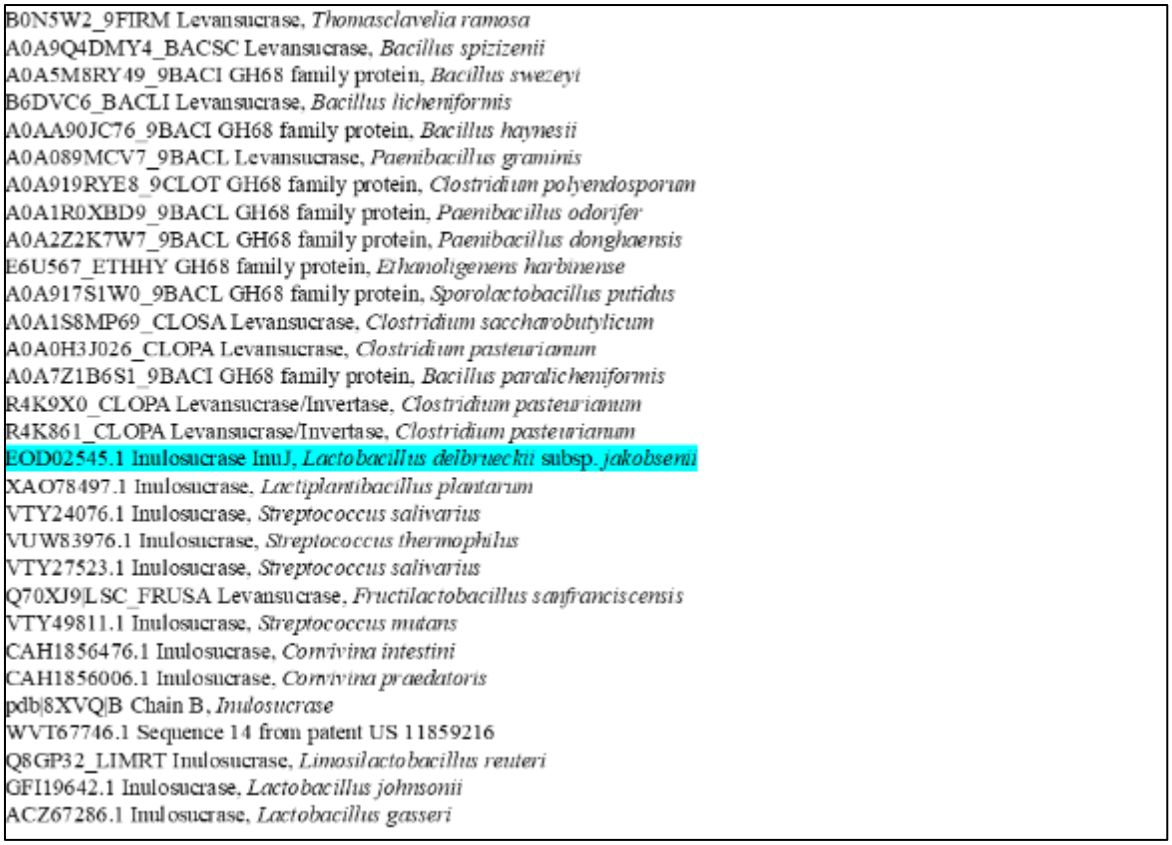


Figure 3 'Mix and Match' MSA of all three groups of fructosyl transferases: levansucrases, inulosucrases and GH68 family glycoside hydrolases from Gram-positive and Gram-negative bacteria

4. Analyses of active site amino acids in the hydrolytic and transferase domains

The amino acids in the hydrolytic and transferase active sites are confirmed by SDM experiments, X-ray crystallographic and MSA analyses. All three groups, viz. the levansucrases, inulosucrases and other GH68 family of glycoside hydrolases invariably use three highly conserved amino acids in their active sites, as catalytic triad, for hydrolysis of sucrose molecule and another catalytic triad for subsequent fructosyl transferase activity.

4.1. Catalytic site amino acids in the hydrolase domain of levansucrases and inulosucrases

As discussed elsewhere, the hydrolytic domain's catalytic triad consists of two invariant Ds and an E in all three groups of enzymes. *B. subtilis* levansucrase (SacB) uses D^{86}, D^{247} and E^{342} as the catalytic triad and they are shown to act as the nucleophile, transition-state stabilizer and the general acid-base catalyst (proton donor), respectively. On modification of the above amino acids to Ala by SDM, the enzyme lost its activity and thus, confirming their direct involvement in catalysis [18].

In the same way, the levansucrases from *L. reuteri* uses D^{249}, D^{404} and E^{503} as the catalytic triad in its hydrolytic domain. On modification of the above amino acids to their amide forms, viz. D^{249}→N, D^{404}→N and E^{503}→Q by SDM, about 10,000-fold reduction in sucrose hydrolytic activity was observed and thus, confirming their role on hydrolysis of sucrose molecule [34].

In *B. megaterium* levansucrase, the residues Asp^{95}, Asp^{257} and Glu^{352} are proposed to form the catalytic triad. After the substitution of Ala to the proposed catalytic residues by SDM (Asp^{95}→A, Asp^{257}→A, Glu^{352}→A), it was found that the enzyme had lost its activity [35] and thus, confirming their direct involvement in the sucrose hydrolysis. Interestingly, the variants R^{256}→A and E^{350}→A were also nearly inactive.

Polsinelli *et al*. [36] have determined the 3D structure of a levansucrase from *Erwinia tasmaniensis* and found the catalytic triad involved in catalysis are, Asp^{46}, Asp^{203}, and Glu^{287}.

Not only the levansucrases, but also the inulosucrases also use the same active site amino acids in their hydrolytic domain. Ozimek *et al*. [34] proposed that the inulosucrase of *L. reuteri* might use D^{272}, D^{424} and E^{523} in the catalytic site of its hydrolytic module. This was further confirmed by SDM experiments. For example, the substitution of Ala to the proposed catalytic residues (Asp^{272}→A, Asp^{424}→A, Glu^{523}→A), led to the loss of enzyme activity.

Pijning *et al*. [37] found that the three putative catalytic residues (D^{272}, D^{425}, and E^{524}) in an inulosucrase from *Lactobacillus johnsonii*. These three amino acids superposed almost perfectly with those of *B. subtilis* SacB and thus, confirming both the enzymes use the same amino acids at the active sites of their hydrolytic domains. Furthermore, they found that D^{272}→N, a nucleophilic mutant of the enzyme, exhibited only a very low residual activity and the mutant/ native enzyme activity ratio was about 8×10^{-6}.

The crystal structure of an inulosucrase from a halophilic archaeal strain, *Halalkalicoccus jeotgali*, also exhibited the same pattern of amino acids at their hydrolytic domain. The catalytic triad is found to be composed of resides D^{47}, D^{197}, and E^{268} [38]. Furthermore, the 3D structure of the halophilic enzyme resembled closely to other fructansucrase structures.

The crystal structure of a fructosyl transferase from the fungus, *Aspergillus japonicus*, has shown that the amino acids in the catalytic triad are, Asp^{60}, Asp^{191}, and Glu^{292} and these three act as nucleophile, transition-state stabilizer, and general acid/base catalyst, respectively in the fungal enzyme and further suggested that they also governed the binding of the terminal fructose at the -1 subsite for the catalytic reaction. The active site amino acids in the fungal enzyme were further confirmed by SDM analysis. It was found that the mutants D^{60}→A, D^{191}→A, and E^{292}→A completely lost their activity [27].

MSA analysis has also confirmed that these three completely conserved amino acids constitute the catalytic triad in the hydrolytic site of all these fructosyl transferases (Fig. 3).

4.2. Catalytic site amino acids in the transferase domain of levansucarses and inulosucrase

The catalytic site amino acids in the transferase active site are arrived at from SDM experiments, X-ray crystallographic data and MSA analysis. As already discussed elsewhere, the fructosyl transferase from *G. stearothermophilus* was found to be inactive due to replacement of a K^{363} to Q^{363} in the proposed transferase domain. The importance of this highly conserved basic amino acid in this domain was further confirmed from analysis of hundreds of fructosyl transferase sequences from levansucrases, inulosucrases and other GH68 family glycoside hydrolase enzymes from a large number of organisms (Fig. 3). It is interesting to note that it has been shown a positively charged amino acid, viz. K/R/H is crucial for the functioning of nucleotidyl transferase catalytic reactions in DNA/RNA polymerases [1-3] where it initiates the catalytic reaction by abstraction of a proton from the substrate. Furthermore, in both the types of transferases, an invariant R at -3 or -4 from the proton abstractor (Tables 1 and 2) acts in the donor selection. The involvement of a basic amino acid as the proton abstractor and an R at -3 or -4 from the proton abstractor as nucleotide/fructose donor selection amino acid are further confirmed by SDM experiments, MSA analysis, and also from X-ray crystallographic data. The third template-binding pair (L/I/V/YG) in fructosyl transferases is suggested based on the sequence similarity from a large number of nucleotidyl transferases (DNA/RNA polymerases) (Tables 1 and 2 [1-3].

The above conclusions are further substantiated by SDM experiments on large number of fructosyl and nucleotidyl transferases. Meng and Fütterer [18] found that SDM studies on the levansucrase of *B. subtilis* SacB revealed that modifications of the residues N^{242}, K^{363}, and Y^{237} (numbering of *B. subtilis* levansucrase), which are located on the surface of the catalytic cavity affect the enzyme catalytic efficiency, transfructosylation, and hydrolysis ratio by interrupting the polymerization process. Therefore, they suggested that these residues could control the chain lengths of levans. However, they concluded that the interactions mediated by these residues had not yet been identified, and the existence of additional external acceptor-binding subsites, acting as structural determinants in the elongation of levans had not yet been clarified. Furthermore, in chain A, the interactions might resemble an acceptor anchoring state, with R^{360} and K^{363} maintaining direct contact with fructosyl-2 of the ligand, while Y^{411} might coordinate a water-mediated contact. Therefore, it is clear that these two residues, R^{360} and K^{363} play an important role in the transferase active site, which is also further supported by the present MSA analysis.

Further SDM experiments have conclusively proved that these two amino acids in the transferase catalytic site play a crucial role in levan synthesis. For example, the *B. megaterium* levansucrase SacB variants, viz. Y^{247}→A, Y^{247}→W, N^{252}→A, D^{257}→A, and K^{373}→A revealed novel surface motifs remote from the sucrose-binding site with distinct influence on the polysaccharide product spectrum. Consistent with the eliminated polysaccharide synthesis, the K^{373}→A mutant showed an increased hydrolytic activity of almost 33%. Furthermore, the five structures of the SacB variants (Y^{247}→A, Y^{247}→W,

N^{252}→A, D^{257}→A, and K^{373}→A) obtained at resolutions between 2.0 and 1.75 Å supported a surface-modulated transfructosylation mechanism [39]. It is interesting to note that the D^{257} is in the highly conserved –LRD^{257}P- motif of Fru-binding site and the others are in its vicinity and the K^{373} is implicated as the proton abstractor in the transferase active site. Homann *et al*. [35] also found that the R^{370} at -3 from the proton abstractor is crucial in the levansucrase of *B. megaterium* for the transferase activity. Furthermore, they found that the residues R^{370} and N^{252} seem to be crucial for polyfructan synthesis and are conserved in all levansucrases from Gram-positive bacteria.

Yanase *et al*. [33] found that the H^{296}, the proposed proton abstractor in the transferase active site is crucial for catalysis of the transfructosylation reaction in the levansucrase of *Z. mobilis* (Table 2).

Del Rio *et al*. [26] studied the transferase active site of an inulosucrase from *L. citreum* by SDM experiments. They found that R^{618} is an invariant amino acid in all inulosucrases and is placed at -3 from the proton abstractor K^{621} and hence, suggested it could play an important role in fructose donor selection. The transfructosylation activity and product selectivity factor found in the R^{618}→K mutant was only 50% with an order of magnitude decrease, respectively.

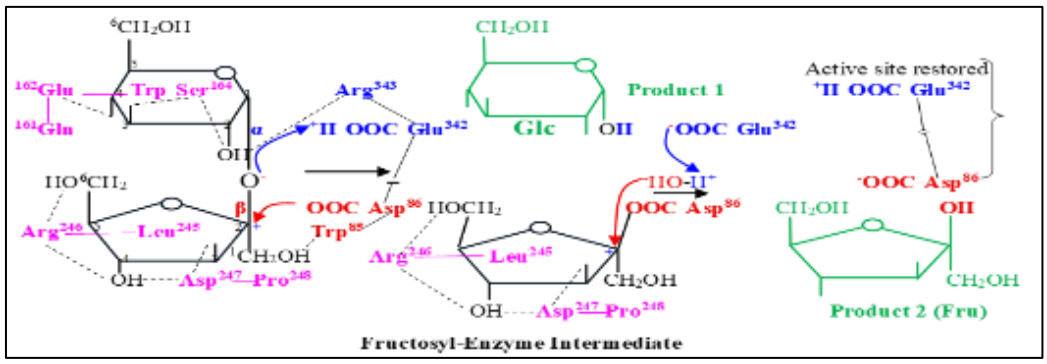
The above SDM data and the MSA analysis confirm that the highly conserved K and the R (at -3) play important roles in the transferase reactions in levan- and inulosucrases, where they act as proton abstractor and donor selection, respectively.

5. Mechanism of Hydrolytic and Transferase Reactions in Fructosyl Transferases

5.1. Mechanism of action of the hydrolytic reaction

It is interesting to note that both the hydrolytic and transferase modules in all three groups of fructosyl transferases invariably use catalytic triads for hydrolysis and transferase reactions. Furthermore, the sucrose-binding sites and the hydrolytic and transferase reactions are virtually identical in all three groups of enzymes, confirming that they use a fully conserved structural framework for the sucrose-binding, cleavage and transferase reactions. In bacteria, the fructan synthesis is somewhat simpler as compared to plant systems, because only one enzyme with the bifunctional property (exhibiting both hydrolytic and transferase activities) is involved. By SDM and mutational analyses, Chembert and Petit-Glatron [40] found that the hydrolytic and transferase activities could be separated in levansucrases, suggesting that they are two independent activities.

The hydrolytic domain is composed of a catalytic triad: a nucleophile (Asp), a general acid/base catalyst (Glu), and a transition-state stabilizer (Asp). The hydrolytic reaction follows a double-displacement mechanism involving two steps, (i.e.) i) protonation of the glycosidic oxygen and breaking the glycosidic linkage, and ii) a nucleophilic attack on the anomeric carbon of the donor resulting in the formation of the fructosyl-enzyme intermediate. In the latter step, a water, or a sucrose molecule or a fructan serves as an acceptor resulting in the hydrolysis or transfer of the fructose to the acceptor molecule, where the acceptor is elongated by one fructosyl unit, respectively. The reaction occurs with an overall retention of the anomeric configuration of the fructosyl residue.



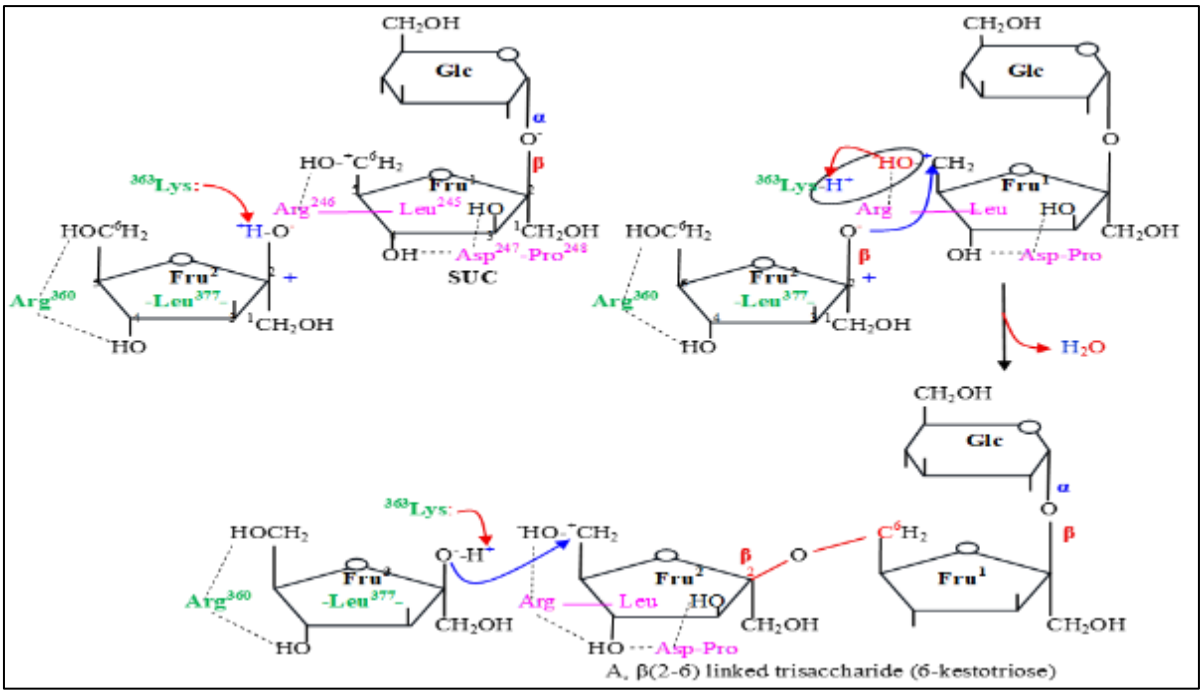
The amino acids in the catalytic triad involved in the hydrolysis of the sucrose molecule are; Asp^{86}, Glu^{342} and Asp^{247} (they act as nucleophile, proton donor and transition state stabilizer, respectively) [18]. The nucleophilic amino acid pair, 'Trp-Asp' and the catalytic acid-base amino acid pair 'Glu-Arg', are conserved in all levansucrases. The highly conserved –E^{162}WSGS- and –L^{245}RDPH- motifs are involved in sucrose-binding and shown in magenta.

Figure 4 A schematic diagramme showing the hydrolytic active site amino acids and its proposed mechanism of action (numberings from the *B. subtilis* levansucrase, SacB)

During the first step of the protonation reaction, the acid/base catalyst, Glu, donates a H^+ to the glyosidic bond resulting in its cleavage, followed by a nucleophilic-electrophilic attack on the anomeric carbon of the fructose unit resulting in the formation of a fructosyl-enzyme intermediate, where the C-2 of fructose forms a covalent bond with the nucleophilic Asp residue [14, 13, 18, 25]. The fructosyl-enzyme intermediate formation is supported by kinetic analysis under steady–state conditions and was also successfully isolated and studied by Chambert and Treboul [41], where they found that the β-carboxyl group of the Asp formed a covalent bond with the fructose (Fig. 4). The second Asp acts as a transition-state stabilizer in both steps, and it is based on the observation that it forms strong hydrogen bonds with the C3 and C4 hydroxyls of the fructosyl unit [34] (Fig. 4).

5.2. Mechanism of action of the fructosyl transferase reaction

A second, separate module of the same enzyme accomplishes the fructosyl transferase reaction [40]. The synthesis of fructans starts with a transfructosylation reaction in which the hydrolyzed sucrose molecules in the first step provide the fructosyl donors in which a molecule of sucrose acts as the initial acceptor of the fructosyl units. The transfer of a fructose unit to the acceptor sucrose molecule can occur via O1 to form 1-kestotriose, (the basic unit for the formation of β-(2→1)-linked inulin) or via O6 to form 6-kestotriose, (the basic unit for the formation of β-(2→6)-linked levan (Fig. 5). The transferase module is also composed of a catalytic triad where a basic amino acid acts as a proton abstractor, a hydrophobic/aromatic amino acid (found downstream to the catalytic proton abstractor) forms the template-binding pair and again a basic amino acid (found upstream to the catalytic proton abstractor) involves in the donor sugar selection. The proposed fructosyl transferase reaction is very similar to the well-established nucleotidyl transferase reaction [1] as discussed elsewhere. In *B. subtilis* SacB enzyme, the catalytic K abstracts a proton from the donor fructose in the first step, leading to a nucleophilic–electrophilic attack between the incoming fructose unit and the acceptor sucrose molecule and thus, establishing a glycosidic bond between the donor and the acceptor in the second step. By the same mechanism, the fructosyl units are added sequentially to the growing chain forming a levan polymer. The initial linkage on the kestotriose, either β-(2→6) or β-(2→1) decides the formation of a levan or an inulin polymer, essentially using the same mechanism, but accomplished by two different enzymes, viz. the levansucrases or the inulosucrases, respectively (Fig. 5). For sucrose at concentrations of >100 mM, fructosyl transferases exhibit almost an exclusive transfructosylation activity [27].



The hydrolytic domain's Fru-binding active site amino acids are in magenta and the transferase domain's active site amino acids are in green. The active site amino acids in the transferase active site domain are: Arg^{360}, Lys^{363} and -Leu^{377}GY- (for incoming fructose- selection, proton abstraction and template-binding, respectively).

Figure 5 A schematic diagramme showing the transferase active site and its proposed mechanism action (numbering from the *B. subtilis* levansucrase, SacB)

Like the nucleotidyl transferases (RNA/DNA polymerases), the fructosyl transferases are also multifunctional enzymes and possess two active site domains, one for sucrose hydrolysis (Fig. 1) and the other one for fructosyl transfer, i.e., in the first step, the sucrose molecules are hydrolyzed and in the second step a fructose unit is added to an acceptor sucrose molecule to form a trisaccharide, either 6- or 1-kestotriose (depending on the enzyme type) which is followed by sequential additions of fructose units resulting in levan or inulin polymer, respectively (Fig. 2) [37].

6. Structure and Mechanism of Action of Nucleotidyl Transferases

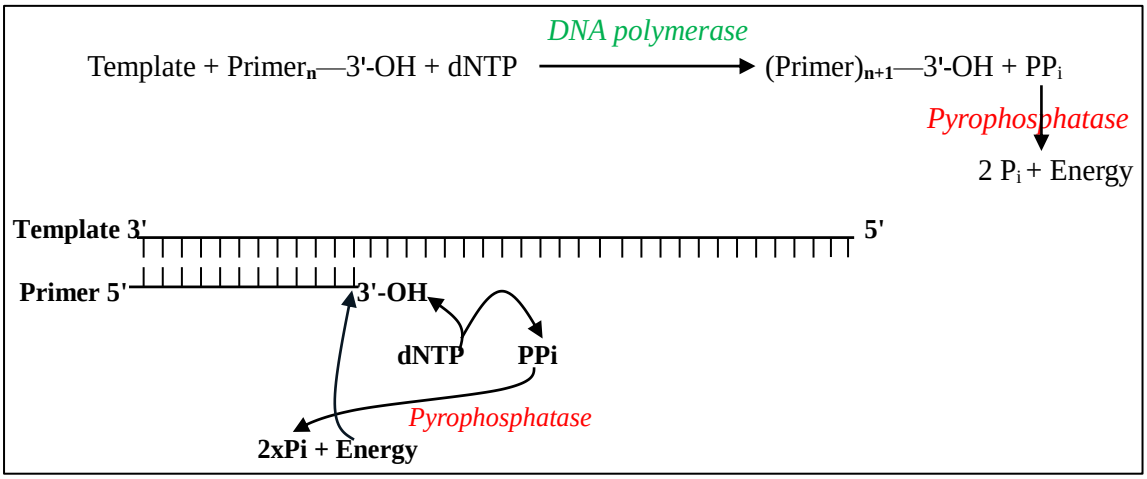
The nucleotidyl transferases also belong to the Main class, 'Transferases', like the fructosyl transferases, but these enzymes involve in the sequential addition of nucleoside triphosphates (NTPs) or deoxynucleoside triphosphates (dNTPs) to synthesize an RNA or a DNA molecule, respectively. DNA polymerases make sequential addition of dNTPs to the 3-OH of a primer on a template, whereas the RNA polymerases make sequential addition of NTPs on a template. Thus, both the polymerases belong to the subclass, nucleotidyl transferases. The properties and mechanisms of action of these crucial catalysts of life are already analyzed in detail and reported by this author [1-3].

DNA polymerases catalyze the polymerization of a new strand of DNA on a given template DNA, using a primer molecule. Except for viruses, there are always more than one enzyme in a living cell. For example, at least three different polymerases have been identified even in the simplest prokaryotes. There are at least five different DNA polymerases reported from *E. coli* and they are named DNA polymerases I, II, III, IV and V. The DNA polymerases, which undertake replication, are known as replicases. The *E. coli* DNA polymerase I is used as an example to explain the mechanism of action here. It was the first enzyme to be discovered and is encoded by *polA* gene. It is a single-subunit enzyme with a molecular mass of 10^3 kDa (928 amino acids). The main functions of this polymerase are: i) removal of the RNA primer after genome replication and subsequent filling of the gap arising due to such removal, ii) DNA repair and iii) removal of TT dimers in UV irradiated cells. Thus, it is a multifunctional enzyme and exhibits three different activities, viz. 5'→3' DNA polymerase activity, 3'→5' exonuclease activity (proofreading function) and 5'→3' exonuclease activity (DNA repair function). There are ~ 400 copies of the enzyme per cell.

6.1. Mechanism of Nucleotidyl Transferases

6.1.1. Dynamics of DNA polymerization

DNA polymerases synthesize a new strand of DNA on a given template using a primer and the four dNTPs (Fig. 6). They add nucleotides one at a time to the 3'-OH end of the primer as shown and thus, the new strand grows from 5'→3'. The overall reaction catalyzed by DNA polymerases can be written as,

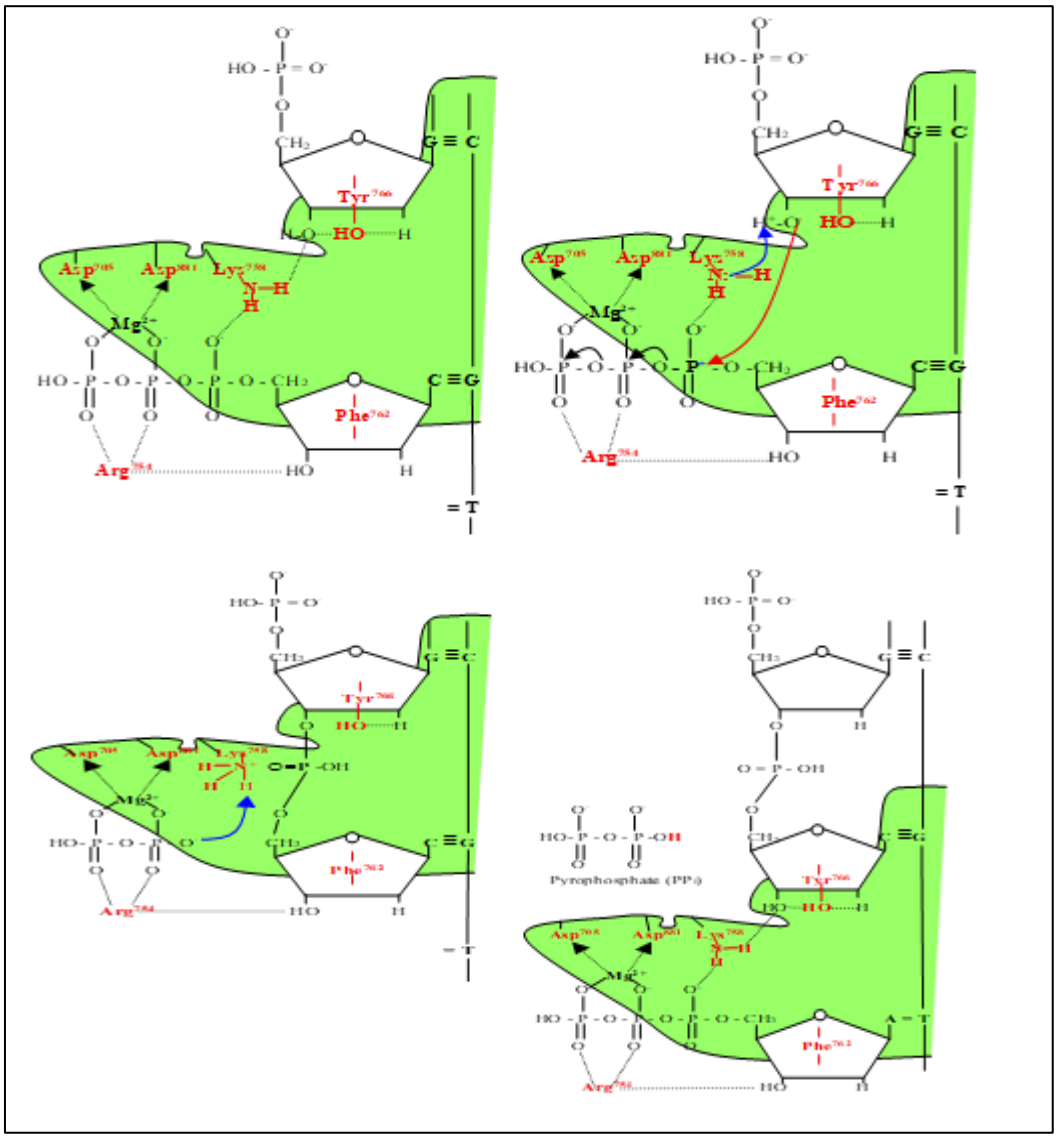


NB: PPi, Inorganic pyrophosphate; Pi, Inorganic phosphate

The high-energy inorganic pyrophosphate is hydrolyzed immediately by the enzyme pyrophosphatase into two molecules of inorganic phosphate resulting in release of energy, which is utilized for the polymerization reaction. Such coupling of reactions drives the polymerization process only in the forward direction, i.e., the polymerized nucleotides cannot be depolymerized.

Figure 6 A schematic diagramme showing the overall reaction of DNA polymerases

As discussed elsewhere, the DNA and RNA synthesis in cells are accomplished by the nucleotidyl transferases where they use either dNTPs or NTPs, respectively. A great deal of information is available on these enzymes from a large number of sources. X-ray crystallographic structures of a large number of polymerases from both prokaryotes and eukaryotes are available. Although they exhibit significant differences in their primary structures (probably due to divergent evolution), their crystal structures are remarkably the same in overall shape. For example, in general, all DNA polymerases, irrespective of their source, have a common protein fold that resembles the shape of a half-opened "right hand" and the polymerase domain exhibits three distinct subdomains, “thumb”, “palm” and “fingers” with analogous functions in all organisms [42]. The mechanism of action of *E. coli* DNA polymerase I is shown in Fig. 7 [1]



Watson-Crick base pairing of incoming dNTP to the template at the active site; Electronic transitions at the active site for proton abstraction and an electrophilic-nucleophilic attack; Proton abstraction by the active site amino acid Lys followed by an electrophilic-nucleophilic attack resulting in the formation of 3’→5’ phosphodiester bond with the concomitant release of pyrophosphate; Transfer of the proton abstracted by the Lys to the pyrophosphate resulting in the formation of inorganic pyrophosphate. The high-energy bond in the inorganic pyrophosphate is hydrolyzed immediately by the enzyme pyrophosphatase (usually associated with enzyme) and the energy released is utilized by the DNA polymerase to move (translocate) to the next nucleotide, already positioned on the template. As the PPi is removed constantly from the reaction, the polymerization reaction is always unidirectional.

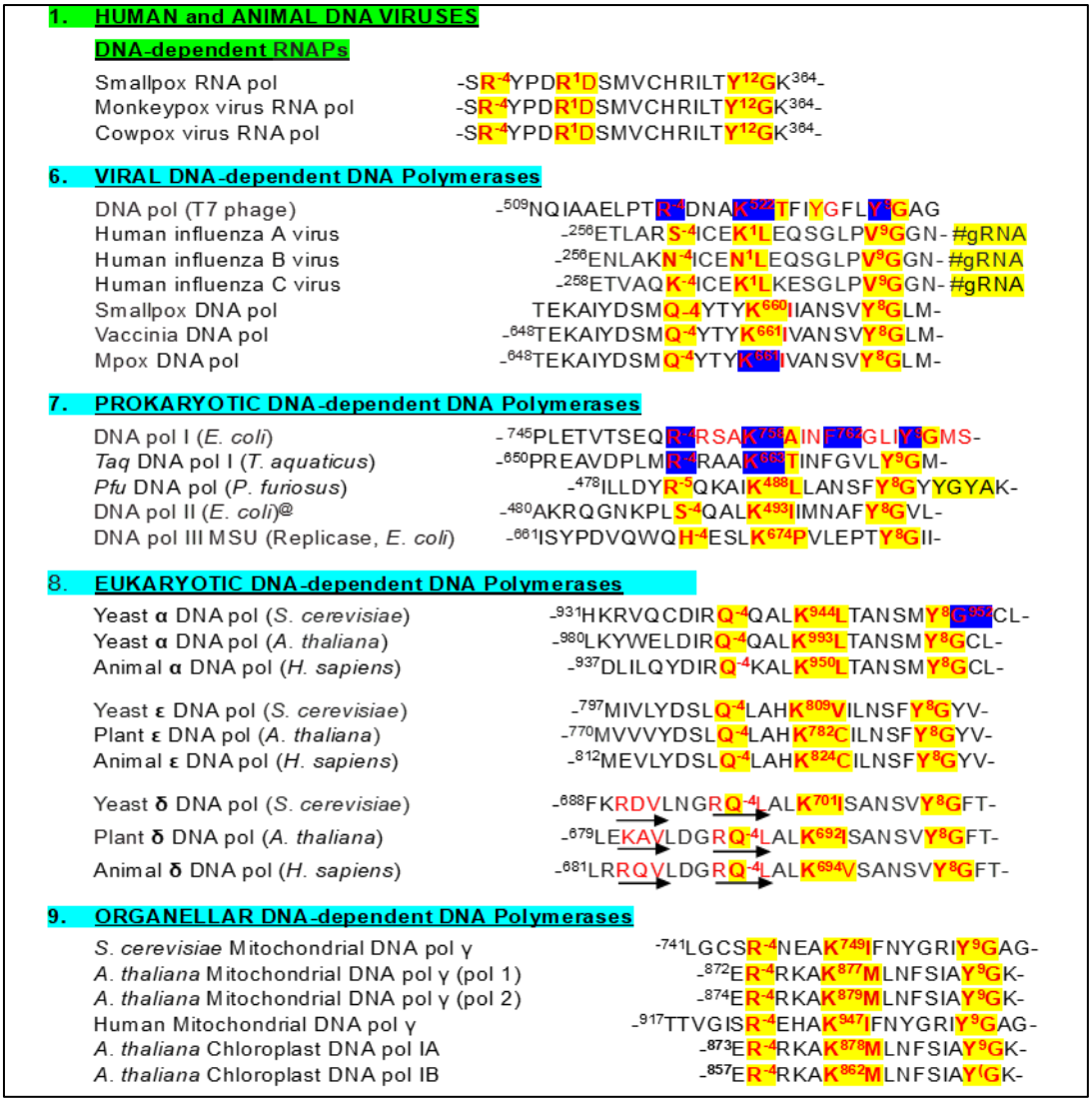
Figure 7 A schematic diagramme showing the steps involved in the polymerization of nucleotidyl transferases (numberings from the *E. coli* DNA polymerase I)

7. Comparative analysis of the active site amino acid domains from the two different transferases

The substrates for the two different transferases, viz. the ribose and fructose have an important similarity in structure, in that, both have a five-membered furanose ring system, suggesting the same active site amino acids could be used for polymerization reactions. This is exemplified in the following two tables. Table 1 shows the highly conserved catalytic amino acids from a large number of DNA/RNA polymerases like, single subunit (SSU) and multisubunit (MSU) DNA polymerases, RNA polymerases, RNA transcriptases from viruses to humans. Irrespective of the source and type of the polymerase, these enzymes use a catalytic triad which consists of a proton abstractor (a basic amino acid, K/R), a template-binding pair located downstream to the proton abstractor (usually an -YG- pair) and a nucleotide selection amino acid (usually a basic amino acid, R, or in some cases a Q or N is also found) at about -4 from the proton abstractor.

Table 1 Conservation of catalytic domain(s) in viral, prokaryotic and eukaryotic RNA/DNA polymerases (Proposed and Confirmed)

Polymerase Type	Polymerase Catalytic Region
1. BACTERIOPHAGES	
SSU RNA Polymerases	
SSU DNA-dep RNAPs	
T7 Viral SSU RNA pol	-620WLAY-8GVTR-4SVTK631RSVMTLAY9GS-
SP6 Viral SSU RNA pol	-612WDSI-8GITR-4SLTK1KPVMTLPY9GS-
T3 Viral SSU RNA pol	-WLAY-8GVTR-4SVTK1RSVMTLAY9GS642-
BPK11 Viral SSU RNA pol	-WLQY-8GVTR-4KVTK1RSVMTLAY9GS634-
2. HUMAN VIRUSES	
(+) Strand RNA Viruses (SARS-Coronaviruses)	
RNA-dep RNAPs (NSP12)	
SARS-CoV-1	-STMTNR-5Q-4FHQK1LLKSIAATRGATVVIGTSKFY22GG597-
SARS-CoV-2	-STMTNR-5Q-4FHQK1LLKSIAATRGATVVIGTSKFY22GG597-
MERS-CoV	-STMTNR-5Q-4YHQK1MLKSMAATRGATCVIGTTKFY22GG598-
(-) Strand, Segmented RNA Viruses	
PB1 Catalytic Subunits of the RNA-dep RNAPs	
Human influenza A virus	-477MSKKK-5SYINR1TGTFEFTSFFYRY14GFV-
Human influenza B virus	-666RTKRN-5RSILN1TDQRNMILEEQCY14AKC-
Human influenza C virus	-464IRRFN-4AVCK1LIGINMSLEKSY13GSL
(-) Strand, Non-segmented RNA Virus	
RNA-dep RNAPs	
Human Respiratory Syncytial Virus	-291CLN-4TLNK1SLGLRCGFNNVILTQLFLY18GD- (catalytic core 1) -640QVQ-5ILAEK1MIAENILQFFPESLTRY17GD- (catalytic core 2)
3. PROKARYOTIC AND EUKARYOTIC MSU DNA-dependent RNAPS (mRNAs)	
a) MSU RNAP Family (Rpb2: Initiation subunits)	
E. coli MSU RNAP β subunit	-539TRER-6AGFEVR1DVHPTHY8GRV568-
S. cerevisiae MSU RNAP II Rpb2 subunit	-851FR-5SLFFR1SYMDQEKKY10GMS869-
S. pombe MSU RNAP II Rpb2 subunit	-840FR-5SIFYR1TYTDQEKKI10GMT858-
A. thaliana MSU RNAP II Rpb2 subunit	-819FRSYR-5DEEKK1MGTLVKEDF10GRP840-
Human MSU RNAP II Rpb2 subunit	-806FR-5SVFYR1SYKEQESKK10GFDQ825-
b) MSU RNAP Family (Rpb1: Elongation subunits)	
E. coli MSU RNAP β' subunit	-833NSV-6DAVKVR1SVVSC6DTDFGVC13AHC16Y17GRDL861-
S. cerevisiae MSU RNAPII Rpb1 subunit	-55DPR-6LGSIDR1NLKC5QTC8QEGMNEC15PGHF19GHI84-
P. pastoris MSU RNAPII Rpb1 subunit	-55DPK-6LGSIDR1NFKC5QTC8GEGMAEC15PGHF19GHM84-
A. thaliana MSU RNAPII Rpb1 subunit	-54DTR-7LGTIDRK1VKC4ETC7MANMAEC14PGHF18GYL83-
Human MSU RNAPII Rpb1 subunit	-59DPR-6QGVIER1TGRC5QTC8AGNMTEC15PGHF19GHI88-
4. ORGANELLAR SSU RNAPs (NEPs)	
Mitochondrial RNA pol (*S. cerevisiae*)	-TR-4KVVK1QTVMTNVY9GV1024-
Mitochondrial RNA pol (*A. thaliana*)	-QVDR-4KLVK1QTVMTSVY9GV762-
Mitochondrial RNA pol (*Nicotiana sylvestris*)	-QVDR-4KLVK1QTVMTSVY9GV788-
Mitochondrial RNA pol (*H. sapiens*)	-TR-4KVVK1QTVMTVVY9GV1001-
Chloroplast RNA pol (*Oryza sativa*, Japonica)	-QVDR-4KLVK1QTVMTSVY9GV739-
Chloroplast RNA pol (*A. thaliana*)	-QVDR-4KLVK1QTVMTSVY9GV779-



Adapted from Palanivelu [1-3, 43, 44]

NEPs, Nuclear Encoded Pols; #gRNA. Genomic RNA (replicase type); Repl, Replicase. Pfu, *Pyrococcus furiosus.*

The confirmed active site amino acids (by SDM, X-ray crystallography and active site-directed inhibitors) are highlighted in dark blue. In *E. coli* pol I, the replacement of K^{758} with Ala caused a 1,000-fold reduction in k_{cat}. The Ala substitutions result in moderate to severe effects on the polymerase activity of the individual mutant enzymes. Severe loss of activity is associated with R^{754}→A, K^{758}→A, F^{762}→A, and Y^{766}→A (highlighted in dark blue) in the *E. coli* DNA pol I. Tyr ^{766} and Phe^{771} are either involved in template-primer binding or are in the vicinity of the DNA-binding track. Residues Arg^{754}, Lys^{758}, Phe^{762}, and Tyr^{766} appear to be required for the binding of Mg.dTTP, while only Arg^{754} and Lys^{758} are utilized in the polymerization of Mn.dTTP [45 and references therein].

In *Taq* pol I, the invariants R^{659} and K^{663} in the active site were found to be immutable [45 references therein].

Table 2 shows the highly conserved catalytic amino acids in the hydrolytic and transferase active site domains of fructosyl transferases, viz. levansucrases and inulosucrases. It is interesting to note, that the fructosyl transferases also follow the same transferase active site amino acid pattern like the nucleotidyl transferases and their active site is also composed of a similar catalytic triad, where a basic amino acid (K/R/H) acts as the proton abstractor, a -L/I/V/YG- could act as the template-binding pair which is placed downstream to the proton abstractor and a donor (Fru) selection amino acid usually an R which is placed at -3 from the proton abstractor. It is interesting to note that the *G. stearothermophilus* enzyme is reported to be inactive where the regular basic amino acid K is replaced by a Q.

Table 2 Proposed amino acids in the active site of hydrolytic and transferase domains of levan- and inulosucrases from different organisms.

Invertase module's active site	Transferase module's active site				
Fru- transferase type	**Nucleophile**	**GBM**	**Proton Donor**	**FBM**	**Fru selection/Proton abstractor/ Template-binding pair**
1. Levansucrases					
Bacteria					
Bacillus subtilis (P05655)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSK^{1}MTIDGITSNDIYML^{15}GYV-
Geobacillus stearothermophilus (P94468)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSQ^{1}MTIDGITSNDIYML^{15}GYV-
Salibacterium salarium (A0A428MZE4)	-WDTW-	-QWSG-	-EIER-	-FRDP-	-AR^{-3}GSK^{1}MTIDGIDDEDIYML^{15}GYV-
Bacillus megaterium (A0A3D8X658)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSK^{1}MTIDGIGQDDVYML^{15}GYV-
Paenibacillus graminis (A0A089MCV7)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSK^{1}MTIDGIGANDVYML^{15}GYV-
Clostridium saccharobutylicum (A0A1S8MP69)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSK^{1}MTIDGISDKDIYML^{15}GYV-
Clostridium pasteurianum (R4K861)	-WDSW-	-QWSG-	-EIER-	-LRDP-	-SR^{-3}GSK^{1}MTIDGIGNKDVYML^{15}GFT-
Gluconacetobacter diazotrophicus	-WDTW-	-EWSG-	-QTER-	-FRDP-	-FT^{-3}ISH^{1}RTTFAAGVDGPDGVY^{16}GFV-
Beijerinckia indica	-WDTW-	-EWSG-	-QTER-	-FRDP-	-FT^{-3}ISH^{1}RTTYAAGVDGPDGVY^{16}GFV-
Erwinia amylovora	-WDTM-	-EWAG-	-QTER-	-FRDP-	-FT^{-3}ISH^{1}KYTFADNLTGPDGVY^{16}GFV-
Erwinia tasmaniensis	-WDTM-	-EWAG-	-QTER-	-FRDP-	-FT^{-3}ISH^{1}KYTFADNLTGPDGVY^{16}GFV-
Zymomonas mobilis	-WDTW-	-EWAG-	-QTER-	-FRDP-	-FT^{-3}ISH^{1}HSTYADGLSGPDGVY^{16}GFV-
2. Inulosucrases					
a) Eubacterial Inulosucrases					
Lactobacillus delbrueckii (EOD02545.1)	-WDSW-	-QWSG-	-EVER-	-LRDA-	-GR^{-3}LTR^{1}ATDTDLLAKANSTIGDNVAML^{22}GFV-
Streptococcus salivarius (VTY24076.1)	-WDSW-	-QWSG-	-EVER-	-LRDP-	-SR^{-3}ISK^{1}STDAEGTVAVRGAVGDDVVML^{22}GFV-
Streptococcus mutans (VTY49811.1)	-WDSW-	-QWSG-	-ELER-	-MRDP-	-SR^{-3}LNH^{1}GSNNDAWNKANEVVGDNVVML^{22}GYV-
Convivina intestine (CAH1856476.1)	-WDSW-	-QWSG-	-EIER-	-LRDS-	-TR^{-3}LNR^{1}GSNDYAWQKANRVIGDNVAM^{22}GYV-
Limosilactobacillus reuteri (Q8GP32)	-WDSW-	-QWSG-	-EIER-	-MRDA-	-TR^{-3}LNR^{1}GSNDDAWMNANYAVGDNVAMV^{22}GYV-
Streptococcus thermophilus (ACZ67286.1)	-WDSW-	-QWSG-	-EIER-	-MRDA-	-TR^{-3}LNR^{1}GSNDDAWMAANKAVGDNVAM^{22}GYV-
b) HaloArchea Inulosucrases					
Halofera gibbonsii	-WDTW-	-QWAGS--ELER-		-FRDP-	-FV^{-3}SSH^{1}VHTFAPGLTGYDALY^{16}GFV-
Natronococcus amylolyticus	-WDTW-	-QWAGS--ELER-		-FRDP-	-FL^{-3}SSH^{1}EHTFAEGLEGYDALY^{16}GFV-
Halalalkalicoccus jeotgali	-WDTW-	-QWAGS--ELER-		-FRDP-	-FV^{-3}SSH^{1}DHTFAPGLEGPDGLY^{16}GFV-
Halomicrobium sp.	-WDTW-	-TWAGS--ELER-		-FRDP-	-FV^{-3}SSH^{1}DHTFAPGLDGYDALY^{16}GFV-
Haloarcula sp	-WDTW-	-QWAGS--ELER-		-FRDP-	-FI^{-3}SSH^{1}LHTFAPGLEGFDALY^{16}GFV-
Halorubrum saccharovorum	-WDTW-	-QWAGS--ELER-		-FRDP-	-FV^{-3}CSH^{1}VHTFAPGVTGPDGLY^{16}GFV-

NB: GBM, Glucose-binding motif; FBM, Fructose-binding motif.

8. Conclusion

The data presented here reveal that the enzyme active sites are designed not at random, but based on the chemical basis of a particular reaction. Therefore, it is imperative that each enzyme active site is designed based on the molecular interactions between specific amino acids at its active site and its corresponding substrate. The present data also confirm that not only the same active site amino acids are employed for the same type of reactions, but are also conserved and adapted from viruses, bacteria, plants to humans during the long evolutionary process. It is likely that the transferase domain of fructosyl transferases harbouring a similar active site amino acid structure as the nucleotidyl transferases might have been acquired from them during the evolutionary process. After tight binding of an enzyme to its substrate, the movements of the subatomic particles, viz. the proton and electron flow, could take place spontaneously between the active site and substrate molecule, effecting transformation in the substrate. A deeper understanding of the evolution of active sites of enzymes could play an important role in enzyme engineering to design novel enzymes for degrading recalcitrant substrates and also in drug development.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declares no conflicts of interest.

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