Journal of Global Biosciences
Peer Reviewed, Refereed, Open-Access Journal
ISSN 2320-1355
Volume 10, Number 5, 2021, pp. 8685-8705
Website: www.mutagens.co.in
URL: www.mutagens.co.in/jgb/vol.10/05/100502.pdf

SCAN ME

Review Paper

PULLULANASE FUNCTIONS IN STARCH HYDROLYSIS AND THE IMPROVEMENT OF ENZYME ACTIVITY USING ACTIVE HYDROGEN BOND NETWORK AS METHOD WITH POTENTIAL INDUSTRIAL APPLICATION OF THE ENZYME

Ndochinwa, O. G.[1], Qing-Yan Wang[2], O. C. Amadi[1], T. N. Nwagu[1], C. I. Nnamchi[1] and A. N. Moneke[1]

[1]Department of Microbiology Faculty of Biological Science,
University of Nigeria, Nsukka, Nigeria,
[2]State Key Laboratory of Biomass Enzyme Technology,
National Engineering Research Center for Non Food Biorefinery,
Guangxi Academy of Sciences, Nanning, Guangxi,
China.

Abstract

The industrial application of pullulanase (EC 3.2.1.41) has recently increased in starch based industries for glucose production and ethanol biorefinaries that uses starch as potential raw mateirlas. The use of crude oil as source of energy has seriously damaged the ozone layer and also leads to climate change which is the major cause of disease outbreaks and pandemic. Pullulanase; an important debranching enzyme that specifically hydrolyses the α 1, 6 glucosidic linkages in starch, amylopectin, pullulan and other related oligosaccharides which enables a complete and efficient conversion of branched polysaccharides into smaller fermentable sugars. The industrial production of glucose is carried out in two different process, the liquefaction and the saccharification process. Pullulanace has been used to increase the final glucose concentration with reduced amount of glucoamylase during the saccharification process, thereby preventing the reverse reaction that involves resynthesis of saccharides from glucose molecules. (i) pullulanase type I, (ii) amylopullulanase, (iii) neopullulanase, (iv) isopullulanase, and (v) pullulan hydrolase type III. are five groups of pullulanase enzymes that have been reported The current paper extensively reviews components of starch, starch-converting enzymes , pullulanase and its function, biotechnology and bioprocess (functions and importance), genetic engineering , protein science, role of hydrogen bond in DNA duplexes, methods for altering protein stability using active hydrogen bond network as methodology.

Key words: Pullulanase; hydrogen bond; amylose; amylopectin; hydrolysis.

INTRODUCTION

1.1 Components of Starch

Starch is a complex polysaccharide made up of a large number of glucose units joined together by glucosidic bonds. It is a major raw material that can processed into variety of products ranging from food industries (glucose and fructose syrup) to textile industries to paper making industries to washing detergent industries and finally to bioethanol refinery (for industries that uses cassava starch as potential raw material) [1]. It is made up of C, H and O atoms in the ratio of 6:10:5 ($C_6H_{10}0_5$). Each of these atom is linked to each other through C1 oxygen as glucosdic bond. Treatment of these bond with alkaline solution makes it stable while acid and enzyme breaks it down into its constituent glucose molecule.

Starch are made up of two polymers with high molecular weight

i) A linear chain molecule of amylose

ii) A branch polymer of amylopectin.

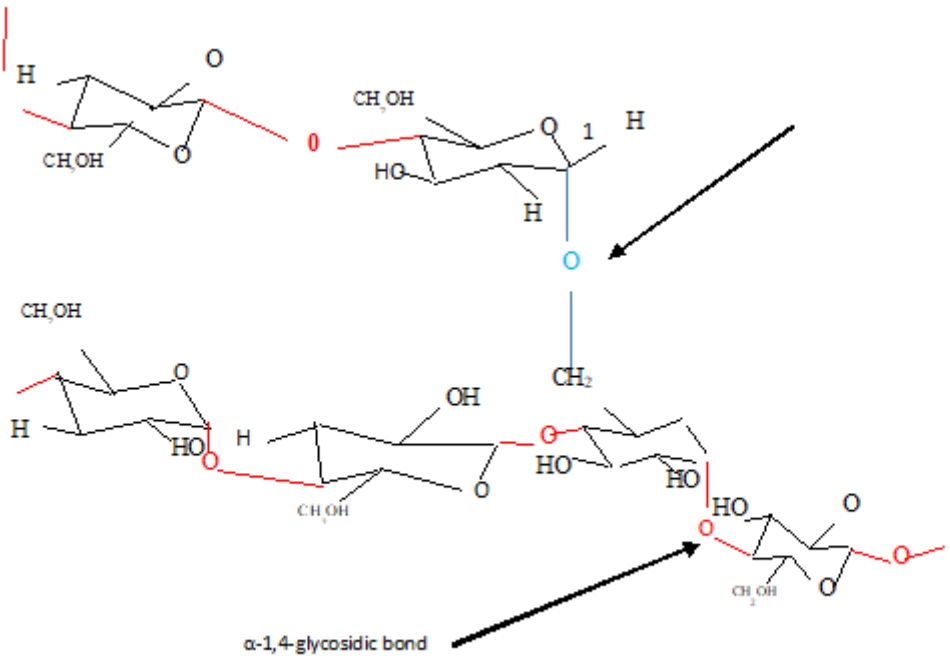

Fig. 1. Section of amylopectin molecules showing α-1,4 and α-1,6 chain linkage in starch [2].

2. AMYLOSE

Amylose consist of linear, helical chains of roughly 500 to 20000 alpha D-glucose monomers linked together through α1, 4 glycosidic bonds. (Tester et al., 2004). A trace amount of branching is also present in the amylose molecule, although the side chains through branches are shorter in amylose than those in amylopectin [3]. The degree of polymerization, level of amylose and weight varies from different type of starch sources [3].

3. AMYLOPECTIN

One of the largest molecule in nature is amlyopectin, it has an average depolymerization of about 2 million. Amylopectin differ from amylose due to the presence of additional α 1, 6 glycosidic bond which occurs every 20-30 anhydroglucuose units. Aqueous solution of amylopectin has the following characteristics; they are very stable, they have high viscosity, they are highly resistance to gelling, they have high clarity and finally they bind weakly with iodine and the complex typically gives a red colour.

Majority of starch used in the manufacture of glucose syrups contains 75% to 85% of amylopectin [4]. Cassava, sorghum, barely, potato, pea and most other plants contains amylopectin ranging from 60% up to 90% and in some cases reached about 100% of the entire cultivars [3].

Due to the complexity of starch polymer, it requires a combination of enzymes (endoamylases and exoamylases) for the depolymerization of starch into oligosaccharides and smaller sugars or the transformation of starch by transferring oligoglucosidic linkages, residues and creation of a new bond [5,6,7].

The enzymes commonly used for starch processing are generally classified as amylases [8,5]. Basically, there are four types of starch converting enzymes: I, endoamylases, II, exoamylases III, debranching enzymes, IV, transferases.

Endoamylases: Endoamylases are those starch converting enzymes that cleave to α-1, 4 glucosidic bond present in the inner part of the amylose or amylopectin chain. Enzyme α-amylase (EC 3.2.1.1) is an example of endoamylase enzyme that hydrolyses α-1,4 linkages in the interior part of the starch polymer which leads to formation of linear and branched oligosaccharides . These enzymes are found in a wide variety of microorganism belonging to Bacteria and Archaea [9,5].

Exoamylases: Exoamylases are those starch converting enzymes that cleave to both α-1,4 and α-1,6 glucosidic bond on the external glucose residues of amylose and amylopectin. Example of these enzymes are glycoamylases (EC 3.2.1.3) and α glucosidases (EC3.2.1.20). Also β amylases (EC 3.2.12) which is another example of exoamylase enzyme cleaves exclusively to α-1,4 bonds produced by maltose and β limit dextrin [5,10,6,11].

Transferases: Transferases are those group of starch converting enzymes that cleave to α-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glucosidic acceptor with a formation of new glucosidic bond . Example of these group of enzymes includes amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyl transferase (EC 2.4.1.19) which forms a new α-1,6 glucosidic bond [5,7].

Finally the last group of starch converting enzyme which is our main interest in this review is the debranching enzyme. Starch debranching enzymes are those enzymes that specifically hydrolyses α-1,6 glucosidic bond in amylopectin or glycogen with other related polymers. The affinity of debranching enzyme for α 1,6 bond distinguishes these enzymes from other amylases which have primary affinity for α-1,4 gycosidic linkages. They are classified into to major groups which are direct and indirect debranching enzyme [12].

Indirect Debranching Enzyme: These are starch debranching enzymes that requires modification of substrate by another enzyme or enzymes prior to their debranching action [13,12] and it is normally present in animal and yeast [3]. These enzymes requires the removal of an oligosaccharide by transglucosylase first (4 –α glucanotransferase E C 2.4.1.25), leaving a glucose residue bound to a tetrasaccharide through an α 1,6 bond [13,8,3]. Amylo 1,6 glucosidase will only hydrolyse an α1, 6 branch point if the side chain consist of a single glucose unit. Branch pentasaccharid-6 α glucosyl maltotetraose is the known smallest substrate that can be hydrolyzed by amylo-1,6 glucosidase and the products are hydrogen and maltotetraose.

Direct debranching enzyme: These enzymes hydrolyes α 1,6 glucosidic bonds of unmodified substrate. They are classified into pullulanase and isoamylases or R-enzymes on basis of substrate specificity but our main interest in this review are pullulanses.

4. PULLULANASE AND ITS UNCTIONS

These enzymes (pullulan-6-glucano hydrolase EC 3.2.1.41) are defined as debranching enzymes which can specifically hydrolyze α 1,6 glucosidic linkages in pullulan, starch, amylopectin and other oligosaccharides [1]. They are divided into two major categories on basis of their substrate specificities and hydrolytic properties. A, Type I pullulanase specifically catalyze the hydrolysis of α-1,6 glucosidic linkages in pullulan and other branched oligosaccharides to form maltotriose or linear oligomers . B,Type II pullulanase (amylopullulanases) catalyzes the hydrolysis of both α-1,6 glucosidic linkages in pullulan and also cleave to α 1,4 glucosidic bond in other polysaccharides such as amylopectin, glycogen and starch (Li et al., 2012). Based on their amino acid sequence similarities, pullulanases have been assigned to glycoside hydrolase (GH) families [14]. These enzyme belongs to the α-amylase family and was first isolated from Aerobacter aerogenes [15]. Pullulanases are widely distributed in bacteria (*Bacillus* spp, *Aerobacter* spp, *Klebsiella* spp) yeasts, fungi, plants and animals; among these, *Bacillus* spp produce majority of pullulanases that are widely used in starch processing industries.

These enzymes are used in different starch processing industries because addition of it during saccharification helps to hydrolyze the α1,6 glucosidic bonds of the branched dextrins which allows maximum usage of glycoamylase and also reduces the saccharification time thereby ensuring well concentrated and converted substrate. Pullulanases are widely used in food.

Pullulanase are also used in food processing industries for the production of high maltose corn syrup, fructose syrup, cyclodextrins etc. [16]. It is also used in Chinese biofuel industries where cassava starch is used as raw material [17]. A number of pullulanases encoding genes have been cloned and characterized with their crystal structures determined examples includes *Bacillus* spp [18] *Geobacillius thermolevorans* [19]. *Bacillius* spp CICIM 263 [20] *Lactococcus lactis* [21], *Bacillus naganoensis* [22,16], *Bacillus naganoensis* BNpulA324 [17]. However, most pullulanases are type II enzymes (Li et al., 2012).

4.1 Biotechnology and Bioprocess (Functions and Importance), Genetic Engineering

Bioprocess engineering covers biocatalysts development and actual application of enzymes in industrial process. In this context, enzyme technology is an important activity that can have major impact on the utilization of enzymes in industrial concepts.

Enzyme technological concept can force enzymatic reaction to proceed in a desired direction, enhance their selectivity and also stabilize enzymes towards the reaction conditions required for large scale synthesis. Enzyme technology complements enzyme engineering which addresses enzyme characteristics at molecular level [23].

The use of enzyme and whole cells as biocatalyst for industrial synthetic chemistry is rapidly growing, because biocatalyst exhibit exquisite catalytic power (high selectivity and high stability) unmatched by conventional catalysts. In the past few decades biocatalyst have been successfully exploited for the synthesis of complex drug intermediates, specialty chemicals and even commodity chemicals in pharmaceutical, chemical and food industries. All these were achieved with recent advances in recombinant DNA technologies.

4.2 Importance of Biotechnology and Bioprocess

It has been demonstrated that enzyme is highly specific both in nature of substrate it utilizes and the type of reaction it catalyzes. The lock and key hypothesis that was proposed long ago proved to be a fruitful way to depict the binding of enzyme to the substrate but with the help of enzyme engineering it has now become possible to alter catalytic activity and specificity on enzyme. The use of enzyme in industry is often limited by lack of stability under extreme conditions. Most of the mesophilic enzyme are often not well suited for the harsh reaction conditions required in industrial processes due to lack of structural stability. Protein engineering is an efficient method to improve the above-mentioned properties in normal mesophilic proteins. Finally, production of enantiomeric pure compounds is of steadily increasing importance to the world market for chiral fine chemicals, pharmaceuticals, agrochemicals and flavour compounds. It is important to know that the growing requirements for the synthesis of optically active compounds can be met either by asymmetric catalysis or biocatalysis using enzyme. It has also been observed that in most of the cases, enantio-selectivity of a given enzyme is not as high as what is required for desired reaction. Therefore, it is necessary to develop novel methods that allow creation of enantioselective enzymes [24].

4.3 Genetic Engineering of Pullulanase

Pullulanase produced by wild type microoraganism has low activity of the enzyme which is not enough to meet the demand for biotechnological application. Molecular cloning of the corresponding genes and their expression in heterologus hosts is one of the possible solution to this problem. Large quantities of specific gene can be isolated in

pure form by molecularcloning and the target DNA can be produced in large quantity under the control of the expression vector. Over expression after the cloning step can increase significantly the enzyme yield by sub cloning the target gene into a suitable expression vector.

Based on this mechanism a complete hydrogen bond network of Bacillius nangneonsis (BNPULA324) was studied and the result showed that the active region of an enzyme is relatively simple and independent. In pullulanases the active region is the place surrounding the three active residues which are Asp 511,Glu 540, Asp 625 and these amino acids are known as catalysis triad at the top of (α/β)8 TIM barrel. Starting from the three active residues (Asp 511, Glu 540 and Asp625) tracing the hydrogen bonds, the active hydrogen bond network (AHBN) of pullulanase BNpulA324 is identified. In AHBN the three active residues (Asp 511, Glu 540 and Asp625), twelve functional residues (Glu 326, Tyr 398, His 445, Thr 477, Arg 509, Trp 542, Asp 570, Arg 573, Tyr 620, His 624, Asn 626, Asn 680) and eighteen water molecules (W 311, W602, W603, W604, W605, W630, W659, W662, W724, W727, W729, W730, W731, W734, W786, W 788 and W 952) are cross linked by hydrogen bonds. In AHBN each water molecule is connected by 2 to 4 hydrogen bonds. Also, in hydrolysis reaction of pullulanase, (BnpulA324) AHBN is responsible for the transportation of protons and water molecules which influence the reaction activity, pH sensitivity and thermal stability of pullulanase (BnpulA324) [17]. The DNA encoding the gene for some functional residues were mutated to cause over expression of the cloned gene.

5. PROTEIN SCIENCE

Pauling and Mirsky in 1936 when the structure of globular protein was beginning to be understood concluded that this chain is folded into a uniquely defined configuration in which it is held by hydrogen bonds between the peptide nitrogen and oxygen atoms [25]. They also suggested that each hydrogen bond would contribute 5 kcal/mol to the stability of the uniquely defined configuration [25]. Pauling et al. [26] made use of restriction derived from structural studies of model compounds and their ideas about hydrogen bonds to discover the most important of these configuration, the α helix and β pleated sheet [26]. In the paper they described the α helix as stated below:

> "the energy of an N-H..O=C hydrogen bond is on the order of 8 Kcal/mol and such great instability would result from the failure to form these bonds that we may be confident of their presence"

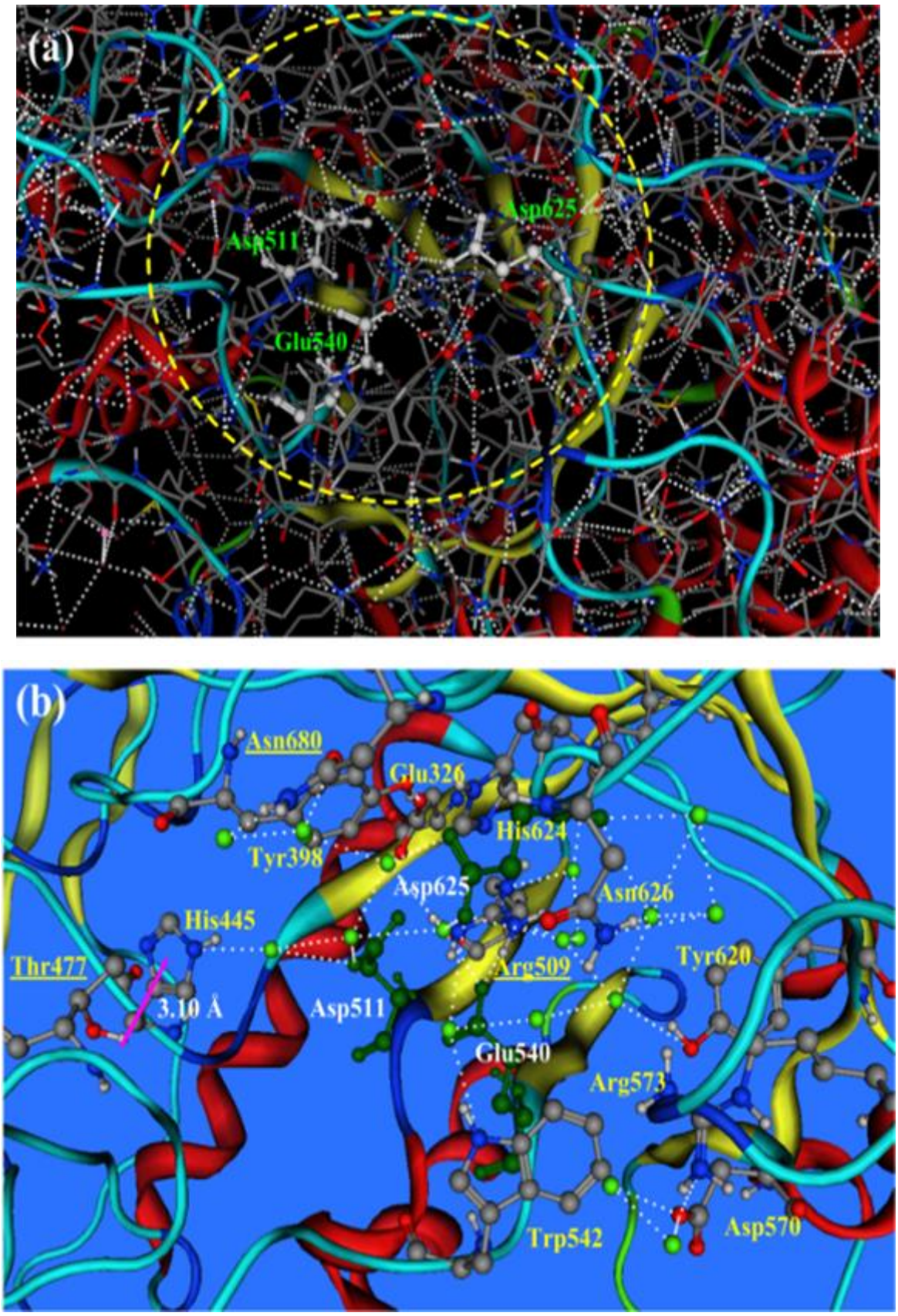

Fig. 2. (A) The complete hydrogen bond network in pullulanse BNPulA324 showing the active residue
(B) Hodrogen bond network of BNPulA324 showing the functional residue [17]

Later the same year when they had reached a better understanding of the β pleated sheet, they suggested that with protein in aqueous environment, the effective energy of hydrogen bond is not great, in as much as the difference between the energy of the system with N-H…O hydrogen bonds surrounded by water and a system with N-H group with the oxygen atom forming hydrogen bonds with water molecules may not be more than 2 Kcal/mol. After sixty years of Paul and Mirsky research paper, there is still uncertainty about the contribution of hydrogen bonds to protein stability (-NH.. O=C-) protein + $2H_2O \longleftrightarrow H_2O..HN- + C=O.H_2O$. It was not clear whether the free energy change for this reaction was favorable or unfavorable based on model compound data. Example Klotz suggested -750 cal/mol and Schellman suggested +400 cal/mol as free energy for this reaction. In 1959 Kauzmann review made a strong case for the importance of hydrophobic bonds but concluded that it is likely that hydrogen bonds between peptide links and hydrophobic bonds are by far most important in determining the overall configuration of protein molecule (Kauzmann). He went further to state that it could be that the peptide hydrogen bond and hydrophobic bonds are barley sufficient to stabilize the native configuration of some protein and that relatively few of the less abundant bonds contribute the decisive increment that stabilizes the native configuration, therefore it is not safe to conclude that any of the bond is less important than other. Progress was made 30 years after the Kauzmann's review when Dill in an excellent review published in 1990 concluded that there is evidence that hydrogen bonding or van der waals interactions among polar amino acids may be important in protein stability but their potential remains poorly understood [27]. Fersht conducted an experiment on engineered enzymes modified inhibitors and synthetic DNA duplexes and concluded that an individual hydrogen bond contributes 0.5 to 1.8 kcal/mol to binding energies which is meaningful contribution to protein stability [28,29]. Since then several studies of hydrogen bonding groups in individual proteins using site directed mutagenesis methods have reached similar conclusion. .

RNaseT1 [30,31,32], Barnase [33] Arc Repressor [34] BPTI [35] RNase sa [31,36,37,38] Staphylococcal nuclease (Green et al.,1992, [39]) human lysozyme (Yamagata et al.,1998, [40]) membrane protein bacteriorhodopsin [41] and pullulanase [17].

It is very important to note that electronegative atom that attached covalently to hydrogen atom play a role of the hydrogen bond donor example O, F,N. Also when carbon atom bound to electronegative atoms as in case of chloroform $CHCl_3$, hydrogen

atom attached to carbon also participate in hydrogen bonding. Finally, in hydrogen bond, the electronegative atom that is not covalently attached to the hydrogen is known as proton acceptor whereas the one covantely attached to hydrogen is known as proton donor.

5.1 Role of Hydrogen Bond

In determining the three dimensional structures and properties adopted by many synthetic and natural proteins, hydrogen bond plays a very vital role. Bonding between parts of the same macromolecules cause it to fold into a specific shape which helps to determine the molecules physiological and biochemical role. Example the double helical structure of DNA consist of due largely hydrogen bonding between its base pair and also the π stacking interaction which link complementary strand to the other and enable replication.

The secondary structure is determined by hydrogen bond between amide group NH2 and the carboxyl group (C=O) of another residue. A hydrogen bond is denoted as X-H.Y where X and Y are the electronegative donor and electronegative acceptor respectively and the strength ranges from 1.0 to about 40kcal. Although the strength of hydrogen bond is relatively weak compared to other covalent bond, a combination of several hydrogen bonds as well as other covalent process may also lead to the formation of highly complex supramolecular aggregates.For complexes of different hydrogen bond, studies have aimed to estimate or determine the total interaction energies, but the evaluation of the individual strength of each hydrogen bond is important, which allows for the main factors contributing to the total binding energy between the fragments to be determined and this particular information is important for rational design of new strategies for molecular recognition.

It was suggested by Dannenberg et al., that an energy of a given hydrogen bond could be determined by computing the binding energy of a hypothetical twisted structure in which two bases are bonded by this hydrogen bond only [42]. The hypothetical structure can be formed by rotating one of the bases with respect to the other about the axis of this hydrogen bond so that other bond is broken (it is important to note that this methodology is not applicable to more complex structure without causing steric interaction). He concluded that since hydrogen bond strength obtained in this way corresponds to the energy of a given hydrogen bond in the absence of others, the difference between the sum of the individual hydrogen bond energies and the total

interaction energy of all hydrogen bond could be used to measure the co operativity of the hydrogen bonding interactions. Very recently, Scheiner proposed that the energy of each individual hydrogen bond and could be approximately taken as the energy difference between the original system and the modified system with some groups being replaced by hydrogen atoms [43].

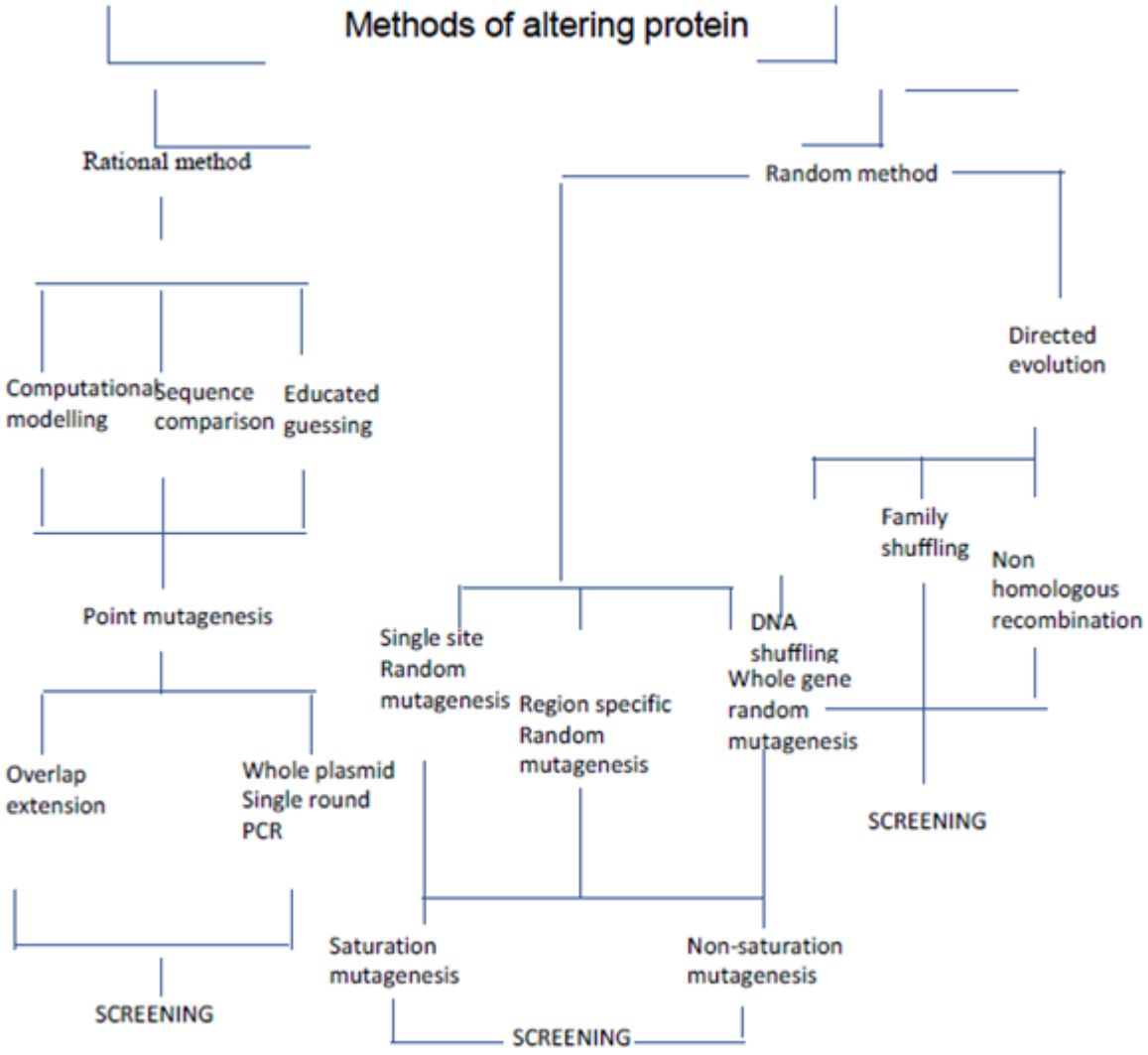

Fig. 3. Method of altering protein selectivity/ stability [44]

Random methods for altering protein selectivity/stability which consist of random mutagenesis and directed evolution. Use of methods for the rational mutagenesis of protein is often limited by difficulty of predicting what specific amino acid replacements will lead to the preferred altered selectivity/stability, even when high resolution structural data are available. Simple substitution can lead to changes in protein tertiary structure and flexibility that affects both the catalytic activity and substrate specificity in unpredictable ways. Accordingly, methods have been designed and developed in

which individual amino acids are randomly muted to any of the other naturally occurring amino acids. Such methods which require little understanding of both the structure and function of the protein leads to the generation of a library proteins that is then screened to identify novel enzyme variants that display the desired phenotype. Critical to the success of these approaches to protein engineering is the availability of an efficient and reliable means of screening the library for activity. When the library size is modest, established assays for a particular enzymatic reaction can often be adapted in relatively straight forward manner to determine the catalytic activity of the mutants. However, when large libraries are produced other procedures must be developed and employed.

5.2 Techniques for Random Mutagenesis and Application

The simplest technique for random mutagenesis is termed saturation mutagenesis and it involves changing one promising amino acid in a protein with each of the remaining 19 amino acids to give every possible variant at that site. This method, which is often used in combination with other techniques is especially useful for gaining insights into the structure -function relationship of a biologically active peptide ligand [45] or an enzyme [46] because the effects of introducing each amino acid at a given position may be explicitly evaluated.

Redesigning enzyme may also be applied to producing enzymes and other proteins that may serve as medicinal drugs that are known as macromolecular human therapeutics [47,48]. Although the use of enzyme as therapeutic agents currently constitutes only a minor portion of pharmaceutical production, such proteins have significant potential for treating a variety of disease states. Several examples will illustrate the essential concepts and some of the possibilities. Thrombin is a serine protease that proteolytically cleaves fibrinogen and protein C, respectively to stimulate coagulant and anticoagulant pathways. Towards the goals of creating a variant having enhanced anticoagulant activity, the crystal structure of thrombin was inspected after identify surface was exposed to polar or charged residues that would likely be involved in electrostatic and hydrogen bond interaction with charged ligands and alanine scanning which was performed at these positions [49], because these alanine replacements at W50, K52, E229 and R223 produced variants favoring the anticoagulant substrate protein C, these residues were individually substituted with 19 naturally occurring amino acid by saturation mutagenesis. The resulting library of thrombin variants was

then screened for increased selectivity for protein C and clotting activity was measured by release of fibrinopeptide A [50]. These studies led to the discovery of the E229K thrombin variant which exhibited a substrate specificity that favored protein C activation over fibrinogen cleavage by 130fold. Thrombin variants such as E229K having improved anticoagulant might be useful in treating strokes or myocardial infraction.

Rational methods for altering protein substrate selectivity/stability Rational approaches directed towards altering enzyme selectivity and stability involve replacing amino acids at specific sites of the protein with another amino acids that are selected based on a combination of mechanistic and structural knowledge [51]. This method depends on a detailed understanding of the determinants of substrate selectivity and catalytic mechanism of the enzyme. Although this requirement limits the rational approach to well define system, an advantage of rational point or site directed mutagenesis relative to random and evolution-based approaches is that only small number of variants must be examined. Hence, the specificities of the mutant can be quickly determined by comparing their respective specificity constants which are defined by ratio of the turnover constant Kcat and the Michaelis constant Km. Rational mutagenesis is also useful for testing hypothesis about the structural and functional roles of specific amino acid residues in a protein.

5.3 Techniques for Rational Method and Application

Two techniques are commonly used to introduce specific amino acid changes into target gene.

The first of these is known as overlap extension method. In this method, four primers which are short sequences of synthetic DNA complementary to a section of the gene of interest are used in the first polymerase chain reaction (PCR) step where two separate PCRs are performed. The primer pairs for these PCRs are 1/3 and 2/4 respectively,with primer 2 and 3 containing the mutant codon with a mismatched sequence. Two double stranded DNA products are obtained. When these double stranded duplexes are heated to denature and cooled to anneal, two heteroduplexes are produced where in each strand of the heteroduplex contains the desired mutagenic codon X. The overlapping 3'and 5'ends of each heteroduplex are then filled in using DNA polymerase and the second PCR step using primers 1 and 4 amplifies the mutagenic DNA. The second method for performing site directed mutagenesis is kown to as whole plasmid single-

round PCR. In this protocol, two oligonucleotide primers containing the desired mutation and complementary to the opposite strand of the double-stranded DNA plasmid template are extended using DNA polymerase. In this PCR step, both strands of the template are replicated without displacing the primer to afford the mutated plasmid containing breaks that do not overlap. Because the original wild type plasmid originates from Escherichia coli and thus methylated on various A and C residues, it may then be selectively digested using Dpnl methylase endonuclease to cleanly give a circular nicked vector containing the mutant gene. When this nicked vector is transformed into competent cells, the nick in the DNA is repaired by cell machinery to give a mutated circular plasmid. High resolution structural data of protein and sometimes its complexes with biologically relevant small molecules have historically been used to provide a starting point for deciding what amino acid residue to vary. However computational methods are also being used to provide insights that facilitate altering the properties of enzymes. There are numerous examples of the use of site directed mutagenesis to create proteins that exhibit novel properties including specificity, function and stability but few case studies will serve to illustrate the features of this method.

Starting from the three active residues (Asp 511, Glu 540 and Asp625) tracing the hydrogen bonds, the active hydrogen bond network (AHBN) of pullulanase BNpulA324 is identified. In AHBN the three active residues (Asp 511, Glu 540 and Asp625), twelve functional residues (Glu 326, Tyr 398, His 445, Thr 477, Arg 509, Trp 542, Asp 570, Arg 573, Tyr 620, His 624, Asn 626, Asn 680) and eighteen water molecules (W 311, W602, W603, W604, W605, W630, W659, W662, W724, W727, W729, W730, W731, W734, W786, W 788 and W 952) are cross linked by hydrogen bonds. In AHBN each water molecule is connected by 2 to 4 hydrogen bonds. Also, in hydrolysis reaction of pullulanase, (BNpulA324) AHBN is responsible for the transportation of protons and water molecules which influence the reaction activity, pH sensitivity and thermal stability of pullulanase (BNpulA324) [17]. Based on this knowedege a site directed mutagenesis was done and the results showed improved thermal stability and pH sensitivity. Mutation at Arg509 (a functional residue of AHBN that belongs to BNpul A324) which is sited at the middle of the fourth β strand in the Tim barrel which directly connects the active residues Asp511 and Glu540, two water molecules (W602 and W603) and indirectly connects active residue Asp625 through functional residue

His624. It was observed that Arg509 plays important role in the hydrolysis catalysis reaction and transportation of protons and water molecules. Saturated mutation experiment done on this position confirmed that any mutation on this position either expropriates bioactivity of pullulanase completely or minimizes its bioactivity seriously Another mutation at functional residue Asn680 which is located at the outer edge of the AHBN that belongs to BNpulA324.

Because of its position, it does not connect directly or has no directive influence on the active residue (Asp511, Glu540 and Asp625) but however it may affect the bioactivity of the enzyme through AHBN (which consist of active residue, functional residue and water molecule) . Site directed mutagenesis at this position (Asn680) Asn680Asp was replaced with Asp. This mutation brought about changes in orientation and atomic charges and these affected the transportation of water molecules and protons to the active centre in certain degree. The mutation of Asn680Asp showed improved thermostability the wild type and also improved bioactivity at p H range 4.5 to 5.2 better than the wild type. Directed evolution Directed evolution is a process that directs molecular evolution in the test tube to produce the desired protein. This has resulted in the development of enzymes with improved properties for established technical application and production of new enzymes tailor-made for entirely new areas of application. A directed evolution approach starts with identification of a target enzyme to be optimized and the cloning of the corresponding gene. An efficient expression system is needed before the target gene is subjected to random mutagenesis and/or in vitro recombination, thereby creating molecular diversity. Subsequently improved enzyme variants are identified, preferably after being secreted into culture medium, by screening or selection for desired property. The gene encoding the improved enzyme are then used to parent the next round of directed evolution.

5.4 Potential Industrial Application of Pullulanase

Production of glucose syrup: For pilot scale production, a suspension of 35% (w/v) of previously extracted mazie starch was prepared . The suspension p H was adjusted using appropriate buffer (0.2M 6.0 phosphate).The suspension was maintained under heating until the starch is solubulized; after heating, the solution was cooled in water bath at 50°C with α amylase for 2 hours for total liquefaction. The saccharification process was carried out on the resulted maltodextrin syrup which was adjusted to p H 4.2 with appropriate buffer (0.2M of acetate). 100 ml of glucoamylase and 200 ml of

pullulanase was added and incubated at 60°C for 48 hours under constant agitation. Glucose production was monitored using HPLC method. After the saccharification process the resulting syrup was purified by ion exchanged chromatography using cathonic resin and anionic resin. The product was concentrated under reduced pressure by evaporation.

Production of Fructose Syrup: fructose syrup can be produced from glucose syrup. The solution p H was adjusted to p H 5.0 using the appropriate buffer (0.2 Tris-HCl buffer containing $MgSO_4$.$7H_2O$, 50 Mm and $CoCl_2.6H_2O$ 0.25Mm) The isomerization was performed with *Streptomyces mycelium* and pullulanase simultaneously . The system was incubated under constant agitation at 70°C for 96 hours. The fructose production was observed using the cysteinen carbazol method [52].

Production of corn syrup: The production of corn syrup in pilot scale could be done using the method described by Srikanta et al. [53]. It starts by preparation of an initial starch using corn starch, this is followed by liquefaction and saccharification (using glucoamylase and pullulanse simultaneously) finally purification is done using ion exchanged chromatography.

6. CONCLUSIONS

Pullulanes capable of hydrolyzing α 1, 6 bonds of different starch polymers are widely used for saccharification processwhich produces various useful end products such as maltose, amylose, glucose, bioethanol (for biorefinery that uses cassava starch as potential raw material) by debranching starch with or without α-amylase, βamylase or glucoamylase respectively. It is very clear that from this rewiew, pullulanase could facilitate a major change in the current strategy for starch processing industries and hence there is strong demand for this enzyme all over the world.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hii S, Tan J, Ling T, Ariff B. Pullulanase: Role in starch hydrolysis and potential industrial application. Enzyme Resource. 2012;1:921362.
2. Madihah M, Ariff A, Sahaid K, Suraini A, Karim A. Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum*. Word Journal of Microbiology and Biotechnology. 2001; 17(6):567-576.

3. Nakamura Y. Some properties, of starch debranching enzymes and their possible role in amylopectin biosynthesis Plant Science. 1996;121(1):1–18.
4. Jensen B, Norman B. *Bacillus acidopullyticus* pullulanase: Application and regulatory aspects for use in food industry. Process Biochemistry. 1984;1:397-400.
5. Van Der Maarel M, Van Der Veen B, Uitdehaag J, Leemhuis H, Dijkhuizen L. Properties andapplications of starch-converting enzymes of the α-amylase family. Journal of Biotechnology. 2002; 94(2):137–155.
6. Haki G, Rakshit S. Developments in industrially important thermostable enzymes: A review. Bioresource Technology. 2003;89(1):17-34.
7. Hobel C. Access to biodiversity and new genes from thermophiles by special enrichment methods. (Ph.D. thesis), University of Iceland, Iceland; 2004.
8. Gerhartz W. Enzymes in Industry: Production and Applications. VCH; 1990.
9. Pandey A, Nigam P, Soccol C, Soccol V, Singh D, Mohan R. Advances in microbial amylases. Biotechnology and Applied Biochemistry. 2000;31(2):135–152.
10. Bertoldo C, Antranikian G. Starch-hydrolyzing enzymesfrom thermophilic archaea and bacteria, Current Opinion in Chemical Biology. 2002;6(2):151-160.
11. Tharanathan R, Mahadevamma S. Grain legumes-a boon to human nutrition. Trends in Food Science and Technology. 2003; 14(12):507–518.
12. Fogarty W, Kelly C. Recent advances in microbial amylases, in Microbial Enzymes and Biotechnology. Elsevier Applied Science Publishers, London, UK. 1990;71-132.
13. Norman B. New developments in starch syrup technology, Enzymes and Food Processing. 1981;15–51.
14. Elleuche S, Qoura FM, Lorenz U, Rehn T, Bru¨ck T, Antranikian G. Cloning, expression and characterization of therecombinant cold-active type-I pullulanase from Shewanellaarctica. J Mol Catal B Enzym. 2015;116:70–77.
15. Wallenfels K, Bender H. Pullulanase (an amylopectin and glycogen debranching enzyme) from Aerobacter aerogenes. Methods in enzymology. 1966;8:555-559.
16. Song W, Nie Y, Mu X. Enhancement of extracellular expression of *Bacillus naganoensis* pullulanase from recombinant *Bacillus subtilis*: Effects of promoter and host. Protein Expression Purification. 2016; 172:1562-1568.
17. Wang Q, Xie N, Du Q, Qin Y, Li J, Meng J, Huang R. Active hydrogen bond network (AHBN) and application for improvement of thermal stability and pH sensitivity of *Pullulanase* from *Bacillus naganoensis*. PLoS ONE. 2017;12(1):e0169080.

18. Kunamneni A, Singh S. Improved high thermal stability of pullulanase from newly isolated thermophilic Bacillus spp. AN-7. Enzyme Microbiology Technology 2006; 39(7):1399-1404.
19. Ayadi D, Ali M, Jemli S, Mabrouk S, Mezghani M, Messaoud E, Bejar S. Heterologous expression, secretion and characterization of Geobacillus thermoleovorans US105 type I pullulanase. Applied Microbiology Biotechnology. 2008; 78:473-481.
20. Li Y, Zhang L, Niu D, Wang Z, Shi G. Cloning, expression, characterization and biocatalytic investigation of a novel Bacilli thermostable type 1 pullulanase from Bacillus sppCICIM263. Journal Agric Food Chemistry. 2011;60:11164-11172.
21. Wasko A, Polak-Berecka M, Targonski Z. Purification and characterization of pullulanase from *Lactococcus lactis*. Prep Biochem Biotechnol. 2011;41:252–261.
22. Nie Y, Yan W, Xu Y, Chen W, Mu X, Wang Y, Xiao R. High-level expression of *Bacillus naganoensispullulanase* from recombinant *Escherichia coli* with auto induction: Effect of Lac operator. PLoS ONE. 2013;8:e78416.
23. Arnold F. Combinatorial and computational challenges for biocatalyst design. Nature. 2001;409(6817):253-257.
24. Reetz M. Controlling the enantioselectivity of enzymes by directed evolution: Practical and Theoretical ramification. Proceeding National Academy of Science of the United State of America. 2004;101(16):5716-5722.
25. Mirsky A, Pauling L. On the structure of native, denatured and coagulated proteins. Proceedings of the National Academy of Sciences of the United States of America. 1936;22:439-447.
26. Pauling L, Corey B, Branson H. The structure of proteins; two hydrogen bonded helical configuration of the poly peptide chain. Proceedings of the National Academy of Sciences of the United States of America. 1951;37:205-211.
27. Dill KA. Dominant forces in protein folding and biological specificity analyzed by protein engineering. Nature. 1990;314:235-238.
28. Fersht A, Shi J, Knill-Jones J, Lowe D, Brick P, Carter P, Waye M, Winter G. Hydrogen bonding and biological specificity analyzed by protein engineering. Nature. 1985;314:235-238.
29. Fersht A. The hydrogen bond in molecular recognition. Trends Biochemistry Science. 1987;12:301-304.

30. Shirely B, Stanssens P, Hahn U, Pace C. Contribution of hydrogen bonding to the conformational stability of ribonuclease T1. Biochemistry. 1992;31:725-732.
31. Hebert E, Giletto A, Sevcik J, Urbanikova, L, Wilson K, Dauter Z, Pace C. Contribution of a conserved asparagines to the conformational stability of ribonucleases Sa, Ba and T. Biochemistry 1998;37:16192-16200.
32. Giletto A, Pace C. Buried, charged, non-ion paired aspartic acid 76 contributes favorably to the conformational stability of ribonuclease T1. Biochemistry. 1999;38: 13379-13384.
33. Serrano L, Kellis J, Cann P, Matouschek A, Fersh A. The folding of an enzyme II substructure of barnase and the contribution of different interaction to protein stability. Journal Molecular Biology. 1992;224:783-804
34. Milla M, Brown B, Sauer T. Protein stability effects of a complete set of alanine substations in Arc repressor. Nature Structure Biology. 1994;1:518-523.
35. Mendoza J, Jarstfer B, Goldenberg P. Effects of amino acid replacements on the reductive unfolding kinetics of pancreatic trypsin inhibitors. Biochemistry. 1994;33: 1143-1148.
36. Pace C, Horn G, Hebert E, Bechert J, Shaw J, Urbanikova L, Scholtz J, Sevick J. Tyrosine hydrogen bonds makes large contribution to protein stability. Journal Molecular Biology. 2001;312:393-404.
37. Takano K, Scholtl M, Sacchettini J, Pace N. The contribution of polar group burial to protein stability is strongly context dependent. Journal Biology Chemistry. 2003;278:31790-31795.
38. Pace C, Trevino S, Prabhakaran E, Scholtz J. Protein structure, stability and solubility in water and other solvents. Philos Trans R Soc Lond B Biol Sci. 2004; 359:1225-1234; discussion 1234-1235.
39. Byrne M, Manuel R, Lowe L, Stites W. Energetic contribution of side chain hydrogen bonding to the stability of *Staphylococcal nuclease*. Biochemistry. 1995;34:13949-13960
40. Takano K, Yamagata Y, Kubota M, Funahashi J, Fujii S, Yutani K. Contribution of hydrogen bonds to the conformational stability of human lysozyme: Calorimetry and X-ray analysis of six Ser --> Ala mutants. Biochemistry. 1999;38:6623-6629.

41. Joh N, Min A, Faham S, Whitelegge J, Yang D, Woods L, Bowie J. Modest stabilization by most hydrogen-bonded side chain interaction in membrane proteins. Nature. 2008;453:1266-1270.
42. Asensio A, Kobko N, Dannenberg J. Cooperative hydrogen-bonding in adenine−thymine and guanine−cytosine base pairs. Density Functional Theory and Møller−Plesset Molecular Orbital Study. Journal Physical Chemistry. 2003;107(33): 6441-6443.
43. Scheiner S. Contributions of NH. O and CH. O hydrogen bonds to the stability of β-sheets in proteins. Journal Physical Chemistry. 2006;110(37):18670-18679.
44. Antikainen NM, Martin SF. Altering protein specificity: Techniques and applications. Bioorganic and Medicinal Chemistry. 2005; 13:2701-2716.
45. Tsutsumi M., Claus T, Liang Y, Li Y, Yang L, Zhu J, Dela Cruz F, Peng X, Chen H, Yung S, Hamren S, Livingstone J, Pan C. A potent and highly selective VPAC 2 agonist enhances glucose-induced insulin release and glucose disposal. A potential therapy for type 2 diabetes. Diabetes. 2002;51(5):1453-1460.
46. Miyazaki K, Wintrode P, Grayling R, Rubingh D, Arnold F. Directed evolution study of temperature adaptation in a psychrophilic enzyme Journal Molecular Biology. 2000;297:1015-1026.
47. Cho M, Juliano R. Marco molecular versus small molecule therapeutics; Drug discovery, development and clinical considerations. Trends Biotechnology. 1996;14:153-158.
48. Weng Z, De Lisi C. Protein therapeutics: Promises and Challenges for the 21st century. Trends Biotechnology. 2002; 20(1):29-35.
49. Tsiang M, Jain A, Dunn K, Rojas M, Leung L, Gibbs C. Functional mapping of the surface residues of human thrombin. Journal Biology Chemistry. 1995;270(28): 16854-16863.
50. Tsing M, Paborsky L, Li W, Jain A, Mao C, Dunn K, Lee D, Matsumura S, Matteucci M, Coutre S, Leung L, Gibbs C. Protein engineering thrombin for optimal specificity and potency anticoagulant activity *in vivo* Biochemistry. 1996;35(51):16449-16457.
51. Cedrone F, Menez A, Quemeneur E. Tailoring new enzyme functions by rational redesign. Current Opinion Structural Biology. 2000;10:405-410.

52. Dishe Z, Borenfreud E. A new espectro-photometric method for the detection and determination of keto sugars and trioses. The Journal of Biological Chemistry. 1951; 192(2):583-587.
53. Srikanta S, Jaleel S, Kunhi A, Rehana F, Childyal N, Lonsane B, Karanth N. Downstream processing of the hydrolysate form cassava fibrous waste in the production of confection syrup. Starch. 1989;41(5):196-200.