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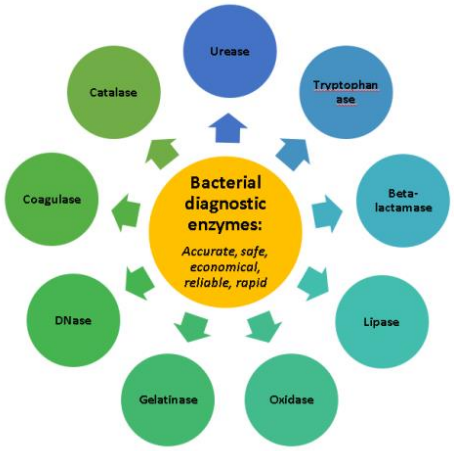
Bacterial enzymes as diagnostic tools for various human pathogens

[1]Meenakshi Vachher, [2]Aparajita Sen, [3]Archana Burman and *[4]Arti Nigam

[1]Department of Biochemistry, Institute of Home Economics, University of Delhi, New Delhi-110016, India; meenakshi.vachher@ihe.du.ac.in
[2] Department of Genetics, University of Delhi, South Campus, New Delhi-110021, India; senaparajita99@gmail.com
[3]Department of Biochemistry, Institute of Home Economics, University of Delhi, New Delhi-110016, India; archana.burman@ihe.du.ac.in
*[4]Department of Microbiology, Institute of Home Economics, University of Delhi, New Delhi-110016, India; arti.nigam@ihe.du.ac.in

Abstract: **Rapid and accurate diagnosis of diseases is essential for early therapeutic intervention and efficacious treatment. Infectious diseases can be devastating with very high mortality and morbidity rates. These are caused by diverse organisms which replicate in their hosts. Enzymes are proteinaceous biocatalysts in nature, except ribozymes, which catalyze diverse reactions indispensable for life. Enzymes have numerous applications owing to their unique features like high substrate specificity, immense catalytic efficiency, diverse pH and temperature optima and the ability to be regulated. Enzymes play an essential and inimitable role in clinical diagnosis and management. Detection of enzymes produced by certain pathogens can help in apt and timely diagnosis of the infectious agent. This can favorably result in a positive outcome and successful therapeutic intervention and thus save lives. In this review, we attempt to summarize microbial enzymes including urease, coagulase, DNase, gelatinase, catalase, oxidase, β-lactamase, tryptophanase and lipase, which are being used in the clinical diagnosis of diseases caused by pathogens and thus they positively influence the disease outcome. Owing to the various novel characteristics possessed by these enzymes, they have become a method of choice for rapid, accurate, economical and simple diagnostics.**



Index Terms: **β-lactamase, Catalase, Clinical Diagnosis, Coagulase, DNase, Gelatinase, Lipase, Oxidase, Pathogens, Tryptophanase, Urease.**

I. INTRODUCTION

Infectious diseases are driven by intricate and multifaceted factors, and represent one of the biggest healthcare challenges and threats to mankind in the present century. Rapid and accurate diagnostic technologies emerge as the first line of defense in the never ending rift between infectious organisms and their human hosts. The ability to identify disease causing pathogens with greater precision and sensitivity is a critical instrument in healthcare. It provides indispensable information to the clinicians to decide treatment options, to update epidemiological models and to monitor therapeutic and prognostic responses of patients. Enzymes are biocatalysts which accelerate the rate of reaction by several orders of magnitude and possess many unique characteristics like high substrate specificity, ability to work at mild pH and temperature conditions and be regulated. The existence of these biocatalysts makes life practicable. Certain enzymes in the diagnostic premises are labeled as biomarkers, which aids in quantifying any biomolecule that acts as an indicator of disease burden. Diagnosis of infectious organisms using enzymes has proven to be one of the most effective and reliable approaches till date. Detection of pathogens at the genomic level using enzyme-assisted nucleic acid diagnostics like different kinds of polymerase chain reactions (PCR) is also a very powerful diagnostic tool (Suea-Ngam et al, 2020). Besides, ELISA (Enzyme-linked Immunosorbent Assay) that measures antigens or antibodies in a patient's blood also relies heavily on enzymes for its application in diagnostics. Thus, multiple enzyme based technologies are proving to be effective in pathogen identification. Additionally, enzymes exhibit varied applicability, ranging from analytical detection and immunoassays to enzyme based biosensors for various non-communicable disorders too (Fahad Ullah et al., 2019; Hemalatha et al., 2013).

The pertinent usage of diagnostic information can substantially influence and assist in initial intervention, leading to reduced burden on healthcare infrastructure and systems, and eventually saving more lives. Furthermore, analyzing and tracking the diagnostic data of infectious diseases can aid biomedical researchers to establish underlying disease models, decipher advanced drug targets and study the potency of existing drugs. Accordingly, rapid and accurate diagnostics of infectious diseases is of supreme significance. A unique program of WHO called Research and Training in Tropical Diseases (WHO/TDR) issued norms for *in-vitro* infectious diseases point of care diagnostics in 2003. They have been categorized as ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment free and Delivered to the end users. Further, the proposal of adding two more innovative norms of "Real time connectivity" and "Ease of specimen collection" led to a newer version of "REASSURED" standards of pathogen detection to reinforce medical management and ameliorate patient upshots (Land et al., 2019). Enzyme based diagnosis of infectious diseases facilitates rapid and accurate recognition of the pathogen, promising an effective strategy against ever-increasing threats of infections. Concerted and multi-disciplinary action using enzyme engineering and molecular biology tools is the need of the hour since accurate diagnosis is the key to successful disease treatment. Various microbial enzymes are employed in clinical diagnostics. Some enzymes used commonly as a part of routine biochemical tests to identify pathogens or to differentiate among various groups of microbes are Urease, Catalase, Oxidase, Gelatinase, DNase, Lipase, Tryptophanase, β-lactamase and Coagulase. Each of the aforementioned enzymes acts on their respective substrates, yielding products that help to differentiate between microbes that are enzyme producers and non-producers. Immobilization of these diagnostic enzymes while maintaining their selectivity, stability and kinetic properties to create efficient miniaturized biosensors is an area of widespread research. These biosensors consist of immobilized enzymes on the surface of a signal transducer. In the present review, we attempt to emphasize on the importance of various microbial enzymes that are routinely used in clinical diagnosis.

II. UREASE

Urease (urea amidohydrolase, EC number 3.5.1.5) was the first metalloenzyme to be crystallized with nickel as its prosthetic group. Urease is a multi-subunit enzyme ubiquitously present in bacteria, fungi and plants but not in animals. It catalyzes the hydrolysis reaction of urea, leading to formation of ammonia and carbamate, which further are spontaneously hydrolyzed to ammonia and carbonic acid (Mobley et al., 1995; Mobley & Hausinger, 1989). In aqueous solutions, carbonic acid and ammonia are present in equilibrium with their unprotonated and protonated forms respectively, leading to a rise in pH. Catalytically, urease accelerates the rate of urea hydrolysis by a factor of 10^{14}, as opposed to uncatalyzed urea degradation by elimination reaction (Kappaun et al, 2018).

Urease is formed by various taxonomically distinct bacterial species, comprising of pathogens, non-pathogens and normal human microflora. Urease synthesis may be constitutive or stress induced to counteract low pH environments, and helps in the use of ammonia as a source of nitrogen (Cotter & Hill, 2003). Urease plays a critical role in the pathogenesis elicited by various bacterial species, by acting as a virulence factor (Rutherford, 2014). Pathogenic ureolytic bacteria *Helicobacter pylori* (*H. pylori*) are associated with gastric ulcers, non-ulcer dyspepsia, gastritis and stomach cancer. It colonizes the stomach and produces huge quantities of urease to degrade urea for its survival in the acidic environments of the stomach, by neutralizing the same (Amieva & Peek, 2016). *H. pylori* urease (HPU) has also been shown to be an inducer of endocytosis besides being angiogenic and pro-inflammatory in nature. It activates human neutrophils leading to generation of damaging reactive oxygen species which further lead to infection and carcinogenesis (de Jesus Souza et al., 2019). *Streptococcus salivarius* generates ammonia in the oral cavity using its urease to lower the surrounding pH. This creates dental plaques as well as calculus depositions (Sissons & Yakub, 2000). *Klebsiella pneumoniae* and *Proteus mirabilis* exhibit high levels of urease and are causative agents of pneumonia, urinary tract infections and kidney stone formations, leading to the pathogenesis of pyelonephritis and catheter covering (Paczosa & Mecsas, 2016). Urease production by pathogens like Shiga toxin generating *E. coli* (Steyert & Kaper, 2012), *Yersinia enterocolitica* (de Koning-Ward et al., 1995), *Brucella abortus* (Sangari et al., 2007) and *Haemophilus influenzae* (Murphy & Brauer, 2011) helps the pathogens to acquire acid resistance and assists in colonization. Ureases produced by few fungi also aid in the pathogenesis of various diseases like Cryptococcosis in humans caused by *Cryptococcus neoformans* and *C. gatti* (Feder et al., 2015). Urease is emerging as a potential therapeutic target since there are no other known nickel metalloenzymes in humans till date (Follmer, 2010). Pathway for the maturation of urease with nickel at its active site is a complex process and is emerging as a prospective pathway for chemical intervention, as well as novel drug targets like substrate analogs and metal chelators (Follmer, 2010; Svane et al, 2020).

Urease induced hydrolysis of urea can be applied for bacterial identification and aid in disease diagnosis. Such assays rely on analysis of activity and kinetic behavior of the enzyme. Majorly, they are indirect assays based on colorimetric estimation of ammonia produced while incubation in a buffered solution of urea (Hamilton-Miller & Gargan, 1979). Earliest method for determining the ureolytic activity was based on the growth of microbes in a urea rich medium (Christensen's urea medium) (Christensen, 1946). This method enjoys the status of being the most widespread qualitative test for urease producing pathogens like *Proteus* sp. A modification of Christensen technique rapidly determines urea hydrolysis and reduces assay time to 30 minutes, yielding quicker results (Qadri et al, 1984). Ureolytic activity of *H. pylori* has emerged as a biomarker to diagnose infection and to monitor therapeutic response (Bell et al, 1987; Best et al, 2018). Globally, about 13 to 81% of the population has *H. pylori* infection (Peleteiro et al, 2014). Both invasive and non-invasive tests are a part of the protocol to diagnose infection, depending upon the need of endoscopy. The confirmatory diagnostic test is an endoscopic biopsy followed by Haematoxylin and Eosin staining or special staining using Giemsa or Warthin-Starry stain for histopathological examination. The most widespread and common invasive test is a Rapid Urease Test (RUT). It is an indirect test for *H. pylori* infection based on the absence or presence of urease in or on the gastric mucosa, which requires biopsies. RUT is advantageous over serological tests as it identifies active infection only. Besides, tissue samples implanted in a RUT gel

can be used for other molecular biology tests too. Various highly sensitive and specific versions of this test have been approved for human use (Uotani & Graham, 2015). While performing a RUT test, the gastric mucosal sample is added to a urea-containing gel or tube, and the products of urea hydrolysis (ammonia and carbon dioxide) are detected. These methods include assessment of labelled carbon dioxide in breath or serum samples. pH alterations induced by ammonia formation are detected either by a color change or with the help of a pH meter. The original test consisted of phenol red as a pH indicator in the medium, which exhibited a change in color from yellow to pink/red as the pH increased due to alkalization of the medium (Foroutan et al, 2010). Other indicators that can eliminate contaminating urease producing oral bacteria, by starting to change color at a lower pH of 5.4, are also available commercially (HP*fast*®). Among non-invasive tests, Urea Breath Test (UBT) is common. This is a simple test and relies on oral administration of non-toxic isotope containing (C^{14} or C^{13}) urea. Urea hydrolysis yields ammonia and isotopically labelled carbon dioxide (CO_2). This CO_2 is detected in the exhaled air in the breath samples. Breath samples are collected for up to half an hour as the CO_2 gets dissolved in the blood, removed via lungs and collected into a CO_2 trapping agent. The proportion of carbon recovered for the duration of the collection time per minute is one of the threshold criteria used. UBT has become a method of choice for the diagnosis of *H. pylori* infection in medical practice, as it offers higher accuracy as compared to serology or stool antigen test (Artiko et al, 2001; Ferwana et al, 2015). Some studies also point towards the usage of UBT for tuberculosis diagnosis (Jassal et al, 2010). Other methods based on the ureolytic activity of urease are also available and are mostly used in research laboratories. These include native gel electrophoresis to detect the presence of active urease (Shaik-M et al, 1980), phenol hypochlorite assay which measures ammonia when it reacts with phenol hypochlorite and forms indophenol (Weatherburn, 1967), Nesslerization reaction (Y.-L. Lin et al, 2000), coupled enzyme assays based on combination of ammonia to α-ketoglutarate in the presence of the enzyme glutamate dehydrogenase (Kaltwasser & Schlegel, 1966) and potentiometric assays with ammonia sensitive electrodes (Montalvo, 1970). Other methods to assess ammonia released by the action of urease include vacuum distillation, electro-conductivity measurements and Fourier Transform Infrared spectroscopy (Karmali et al, 2004). The significance of urease as a virulence factor that prevents triggering host's immunity is clearly evident in case of *H. pylori* (Olivera-Severo et al, 2006), *M. tuberculosis* (W. Lin et al, 2012) and *P. mirabilis* (Coker et al, 2000) infections, as it causes alkalization of their microenvironments, and facilitate their persistence in the host cells. It affects host's immune system (Dunn & Phadnis, 1998) and helps the bacterium to survive when urea is the only nitrogen source (W. Lin et al, 2012). Thus owing to its central role in establishing infections, it has a great potential as a diagnostic tool.

III. COAGULASE

Coagulase (Coa, EC Number 3.4.2.3) is an enzyme that catalyzes the conversion of soluble fibrinogen present in blood plasma to insoluble fibrin. Coa is a polypeptide of 670 amino acids, varying in length in different strains. Blood coagulation is generally reflected as an innate and early defense mechanism against microbial pathogens, by trapping and immobilizing the invading pathogen into a clot (Loof et al, 2011). Nonetheless, as for various other host defense pathways, it can also act as a mechanism of bacterial immune evasion (Loeb, 1903). *Staphylococcus aureus* is a commensal microorganism inhabiting the skin and nostrils of humans, but it can often invade skin through wounds, causing infections of the soft tissue. It also acts as a causative organism for deadly invasive infections including pneumonia, sepsis, osteomyelitis, endocarditis and toxemia of the intestinal as well as reproductive tracts (Lowy, 1998). In health care settings, intrusive infections of *S. aureus* are hospital acquired and rampant (Boucher & Corey, 2008). Some *S. aureus* strains have developed resistance to the commonly used drug methicillin, which gave rise to Methicillin Resistant *Staphylococcus aureus* (MRSA), which has enhanced mortality rate and increased the cost of treatment, making it a great health hazard. It causes additional serious lethal complications like bacteremia and toxic shock syndrome. *S. aureus* secretes two coagulation promoting proteins, coagulase and von Willebrand factor binding protein (vWbp). These two activate prothrombin non-proeolytically, acting as clotting factors (Friedrich et al, 2003). The N-terminal regions of each Coa and vWbp associate with a prosite found in prothrombin, leading to completion of an active site that is exclusively present in thrombin. Human prothrombin is also directly bound by Coa leading to formation of a prothrombin-staphylocoagulase complex. As a consequence of its interaction with prothrombin, the resulting conformational change in prothrombin generates an active site proficient in catalyzing the conversion of soluble fibrinogen into fibrin (Cheng et al, 2010). The dynamic complex of the host's prothrombin with either Coa or vWbp is called "Staphylothrombin" (Vanassche et al, 2011). Coagulase is a surface bound protein of the bacterium *S. aureus* and coats the bacterial cell through fibrin as it comes in contact with blood. This fibrin clot protects the bacterium from phagocytosis by host immune cells. The fibrin coat thus increases the bacterial virulence, and hence Coagulase is known to be a virulence factor (Ghassemi et al, 2017). It produces abscesses in cells of the host which it uses to replicate at the center of such lesions. Thus, it is protected from the immune system of the host through this psuedocapsule.

One of the first identifiable features of staphyloccal infection was its ability to bring about clotting of human blood. Even today, the coagulase activity is employed in a clinical laboratory, routinely to differentiate between different isolates of *Staphylococcus*, or to distinguish between *S. aureus* and clinically less pathogenic strains of coagulase negative *Staphylococcus*. *S. aureus* is usually coagulase-positive, along with eleven other species of *Staphylococci*. A negative coagulase test indicates presence of organisms such as *S. epidermidis* or *S. saprophyticus*, commonly known as Coagulase negative Staphlococci (CoNS) which are relatively less virulent and have a different disease spectrum (Becker et al, 2014). However, not all *S. aureus* strains test positive for coagulase.

Most pathogenic strains of *Staphylococcus* produce coagulase. Coagulase test is a recommended confirmatory test to detect *Staphylococcus aureus* and differentiate it from other *Staphylococcus* species (Silva et al, 2020). Detecting MRSA through the traditional Coagulase Test takes long. Now, certain chromogenic media are available in which detection is achieved after 24-48 hours of incubation. *Staphylococcus aureus* can produce two types of coagulase, namely free and bound coagulase. Free coagulase is extracellular and can react with

prothrombin and its derivatives, whereas bound coagulase reacts with the α- and β-chains of fibrinogens to form a coagulate, and is localized on the surface of the cell wall. Seven antigenic types of free coagulase are known, and one antigenic type of bound coagulase exists. Bound coagulase is heat stable, whereas free coagulase is not. It is also produced by bacteria like *Yersinia pestis* and *Bacillus cereus* (M et al, 2013).

The most important application of coagulase is as a routine laboratory test (Sperber & Tatini, 1975). The coagulase test indicates whether or not an organism can produce this enzyme. It is also a good indicator of the pathogenicity of *S. aureus* (Qian et al, 2007). In this test, the microbial sample whose pathogenicity is to be determined is added to rabbit plasma and left at 37°C for a specified time period. If a clot is formed within four hours, it is interpreted as a positive result, indicating the presence of a virulent strain of *S. aureus* (Khusro et al, 2020). The absence of any coagulation even after twenty four hours of incubation is a negative result, indicating an avirulent strain. Various variants of coagulase test like TCT (Tube Coagulase Test) that uses rabbit and human plasma, culture media like MSA (Mannitol Salt Agar) or DNase (Deoxyribonuclease) media and Hiaureus™ Coagulase Confirmation kit (HACCK) are routinely used in clinical diagnosis and hold great promise in identification of virulent stains of *S. aureus* (Subramanian et al, 2017).

IV. CATALASE

Catalase (EC number 1.11.1.6) is an oxidoreductase metalloenzyme ubiquitously found in almost all oxygen-exposed living organisms. It serves as an antioxidant enzyme catalyzing the transformation of hydrogen peroxide (H_2O_2) to water and oxygen ($2\ H_2O_2 \rightarrow 2H_2O + O_2$) (Chelikani et al, 2004; Glorieux & Calderon, 2017). It plays a significant role in cells as it protects them from oxidative damage caused by harmful reactive oxygen species (ROS). Catalase exhibits catalytic perfection as it has one of the highest turnover numbers among various enzymes. H_2O_2 is a detrimental byproduct of aerobic metabolism that can cause oxidative damage to the cells. To prevent this, H_2O_2 should be converted into substances like water and oxygen rapidly. This rapid decomposition of H_2O_2 is catalyzed by catalase in two stages, involving one electron transfer and a high valent iron intermediate, eventually producing water and oxygen. Catalase can also catalyse H_2O_2-mediated oxidation of various metabolites (R) and toxins such as phenols, alcohols, formic acid, formaldehyde and acetaldehyde, by the reaction $H_2O_2 + H_2R \rightarrow 2H_2O + R$. Catalase finds diverse commercial applications in food industry, cheese production, textiles, packaging and in contact lens cleaning solutions (Heck et al, 2010; Hiti et al, 2002; Mehra & Chadha, 2020). Also low levels of catalase have been shown to cause greying of human hair, as H_2O_2 interferes with melanin production (Wood et al, 2009).

Catalase is produced by many human pathogens including *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Legionella pneumophila* and *Edwardsiella tarda* that helps inactivate the H_2O_2 produced by phagocytic cells in the host, allowing themselves to survive despite the host defense mechanisms (Mandell, 1975; Srinivasa Rao et al, 2003). Almost all aerobes use catalase. In eukaryotes, catalase is primarily found in the peroxisomes and mitochondria. Some anaerobes like *Methanosarcina barkeri* also possess catalase (Brioukhanov et al, 2006). Catalase is also present in most fungi and plants (Hansberg et al, 2012). Hyperthermophilic archaea like *Pyrobaculum calidifontis* also produce catalase that optimally functions at 90°C (Amo et al, 2002). Some other catalase producing microorganisms include bacteria like *Burkholderia cepacia*, *Corynebacterium diphtheriae*, *Listeria*, *Staphylococci*, *Micrococci*, *Nocardia*, Enterobacteria (*E. coli*, *Citrobacter*, *Enterobacter*, *Yersinia*, *Klebsiella*, *Proteus*, *Shigella*, *Salmonella and Serratia*), *Mycobacterium tuberculosis*, *Pseudomonas* and *Rhodococcus equi*, and fungi like *Aspergillus* and *Cryptococcus* (Amo et al, 2002).

The catalase test (CT) plays a major role in diagnosing bacterial pathogens (Iwase et al, 2013). H_2O_2 is added to the bacterial sample and the reaction is observed. If the bacteria are catalase-positive, oxygen bubbles (effervescence) are observed as in indication of the presence of catalase. In the absence of any bubbles or froth, the bacteria are concluded to be catalase-negative, such as *Streptococci* and *Enterococci* (Ruoff, 2002). Thus, catalase test can be used to distinguish *Staphlococci* (catalase positive) from *Streptococci* (catalase negative) and greatly help in pathogen identification.

V. DNASE

Deoxyribonuclease (DNase-I, EC Number 3.1.21.1) is a nuclease that catalyses the degradation of DNA, by hydrolytically cleaving the phosphodiester bonds in the backbone of DNA. Various deoxyribonucleases have been identified till date, differing in properties like mechanism of catalysis, substrate specificities and biological functions (Meuleman et al, 2020). Some DNases are exonucleases, and cleave residues only at the ends of DNA molecules, and others are endonucleases, and can randomly cleave anywhere along the chain. Some are indiscriminate about the cleavage site, while others such as restriction endonucleases are very specific to the sequence of the cleavage site. Some DNases are specific for double-stranded DNA, whereas others only cleave single-stranded molecules and some others can act on both (Yu et al, 2015).

Bacterial DNases hydrolyze external DNA into nucleotides that can easily move via transport proteins across the cell membrane to be utilized by the bacteria for nucleic acid synthesis or as carbon, nitrogen and phosphate sources. Extracellular DNases can also be produced by many human pathogens (Buchanan et al, 2006). *Staphylococcus aureus*, *Serratia* sp, and *Moraxella catarrhalis* are some of the bacteria that produce the enzyme DNase (Pourbabaee et al, 2020). DNase can be inhaled by patients suffering from cystic fibrosis, using a nebulizer. In cystic fibrosis, white blood cells accumulate in the patient's mucus, degrade and release DNA making the mucus more sticky and viscous. This is prevented by DNase enzymes (Deacon et al, 2015). DNases are also widely used in molecular biology and research.

Another important application of DNase is as a diagnostic tool. The DNase Test tests if an organism is able to produce DNase. An organism is grown on a DNase Test Agar plate to test for DNA

hydrolysis. It helps to detect DNase activity of microbes, and to identify pathogenic strains of *Staphylococci* (Asha & Krishnaveni, 2020). The DNase Test Agar plate also contains Methyl Green as an indicator. Deoxyribonuclease breaks DNA in the medium into smaller fragments which can no longer bind to Methyl Green, and thus a clear halo appears around the colonies of DNase-producing bacteria (Patel et al, 2019). This test is used to differentiate between DNase producers like *Staphylococcus aureus*, *Serratia* etc. from non-DNase producers like *Enterobacter*, *Neisseria* and other *Staphylococci* (Kateete et al, 2010).

VI. GELATINASE

Gelatinases (Gelatinase A, EC Number 3.4.24.24) belong to a broad family of extracellular metalloenzymes and are Ca^{2+} and Zn^{2+}-dependent neutral endopeptidases (Gioia et al, 2007). They catalyze the hydrolysis of various components of the extracellular matrix including collagen, gelatin, fibrinogen, casein and pheromones (Mäkinen & Mäkinen, 1994) into constituent amino acids and smaller peptides, which can easily cross the plasma membrane and be used as nutrients by the producer microorganism (Balan et al, 2012). When gelatinase binds to specific domains of the triple helical structure of collagen, it brings about a mild alteration in its assembly, leading to a chain-specific cleavage. Gelatinases are more involved in disruptive events that are associated with pathological processes. Gelatinase is produced by many microbes as a virulence factor, in order to help them degrade the extracellular matrix of the host and aid in invasion. *Clostridium perfringens, Enterococcus faecalis, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus* and *Serratia marcescens* are some such human pathogens that are known to produce Gelatinase (Balan et al, 2012).

Gelatinases have recently emerged as potential novel drug targets owing to their capacity to degrade connective tissue and their association with cancer metastasis. The most important application of Gelatinase in clinical diagnostics is the Gelatinase Test used for bacterial characterization, to identify gelatinase producers and distinguish them from non-producers. If Gelatinase is present, the protein gelatin liquefies as it is broken down into monomeric amino acids. It is used as a presumptive test to identify pathogenic organisms like *Serratia*, *Pseudomonas, Flavobacterium* and *Clostridium.* The Gelatin Agar Medium is stabbed with the bacterial culture, and if liquefaction of the media occurs even at 4°C, the bacteria is said to be Gelatinase positive.

VII. OXIDASE

Oxidases are a subclass of oxidoreductases which catalyze oxidation-reduction (redox) reactions, which use oxygen (O_2) as the electron acceptor and produce water (H_2O) or hydrogen peroxide (H_2O_2) as by-products. Usually they contain flavin nucleotides and iron or copper as cofactors at the active site. Cytochrome c oxidase (EC Number 7.1.1.9) is a key enzyme that uses oxygen for energy production via oxidative phosphorylation using electron transfer reactions. It plays an indispensable role in the cellular respiration and the electron transport chain. It is present in the inner mitochondrial membrane of the eukaryotes and in the plasma membrane of the prokaryotes. It is a large transmembrane protein complex (Complex IV) that pumps protons across the plasma membrane from the cytosol to the extracellular space in bacteria or across the inner mitochondrial membrane from the mitochondrial matrix to the intermembrane space in eukaryotes. This results in the generation of a transmembrane electrochemical gradient (Wikström et al, 2018).

Oxidases are a large group of enzymes, which have slightly different mechanisms of action. In oxygen utilising enzymes like cytochrome c oxidase, oxygen binds to the metal cofactors in the enzyme. One oxygen atom binds the enzyme bound Fe^{2+} of a heme of the cytochrome a3, and the other oxygen atom binds Cu^{+} of CuB. All intermediates remain enzyme bound. Four different cytochrome c molecules serve as mobile electron carriers, and add four electrons as the substrates (Kadenbach, 2020).

All aerobic bacteria and facultative aerobes where oxygen acts an ultimate electron acceptor during respiration can produce oxidase. If an organism does not produce oxidase, it means it does not have the cytochrome c oxidase. Few oxidase positive bacteria include *Alcaligenes, Aeromonas, Brucella, Campylobacter, Pseudomonas, Neisseria, Vibrio, Pasteurella, Moraxella, H. pylori, Legionella pneumophila* etc. (Bezanson et al, 2008).

Oxidases find numerous applications in varied industries such as food, textile, paper and pulp and biodiesel production. However, the most relevant application in terms of the present review is the use of oxidase as a biochemical diagnostic test to detect bacterial species possessing cytochrome c oxidase. Most commonly, it helps in characterizing gram negative bacteria. In the presence of cytochrome c oxidase, the reagent N,N,N,N-tetramethyl-p-phenylenediamine (TMPD) used in the test gets oxidised to indophenols which imparts a purple color. Thus organisms that are oxidase positive turn the reagent blue/purple. If the enzyme is absent, the reagent remains colorless as it is colorless when reduced. This test distinguishes oxidase-negative Enterobacteriaceae from oxidase-positive genera like *Pseudomonas* sp., *Aeromonas* sp., *Campylobacter* sp., *Neisseria* sp. and *Pasteurella* sp. (Tarrand & Gröschel, 1982). Oxidase strips (Oxystrips™) and oxidase swabs (Oxysticks ™) are available commercially to distinguish oxidase positive bacteria from negative ones.

VIII. B-LACTAMASE

β-lactamases (EC Number 3.5.2.6) are bacterial enzymes that protect the bacteria from β-lactam antibiotics including pencillin, cephamycin, cephalosporin as well as carbapenem. β-lactam antibiotics are commonly employed to treat a wide variety of bacterial infections caused by both gram positive as well as gram negative bacterial species (Tooke et al, 2019). β-lactamases are produced and secreted by Gram-negative organisms when β-lactam antibiotics are present in their vicinity (Majiduddin et al, 2002). β-lactamase can generate antibiotic resistance in such bacterial species by breaking the structure of β-lactam antibiotics, which have a common four-membered ring called the β-lactam ring. The enzyme catalyzes the hydrolysis of β-lactam

ring, leading to its opening and deactivation of the antibacterial properties of the drug. β-lactamases can be further subdivided into various classes and subclasses based on activities and peptide sequence information. Presently, more than three hundred β-lactamase enzymes are known, belonging to four separate classes, namely A, B, C and D, present in the Beta- lactamase database (BLDB) (Naas et al, 2017). β-lactamases may be chromosomal or plasmid encoded in different bacteria. β-lactamase induced antibiotic resistance initially was observed only in a few bacterial species like *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa*, which underwent mutation and hyperproduced β-lactamase. In a few years' time antibiotic resistance also started to appear in several bacterial species that did not produce these enzymes naturally, like *Klebsiella pneumoniae*, *Salmonella* sp. as well as *Proteus mirabilis*, as they started producing ESBLs (extended spectrum beta lactamases) (Jacoby, 2009).

β-lactamases are used to test the antibiotic resistance or susceptibility of microbes. In this, a substrate called nitrocefin is used, which is a chromogenic cephalosporin having wide spectrum susceptibility to the commercially available β-lactam antibiotics. Disks are impregnated with nitrocefin. When a β-lactamase hydrolyzes the amide bond in a β-lactam ring, the nitrocefin on the disk changes its color from yellow to red. Bacteria that produce significant amounts of β-lactamases can change the yellow color of the Nitrocef Disk to red. These β-lactamases can inactivate amoxicillin, penicillin, ampicillin, mezlocillin, carbenicillin and piperacillin. β-lactamase tests yield results much faster than an MIC (Minimum Inhibitory Concentration) or disk diffusion test (Livermore, 1995).

IX. TRYPTOPHANASE

Tryptophanase (tryptophan-indole lyase, EC Number 4.1.99.1) is an enzyme that uses pyridoxal 5′-phosphate (PLP) as a cofactor to catalyse the hydrolysis of L-tryptophan (L-Trp) to ammonia, pyruvate and indole (L-tryptophan+ H_2O→ Indole+ pyruvate + ammonium). This reaction triggers certain physiological changes which in turn aid in regulation of host-pathogen interactions between the bacteria and their mammalian hosts. It is a tetramer with has four identical monomers of 52kDa, with one molecule of PLP present in each monomer. An internal aldimine bond is formed between the lysine present in the enzyme's active site and PLP (Ku et al, 2006). It is one of the enzymes with highest stereospecificity for optical isomers, and thus tryptophanase is not very active towards D-tryptophan (D-Trp). Production of tryptophanase is induced by the presence of external tryptophan, and it allows tryptophan to be used as a carbon and nitrogen source. Tryptophanase operates its catalytic mechanism via an α,β elimination reaction with several intermediates, starting with its association with tryptophan to form the Michaelis complex, followed by external aldimine formation (transaldimination), quinonoid formation from the external aldimine by alpha-proton abstraction and tautomerisation of the indolyl group. Next, indole is eliminated and aminoacrylate Schiff base intermediate is formed. A second transaldimination restores the internal aldimine. Finally, aminoacrylate is released and decomposed to ammonia and pyruvate (Snell, 1975).

Tryptophanase is produced by many Gram-negative and Gram-positive bacteria like *Aeromonas hydrophila*, *A. punctata*, *Bacillus alvei*, *Clostridium* sp., *Escherichia coli*, *Edwardsiella* sp., *Flavobacterium* sp., *Haemophilus influenzae*, *Klebsiella oxytoca*, *Proteus* sp. and *Bacteroides* sp. (DeMoss & Moser, 1969; Lee & Lee, 2010; Roager & Licht, 2018).

The most widespread application of tryptophanase in diagnosing pathogens is the Indole Test, which constitutes a part of Indole, Methyl red, Voges-Proskauer and Citrate (IMViC Test) widely employed to differentiate between various members of the Enterobacteriaceae family, and particularly to distinguish *E. coli* from *Enterobacter* and *Klebsiella*. It is also used to differentiate *Proteus mirabilis* from various other *Proteus* species. Indole is produced from the substrate tryptophan only if the bacteria possess tryptophanase. This indole produced reacts with Kovac's reagent containing p-dimethylaminobenzaldehyde, concentrated hydrochloric acid and isoamyl alcohol, to give a cherry red color. A red layer of isoamyl alcohol is formed at the top of the broth, as isoamyl alcohol is insoluble in water. The formation of cherry red ring above the medium indicates a positive result. A negative result is indicated by absence of any coloration. Spot test may also be used for distinction of indole positive and indole negative microorganisms using a filter paper saturated with Kovac's reagent (Trepeta & Edberg, 1984).

X. LIPASE

Lipases (EC Number 3.1.1.3) are serine hydrolyases belonging to the triacylglycerol ester hydrolase superfamily, that catalyze the hydrolysis of fats or lipids. They are indispensable enzymes of living organisms required for digestion and transport of lipids. Most lipases act on the glycerol backbone of a lipid substrate. They catalyze the hydrolysis of long chain triacylglyerols to diacylglycerols, monoacylglycerols, fatty acids and free glycerol (Javed et al, 2018). Various types of lipases exist in nature, such as phospholipases and sphingomyelinases. Lipases are monomeric proteins with molecular weight ranging from 19-60kDa. Some lipases are secreted by pathogens during infection, such as by *Candida albicans.* One of the significant factors contributing to the persistence and virulence of *C. albicans* in humans is lipase acting as a virulence factor (Chandra et al, 2020). Lipase is produced by various bacteria like *Staphylococcus aureus*, *Clostridium sporogenes*, *Bacillus* sp., *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp. and fungi like *Penicillium* sp., *Fusarium* sp. and *Aspergillus* sp. (Stehr et al, 2003).

Lipases find varied applications in several industries such as foods and drinks, biodiesel, leather, detergents, textiles and pharmaceuticals (Sharma et al, 2011). However, from the diagnostic point of view, it is used for the detection of lipase producing bacteria. Tributyrin is used as the substrate as it is the simplest naturally occurring triglyceride. Tributyrin needs a lipase as it is too large to enter the cell, so that it is broken down prior to cellular uptake. Upon hydrolysis, the glycerol is converted to dihydroxyacetone phosphate, a glycolytic intermediate, and the fatty acids undergo β-oxidation and are converted into end-products that can be used for energy production by the cell. Tributyrin agar tests the ability of an organism to hydrolyze

tributyrin oil due to production of lipase. It is a differential medium that is prepared as an emulsion to make the agar opaque. On inoculation and incubation, a lipase-positive organism will give clear zones around its colony. Absence of clear zones indicates a lipase-negative organism.

Thus, these bacterial enzymes each with its unique properties, either alone or together with other enzymes, can greatly help in the identification of producer bacterial species. Table 1 summarizes the above described bacterial diagnostic enzymes routinely used in a clinical laboratory.

Table 1: Various Bacterial enzymes used in Clinical diagnostics

Enzyme	**Reaction catalyzed**	**Microorganism identified**	**Reference**
Urease	Conversion of urea into ammonia and carbonic acid	*H. pylori, Haemophilus influenzae, M. tuberculosis*	Best et al, 2018, Noh et al, 2020
Coagulase	Binds to prothrombin to form the staphylothrombin complex that converts fibrinogen into fibrin.	Virulent strains of *S. aureus*	Cheng et al, 2010, Kumari et al, 2020
Catalase	Breakdown of hydrogen peroxide into water and oxygen	*Burkholderia cepacia, Corynebacterium diphtheriae, Listeria sp., Micrococcus sp., Nocardia sp., Staphylococcus sp.*	Srinivasa Rao et al, 2003
DNase	Hydrolytic cleavage of phosphodiester bonds of DNA	*Moraxella catarrhalis, Serratia* sp., *Staphylococcus aureus*	Patel et al, 2019
Gelatinase	Cleave components of extracellular matrix like gelatin and collagen	*Clostridium perfringens, Enterococcus faecalis, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Serratia marcescens*	Balan et al, 2012, Iseppi et al, 2020
Oxidases	Catalyzes the reduction of molecular oxygen to water or hydrogen peroxide	*Aeromonas sp., Alcaligenes sp., Brucella sp., Campylobacter sp., Legionella pneumophila, H. pylori, Neisseria sp., Moraxella sp., Pseudomonas sp., Pasteurella sp., Vibrio sp.*	Bezanson et al, 2008
β-lactamases	Hydrolyse the β-lactam ring consequently de-activating β-lactam antibiotics like pencillin	*Citrobacter freundii, Enterobacter cloacae, Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa*	Jacoby, 2009
Tryptophanase	Hydrolytic β-elimination of L-tryptophan to indole, pyruvate and ammonium	*E. coli, Proteus mirabilis*	DeMoss & Moser, 1969; Roager & Licht, 2018
Lipase	Hydrolysis of fats into free fatty acids and glycerol	*Achromobacter sp., Alcaligenes sp., Arthrobacter sp., Bacillus sp., Clostridium sporogenes, Staphylococcus aureus, Pseudomonos sp. Aspergillus sp., Fusarium sp., Penicillium sp.*	Sharma et al, 2011

CONCLUSION

Recent progress in engineered Activity based diagnostics (ABDx) empowers enzymatic activity to measure or produce biomarkers (Soleimany & Bhatia, 2020). Owing to catalytic signal augmentation, high substrate specificity, no residual toxins, cost-effectiveness, timely results and ability to be reused when immobilized, enzymes offer great potential as highly specific and sensitive diagnostics for detecting infectious as well as non-communicable diseases. There has been a rapid upsurge in the application of microbial enzymes as diagnostic tools. These enzymes act on their respective substrates and yield different results for the bacteria that produce the enzymes and those who do not, thus allowing easy detection and diagnosis of pathogens. Effective clinical management critically depends upon accurate detection of diseases and monitoring therapeutic response. Integrating tools from a plethora of disciplines like enzymology, molecular biology, orthogonal chemistry, machine learning, nanobiotechnology, artificial gene circuits and genome editing for the development and improvisation of enzyme based diagnostics holds promise to help realize the goals of precision medicine.

AUTHORS CONTRIBUTIONS

All authors participated in the designing, drafting, revising and approval of the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare the lack of any commercial or economic relationships that could be considered as a potential conflict of interest.

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