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THE ROLE OF ENZYMES IN BIOCHEMICAL CATALYSIS ADVANCES AND ITS APPLICATIONS.

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ABSTRACT

Enzymes are pivotal in biochemical catalysis, driving reactions essential for life and numerous industrial processes. This review explores the fundamental principles of enzyme catalysis, including their structure, mechanisms of action, and factors influencing activity. Advances in enzyme engineering, such as site-directed mutagenesis, directed evolution, and computational approaches, have revolutionized the field, enabling the development of enzymes with enhanced efficiency, specificity, and stability. High-throughput screening and metagenomics have further facilitated the discovery of novel enzymes, while advanced techniques like X-ray crystallography and cryo-EM provide deeper mechanistic insights.The applications of enzyme catalysis are vast, spanning industries such as pharmaceuticals, food processing, biofuels, and environmental sustainability. Enzymes are employed in drug synthesis, chiral compound production, waste management, and green chemistry initiatives, showcasing their versatility and ecological benefits. Despite these advancements, challenges such as stability under extreme conditions, substrate specificity, and production costs remain significant barriers.Looking forward, emerging technologies like synthetic biology, nanotechnology, and artificial intelligence offer promising solutions to overcome these limitations and expand the potential of enzyme applications. This review highlights the transformative impact of enzymes in science and industry, emphasizing their continued importance in advancing sustainable and innovative solutions.

INTRODUCTION

History:

From about 1894 onwards, Emil Fischer investigated in a series of experiments the action of different enzymes using several glycosides and oligosaccharides; the results revealed specificity as one of the key characteristics of enzymes. In 1894, he compared invertin and emulsin. He extracted invertin from yeast, a

usual procedure, and showed that it hydrolyzed α-, but not β-methyl-D-glucoside. In contrast, emulsin, a commercial preparation from Merck, hydrolyzed β-, but not α-methylD-glucoside [1]. Among a series of tests with different saccharides, he observed that invertin hydrolyzed sucrose and maltose, but not lactose. An extract from a “lactose-yeast,” however, was able to hydrolyze lactose but not maltose. These observations are the most essential from a major range of which Fischer derived his famous theory on specificity (see below) (summarized by Fischer [2]. In his first paper on alcoholic fermentation without yeast cells (1897), Buchner described, in a remarkably short and precise manner, the separation of the (alcoholic) fermentation from the living yeast cells by an extract. He published the experimental details for the preparation of a cell-free press juice from yeast cells, with disruption, filtration under high pressure, and further filtration. He then described the formation of carbon dioxide and alcohol from carbohydrates, sucrose, glucose, fructose, and maltose. No microscopic organisms were present. At elevated temperature, protein was precipitated, the activity reduced, and finally destroyed. From these and further results, Buchner derived essential new insights, both into the nature of alcoholic fermentation and enzymatic activity governing the transformations observed.In subsequent papers, he communicated further important experiments, which also led to immediate objections of other scientists active in the field (see e.g.,[3]). These findings (with many more details published by Buchner et al.) deserve special attention since they represent the demarcation of a breakthrough, which reduced all reactions in physiological (or bio-) chemistry to chemistry. Further findings relevant for the establishment of the chemical nature of enzymatic catalysis and technical application followed shortly thereafter. Croft and Hill performed the first enzymatic synthesis, that of isomaltose, in 1898, allowing a yeast extract (α-glycosidase) to act on a 40% glucose solution [4]. In 1900, Kastle and Loevenhart found that the hydrolysis of fat and other esters by lipases is a reversible reaction and that enzymatic synthesis can occur in a dilute mixture of alcohol and acid [5]. This principle was utilized for the synthesis of numerous glycosides by Fischer and coworkers in 1902.

Theoretical Developments:

Fischer [2]in his work elaborated the essential aspects of enzyme catalysis during the 1890s. The first aspect is specificity. The agents of the living cell (enzymes) are optically active, and therefore one might assume that the yeast cells with their asymmetric agents can utilize only those sugars whose geometry is not too far from that of glucose. From there, Fischer deduced the famous 18 2 Enzyme Technology: History and Current Trends picture of lock and key; he assumed that the “geometrical form of the (enzyme) molecule concerning its asymmetry, corresponds to that of the natural hexoses” (sugars). The second aspect refers to the protein nature of enzymes. In 1894, Fischer [6] stated that among the agents that serve the living cell, the proteins are the most important. He was convinced that enzymes are proteins. Controversies on the nature of enzymes and proteins continued for long [7]. Proteins, such as albumin and casein, were included in the group of colloids, which were attested a dynamic state of matter. “…The colloid possesses ENERGIA, …the probably primary source of the force … of vitality.” Protoplasm was given mystical and even magical properties.” To the contrary, Béchamp, also referring to his former work in 1853–1857, had demonstrated that soluble ferments are a basic or original principle (pure principei immediat) [8]. But Willstätter, still in 1927, denied that enzymes were proteins [9]. Buchner initiated a new paradigm, which, in strict contrast to that of Pasteur, stated that enzyme catalysis, including complex phenomena like that of alcoholic fermentation, was a chemical process not necessarily linked to the presence and action of living cells. In his first paper, he wrote that he presented the proof that (alcoholic) fermentation does not require the presence of “such a complex apparatus as is the yeast cell.” The agent is a soluble substance, without doubt a protein body, which he called zymase [10]. Buchner’s findings marked a new – biochemical – paradigm leading research and theory on enzymes. It displaced an established paradigm which taught that processes in living organisms – alcoholic fermentation being the most important example – were not of pure chemical nature but required a vis vitalis, a vital force. Now, the chemical paradigm, which reduced all reactions in physiology (or bio-) chemistry to the laws of chemistry without further hidden forces, began to play the dominant role. Technical development also got a new scientific basis on which to proceed rationally.

Enzymes allow organisms to channel the flow of matter to their own advantage, allowing some reactions to proceed rapidly compared with other reactions that offer no selective advantage. After a substrate is bound at an enzyme's active site, its half-life is usually a small fraction of 1 s. Rapid turnover is necessary if any enzyme is to produce a significant rate of reaction at the limited concentration at which enzymes are present within the cell. Many enzymes are known to have evolved to work nearly as efficiently as is physically possible, with second-order rate constants that approach their rates of encounter (~109 s^{-1} M^{-1}) with the substrate in solution (Figure 1)[11]

How rapidly would biological reactions occur if an enzyme were not present? Until recently, some reactions were known to require several years,[12] and everyday experience suggests that some reactions are slower still. The survival of paper documents and ancient ships for long periods under water implies that the glyosidic bonds of cellulose, for example, are very resistant to hydrolysis in the absence of celluloses that catalyze their hydrolysis. Why would one wish to know the rate of a biological reaction in the absence of an enzyme? That information would allow biologists to appreciate what natural selection has accomplished in the evolution of enzymes as proficient catalysts and would enable chemists to compare enzymes with artificial catalysts produced in the laboratory. Such information might also be of value in considering the design of enzyme antagonists: the greater the rate enhancement that an enzyme produces, the greater is its affinity for the altered substrate in the transition state compared with its relatively modest affinity for the substrate in the ground state (Figure 1)[13].

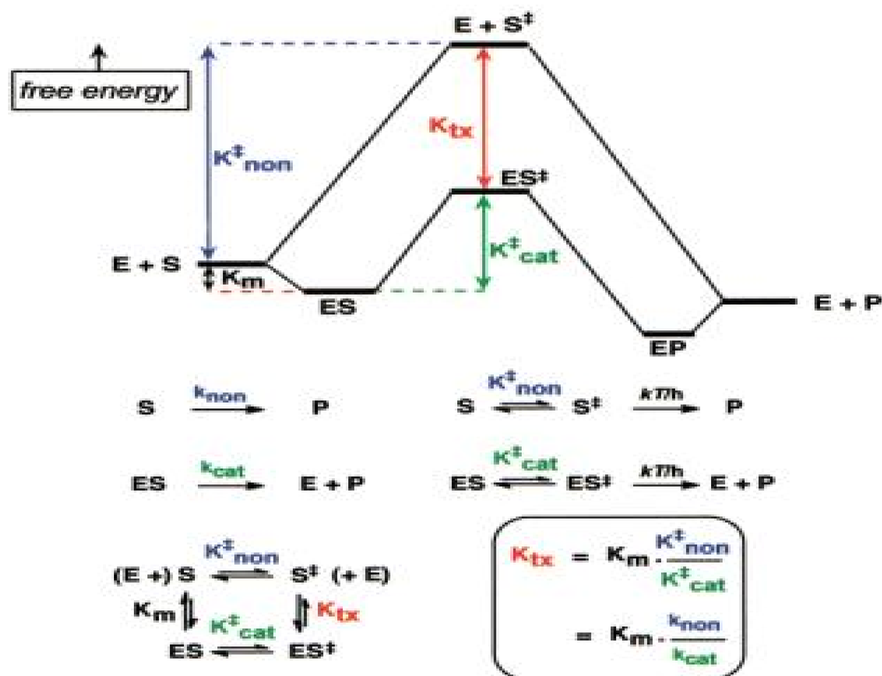


FIGURE 1: Suppose equilibrium is maintained between the ground state and the transition state in dilute solution. In that case, the formal dissociation constant of the altered substrate in the transition state (Ktx) is expected to be less than that of the substrate in the ground state (Km), by a factor matching the factor by which the rate constant of the catalyzed reaction (kcat) exceeds that of the uncatalyzed reaction (known). Effects of desolvation, charge separation, or proximity in multisubstrate reactions can be considered to involve subpopulations of ES that depart from the mean in the usual statistical description of molecules in the ground state. At equilibrium, any of these subpopulations can be more reactive than ES but can do so only to the extent that it is rare. Transition-state affinity may be underestimated if the mechanism of reaction in solution differs fundamentally from the mechanism of reaction at the enzyme's active site, or if kcatKm is limited by the enzyme-substrate encounter[14].This relationship does not apply to reactions involving tunneling and requires modification for reactions proceeding through covalent intermediates [15].

That principle has furnished a basis for the design of transition-state analogs, stable molecules exploiting that special affinity. Examples have now been discovered for enzymes of every class, including agents currently used to control hypertension, the spread of HIV, the maturation of insects, and the growth of weeds [16]. By allowing "snapshots" of enzymes in action, transition-state analogues have also provided valuable tools for investigating enzyme structures and mechanisms, most recently that of the peptide bondforming center of the ribosome [17]. Those enzymes that produce the largest rate enhancements and transition state affinities should offer the most sensitive targets for inhibitor design. This Account describes experiments exploring enzymes' ability to enhance the reaction rate above the rate at which it would occur spontaneously in neutral aqueous solution. This work has uncovered several of the slowest reactions whose progress has ever been determined in water, with rate constants spanning a range of at least 16 orders of magnitude. The energetic terrain of biological chemistry in the absence of enzymes is much more rugged than had been appreciated, and the catalytic power of some enzymes is remarkable.
The study aims to fill up the gaps in the knowledge of enzymatic mechanisms, narrow substrate specificity, high production costs, and low stability under extreme conditions. It aims to create cutting-edge enzyme engineering techniques to improve effectiveness, stability, and adaptability. In addition to cost-effective manufacturing techniques for industrial scalability, computational methods such as artificial intelligence and molecular modeling will be investigated for enzyme design. Synthetic biology and metagenomics will be used to find new enzymes. The ultimate objective is to increase the use of enzymes in medicinal, industrial, and environmental domains while encouraging creative and sustainable solutions.

2 structures of enzyme

The crystallographic structure of many enzymes from the GH-32 family have been determined in both of prokaryotes [18]and eukaryotes including bacteria ,(fungi), yeast and plants [19].The structure of the invertase enzyme from Saccharomyces cerevisiae has a close phylogenetic relationship with the structure of the inulinase enzyme from Shanomycesis oxidantalisis. GH32 and GH68 enzymes share a common catalytic 5-fold β-pro peller domain. GH-32 enzymes have an additional C-terminal β-sand wich domain attached to the N-terminal catalytic domain. Unlike the GH-68 enzymes, the GH-32 enzymes have an additional beta sandwich attached to the active site [20]. Circular dichroism (CD) spectroscopy reveals that the invertase structure contains mostly the secondary structures of beta-sheets and loops, and has relatively small amounts of R-helical structures [21]. Sainz-Polo et al. reported the crystal structure of recombinant Saccharomyces Invertase (SInv) at 3.3 Å resolution. They showed that the enzyme has a catalytic propeller and sandwich domains like the GH-32 enzymes, but SInv shows an uncommon quaternary structure. Monomers make up two kinds of dimers, and these dimmers ultimately form an octamer or correctly a tetramer of dimers. Dimer ization determines the specificity to the substrate because makes a space constraint that limits the access of oligosaccharides of more than 4 units to the active site. The dimers are placed at the vertices of a rectangle and can be in an opened or closed state which open dimer is more stable than closed type dimer. Crystal structure of Schwanniomyces occidentalis invertase at 2.9 Å revealed a 5-fold beta-propeller catalytic domain in N-terminal and a beta-sandwich domain in C-terminal [20]. Also, crystallography of Thermotoga maritima at 2 Å resolution showed a five-bladed-propeller connected to a β-sandwich domain by 10-residuelong linker regions, like another glycosidase from GH-43 and GH-68 families. β-sandwich including two sheets of 6 β-strands at the position of 306 to 432 in C-terminus [22]. The catalytic domain (The amino acid residues Asp-17, Asp-138, and Glu-190) of Thermotoga maritima invertase is excellently superimposable to GH43 and GH68 family enzymes. Three carboxylates groups at the bottom of funnel-shaped deep of the B-propeller are crucial for nucleo philic activity, acidic properly, and stabilizing the transition state, respectively [18]. Despite many efforts to produce high-resolution crystallography, only a few GH-32 enzyme structures have been put in the PDB. Aspartate (D) and glutamate (E) close to the N terminus in motifs of NDPNG, RDP, and EC act as a nucleophile and acid/base catalyst, respectively [23].

Mechanism of enzyme:

At the outset it is worthwhile to enquire as to what is to be meant by mechanism as applied to enzyme catalysis. It is generally agreed that the first event in an enzyme catalysed reaction is the stoichiometric combination of substrate or substrates and enzyme to give a complex, usually called the Michaelis complex, whose formation does not involve covalency changes. The rates of formation of such complexes may, in favourable cases, be only a few orders of magnitude less than the encounter rate. In the simplest model the Michaelis complex breaks down directly to give products and free enzyme at a rate which is considerably less than the rate of its formation. However, this is to suppose that the enzyme acts as a classical "noble" catalyst and it is abundantly clear that this model is much too simple and that in all real cases, the Michaelis complex generates other intermediates, some of which may involve covalent bonding between substrate, or some fragment of the sub- strate, and the enzyme. The number of intermediates lying on the reaction path between factors and products may be considerable: for example, in the case of aspartate aminotransferase (see below) the generally accepted mechanism involves four intermediates excluding Michaelis type intermediates and this is known to be an oversimplification. The number and structures of the intermediates lying on the reaction path is, therefore, the minimum information required to specify an enzyme mechanism. Structure, in this context, normally refers to the so-called active site, that is, defines the structures and relative positions of the groups which interact with the substrate or substrate fragment and specifies the nature of these interactions. The more stringent requirement that the total structure of the polypeptide chain be known for each intermediate is obviously, in general, unattainable: it could be satisfied, in any case, only where crystallographic study is possible. In these terms, the mechanisms of a number of enzyme-catalysed processes are now more or less understood but the data required for a more complete specification, i.e. the values of the rate coefficients for all the unit steps, is, in no case, as yet available. However, even when the problem of mechanism is solved, the problem of catalysis remains. The distinction between these two problems, although often overlooked, is important and implies the idea that the nature of the forces responsible for the rate enhancement produced by an enzyme cannot be deduced simply by inspection of the sequence of events which make up the mechanism. In a sense mechanism has to do with chemistry, catalysis with physics. An example best elaborates the point: it is generally agreed that the so-called transamination reaction (equation (1)) catalysed by aspartate aminotransferase involves prototropic rearrangements of azomethine intermediates formed by condensation of bound cofactors (pyridoxal and pyridoxamine-5'-phosphates) and substratest (equation (2), where R and R' represent the amino acid side chains). Since the rearrangements formally involve proton removal and addition it is to be presumed that the enzyme contains suitably oriented groups, (G~, G2) which donate and accept protons. In the currently used symbolism this would be represented as in Figure 2 where the continuous line represents the enzyme surface. If the chemical nature of the groups G1 and G2 were known then this part of the mechanism could be said to be specified. However, the catalysis would still remain to be explained. It is known (Banks et aL, 1968a) that the rate enhancements of the prototropic rearrangements produced by the enzyme are of the order of 109 and there is nothing in the scheme given in Figure 2 which explains this. It is customary to classify this kind of process as an example of general-acid, general-base catalysis and to imply that the rate enhancement arises, at least in part, from the concerted nature of the process. This view seems to be based on a misinterpretation of the significance of the arrows. They merely indicate the movements of electron pairs and, in effect, enable the structures of several intermediates to be included in one figure. Whether the chemical processes they represent are concerted or not and, if concerted, whether this is sufficient to explain the catalysis are matters which are not easily decided and, in any case, are not derivative of the mechanistic arguments which lead to diagrams of which Figure 2 is a rather simple example. The subject of the relevance of concerted processes to enzyme-catalysed reactions is discussed in greater detail below [24].

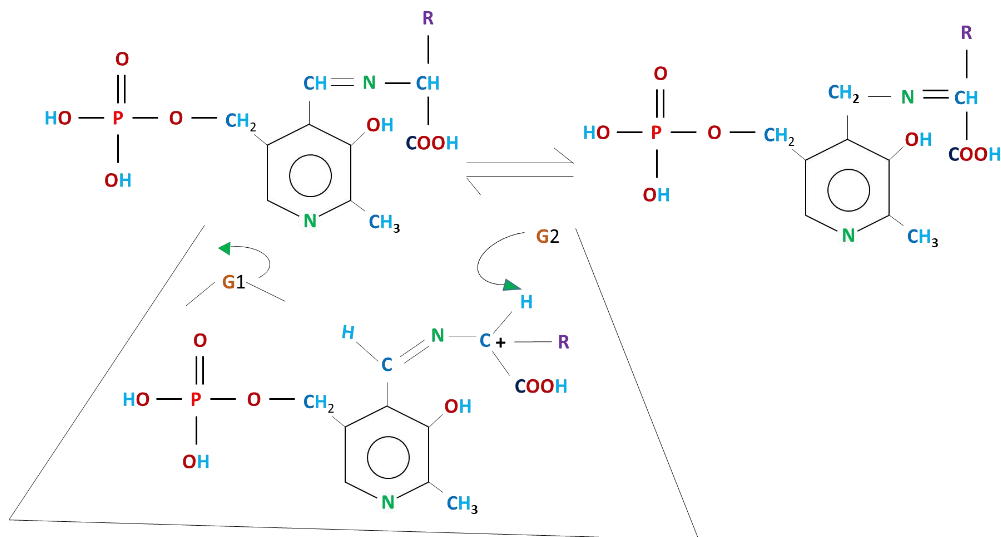
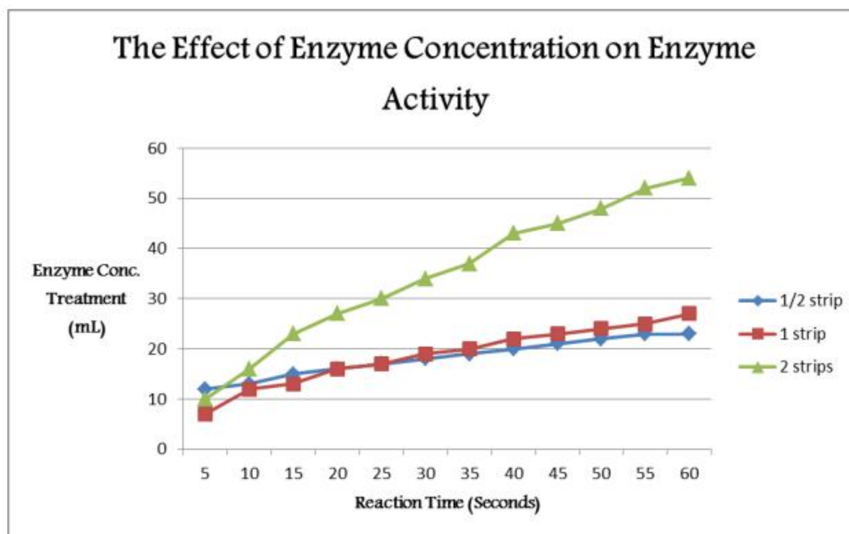


Figure 2: Rearrangement of azomethine intermediates in reaction catalysed by aspartate aminotramferase.

Factors that affect the activity of enzyme:

• Enzyme concentration

The activity of an enzyme increases as the concentration of the enzyme increases. This is because more enzymes are available to bind to the substrate. In turn, the reaction speed increases. The rate of reaction is proportional to the amount of enzyme present. Therefore, we see a straight line in the graph, where the x-axis is enzyme concentration, and the y-axis is the rate of reaction.

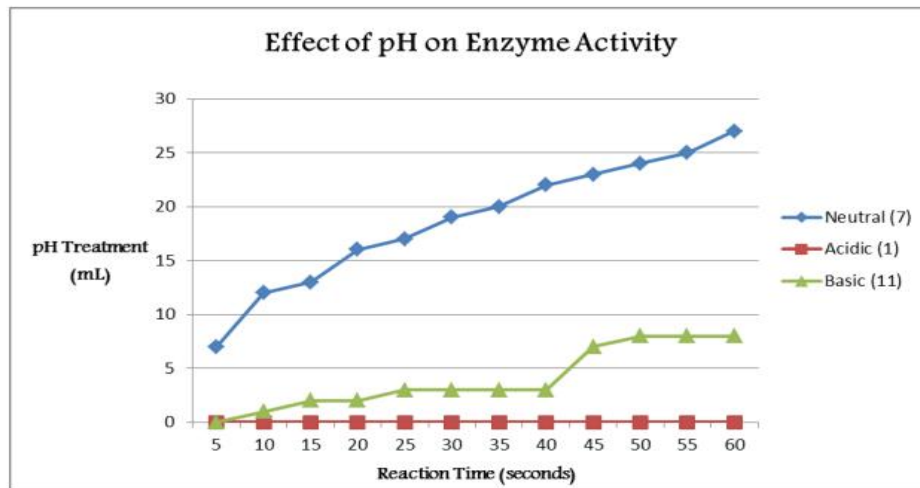


• Substrate concentration

An enzyme's activity increases with the rise in substrate concentration. The enzyme activity rises until it reaches a maximum limit. In other words, the enzyme molecules are completely saturated with the substrate. This means that all enzymes' active sites are occupied. The surplus substrate molecules will not react until the substrate that has already been bound to the enzymes has reacted and has been released or released without reacting. To help you visualize, the rate of reaction increases initially. However, the reaction rate reaches a plateau when all enzymes are occupied. For an enzyme-catalyzed reaction, there is usually a hyperbolic relationship between the reaction rate and the substrate concentration.

• Effect of pH

on the rate of reaction pH has an impact on enzyme activity. A bell-shaped curve emerges when enzyme activity is plotted versus pH. Each enzyme has a certain optimal pH at which the reaction rate is the fastest. The optimal pH is when a specific enzyme's activity is at its peak. The enzyme activity is greatly reduced below and above the optimal pH, and at high pH, the enzyme becomes completely inactive. Acidic carboxylic groups (COOH–) and basic amino groups (–NH2) are found in enzymes. As a result, changing the pH value affects the enzymes. For most enzymes, the optimum pH for the enzymatic activity of ranges from 6 to 7.



• Effects of temperature

on the rate of reaction An optimum temperature range is required to maximise the enzyme activity. Temperatures that are either higher or lower than the optimal temperature causes a decrease in the enzyme activity and can lead to denaturation (when the temperature is too high). The temperatures between 37 and 45°C are referred to as the optimal temperature for most enzymes. At this given temperature, most enzymes become more active. Extremely high temperatures can cause an enzyme to lose its denature form and eventually stop functioning. The majority of enzymes in the human body have a temperature optimum of 37 °C and are denatured or destroyed at higher temperatures. However, there are some exceptions, for example, in extremophiles. Few enzymes, such as Taq DNA polymerase found in thermophilic bacteria, are active at temperatures as high as 100°C [25].

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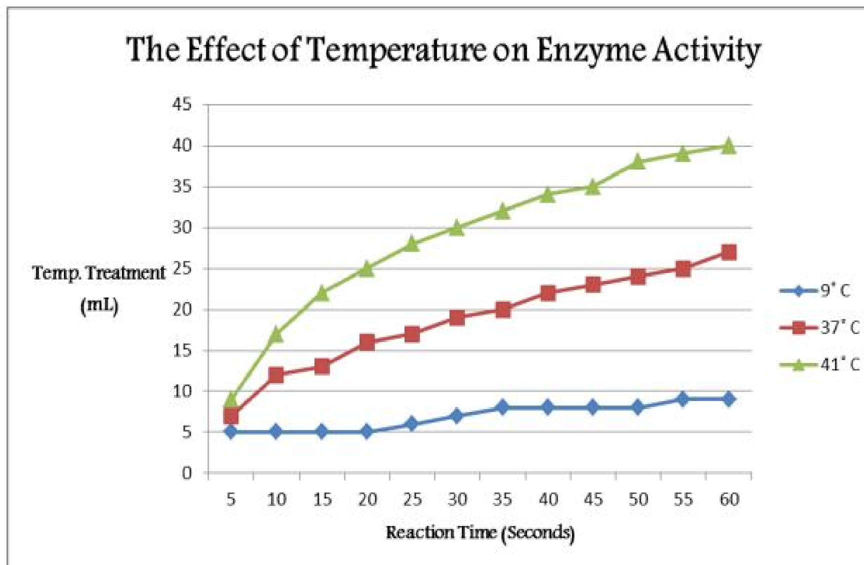

Engineering Enzymes for Enhanced Catalysis

Traditional approaches to enzyme engineering by rational design generally target residues in or very near active sites, changing important amino acids to alter substrate binding affinity, molecular selectivity or catalytic reaction rate. Directed evolution has been particularly successful for the generation of enzymes with activities towards new substrates, enhancing selectivity, or stability, also through active site mutations as well as mutations distributed throughout the protein molecule. These enzyme engineering strategies are well-developed, have been discussed thoroughly elsewhere, and do not fall into the scope of this tutorial [26]. One approach that our research groups have explored is the rational design of intermolecular interactions between a nanostructured enzyme and its substrates [27].This approach was inspired by enzymes like superoxide dismutase (SOD), one of the fastest known enzymes, that exploits electrostatic interactions between a charged substrate and oppositely charged residues on the surface of the enzyme. In the case of SOD, its superoxide substrate is directed to the opening of the active site tunnel by a positively charged patch of surface residues [28]. Our engineering strategy mimics this effect by introducing substrate–enzyme binding interactions far from the active site that result in increased local substrate concentrations, effectively reducing the apparent Michaelis constant (KM,app) and driving higher catalytic rates at low bulk substrate concentrations (Figure 3).

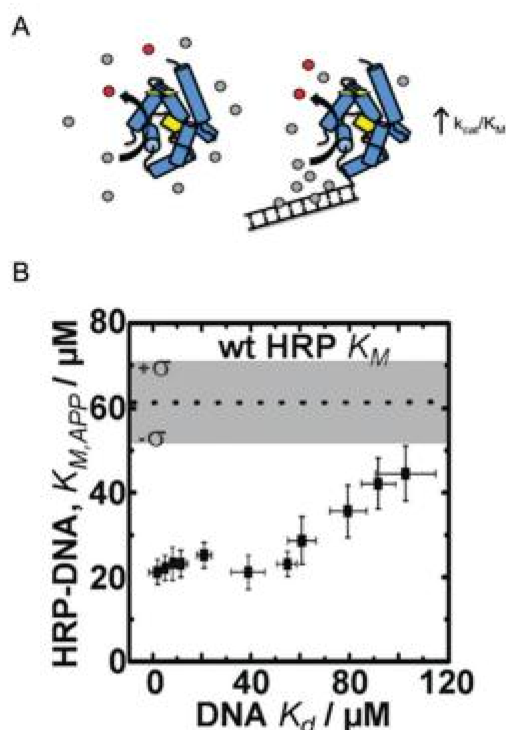

Figure 3: Enzyme engineering through the rational design of intermolecular interactions. (A) A schematic diagram of the approach. Conjugation of a DNA fragment with micromolar binding affinity to the enzyme's substrate can increase the local substrate concentration and increase catalytic efficiency. Reproduced with permission from ACS Catal., 2015, 5(4), 2149–2153. Copyright 2015, American Chemical Society.[29] (B) The relationship between DNA binding of the HRP substrate TMB and the apparent KM of HRP–DNA. The DNA sequence-dependent binding of TMB allows for creation HRP–DNA nanostructures with TMB binding affinities ranging from B1 to B100 mM. Reproduced with permission from ChemBioChem, 2016, 17(15), 1430–1436.18

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Working with the model enzyme horseradish peroxidase (HRP), we created a series of enzyme–DNA structures. HRP was used because it oxidizes a range of different substrates that we identified as micromolar range binders to double stranded DNA [30]. DNA was selected as the binding interface because recent advancements in DNA nanotechnology have enabled precise control over molecular level chemical and physical features and user-defined sequences are easily obtained through commercial vendors [31]. DNA was also selected as the structural modifier because it is known to have affinity towards many different small molecules, including anti-cancer drugs, polymer precursors, chemical nerve agents, and DNA imaging fluorophores among others. To demonstrate our approach, we first screened multiple HRP substrates for DNA binding affinity using Autodock simulation software. Two substrates were selected based on the binding energies predicted by the software, which were later confirmed with ligand binding assays[32]. Tetramethylbenzidine (TMB) was selected for its DNA sequence-dependent binding and the common colorimetric substrate ABTS was selected as a control since it has no binding affinity to double stranded (ds) DNA.6 Conjugation of 20 bp dsDNA fragments to free primary amine groups on the surface of HRP by standard bifunctional crosslinking chemistry yielded HRP–DNA structures with B1 dsDNA fragment per enzyme. The conjugation of DNA fragments of varying sequence and structure (dsDNA and DNA DX tiles; see ref. [33]) produced a series of HRP–DNA complexes with a range of binding affinity to TMB (B1 to 100 mM). Full kinetic analysis of the HRP–DNA structures revealed that the binding affinity of the DNA to TMB decreased KM,app from 60 16 mM to 23 3 mM (a 2.6-fold decrease), but did not significantly alter kcat (B80 s1). As shown in Fig. 1B, the effect increased with stronger TMB binding, reaching a maximum effect when the Kd of TMB to DNA was less than B20 mM. This trend was not observed with the control

substrate ABTS, which has no affinity to the HRP conjugated DNA. Kinetic analysis of HRP–DNAs with 4-aminophenol (4-AP) as a substrate confirmed the effect of the designed intermolecular interactions. The KM of 4-AP was reduced from 7 1 mM to 2.9 0.5 mM when a DNA DX tile with a measured Kd of 2 1 mM to TBM was attached.18 Short dsDNA fragments were also attached to the alcohol dehydrogenase D (AdhD) from Pyrococcus furiosus to explore whether the DNA binding effect could be used to enhance cofactor utilization. We had previously engineered AdhD to accept the NAD(H) mimic nicotinamide mononucleotide (NMN(H)) in place of NAD(H).25 This provided an enzyme system to test our enzyme–DNA strategy because NMN(H) has micromolar binding affinity to DNA, while NAD(H) does not.6 Conjugation of a 20 bp dsDNA fragment to AdhD did not significantly alter kinetic parameters with NAD+ and 2,3-butanediol as co-substrates, but binding of NMN+ was enhanced (Kd,app for NMN+ decreased from 534 30 mM to 294 18 mM). In this case, the DNAcofactor binding effect did not enhance catalysis as the increased local cofactor concentration led to substrate inhibition, a characteristic common to ordered bi–bi NAD(H) cofactor dependent enzymes.26 With both HRP and AdhD, the result of attaching a designed DNA nanostructure was increased substrate binding. AhdD–DNA constructs exhibited a reduced Kd,app towards the cofactor mimic NMN+ . With HRP–DNA, KM,app for the substrate TMB was reduced. These experimental results were supported by Brownian dynamic simulations. Simulations of HRP–DNA suggested that the presence of DNA results in an increased local residence time of the substrate, as well as an increased substrate on-rate (kON) [27] The latter effect was also confirmed experimentally with a 3.7-fold increase in kON of TMB to HRP–DNA in comparison to unmodified HRP.

DE NOVO ENZYME DESIGN AND EVOLUTION

The ultimate goal in enzyme design is to engineer entirely new enzymes equipped with catalytic capabilities that match or even surpass those of their natural counterparts while catalyzing novel chemical reactions. Substantial progress has been made toward this goal over the past few decades, largely driven by the development of computational enzyme design strategies [34]. Typically, de novo enzyme design begins with the creation and fine-tuning of “enzymes”, which comprise essential side chains positioned to stabilize reaction transition states. This initial design is followed by the selection of enzyme scaffolds capable of accommodating the enzyme configurations, forming the active site of the designed enzyme; recent development even allows the design of the scaffold proteins through deep learning approaches. Subsequently, sequence optimizations are performed to maximize the compatibility of the active site with the reaction mechanism of the enzyme while preserving the overall stability of the designed enzyme. Sequences produced through these processes are then subjected to experimental characterization to evaluate their catalytic efficiencies, often measured as the specificity constant kcat/KM. When necessary, the selected sequences, once their catalytic efficiency has been measured, undergo further refinement to enhance their catalytic performance and improve properties like thermos ability. These strategies have been successfully employed to generate de novo enzymes with the capacity to catalyze a range of organic reactions, including Kempeliminase, retro-aldolase, Diels−Alderase, ester-ase, and luciferase [35]. Alternatively, existing enzymes or sequences are repurposed to catalyze increasingly complex chemical reactions, including poly (ethylene ter-ephthalate) (PET) hydrolysis. Despite these successes, the designed enzymes have shown relatively modest catalytic activities, with kcat/KM values being several orders of magnitude lower than those of natural enzymes. To further enhance the catalytic potential of these designed enzymes, experimental approaches, e.g. Directed evolution, have been employed [36]. This combined approach, starting with computational design followed by experimentally directed evolution, has resulted in designed enzymes with catalytic efficiencies approaching those of their natural counterparts. It also provided valuable insights into the structural determinants of efficient catalysis. In particular, directed evolution introduces variations of active site residues, including the catalytic residues that were originally designed. Amino acid replacements from directed evolution can improve catalysis, for example, by introducing new catalytic groups, optimizing catalytic contacts and ligand-binding modes and enhancing transition-state complementarity within the binding pocket [37]. In some cases, replacements are introduced at positions remote from the active site, affecting ligand-binding properties and/or populations of conformational sub-states [38]. These observations,

together with relatively poor selectivity of substrates in the designed enzymes, point to potential limitations of current computational methods and highlight the need for further development of computational enzyme design algorithms [39]. Such improvements should aim to improve the accuracy of modeling catalytic and ligand-binding interactions while allowing the efficient exploration of the vast sequence space. In addition, the full spectrum of mechanisms contributing to enzyme catalysis, beyond the simple transition state stabilization, needs to be considered in the design of new enzymes to reach the catalytic activity of natural enzymes. In recent years, the application of machine learning techniques has revolutionized the field of protein structure prediction, protein design, and enzyme engineering. These techniques focus primarily on either structure prediction or activity prediction. On the structure side, achievements such as AlphaFold2359 andRoseTTAFold2360 have significantly improved the accuracy of protein structure prediction. These structure prediction techniques have the potential to greatly improve our ability to design robust enzymes with high success rates. Nevertheless, challenges remain, including the need to incorporate aspects of protein dynamics, allosteric and entropy-enthalpy com-sensation into enzyme design principles, as well as the design of enzymes capable of catalyzing multistep reactions. From the perspective of computational enzymology, the physics-based computational methods discussed above have proven instrumental in exploring these complexities of enzymes and understanding their mechanisms. Consequently, these methods need to be fully integrated with machine learning techniques into the enzyme design framework to streamline the effective refinement of the catalytic properties of the designed enzymes. On the activity prediction side, regression models, including linear regression and neural networks, have long been used to decipher the sequence─activity relationship of enzymes. Such relationships have then been used to guide (a) the optimization of enzyme variants and (b) the directed evolution of enzyme activity and product enantio selectivity. A compelling question related to enzyme design principles is how nature evolved enzymes to achieve the remarkable catalytic abilities found in modern enzymes [40].To address this question, ancestral sequence reconstruction (ASR) algorithms, can be combined with protein structure prediction algorithms to test evolutionary design principles, such as those governing their catalytic activities and thermal adaptation, and to rapidly evaluate hypotheses [41]. This idea has already been adopted, where the high accuracy of protein structure predictions such as AlphaFold2 and RoseTTAFold2 have been used to evaluate the structures of the ASR-generated sequences, followed by biochemical/biophysical characterization and structural elucidation [42]. Notable examples of such studies include the study of monooxygenases' functional diversification, which revealed relatively few mutations could be insufficient to induce diversification of the catalytic function of the enzyme, and the development of multiple xylose isomerases by combining big data, ASR, and adaptive laboratory evolution techniques for biotechnology. We expect this combined approach will be used more frequently to provide new research opportunities. Furthermore, these approaches can also be applied to further evolve enzymes for biomedical and biotechnological applications. In these applications and the development of new enzyme-design approaches, advanced physics-based computational methods, such as those discussed in this review, can provide a fundamental understanding of their working mechanisms and thereby help advance the field ofenzyme design and its biotechnological applications.

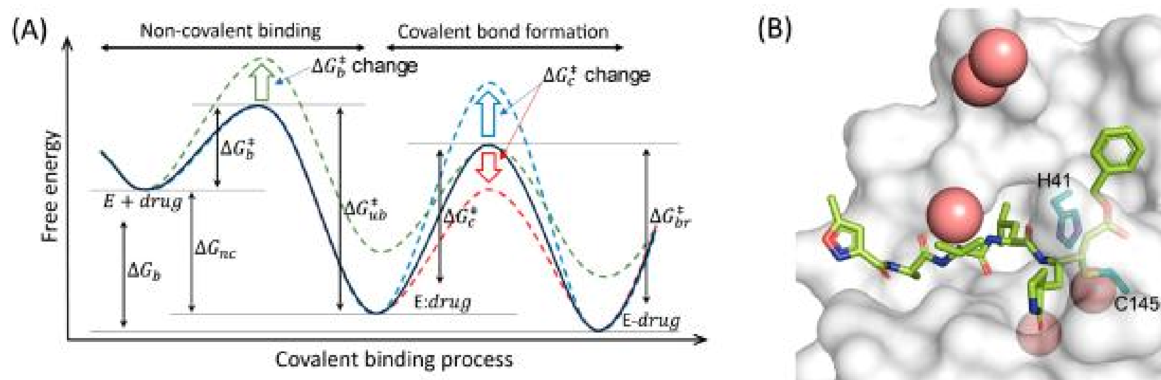

Figure 4. (A) Proposed two-step binding of a covalent ligand. The first step is noncovalent binding with barrier ΔGb ‡, and the second step is covalent bond formation with ΔGc ‡ as the associated barrier. The effectiveness of the covalent binders can be determined by the free energy barrier (ΔGc ‡) of the second step relative to the noncovalent binding free energy (ΔGnc) and/or the unbinding free energy barrier (ΔGub ‡). Too high a barrier can result in premature release of the binder prior to covalent bond formation, while too low a barrier renders the binder reversible or ineffective as a covalent inhibitor. In the latter case, the warhead may be too reactive, leading to nonspecific binding. Therefore, modulation of both ΔGnc and ΔGc ‡ is critical for the design of effective covalent binders, where the binding pocket of the target protein/enzyme provides an environment for nonspecific binding and functional modifications of the warhead control the reactivity of the binders. (B) SARS-CoV-2 Mpro with bound ligand, N3 inhibitor (PDB ID: 7BQY). The bound ligand is colored yellow, and water molecules are pink. The protein surface appears in gray. The Cys 145-His41 dyad is shown in deep teal. Adapted with permission from ref 404. Copyright 2021 American Chemical Society.

Mechanistic Insights

Conformation and stability of the molecular structure of enzymes are ensured by hydrogen bonds, hydrophobic interactions, disulfide and ionic bonds and van der Waals forces. The greatest paradigm of structural studies of macromolecules is that knowledge of the three-dimensional structure is a prerequisite to understand how these molecules act [43]. The catalytic activity as well as the stability and specificity of enzymes depend on their three-dimensional structures. Environmental conditions such as pH, temperature and ionic strength, among others, affect enzyme structure and, consequently, its properties. Enzyme properties can be modified by treatments (e.g., heat, pH, or pressure) resulting in inhibition or activation of the catalytic activity. Recent studies have reported an increase in the catalytic activity of several enzymes submitted to different treatments. Modulation of enzyme activity has been observed after exposure to pressurized fluids, ultrasound irradiation, simultaneous combination of compressed fluids and ultrasound and after exposure to magnetic field [44]. There is, however, a lack of studies focusing on the effects of such treatments on the structure of these enzymes. Structural aspects of enzymes that have undergone ultrasound treatment were addressed by fluorescence, circular dichroism (CD) spectroscopy, UV-visible and Fourier transform infrared (FT-IR) spectroscopy. Structural changes of cellulose from Trichoderma varied submitted to magnetic field treatment were also studied by UV spectrophotometry and far-UV circular dichroism [43]. Structural studies of commercial enzymes submitted to supercritical fluid treatment were carried out using circular dichroism and fluorescence spectroscopy , FT-IR and fluorescence spectroscopy [45]. Recently, a few studies have addressed conformational changes in the structure of treated enzymes via molecular dynamic (MD) simulations. However, very few enzymes submitted to high pressure, magnetic field or heat treatments had their 3D structures determined before and after treatment. Such structural studies would certainly help to elucidate the biochemical phenomenon involved in enzymatic activity changes.

Applications:

Natural Gas Conversion:

The composition of natural gas is 80–95% methane with varying degrees of heavier hydrocarbons, but methane has a relatively low market value due to difficulties in storage and transportation as well as limited use as fuel [46]. The low market value and high greenhouse gas potential of methane have initiated a surge in research and development of technologies that can be employed to convert methane to high-quality, value-added chemicals.

Much current research on the economically viable use of methane as feedstock is focused on conversion to methanol, which can be more readily converted to olefins and other valuable hydrocarbons. Current processes for a traditional chemical conversion of methane to methanol, such as steam reformation and the Fischer-Tropsch process, are limited by several significant drawbacks. The chemical conversion route requires the use of high-temperature, high-pressure unit operations as well as noble metal catalysts, resulting in a poor selectivity of methanol. The low yield of methanol necessitates a large process throughput in order to overcome large capital cost investments, thus the process is only profitable at a massive scale, thus placing further constrictions on process employment due to the difficulty of transporting methane from an extraction site to a production [47].The use of the biocatalyst methane monooxygenase (MMO), for the conversion of methane to methanol, has recently gained interest in the wave of expanding natural gas extraction. MMO has been shown to convert methane to methanol at ambient conditions with selectivity approaching 100% and thus has been researched for the scale-up of methane conversion, considering its multiple advantages over the chemical conversion route. The high selectivity of MMO-catalyzed methanol production eases the intensity of product separation and could significantly reduce the number of steps required in the conversion process. Furthermore, the enzymatic reaction occurs at mild reaction conditions, and could thus cut back on costs associated with heating, pressurization, and other feedstock conditioning steps. Much lab-scale research remains to assess the MMO-catalyzed conversion of methane for industrial applications because isolation of MMO is an intensive process, suggesting it may be beneficial to immobilize the enzyme for reuse. Blanchette et al. reported the use of a 3D printed micro bioreactor with immobilized MMO as packing for continuous methane conversion to methanol. Although immobilized MMO retained good activity through 20 consecutive reuses, the overall product yield was significantly less than the bio catalytic mass required for methane conversion. Ultimately, enzymatic conversion of methane to methanol is a developing technology with several major hurdles to overcome before successful economic scale-up. Due to the inexpensive costs of methane and methanol, enzyme-catalyzed process must be efficient to ensure economic viability. Currently, low catalytic activity of MMO is a significant limiting factor. Furthermore, a more intensive examination of process configurations is required to mitigate the mass transfer limitations that arise from the low solubility of oxygen and methane in aqueous media [47].

Food and Beverage Industry

In many instances, traditional chemical synthesis routes are not viable for food products due to reagent toxicity and complex reaction chemistries that result in unfavorable process economics. Biocatalysts, on the other hand, present an opportunity for simplified, efficient production routes that mitigate the need for harsh substances, and thus are more economically competitive. As such, the use of biocatalysts in food and beverage processes dates back thousands of years to the advents of culinary practices like wine and cheese making.In modern times, the widespread use of enzymes in food and beverage industries for food quality preservation or modification is one of the earliest successful industrial applications of biocatalysis, observed in beer fermentation, juice debittering, and bread baking. The replacement of conventional chemical treatment with enzyme-catalyzed pathways for conversion of starch to glucose and fructose first took place several decades ago.The conventional production route requires temperatures up to 175 °C and considerable pressurization, whereas biocatalytic processes can be carried out at temperatures near 100 °C and at ambient pressure via sequential α-amylase-catalyzed reactions encompassing both liquefaction and saccharification steps.In addition to milder reaction conditions, the multi-enzymatic process resulted in higher product selectivity and therefore allowed for better defined production routes for varying sugar products like maltose,

fructose syrup, and crystalline sugar, as dictated by biocatalyst selection [48].An emerging trend is the use of enzyme catalysis for commercial-scale production of probiotics, artificial sweeteners, and rare sugars.Probiotics, such as oligosaccharides, lactulose, lactilol hydrolysates, and inulin, are non-digestible food additives that stimulate growth of gut bacteria and can reportedly improve human health. Dietary supplement producers have become particularly interested in simple, efficient enzyme-catalyzed synthesis routes of probiotics due to above-average projected market growth and accompanying increase in demand. Yakult Honsha Co. Ltd. of Japan and Friesland Food Domo of The Netherlands, among others, have carried out commercial-scale, enzyme-catalyzed production of galacto-oligosaccharides (GOS), a lucrative probiotic with digestive health benefits and use as low-calorie sweeteners. GOS are produced by transgalactosylation simultaneous to hydrolysis of lactose via β-galactosidase; lab-scale results have shown GOS yields near 40% for free enzyme, while immobilized enzymes show the potential for larger yields of up to 50% through implementation in a continuous system resulting in decreased product inhibition [49].
The enzymatic production of protein hydrosylates for use as nutritional supplements and flavor enhancers has been developed due to milder reaction conditions and increased control over product formation relative to traditional chemical routes. When hydrolyzed, a parent protein forms biofunctional peptides exhibiting antioxidant, antimicrobial, and antihypertensive properties, among other therapeutic effects. The production of fish protein hydrolysates from seafood processing waste via papain, a proteolytic enzyme derived from papaya that has found widespread industrial application, has garnered attention recently because the process is a potential solution for minimizing pollution from fishing industries.Additionally, papain has been researched as a biocatalyst for production of protein hydrosylates from Chinese walnuts; lab-scale work on papain catalysis has shown moderate yields and purities of hydrosylates, and peptides obtained from produced hydrosylates showed good antioxidant properties [50].

Detergents Industry
Successful employment of biocatalysts is cited as the driving force of production of cost effective, environmentally benign detergents.In the instance of the detergents industry it should be noted that enzymes are a product rather than a chemical process-specific catalyst. Nonetheless, favorable market trends in the detergents' industry reinforce the underlying view that biocatalytic products are inherently safer and more sustainable than traditional chemical products that pose health and safety risks. Alkaline proteases—which are effective in the removal of protein stains and the cleaving of damaged cotton fibers—isolated from microbial sources comprise significant portions of multiple detergents produced and sold at commercial scale by manufacturers like Novozymes SA, Kao Corporation, and Genecor International.The high reaction specificity of enzymatic reactions further mitigates damage to fabrics and surfaces that is characteristic of chemically harsh detergent agents.Furthermore, the ratio of catalytically active enzymes in detergent mixtures are optimized for specific detergent applications; for instance, dishwashing detergents often contain varying degrees of amylase and lipase intended for the removal of starch food deposits and fats and oils, respectively [51].

Electrocatalytic applications:
Electrochemical water splitting generates hydrogen (H2) and oxygen (O2) from water by the hydrogen evolution reaction (HER) and the oxygen evolution reaction (OER) with a number of advantages such as high energy conversion efficiency, negligible environment pollution, and potentially wide range of applications. For this reason, it represents one of the greenest electrocatalytic reactions. At the anode, water is split into oxygen gas with accompanying protons and electrons. At the cathode, hydrogen gas is obtained by recombination of the protons and electrons. In the last years, novel water electrolysis technologies have been developed. Nowadays, proton exchange membrane electrolyzer cells (PEMECs) represents one of the most advanced technologies in the field of water electrolysis. Among the traditional water electrolysis methodologies, PEMECS constitute a versatile approach to generate fuels which in turn can be coupled to several sustainable energy sources such as solar energy, wind energy, wave power, geothermal energy and bioenergy. Additionally, they possess a lot of advantages including a fast dynamic response time, a

favourable energy efficiency/density, high hydrogen purity, and a more robust design [52]. The main objective of a PEMEC is to electrochemically split water into hydrogen and oxygen. In this process, water is circulated at the anode for a flow field to the membrane electrode assembly (MEA), where the electrochemical reaction occurs with the catalyst and is split into oxygen, protons, and electrons. Thus, protons are movedto the membrane, react with electrons from an external electrical force, giving rise to hydrogen at the cathode. It is worth to point out that electrochemical reactions at PEMEC occur only on "triplephase boundaries" meaning locations with electron conductors, active catalysts, protoncarriers, and pathways for reactants/products. For example, a water splitting at the anode cathode requires (1) a pore to the outside transport of the liquid water in an molecular oxygen, (2) a catalyst and electron conductors for the reaction, and (3) an electrolyte for proton transport. The electrochemical reactions which are taking place in both sides of PEMEC are (Figure 15):

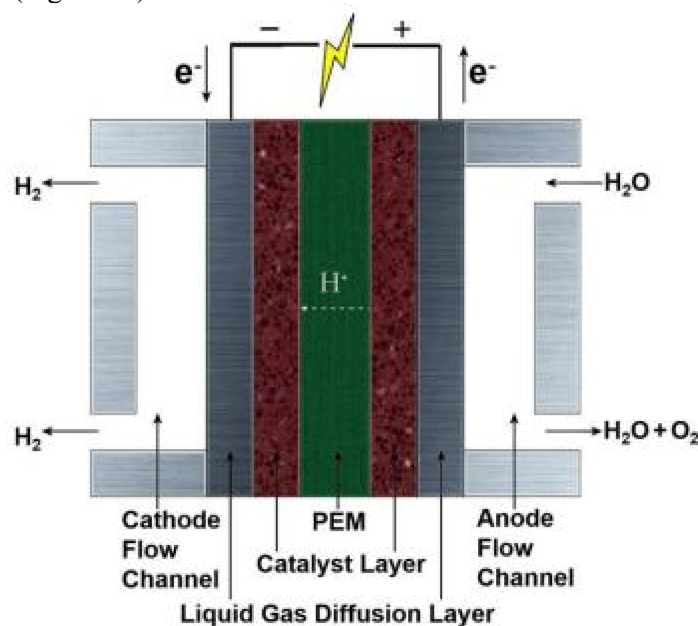

Figure 5: Schematic representation of a PEMEC. Reproduced with permission of ref. [53].Copyright, 2016 Royal Chemical Society.

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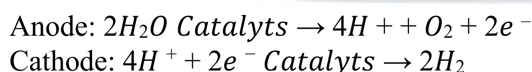

Anode: $2H_2O\ Catalyts \rightarrow 4H + + O_2 + 2e^-$
Cathode: $4H^+ + 2e^-\ Catalyts \rightarrow 2H_2$

In summary, PEMEC are promising electrochemical energy devices for a sustainable future since they can be integrated with high efficiency to a number a sustainable energy sources in a myriad of environmentally benign manners.
The electrochemical reduction of CO2 to other carbon compounds with higher chemical energy is becoming in a useful strategy to generate chemical fuels with reduced carbon emissions. A sustainable alternative towards the conversion of carbon dioxide into value-added compounds such as the synthesis of lowdimensional carbon nanomaterials and electrocatalytic enzymes systems, commented above, have been proposed in the last years. Otherwise, is well-known that these electrocatalytic processes could be economically improved by the generation of electricity from renewable energies (Figure 16), which appear to be a useful method to assure their sustainability. Specifically, it has been proposed that most of the electrochemical CO2 conversion systems will generate CO2 emissions if they do not integrate with renewable-energy sources as well as the renewable energy technologies can trigger the scale-up of several electrochemical CO2 systems for industrial applications. In this sense, the couple of renewable energies to the electroreduction of CO2 is central for the development of most sustainable electrochemical CO2

conversion technologies. Therefore, future endeavours should be focus towards the design of efficient electrocatalytic systems powered by renewable energies

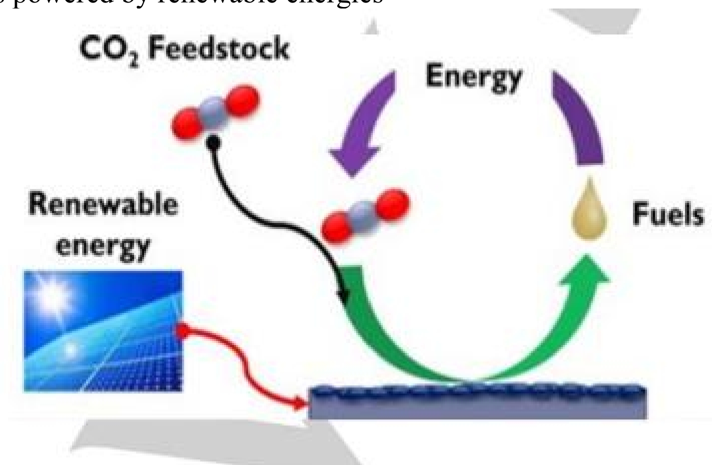

Figure 6: Overview of an electrochemical CO2 conversion system powered by renewable energy. Reproduced with permission of ref. [54] Copyright, 2016 Elsevier

Biomedical applications:

The catalytic properties of iron oxide nanoparticle-based nanozymes in addition to their biocompatibility, versatility in surface modification, and magnetic separation capability could find use in many areas of biomedical applications. Some recent progress in niche application as well as biosensing and disease treatment are briefly reviewed in this section. First of all, the excellent stability and performance in the catalytic activity of iron oxide nanoparticles have enabled the development of advanced biosensors[55]. For example, Vallabani et al. reported enhanced peroxidase-mimetic activity of Fe3O4 nanoparticles over wide ranges of pH and temperature in the presence of adenosine triphosphate (ATP) [56]. It was suggested that the complexation between ATP and Fe3O4 nanoparticles could contribute to the production of OH at physiological pH via electron transfer reactions. They also demonstrated that the interaction between ATP and Fe3O4 nanoparticles can be applied to the single-step detection of glucose in human blood serum. Duan et al. reported a novel type of immunochromatographic strip that uses Fe3O4 nanoparticles for rapid and highly sensitive diagnosis of Ebola virus (EBOV).They utilized the antiEBOV antibody-functionalized Fe3O4 nanoparticles as nanozyme probes for recognition, separation, and naked-eye visualization of EBOV on the strip. The peroxidase-mimetic activity of the Fe3O4 nanoparticles could lower the detection limit by 100-fold for the detection of the glycoprotein of EBOV compared with that of the standard colloidal gold strip. Moreover, they showed that the Fe3O4 nanoparticle-based strips can detect other infectious viruses if paired with corresponding antibodies, demonstrating the versatility of the strips as a rapid, simple, and accurate diagnostic tool (Fig. 5A).

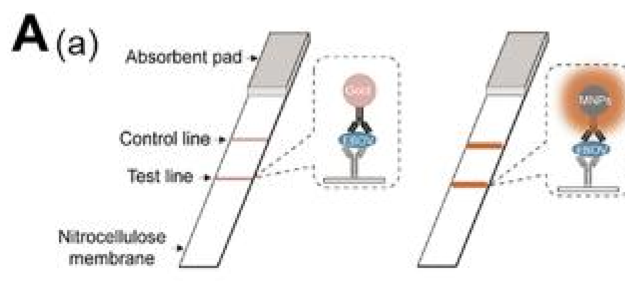

Figure 7: (A) Schematic illustration of the standard colloidal gold strip (left) and Fe3O4 nanoparticle-integrated nanozyme-strip (right) (a). EBOV-glycoprotein (GP) detection using the nanozyme strips (left), and standard gold strips (right) (b). pseudo-EBOV in human serum in the presence of pfu/mL influenza A

virus (non-specific target). The asterisks (*) show the limit of optical detection (c). Reproduced with permission from Ref. [57]. Copyright 2015 Elsevier.

Conclusion:

Enzymes are at the core of biochemical catalysis, facilitating complex reactions with exceptional specificity, efficiency, and minimal energy input. Their natural ability to act as biological catalysts has been harnessed and enhanced through advances in enzyme engineering, paving the way for a multitude of applications across diverse fields. Techniques such as directed evolution, site-directed mutagenesis, and computational modeling have revolutionized the development of enzymes with improved stability, activity, and substrate specificity. These engineered enzymes are now widely employed in pharmaceutical synthesis, where they enable the production of high-purity drugs, and in the food industry, where they enhance processing efficiency and product quality.Moreover, enzymes have become central to sustainable practices, such as biofuel production and bioremediation, offering eco-friendly alternatives to conventional chemical processes. Their integration with emerging technologies, including nanotechnology and synthetic biology, has further broadened their scope, enabling the creation of multifunctional biocatalysts and enzyme-based systems for diagnostics and therapeutics.Looking ahead, the potential of enzymes remains vast, particularly in addressing global challenges like environmental sustainability and resource scarcity. Continued research and innovation in enzyme technology will undoubtedly drive transformative solutions, reinforcing their critical role in advancing science and industry while contributing to a more sustainable future.

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