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REVIEW

Biochemical Characteristics of Microbial Enzymes and Their Significance from Industrial Perspectives

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Abstract
Microbes are ubiquitously distributed in nature and are a critical part of the holobiont fitness. They are perceived as the most potential biochemical reservoir of inordinately diverse and multi-functional enzymes. The robust nature of the microbial enzymes with thermostability, pH stability and multi-functionality make them potential candidates for the efficient biotechnological processes under diverse physio-chemical conditions. The need for sustainable solutions to various environmental challenges has further surged the demand for industrial enzymes. Fueled by the recent advent of recombinant DNA technology, genetic engineering, and high-throughput sequencing and omics techniques, numerous microbial enzymes have been developed and further exploited for various industrial and therapeutic applications. Most of the hydrolytic enzymes (protease being the dominant hydrolytic enzyme) have broad range of industrial uses such as food and feed processing, polymer synthesis, production of pharmaceuticals, manufactures of detergents, paper and textiles, and bio-fuel refinery. In this review article, after a short overview of microbial enzymes, an approach has been made to highlight and discuss their potential relevance in biotechnological applications and industrial bio-processes, significant biochemical characteristics of the microbial enzymes, and various tools that are revitalizing the novel enzymes discovery.

Keywords Microbial enzymes · Biochemical characteristics · Recombinant DNA technology · Industrial applications

Introduction

Microbes constitute about 60% of the total earth biomass. It is estimated that the amount of carbon assimilation fixed through microbial activity is even higher than those by green plants [1]. Animals and plants depend on the symbiont microbial species to carry out some of the metabolic activities particularly in terms of nutrient provision. Archetypes include but not limited to cellulose degradation in ruminants, cattle, and termites [2, 3]; nitrogen fixation in legumes [4]; photosynthesis in micro-algae in corals and sponges [5]; and oxidation of inorganic compounds

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in deep-sea invertebrates [6]. The gut microbiota in human are involved in various metabolic functions, breaking down toxic compounds, and defense against pathogenic organisms via enhancement of the immune system. Thus, the gut microbes in human body are considered as a potential source of novel therapeutics [7, 8]. Microbes associated with plant roots also provide significant contribution to nitrogen metabolism, phosphate uptake, and enhancing access to organic matter and water [9].

The function of microorganisms in various sectors can be classified into three major phases. Traditional industrial microbiology, being the first phase covers the fields that include but not limited to the applications of microorganisms to preserve milk and vegetables, and to produce various food products such as cheese, bread, pickles, beer, wine, and vinegar. An epitome of this field is the production of alcoholic beverages from barley through the application of yeast during the early 6000 BC [1, 7]. Modern industrial fermentation, also referred to as the second major phase, focuses on large-scale fermentation facilities to process various enzymes, vitamins, organic solvents, antibiotics, and other value-added products. Microbiological engineering came into existence through the development of penicillin and streptomycin production. The microbial recombinant DNA technology is the third phase and is also known as modern biotechnology. Modern microbial biotechnology has a wide spectrum of industrial applications ranging from agriculture, food processing, detergents, dairy, beverages, paper/pulp industry, leather, petroleum, mining, textiles, polymer, cosmetics, waste treatment to health care, diagnostics, pharmaceuticals, and human/animal medicine [7]. The advent of protein engineering, directed evolution, and 'omics' approaches has further revolutionized the discovery of new microbes and their enzymes in industrial applications [10, 11].

Enzymes, also known as bio-catalysts, are the biological macromolecules offering a crucial role to accelerate both the rate and specificity of various chemical reactions and metabolic processes [12]. Most of the enzymes are proteinaceous in nature except ribozymes. In recent years, the use of enzymes in the development of eco-benign and efficient chemical syntheses has increased tremendously. Biological enzymes are of tremendous interest due to their special physio-chemical attributes such as low cost, low-energy input/intake, environmentally safe or non-toxic, and high-efficiency rate. These attributes have led to a competitive production phenomenon and subsequently enzymes have been employed extremely in the development of numerous industrial bio-processes [13, 14].

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has classified the enzymes into six major classes on the basis of the reactions they catalyze. The class of enzymes, types of reactions, and selection of the enzymes in industrial processes are enumerated in Table 1.

The term "enzyme" comes from a Greek word "*ε]ζυμο*" meaning in leaven [17]. It was first coined by Wilhelm Friedrich Kuhne in 1877 albeit the term enzyme and its use were already been seen to be widely involved since ancient times [13]. Diastase is the first enzyme discovered by French chemist, Anselme Payen. In 1947, James B. Sumner isolated and crystallized the enzyme urease from jack bean, the first enzyme in pure form, which earned him the Nobel Prize for the year 1947 (https://www.nobelprize.org/prizes/chemistry/1946/summary/) [13]. NOVO used *Bacillus licheniformis* for the commercial production of protease in 1960. Stimulated by the recent advancement in genetic engineering, protein engineering, use of additives, and immobilization, these techniques should favor the enhancement in enzyme production with better yield [18]. The

Table 1 A selection of class of enzymes used in industrial bio-processes, and types of reactions

S. N.[a]	Class of enzyme	Reactions	Industrial enzymes
1.	Oxidoreductase	Involves in oxidation and reduction in between molecules	Alcohol dehydrogenases, catalases, laccases, oxygenases, peroxidases
2.	Transferases	Involves in the transfer of functional groups from one molecule to another (e.g., amino or phosphate groups)	Transketolases, acyltransferases, fructosyltransferases, glycosyltransferases, transaminases
3.	Hydrolases	Catalyze the hydrolysis of a substrate in the reaction	Proteases, amylases, cellulases, xylanases, pectinases, lipases, phytases, phosphatases, cutinases
4.	Lyases	Catalyze the addition or elimination of water, ammonia, or carbon-dioxide to and from double bonds	Decarboxylases, aldolases, dehydratases, pectate lyases, fumarases
5.	Isomerases	Catalyze geometric/structural rearrangement of atoms within one molecule	Racemases, glucose isomerases, epimerases, mutases, tautomerases, cycloisomerases, topoisomerases
6.	Ligases	Join two molecules with the hydrolysis of a diphosphate in ATP or similar triphosphates	Synthetases, argininosuccinate synthase, glutathione synthase, carboxylases

Note: Information about the types of reactions and the enzymes used in various industrial processes taken from Refs. [15, 16]

[a]Serial number

global industrial enzymes market in 2015 was estimated to be approximately $4.6 billion, which is expected to witness rigorous growth of $6.3 by 2021 at a compound annual growth rate of 4.7% for 2016-2021 (https://www.bccresearch.com/market-research/biotechnology/enzymes-industrial-applications-report-bio030j.html). Currently, enzymes have been used for the synthesis of over 500 industrial products across an extensive range of biotechnological applications [19]. Most of the hydrolytic enzymes (about 65%) have diverse industrial applications such as laundry detergents, agro-chemical intermediates, textiles, starch, pulp and paper, leather, and personal care products. About 25% of the enzymes are used in food processing and the remaining 10% are prepared as animal feed supplements [20–22]. The versatility of hydrolases promotes its predominant applications in the biodegradation of natural polymers such as starch, cellulose, proteins, and other chemicals [13, 23]. Protease is the most dominant hydrolytic enzymes in terms of their physiological and biotechnological exploitation and accounts for approximately 57% of the industrial enzymes market [24]. The other enzymes include but not limited to amylase, lipase, xylanase, ligninase, cellulose, isomerase, pullulanase, laccase, and catalase [1].

Nature bestows a bulk of microbial enzyme resources (Table 2). Microorganisms are considered as the principal source of enzymes due to their broad availability, rapid growth rate, and cultivability through genetic manipulation in order to enhance the desirable qualities and yield of targeted enzymes [25]. Yeasts and molds contribute more than 50% of the industrial enzymes. Similarly, 30% of the industrial enzymes are made from bacteria, 8% from animals, and around 4% from plant sources (http://www1.lsbu.ac.uk/water/enztech/sources.html). Some of the archetypes of the microbial enzymes used for industrial applications incorporate (i) the use of *Escherichia coli* amidase for the production of 6- amino-penicillanic acid (6-APA) at 40,000 tons/year; (ii) the use of *Pseudomonas chlorapis* nitrile hydratase to manufacture acrylamide from acrylonitrile at 30,000 tons/year; and (iii) the use of *Streptomyces* xylose isomerase to isomerize D-glucose to D-fructose at 100,000 tons/year [26]. In comparison to the enzymes obtained from plants and animals, the microbial enzymes are contemplated to be more stable and active and thus provide superior performance under diverse physio-chemical conditions [14, 27].

Table 2 List of some enzymes from microbial sources

Source	Enzyme	Microorganism	References
Bacterial	Cellulase	*Bacillus sphaericus*	[28–34]
	Amylase	*Bacillus subtilis*	
	Protease	*Bacillus intermedius*	
	Xylanase	*Bacillus* sp.	
	Lipase	*Bacillus megaterium*	
	Esterase	*Bacillus lichenoformis*	
	Pullulanase	*Bacillus* sp.	
	Penicillinase	*Bacillus subtilis*	
	Pyrophosphatase	*Bacillus subtilis*	
Fungal	Amylase	*Aspergillus oryzae*	[35–39]
	Glucosidase	*Aspergillus flavus*	
	Catalase	*Aspergillus terreus*	
	Pectinase	*Aspergillus niger*	
	Cellulase	*Trichoderma reesei*	
	Lipase	*Rhizopus oryzae*	
	Beta-xylosidase	*Aspergillus niger*	
	Laccases	*Coriolopsis* sp.	
Yeast	Lipase	*Candida rugosa*	[40–42]
	Lactase	*Kluyveromyces lactis*	
	Invertase	*Saccharomyces cerevisiae*	
	Uricase	*Aspergillus flavus*	
	Ribonuclease	*Saccharomyces cerevisiae*	

Applications of Enzymes in Industrial Processes

Enzymes play a critical role to accelerate both the rate and specificity of various biotechnological reactions and metabolic processes [1, 12]. This ranges from food digestion to DNA synthesis. Moreover, bulk of enzymes are used in various industrial processes which include animal feed, food processing, polymer synthesis, paper and pulp industries, detergents, pharmaceutical industries, textiles, and bio-fuel industries. Proteases, cellulases, xylanases, amylases, and lipases are some of the commercially exploited enzymes in broad range of industrial processes. Most of the industrial enzymes are hydrolytic in nature (used for the degradation of natural substances). Among them, α-amylase is considered as the most versatile enzymes with industrial applications ranging from the starch conversion processes to the production of cyclodextrins in pharmaceutical industry. Likewise, proteases constitute about 60% of the global enzyme market [14]. Microbial proteases possess broad range of applications in bio-based industries such as detergent industry, leather processing, food industry, and pharmaceutical industry. In addition, microbial proteases are also used for various research and studies in molecular biology, genetics, and peptide synthesis [43]. Herein, we have discussed a range of enzymes application in various industrial processes.

Animal Feed Industry

The use of enzymes in animal nutrition is well established. Due to the presence of anti-nutritional factors such as phytic acid, and non-starch polysaccharides (NSP), animals cannot digest 15–25% of the consumed feed [44]. In general, feed enzymes enhance the nutrient digestibility which leads to increase in efficiency of feed utilization by animals [45]. For instance, carbohydrases improve the digestibility of carbohydrates, thereby increasing efficiency of animal feeds. In addition, they are used in animal diet formulation to degrade harmful components in the feed [45]. The use of feed proteases reduces the content of non-protein nitrogen supplement in animal diets and thus decreases the urea secretion into the nature [46]. The global market for feed enzymes in 2014 was estimated around $899 million and is expected to reach approximately $1.3 billion by 2020 [1].

Approximately, 85–90% of natural plant phosphorous is bound in phytic acid in cereal-based feed for monogastric animals. The addition of enzyme phytase (the largest enzyme segment in the feed industry) in animal feed contributes to a reduction of phosphorous from monogastric animals; thereby reducing phosphorous pollution and other environmental concerns derived from it such as eutrophication [47, 48]. Proteases break down complex proteins into peptides and amino acids. These are used as feed enzymes to enhance dietary protein hydrolysis and hence enable better nitrogen utilization. This leads to a significant reduction in nitrogen emissions from livestock production [49]. To date, commercially available feed enzymes include phytases, proteases, subtilisin, α-galactosidase, xylanase, glucanase, α-amylase, and polygalacturonase. These feed enzymes are particularly applied in swine and poultry industries [50]. The search for the development and production of thermostable enzymes with high-specific activity, some novel non-starch polysaccharide degrading enzymes, along with the reliable and cost-effective assays for measuring enzyme activity have always been the focus among researchers working in the field [45].

Food Processing Industry

The search for the development of enzymes useful in food processing industries has been intensified rapidly due to the consumer's demand for the quality of foods in terms of flavor and taste. The enzymes for food bio-molecules are efficiently used in yielding better food production and enhancing the various food components such as flavor, taste, texture, digestibility, aroma, functionality, and nutritive values [51]. In addition, the profound use of food enzymes in food and ingredients processing industries has offered better markets along with safer and higher quality of food products. The food and beverage enzymes dominated the industrial enzymes market with the total value of approximately $1.3 billion in 2015 and are projected to grow around $23 billion by 2020 [52]. Some of the common food enzymes used in food processing are cellulase, protease, lipase, amylase, and pectinase. These food enzymes are mainly used in dairy industry (cheese manufacturing), baking industry, wine making and brewing, juice production, and starch processing.

Most of the enzymes used in food industry are categorized as enzymes used for food processing aids except for few of them which are divided into food additives, namely lysozyme and invertase. Those enzymes used in food processing aids lack technological functions in the final food product; nevertheless, have significant role during the food manufacturing process [14, 46]. All these materials are regulated and considered to be safe under the guidance of good manufacturing practice (cGMP). As of today, based on FDA regulations, there are about nine recombinant microorganisms that are regarded as 'Generally Recognized as Safe' (GRAS). It comprises small number of bacterial and fungal species such as *A. oryzae, A. niger, Mucor miehei, Mucor pusillus Lindt, B. subtilis, and B. licheniformis* [53].

Oil and fat modification and sweetener technologies are the prime avenues in food industry that have gained increased attention for the use of food processing enzymes. As microbial lipases are regiospecific and fatty acid specific, they are of immense significance to be exploited for retailoring vegetable oils [54]. Earlier, lipases were used during bio-lipolysis. Lipases were also used in refining rice flavor, modifying soybean milk, yielding better aroma, and stimulating the fermentation of apple wine [55, 56].

The microbial enzymes are mostly used in beverage industry, followed by the dairy industry. The food enzymes such as proteases, pentosanases, α-amylases, β-glucanases, pullanases, amyloglucosidases, and α-acetolactate-decarboxylases (ALDC) are the most significant enzymes used in brewing industry. The brewing enzymes help to control the overall process and thus yield better quality beer and other beverages. For instance, β-glucanases hydrolyze glucans into soluble oligomers and thereby offer lower viscosity and improved filterability; proteases boost malt improvement and enhance yeast growth; ALDC avoids the formation of diacetyl and hence reduces the time of fermentation while providing right taste to the beer [46].

Some of the common dairy enzymes used in dairy industry include β-galactosidases, lactases, esterases, lipases, lysozymes, catalases, chymosin, and lactoperoxidases. Basically, these enzymes are used to improving the shelf life and quality of dairy products. The dairy enzymes namely β-galactosidases and lactases are applied to catalyze the hydrolysis of lactose to glucose and galactose in milk processing. This is of great significance for lactose-intolerant people and thus controls tissue dehydration and other fatal diseases [57, 58]. Chymosin, lipases, and lysozymes are generally applied for the production of cheese, yogurt, and other various milk products. Proteases are used to reduce the allergenic properties of milk products [57].

In a research report published by Freedonia Group, it was stated that the baking enzyme industry is expected to grow around $9 billion by 2020 [59]. Most baking enzymes possess a broad range of applications. β-xylanases are used to improve dough stability; oxido-reductases increase gluten strength; lipases enhance the stability of the gas cells in dough and improve the flavor content through the synthesis of short-chain fatty acids through esterification [60]. Other enzymes such as proteases reduce the concentration of protein in flour, decrease mixing time, reduce dough consistency, and control gluten strength in bread. Similarly, α-amylases degrade starch into smaller dextrins when added to the dough of bread [61]. The addition of α-amylases to the dough decreases the viscosity of dough and increases the rate of fermentation. This yields an enhanced texture, improved taste and volume of the loaf, and better crust color of the dough. It also improves the shelf life of the products and acts as anti-staling agent. A thermostable maltogenic amylase derived from *Bacillus stearothermophilus* is currently used in the bakery industry [61]. Likewise, baking enzymes also aid in yielding better texture, color, and uniformity in addition to prolonging freshness and softness of bread [1].

Several food enzymes are used in juice production industry. Amylases are applied to hydrolyze starch into glucose and to clarify cloudy juice (such as in apple juice production); pectinases to improve the overall juice production; laccases to better enhancing the susceptibility of browning during storage; naringinases and limoninases to act on compounds that cause bitterness in citrus juices [62]. The use of enzymes in juice production can increase the efficiency of operation such as peeling, extraction, maceration, juicing, and clarification and in improving sensory characteristics [63]. This ultimately results in better and cost-effective juice yield.

Polymer and Textile Industry

Polymeric materials are an indispensable part of the modern society and are broadly exploited in several fields that include food packaging industry, agricultural industry, automotive, electronics, pharmaceuticals, medical, and therapeutic industries. Enzymatic polymer modifications and enzymatic monomer synthesis/polymerizations are eco-friendly and benign alternatives to chemical and physical modifications of polymers for the synthesis of novel macromolecules as well as commodity plastics [64]. This is because of the high reaction specificity of enzymes, high enantio-, regio-, chemo-selectivity, non-toxic transformation on the surface of polymer. In addition, the enzymatic reactions occur under mild conditions without the involvement of toxic reagents and hence offer greater global sustainability. Enzymatic polymer synthesis offers tremendous opportunity to advance green polymer chemistry [65].

The production of genetically engineered eco-friendly polyester (3G+) [66] and the development of polylactide (PLA) through lactic acid fermentation [67] are the epitomes of the bio-polymers with industrial applications. Polymeric biomaterials developed through enzymatic polymer modification are biodegradable in nature and thus are able to be broken down and removed easily after the completion of their functions [68]. For instance, the degradable polymers are clinically used as surgical sutures and implants. Similarly, the bio-polymers namely polycarbonates, polyacetals, polycaprolactones, polyurethanes, polyphosphates, polyphosphazenes, and polyesters have tremendous applications in biomedical fields such as orthopedic devices, tissue engineering, drug delivery, vaccine adjuvant, and therapeutic approaches [68].

Lipase, laccase, peroxidase, and transglutaminase are the prime enzymes used in polymer industry. Lipase is a renewable biocatalyst and is benign in nature. Polyester synthesis through the polymerization of lactones, cyclic diesters, and cyclic carbonates (using enzyme, mainly lipase) normally occur at lower temperature, normal pH, optimum pressure, and are highly selective in all respects (i.e., enantio-, regio- and chemio-selectivities). Thus, developed polyesters using lipases are often rated as highly value-added products for biomedical and pharmaceutical applications [64, 69]. The direct esterification of butanol and oleic acid using lipase produces 1-butyl oleate. This reduces the viscosity of biodiesel in winter use [55]. Gurung et al. stated that laccase, peroxidase, and transglutaminase play a significant role to forming cross-links in bio-polymers to provide materials in situ through polymerization [13]. Laccases (EC 1.10.3.2) are group of oxidative enzymes, belonging to the multicopper oxidase family [70]. They are versatile bio-catalysts in organic synthesis, used to produce polymers in air without the use of H_2O_2 [71]. Lacasses induce radical polymerization of acrylamide with and without mediator and also play a crucial role to polymerize different amino and phenolic compounds [72, 73].

Textile industry is one of the largest contributor to environmental pollution [74]. The application of various enzymes in the textile industry has promoted the development of eco-friendly fiber processing technologies and strategies to enhance the quality product [75]. Hydrolase and oxidoreductase are the two different classes of enzymes mostly used in textile industries. The hydrolase enzymes (amylase, cellulase, pectinase, cutinase, protease, and lipase/esterase) are applied to removing starch size, bio-polishing and bio-scouring of fabric, cotton softening, fabric finishing in denim, enhancement of color and surface vividness, resistance to wrinkles, and treatment of wool [76–79]. Similarly, the use of oxidoreductase enzymes (catalase, laccase, peroxidase, and ligninase) includes bio-bleaching, dye decolorization, wool finishing, dye cotton, and wool fabrics, improvement of the whiteness during cotton bleaching, and bleach termination [80, 81].

Paper and Pulp Industry

The use of various enzymes has grown rapidly in paper and pulp industries to decrease the adverse effect on natural ecosystem. Besides, the use of enzymes aids in reducing the processing time, energy consumption, and the use of toxic chemicals in processing. Srivastava and Singh in 2015 stated that the various enzymes are employed in paper and pulp industry to enhancing de-inking, bleaching, and waste treatment by increasing biological oxygen demand (BOD) and chemical oxygen demand (COD) [82]. The separation and degradation of lignin during industrial paper production was done conventionally using chlorine- or oxygen-based chemical reagents [83]. The pre-treatment of wood pulp using laccase offers milder and cleaner strategies of delignification and brightening. Other potential applications of bacterial and fungal laccases include pitch removal, pulp grafting to enhance its physio-chemical properties, and de-inking (a significant step in fiber recycling) of old newsprint (ONP). Old newsprint is one of the prime materials reused for papermaking [83, 84]. Lipases are used in pulp and paper industry to de-inking and improving the pitch control in pulping processes [85]. Xylanases are employed to enhancing the pulp bleaching, water retention capacity, and freeness in recycled fibers, while cellulases and hemi-cellulases are used to improving fiber softness and flexibility and enhancing water drainage [86]. Moreover, mannases are exploited for degrading the residual glucomannan and thus boosting brightness of products in paper industry [87, 88].

Detergent Industry

Detergent industry represents one of the largest industrial application of enzymes with around 25–30% of the total sales of enzymes [89]. The most crucial field of application for enzymes (such as amylase, lipase, protease, cellulase, cutinase) is their addition to detergents, which are used mainly in dishwashing, laundering, and industrial and institutional cleaning. The enzymes in laundry detergents basically help to improve fabric whiteness, its color, soften cotton, and increase the efficiency on stain cleaning and classical soilings such as grass, animal and vegetable fat, and blood. The enzymes used in detergent industry should have alkaline pH, high catalytic activity, and stability at low temperature [24]. Enzymes used in detergent industry mainly belong to hydrolase group. Cold-active serine protease (CP70) produced from *Flavobacterium balustinum* and cold-active alkaline protease isolated from *Stenotrophomonas maltophilia* are the epitomes of such enzymes. Proteases exhibit exceptional stability and compatibility with laundry detergents. Hence, they are used in detergent industry to enhance washing efficiency and remove proteinaceous materials from stains [90]. An alkaline protease obtained from a marine shipworm bacterium is used to clean contact lens at lower temperatures [91, 92]. Amylases are used to remove insoluble starch residues during dish washing [93]; cutinase (EC 3.1.1.74) is mainly employed for dish washing and laundry detergents [94]; lipase isolated from *Pseudomonas putida* ATCC 53552 facilitates the removal of oil and fatty materials (lipid stain) from the surfaces of fabric [55]. Cellulases contribute to modifying the structure of cellulose fiber to enhance the color brightness and overall fabric care [78, 95]. The use of enzymes in detergent industry offers beneficial impacts on natural environment and public health as they contain less bleaching agents, and phosphates in comparison to current surfactants.

Pharmaceutical Industry and Medicine

Microbes are considered as the obvious forefront leader in yielding useful natural products such as antibiotics, immune-suppressants, anti-cancer agents, enzyme inhibitors, vaccines, and anthelmintic [96]. Enzymes are exploited as therapeutic drugs in the treatment of enzymatic deficiency and digestive disorders, and removal of dead skin. In addition, the enzymes have crucial role in clinical diagnostic procedures such as ELISA (enzyme-linked immunosorbent assay), and diabetic testing kits [97]. Microbial enzymes have a prominent place among bio-catalysts and have a broad-spectrum application in biotechnological industries and pharmaceuticals [98]. The enhanced stability, biochemical diversity, and potential susceptibility nature of enzymes derived from microbial sources have enabled them to be among the top groups of candidates in designing novel bio-active compounds, oleochemicals, and drugs [98].

The commercial production of microbial enzymes especially from bacteria and fungi is a major area of interest for process engineers, bio-chemists, and medical microbiologists. These chemicals and drugs are used in the treatment of various human diseases. These include (but not limited to) acne, malaria, prion diseases, diabetes, obesity, ulcers, and even alumina in kidney dialysis patients [96].

Microbial lipases are useful in the preparation of chiral synthons and are potent biocatalyst for the synthesis of active analogues of bio-active molecules such as antagonists or inhibitors in biological system [98]. Moreover, lipases are employed in the regioselective modification of castanospermine, a potent drug for AIDS treatment (http://www.au-kbc.org/beta/bioproj2/uses.html). The detection of severe health conditions such as acute pancreatitis and pancreatic injury is possible using the level of lipase in the blood serum as a diagnostic tool [99]. A research study done by Y. Sokurenko et al. [100] stated that extracellular ribonuclease from *B. licheniformis* has anti-tumor effects. *Streptomyces* are the predominant gram-negative soil bacteria. In addition to the soil ecology potential, *Streptomyces* are of prominent source of novel bio-active secondary metabolites with pharmaceutical prospective [101]. A *Streptomyces* strain named as *S. collinus* Tu 365 is a producer of kirromycin, which possesses activity against bacterial pathogens and malaria parasite *Plasmodium falciparum [*102*]*. Proteolytic enzymes are used to cure burn, whereas fibrinolytic enzymes (streptokinase, urokinase, serrapeptase, bacillokinase II) are used in clot busting and in the treatment of cardiovascular diseases such as atherosclerosis, stroke, angina, and peripheral vascular diseases [103]. For instance, Nattokinase (EC 3.4.2.62) is a favorable agent for thrombosis therapy [104]. Rhodanase (EC 2.8.1.1), dextranase (EC 2.4.1.2), and acid protease are explored in the treatment of cyanide poisoning, tooth decay, and alimentary dyspepsia [105]. Cholesterol oxidase (EC 1.1.36) has a potential application in testing and control of cholesterol level. Likewise, putrescine oxidase helps to determine biogenic amines, such as putrescine, a well-known marker for food spoilage [106]. Tyrosinase (EC 1.14.18.1) is used in the production of L-dihydroxy phenyl alanine (L-DOPA), a precursor for the production of dopamine. Dopamine is an effective drug to control myocardium neurogenic injury and also for the treatment of Parkinson's disease [107]. Uricase obtained from *Aspergillus flavus* is used for the treatment of gout; penicillin oxidase, rifamycin B oxidase produced from *Penicillum* sp. are used in antibiotics synthesis.

Like terrestrial organisms, the marine inhabitants produce many species of natural products that would be useful in pharmaceutical and health care industries. 40% of the marine sponge biomass is occupied by bacteria. Sponge is a reservoir of biologically active compounds due to the presence of symbiotic bacteria. The marine sponge, *Theonella swinhoei* produces metabolites such as onnamides and theopederins with anti-tumor activity [108].

Marine cyanobacteria contribute to the production of various chemical compounds and drugs with anti-viral, anti-bacterial, and anti-cancer activities. Cytarabine, trabectedin, vidarabine, and ziconotide are some of the prominent examples of approved marine products explored in the treatment of various human diseases [96]. Yet, there is still a need for the development of new approaches pertaining to the use of microbial enzymes in pharmaceuticals and human medicine.

Bio-fuel Industry

Cellulosic biomass is the most ubiquitously available natural resources on earth. Lignocellulosic biomass is a type of carbon-rich biodegradable materials generated by plants, and also present in industrial and municipal wastes, forest residues, and wastewater treatment plants [109]. Cellulosic biomass have received significant attention as sustainable feedstock for bio-fuel industry [110]. However, the rudimentary understanding of the mechanistic and biochemical attributes of commercial enzymes, its costly nature, and the slow specific enzymatic hydrolysis are the major impediments for large-scale bio-fuel production [111].

The different cellulolytic enzymes, endoglucanase, exoglucanase, and xylanases derived from various cellulolytic and xylanolytic bacteria, fungi can be exploited for the biomass conversion to feedstock chemicals. The bacterial species present in soil, marine, and herbivore guts possess multi-functional novel enzymes that can efficiently hydrolyze plant cell wall constituents [112]. A study done by Benedict C. Okeke stated the strain of *P. janthinellum* FS22A and *T. virens* FS5A proved to be promising for the co-production of cellulolytic and xylanolytic enzymes in a research lab scale; yet further investigations are required to enhance their enzyme productivity [113]. The holistic approach in engineering the microbial enzymes, their proper isolation, identification, expression, characterization, and final assay can aid further to achieve tailor-made cellulases and xylanases for various bio-fuel industrial applications.

The removal of lignin through pre-treatment is an important step for the efficient hydrolysis of polysaccharides. Laccases in combination with other oxidative enzymes play a crucial role in lignin biodegradation [114]. A research study conducted by Fang et al. found that a novel laccase obtained from white rot fungus (*Ganoderma lucidum*) was used in the detoxification of lignocellulosic hydrolysates and further enhanced the bioethanol production by removing phenolic compounds [115].

The enzymatic hydrolysis of plant cell wall takes place through the combined action of three different glycolhydrolyze (GH) enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase also known as cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). All these enzymes hydrolyze the β-1, 4 covalent bonds where the glucose units are connected in the cellulose fiber. Endoglucanases belong to families GH5, GH6, GH7, GH9, GH12, GH45, and GH74. β-glucosidases belong to families GH1 and GH3. The synergistic action from endoglucanases and cellobiohydrolases, and the exo–exo between two cellobiohydrolases are of phenomenal importance during the hydrolysis of cellulose. Hemi-cellulose hydrolysis also requires the intervention of several functional enzymes along with the complementary activities at various levels. GH and carbohydrate esterase (CH) are involved in the hemi-cellulose hydrolysis by cleaving ester bonds between the acetyl groups and hemi-cellulose chains [116]. In other words, the hydrolysis of xylan requires the combination of hydrolytic enzymes namely endoxylanases, beta-xylosidases, and arabinofuranosidases. The production of better competitive enzymes cocktails through the exploration of fungal biodiversity with their secretomes is one of the new approach in isolating these multi-functional enzymes to increase the saccharification efficiently in biomass conversion [117].

Important Biochemical Attributes of Microbial Enzymes

The utilization of microbes such as bacteria, yeasts, fungi, molds, and their enzymes as biological catalysts at the industrial scale is a major component in a bio-based economy (http://www.bio-economy.net/). Enzymes with higher catalytic strength and effective stereo-selectivity and specificity are touted as a suitable alternative to chemical catalysts [118]. Owing to the harsh environmental conditions such as low/high pH, high temperature, high pressure, oxidative conditions, high shears, or short delays, the development of resistant enzymes is of pivotal significance for better industrial performance [119, 120]. Thus, tailored biotechnological enzyme catalysts should possess unique characteristics such as thermostability (thermo-tolerant), pH stability, high chemo- and stereo-selectivity, and multi-functionality. The enzymes with such attributes are required for efficient and cost-effective bio-conversion of cellulosic biomass into ethanol in bio-fuel industry [121]. In addition, such enzymes can also be used in the synthesis of industrial chemical conversion, reducing the energy consumption and the generation of less toxic by-products.

Nature serves as an exceptional origin of thermostable and biotechnologically relevant bio-catalysts. Stability is an important factor that determines not only the functions and biological fitness of macromolecules, but also enhances their evolvability. Microbial enzymes tend to offer higher thermostability, tend to possess neutral or alkaline pH optima, and are more stable than the enzymes produced from plants and animal sources [122]. In order to explore the enzymes with special features, one should consider digging into environmental microbes that require those enzymes for their adaptation. Unlike plants and animals, thermophilic microorganisms such as bacteria, fungi, and archaea are able to withstand high temperature due to their increased disulfide, electrostatic, and hydrophobic interactions in their proteins [123]. In other words, molecular modifications at cellular and subcellular levels aid them to adapt in harsh environmental niches. Bioprospecting for the microorganisms living in hot springs and salt marshes (marine source) led to the discovery of enzymes (i.e., thermozymes and extremozymes, respectively) with thermostability and tolerance to salt conditions [124]. Enzymes with thermostability and pH stability can retain their catalytic activity, specificity in the chemical reaction, and thus have enormous biotechnological applications. One of the quintessential example of thermozymes is the use of Taq polymerase (isolated from *Thermus aquaticus*) in polymerase chain reaction [125]. Other thermozymes that can withstand not only high temperature but also acidic and alkaline conditions include cellulases, chitinases, pectinases, amylases, pullulanases, lipases, glucose isomerase, and proteases [126]. Alkaline protease isolated from *B. mojavensis* in sea water exhibited better stability towards non-ionic surfactants, and was biocompatible to a wide range of liquid and solid detergents [127]. The purified protease from *B.licheniformis* showed an optimum activity at 50–60 °C and higher pH values (9–11) with 98% retention activity at pH 10 and 82% at pH 11 [128]. Such properties of proteases to withstand high temperature could enhance the substrate solubility, reduce the liquid viscosity, and hence has potential application in detergent industry, dehairing, and bating of skin during leather processing [129]. Similarly, in 2009, Mo et al. [130] reported the optimum enzymatic activity (at pH 8.0) of phospholipase purified from a marine *streptomycete*.

A study done by Saxena et al. isolated a highly thermostable and alkaline amylase enzyme from Bacillus sp. PN5. The enzyme demonstrated 65% activity at 105 °C and had 100% stability at temperature 80–100 °C for 1 h [131]. Such enzymes could further facilitate starch saccharification, detergent formulation, amino acid synthesis, and food processing industry. Xylanases from *Actinomadura* sp. FC7, *Nonomuraea flexuosa* have shown better thermal and pH stability and thus are extensively exploited in lignocellulose degradation, paper and pulp industries [132]. Laccases are metalloenzymes offering broad range of applications such as removing polyphenol in wine industry, removal of lignin, and pulp bleaching. Laccase like multicopper oxidase isolated from *Aquifex aeolicus*, a thermo-tolerant bacterium was found to be heat stable even at 80 °C and 90 °C [133].

The enhancement for the thermostability, pH stability, and multi-functionality can be achieved through various techniques such as directed evolution, protein engineering, and immobilization [134].

Directed evolution is a prominent tool to produce efficient bio-catalysts [135–137]. In directed evolution, mutagenesis and screening helps to characterize novel proteins starting from a parental protein (Fig. 1) under particular evolutionary pressure [138]. Enzymes having broad range of substrate and catalytic activity could be reshaped with enhanced catalytic activity and stability (thermal and pH) for improved protein functions in industrial applications through directed evolution approach [139, 140]. Stephens et al. [141] reported the improved activity and thermostability of endo-β-1,4 xylanase isolated from *Thermomyces lanuginosus* by directed evolution technique. The directed evolution led to the generation of a large mutant library and thus making it possible to identify and isolate mutants with specific desirable functions [142].

Protein engineering is another approach used to enhancing activity and stability of enzymes at high temperatures and extreme pH. Using this approach, one can reprogram the enzymatic characteristics using 3-dimensional enzyme

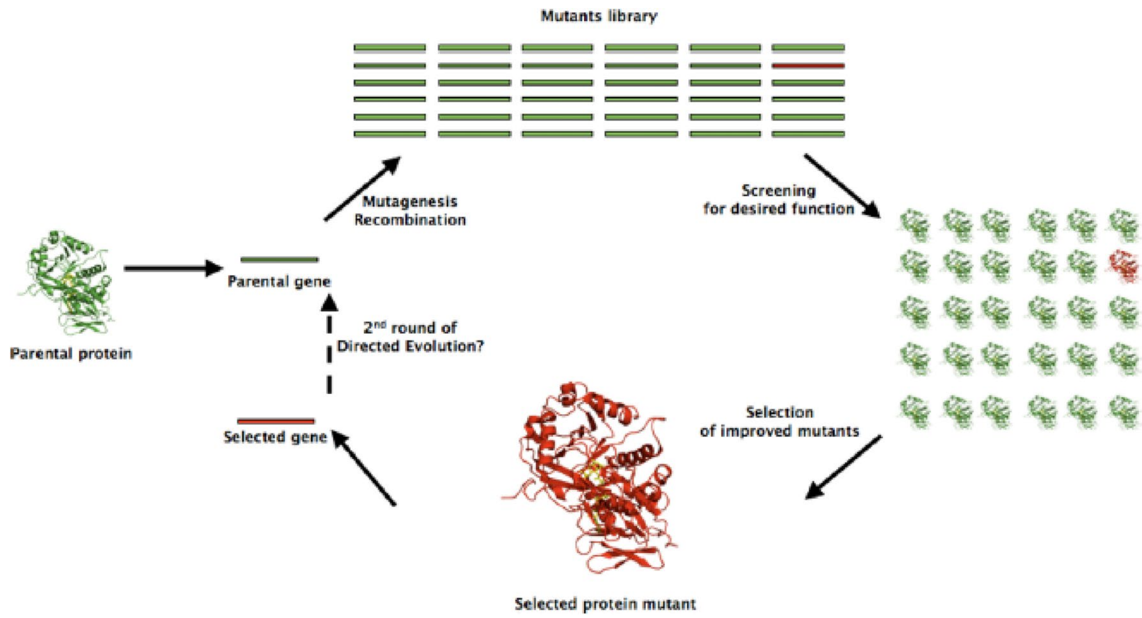

Fig. 1 Schematic representation of directed evolution approach. Gene coding followed by an iterative mutagenesis and screening process is performed for the enzyme of interest. The functional attributes of mutants are screened from the generated mutant library and the mutant with the best functional performance can be used as the parental gene for the next iterative rounds of mutagenesis [121] (adapted with permission)

structure and rational design and hence can tailor the enzymes for specification [143]. This approach involves the introduction of disulfide bridges, replacing N terminus and increasing the number of hydrogen bonds [144]. For example, the development of a protease mutant with increased melting temperature (Tm) of 25 °C and increased half-time at 60 °C (1,200 fold) through DNA shuffling [145]. Unlike directed evolution route, protein engineering incorporates the targeted mutagenesis guided by structural or sequence information [146]. Here, there is no requirement of high-throughput screening approach. As the protein fold is not disturbed by targeted mutagenesis, the chance of obtaining active variants is high.

Thermostability

Thermo-tolerant/thermostable enzymes demonstrate a unique ability to resist irreversible inactivation at high temperatures and are optimally active and perform well at elevated temperature ranging from 60 to 125 °C [120]. Thermodynamic stability and kinetic stability are the two important features of the thermostable enzymes [147]. Thermostable enzymes complement other chemical enzymes in biotechnological applications due to their distinctive properties such as endurance to extreme pH conditions (high alkalinity or extreme acidity), increased substrate concentration, and resistance to chemical denaturants without any loss in their catalytic functionality [126]. Thermostable enzymes limit the microbial contamination and accelerates the chemical reaction; thereby lessening the industrial processing time [148].

The enzymes that are stable at high temperatures (such as glucanase, pectinase, cellulase, amylase, proteases, and esterase) can be derived from various thermophilic bacteria and fungi. The thermophilic bacteria species include but not limited to *Fibrobacter* [149], *Streptomyces* [150], *Bacillus* [151], and *Alicyclobacillus* [151]. Similarly, *Paecilomyces spp* [152]., *Thermoascus aurantiacus* CBMAI-756 [153], and *Talaromyces levcettanus* [154] are some of the thermophilic fungi that can produce/secrete different kinds of hydrolytic enzymes such as glucannase, mannase, and α-galactosidase which possess thermostability and multi-functionality attributes and hence offer potential industrial uses in bio-fuel industry, food, feed, and pulp industry [153, 154].

pH Stability

All biological phenomenon in nature are pH dependent. In other word, pH scale and its stability have tremendous significant effect on almost all the biological functions in nature. pH value of an enzyme provides a clue about the initiation and end of enzyme synthesis. The enzyme functions are influenced by the change in pH. At optimum pH, the enzymes are most active. To the contrary, there is a loss in enzymatic activity at extreme pH conditions like high alkalinity or high acidity. However, the optimum pH value varies depending on the types of enzymes used.

Moreover, the importance of hydrogen ion concentration on enzyme or protein stability is of prime crucial. This is further displayed by acid/base unfolding [155–157], and enzyme pH-dependent stability [158–160]. The interactions among protein–protein [161, 162], protein-membrane [163–165], protein–ligand [162, 166] seem to be highly affected due to the pH activity. The enzymes used for the various biotechnological applications should possess pH stability and optimum pH activity to execute their functions efficiently and with optimum efficacy. The study done by Talley and Alexov [167] benchmarked the idea on the pH optimum of enzymes. The enzyme–enzyme interaction is possible if both the enzymes maintain the same pH stability and hence can bind effectively to one another. The pH stability and pH optimum of activity are co-related.

Multi-functionality

In general, enzymes with multi-functionality properties can dispense multitude physiological and structural functions. Enzymes with multi-functionality characteristic are classified as moonlighting or promiscuous enzymes [168–170]. The interpretation of sequencing and annotation of the protein database from the microbial genome is challenging. This is due to the presence of moonlighting proteins or enzymes. Moonlighting enzymes are believed to possess a single catalytic domain and an additional non-catalytic domain [171]. Each of these domains performs functions independently and the mutation of one domain does not affect the functions of another domain. Such a multi-functionality attribute is of great significance to accelerate the hydrolysis process during the bioconversion of biomass into ethanol. Unlike moonlighting enzymes, the promiscuous enzymes conduct various functions with their catalytic domain. They can utilize the same active site to perform different biotransformations [172]. The promiscuous or prolific nature of the enzymes make them multi-functional, meaning they can recognize more than one substrate or can offer multiple out-products from a given substrate [173]. The multi-functionality of enzymes facilitates the communication and co-operation among various pathways and functions during the efficient substrate conversion mechanism in bio-fuel production. Moreover, the multi-functionality is a notable enzyme characteristic that correlates multiple activities and also maintain the regulation of its own expression during the reaction [169]. In a nut shell, the physio-chemical properties namely charge, hydrophobicity, polarizability, and accessibility to solvents are the key characterization of enzymes with multi-functionality in nature [171].

Approaches in Novel Microbial Enzyme Discovery

Natural ecosystem is a prominent reservoir for obtaining potential novel enzymes with various industrial applications. The traditional method that includes selection, subsequent screening, and sensitive assay of microbial strain is considered as a standard approach [3]. The ability to tap such immense enzyme candidates largely depends on the efficient screening tools and strategy to the possible input of diverse genes. Such strategies include but not limited to (i) metagenomics screening of novel enzymes, (ii) microbial genome mining, and (iii) extremophiles diversity tapping/exploitation.

Metagenomics Screening

Microbes dominate the global biodiversity. The microbial communities such as bacteria, fungi, archaea, and protists represent the largest terrestrial and oceanic biomass. This is further epitomized by the presence of immense microbial diversity of approximately 166,244/24,299 (bacteria/fungi) and 49,102 bacteria operational taxonomic units (OUT) in the Dryland and Scotland data sets [174], respectively, and around 25,000 different microbial genotypes in merely one milliliter of seawater sample in a marine ecosystem [175]. Microbes are regarded as the fore front leader in yielding diverse and novel functional bio-catalysts crucial for wide range of industrial processes. Given the fact that around 1% of the microorganisms in the natural environment can be cultivated through standard laboratory techniques, understanding of the vast microbial genetic insights remains elusive [176]. Molecular metagenomics (independent of cultivability) has emerged as a strategic approach to deliver a significant access in the detection and identification of microbial enzymes and proteins of industrial interest from various environmental habitats including extreme niches. It further provides a plethora of information on the biochemical composition, structure, and functionality of the unclassified enzymes from microbial sources [177].

A metagenomic approach can be classified into two different routes namely function-based metagenomic screening and sequence-based metagenomic screening. Sequence-based metagenomic uses next-generation sequencing (NGS) technology for the exploration and analysis of microbial enzymes and bio-active compounds from the environmental niches [178]. In addition, this approach can be used for genome assembly, gene identification, understanding complete metabolic pathways of various organisms in different communities. Sequence-based metagenomic approach facilitates the discovery of various bio-catalysts, establishing the degree of their natural diversity and thus enhancing the characterization and optimization of bio-catalysts [178]. In general, sequence-based metagenomic involves the construction of metagenomic library and its screening through the amplification of gene of interest through PCR. The clones that contain the gene of interest are then sequenced for further analysis to reveal the ecological diversity and the genetic information.

Unlike sequence-based screening, function-based metagenomic analysis does not require sequence information to the identification of novel class of genes encoding various genomic information [179]. This approach contributes to adding functional information to nucleic acid and protein databases [180]. The induced gene expression [181], phenotypical detection of the desired activity [182], and heterologous complementation of host strains [183] are the three different function-based screening.

Metagenomic studies have facilitated the access to novel enzymes and metabolites from various environmental habitats such as Sargasso Sea [184], Sorcerer II Global Ocean Sampling expedition [185], soil [186, 187, 188, 189], gut of ruminants [190], cow rumen [191], buffalo rumen [192], elephant rumen [193], termite guts [194], hot springs [195, 196, 197, 198, 199], glacier ice [200], and Antarctic desert soil [201]. The microbial enzymes with potential characteristics for industrial application, yielded through metagenomic approach include but not limited to amylase [202], beta-glucosidase [203], [204], lipase [176, 205], oxidoreductase [206], decarboxylase [207], amidase [208], nitrilase [209], epoxide hydrolase [210], and esterase [211]. Biochemical and structural characterizations of a significant number of these proteins, mostly esterases from the α/β hydrolase super-family have revealed the biochemical diversity of adaptation to extreme environmental conditions such as low/high temperature, low/high pH, high pressure, and high salinity [212–214]. The search of novel enzymes through metagenomic strategy in environmental bacteria advances our basic understanding of protein structure, their biochemical functionality, and further enhances the quality of gene annotation in public databases [211]. In addition, it diversifies the bio-catalytic toolbox for synthetic biology, protein engineering, and various other biotransformation reactions.

Metagenomic approaches offer tremendous potentiality in deriving an arsenal of industrial bio-catalysts. Nevertheless, the scarcity of suitable enzymes and a proper host for an efficient gene expression and enzymatic activity are some of the hindrances for biotransformation processes. Similarly, the other limitations while dealing with natural heterogeneity and cross-strain assemblies include low sensitivity and low throughput of the activity-based metagenomics screening [215]. The advancement of fluorescence activated cell sorting (FACS), phenotypic micro-array (PM) [216], community isotype array (CIArray) [217], fluorescence in situ hybridization (FISH), and fluorescence microscopy facilitate the better understanding in biological identification within a single cell [218]. In addition, high-throughput screening strategies such as SIGEX (substrate-induced gene expression) [219], PIGEX (product-induced gene expression) [181], and METREX (metabolite-regulated expression) [220] have proven to be very effective in closing the above-mentioned limitations. To date, there is no defined gold standard for the metagenomic data analysis. Next-Generation Sequencing Simulator for Metagenomics (NeSSM) developed by Jia et al. comes close and is believed to consider both the sequencing errors and sequencing coverage biasness [221]. The betterment of various simulation systems and algorithms further enhances the extraction and analysis of metagenomic sequence data [222, 223].

Microbial Genome Mining

Microbes provide abundant sources of natural products. The natural products obtained from bacterial, fungal, plants, and marine animals make excellent bio-synthetic enzymes, chemical drugs, secondary metabolites, and its derivatives. The recent advancement of the genomics era has enhanced the discovery of secondary metabolites, novel enzymes, and bio-active molecules from various microbial sources. The discovery of novel bio-active molecules and compounds from various microbial sources has an enormous contribution to human medicine, plant protection (such as bio-pesticides, bio-insecticides, herbicides, and plant growth regulators), and animal health. The discovery of novel enzymes and chemical drugs with microbial origin undoubtedly will continue to serve as scaffolds for further human therapeutic discovery (such as anti-infective agents, cholesterol lowering agents, anti-cancer agents, and immunosuppressant) and biotechnological development. Hence, the microbial genome mining is a significant approach to explore novel secondary metabolites for drug discovery for human medicine and animal health [224].

Microbial genome mining is a propitious technology to revitalize new and natural products discovery [225]. Genome mining can be defined as a process that incorporates the translation of secondary metabolites encoding gene sequence data into purified bio-molecules [224]. About two decades ago, owing to the re-isolation of known compounds through low-throughput methodologies such as compound-guided or bio-activity guided approaches, the natural product discovery phenomenon was at a point of diminishing. Nonetheless, the recent development on microbial genome sequencing, genome mining, combinatorial chemistry of natural products, and synthetic and system biology has provided paradigm shift in better understanding the discovery process. Actinomycetes, particularly *Streptomyces*, has been rigorously exploited as the most productive sources for novel enzymes and chemical drugs [226–228]. The classical concept of genome mining stems from the scientific observation that the full genome of *Streptomyces coelicolor* and *Streptomyces avermitilis* encode the unexplored potential secondary metabolites (SMs). A *Streptomyces* genome encodes approximately tenfold more secondary metabolite gene clusters than were known at the time (only two or three secondary metabolites were known at that time) [229, 230]. Later on, the genome mining was further exploited to study the genome sequences of other microbes that includes but not limited to anaerobes, cynobacteria, and myxobacteria [230]. The "Atlas of Biosynthetic Gene Clusters" (ABC), a component of the "Integrated Microbial Genomes" (Platform of the Joint Genome Institute) is the largest collection of automatically mined gene clusters. ABC consists of approximately 960000 putative gene clusters that have been identified in the metagenome datasets and public datasets. Yet, the analysis and characterization of only a fraction of those bacterial gene clusters has been further described [230, 231].

The availability of the wealth of DNA datasets, avalanche of genomic information, high-throughput sequencing technologies, and wide range of genome mining tools and strategies have further enhanced the understanding in the discovery and characterization of the novel enzymes and secondary metabolites such as polyketides (PK), non-ribosomally synthesized polyketides (NRP), aminoglycosides, and many more [230]. Nowadays, the advent of metagenomics and single cell genomics is employed to generating massive microbial genome information to be further analyzed. Furthermore, owing to the reduction in processing time and cost-effective genome sequencing, next-generation sequencing (NGS) provides a potential promise in the search and characterization of novel enzymes and their bio-synthetic pathways. Currently, the two main approaches namely genome hunting, and data mining facilitate the discovery of new bio-synthetic enzymes. Genome hunting incorporates the search for open reading frames within the microbial genome. In this technique, the annotated sequences are subjected to molecular cloning followed by the over-expression and sensitive screening assay. Contrast to this approach, data mining involves the comprehensive bioinformatics tools (BLAST or HMMER) to search the conserved regions among the sequences deposited in the databases [232]. Nadine et al. stated that DECIPHER was the first tool for automated cluster mining. BAGEL, CLUSEAN, and antiSMASH were some of the additional tools developed for data mining in the field of natural products and microbial ecology [230].

Extremophiles Diversity Exploitation/Tapping

Microbial life subsists even in an environment of extreme temperature conditions such as hot springs (60–110 °C), cold polar regions (− 2 to 15 °C), ionic strength (2-5 M NaCl), or pH (< 4, > 9), arid deserts, ocean vents, arctic waters and soil, and increased salt concentration (5% - 30%) [233]. Such extremophiles (belonging to the domains of *Archaea* and *Bacteria*) [234] have adapted to thrive in various environmental niches and come under a number of classes such as thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles, barophiles, radiophiles, metalophiles, and microaerophiles (grow in < 21% oxygen). Extremophiles are considered as the reservoir of enzymes (called as "extremozymes") with novel activities and industrial bioprocesses. The extremozymes have potential industrial applications due to their stable nature, altered specificity, and active functions under conditions in which the enzymes from their mesophilic counterparts were found to be incompatible [235]. Some of the extremozymes exhibit polyextremophilicity [236, 237]. Thus, bioprospecting of microbial enzymes in extreme ecological niches is a promising technique for finding robust extremozymes with greater tolerance under natural conditions [238, 239]. Recent studies demonstrate that the microbial diversity in the extreme environments exceeds what was expected initially [235, 240]. Nevertheless, as the isolation and identification of such extremophiles in pure culture has not been done yet, the determination of the extremozymes stability, substrate specificity, and enantioselectivity still remains elusive [235].

Thermophiles are mainly the microorganisms that can thrive at extreme high temperatures of 65–85 °C, moderate thermophiles live around 45–65 °C, and hyperthermophiles live at above 84 °C. Chemolithoautotrophic archaea *Pyrolobus fumarii* [241, 242] and methanogenic hyperthermophile *Methanopyrus kandleri* [243] are known to sustain at the highest temperature of 113 °C and 122 °C, respectively. Thermophilic amylases, xylanases, cellulases, lipases, proteases, and DNA polymerases have broad range of industrial applications such as paper bleaching, brewing, detergents, baking, textiles, and genetic engineering [244]. In addition, the thermophilic enzymes possess resistivity to denaturing agents and organic solvents, accelerate the reaction rate, and are convenient for separation during the purification processes [245].

Psychrophilic microorganisms are adjusted to grow and maintain their metabolic activities under extremely low temperatures or in cold environments. Psychrophiles (belonging to the domains of *Archaea*, *Bacteria*, and *Eukarya*) undergo photosynthetic, chemoautotrophic, and heterotrophic metabolic pathways [245]. Mykytczuk et al. stated that *Planococcus halocryophilus* Or1, a bacterial strain isolated from high Arctic permafrost, can grow at − 15 °C. Piezo-psychrophiles thrive in biotopes with low temperature and high pressure, whereas halo-psychrophiles live in sea ice with increased salt concentration and low temperature [246]. A research study done by Geolette et al., and Cavicchioli et al. reported the isolation of a broad range of psychrophiles including Gram-negative and Gram-positive bacteria, fungi, and yeast from the cold ecological niches [247, 248]. Currently, owing to the efforts to reduce the energy consumption, psychrophilic enzymes have gained further interest to apply in various industrial bio-processes and biotechnological applications due to their enhanced catalytic efficiency at low or moderate temperatures [249–252]. Notable examples of this include the use of psychrophilic enzymes (amylases) in polymer degradation in laundry detergents at lower temperatures which leads to the reduction in energy consumption and prevention of wearing and tearing of textile fibers, the use of enzymes namely L-glutaminase and L-asparaginase in various food processing industry, and the use of psychrophilic proteases for meat tenderization [253].

Halophiles possess the ability to maintain the osmotic balance and therefore can survive in hypersaline habitats. In other words, they cope with high salt concentrations such as sodium or potassium chloride. Enzymes from such halophiles need to sustain high salt concentrations (e.g., KCl concentration of 4 M and NaCl concentration of > 5 M) [254]. The isolation and production of halophilic amylases, xylanases, lipases, and proteases have been done from halophiles that belongs to the genera *Halobacterium*, *Halothermothrix*, *Haloferax*, and *Halobacillus* and their potential biotechnological applications have been further discussed [254, 255].

Microbes that can thrive under extreme pH values (low pH/high pH) could be a potential source of thermo-acidophilic and thermo-alkaliphilic enzymes. Such enzymes inherit potential characteristics for applications at acidic or alkaline reaction conditions such as in dishwashing detergents and laundry additives [254]. Thermo-alkaliphilic enzymes include proteases, lipases, amylases, and several other enzymes which are active and resistant to high pH; whereas thermo-acidophilic enzymes consist of pullalanases, glucoamylases, and glucosidases which are active at low pH.

Nanotechnology Strategy in Enzyme Development

Enzymes deliver a huge potential in catalytic processes due to their special attributes such as high reaction activity/efficiency, greater stability, unique reaction conditions, etc. The use of enzymes in industrial applications, however, holds some drawbacks. These include high cost for synthesis, isolation and purification, enzyme stability, and recovery from substrates and inhibitory feedback reactions from end products [256, 257]. Nanotechnology introduces the prospect for avant-garde changes across a wide range of applications. The domain of enzyme biotechnology is no exception [258].

Nanotechnology deals with materials of few nanometers to less than 100 nm in size (or more appropriately 0.2 and 100 nm). The National Nanotechnology Initiative (Arlington, VA, USA) defines nanotechnology as the understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications [259]. It uses nanoparticles (NPs)/nanomaterials for the benefit of humankind. The engineering of nanoparticles and delineation of their physio-chemical characteristics holds great prospect to substantially impact the bio-catalysis realm. Unlike their macroscale counterparts, nanoparticles have unique properties such as small size (nano-scale) and bigger surface area-to-volume ratio which would increase their reactivity, efficacy, and selectivity [260].

Recent advancements in nanotechnology pave a wealth of myriad nanoscaffolds that could potentially be very attractive carriers for enzyme immobilization. Immobilization of enzyme is defined as a technique where enzyme is confined to a solid matrix or support different from the one for substrates and products enabling their safe and secure recovery and reusability while maintaining their catalytic activities. The prime components that determine an enzyme immobilization include the matrix, enzyme, and the mode of attachment. The solid matrix used as a carrier matrices for enzyme immobilization include inert polymers and inorganic materials (such as glass, silica, clay, polymers, and gels with) with physio-chemical attributes like stability, physical strength, reproducibility, inertness, biocompatible, capability to enhance enzymatic activity, decrease product inhibition, non-specific adsorption, and microbial contamination [261]. Enzyme immobilization generates several advantages in biotechnological and industrial commercialization. These include (i) economic operation owing to the enzyme pH and thermal stability, enzyme robustness, and recyclability; (ii) ease for the enzyme recovery with enhanced purity level; and (iii) convenience in handling the enzymatic reaction [262] (Table 3).

The several strategies currently employed in enzyme immobilization encompass adsorption, covalent bonding, entrapment and encapsulation, and cross-linking enzyme aggregates (CLEA) (Fig. 2) [279].

Adsorption mechanism for enzyme immobilization utilizes water-insoluble carriers like polysaccharide derivatives, synthetic polymers, and glass [280]. This method is cataloged as physical adsorption (based on the weak forces such as hydrogen bonding, and electrostatic and hydrophobic interactions), electrostatic binding (based on the isoelectric point of enzyme and pH value of the solution), and hydrophobic adsorption (based on the hydrophobic interaction between the enzyme molecules and the solid matrix). Layer by layer deposition and electrochemical doping are the two

Table 3 Some of the enzymes immobilized on nanoparticles and their biotechnological applications

Enzyme Name	Nanoparticles	Applications	References
Glucose oxidase	Thiolate gold nanoparticles	Glucose level estimation	[263]
Laccase	Chitosan magnetic nanoparticles	Removal of pollutants	[264]
B-galactosidase	Con A layered ZnO particles	Hydrolysis of lactose	[265]
Keratinase	Fe_3O_4 nanoparticles	Keratin synthesis	[258]
Lipase	Fe_3O_4 nanoparticles, polystyrene nanoparticles	Hydrolysis of para-nitrophenylphosphate (pNPP), aminolysis, esterification/transesterification	[266, 267]
A-amylase	Cellulose-coated magnetic nanoparticles	Starch degradation	[268]
Diastase	Silica coated nickel nanoparticles	Starch hydrolysis	[269]
Peroxidase	Gold chitosan nanoparticles	Water treatment, pharmaceutical application	[270]
Urease	Silver nanoparticles	Urea content analysis in urine, blood, beverages	[271]
Cellulase	TiO_2 nanoparticles	Carboxymethyl cellulose hydrolysis	[272]
Cholesterol oxidase	Fe_3O_4 nanoparticles	Analysis of cholesterol in serum	[273]
Trypsin	TiO_2 nanoparticles	Refolding, proteomics, cell culture	[274, 275]
Uricase	ZnO nanoparticles	Analysis of serum uric acid	[274]
Lysozyme	Chitosan nanofibers	Anti-bacterial	[276]
Bitter Gourd Peroxidase (BGP)	TiO_2 nanoparticles	Removal of dye and phenol	[277]
Protease	Super paramagnetic nanoparticles	Hydrolysis of proteins in detergent, leather industry, and food supplements.	[278]

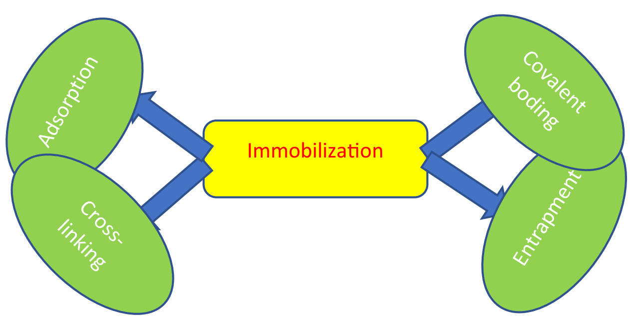

Fig. 2 Pictorial representation of various techniques of enzyme immobilization

common strategies in electrostatic binding strategy that have been extensively exploited to improve enzymatic biosensors [279]. Cunha et al. [281] successfully reported the improved yield and better stability of *Yarrowia lipolytica* lipase immobilized by physical adsorption in comparison to the free lipase. Similarly, *Candida rugosa* lipase immobilized by adsorption on biodegradable poly (3-hydroxybutyrate-co-hydroxyvalerate) retained around 94% residual activity after 4-h incubation at 50 °C and recyclability till 12 cycles [282]. However, the restriction of this technique includes enzyme leaching, non-specific adsorption leading to contamination, and changes in optimal temperature and pH strength.

In covalent bonding immobilization of enzyme, multifunctional reagents such as glutaraldehyde, bisdiazobenzidine, and hexamethylene diisocyanate are used to provide strong bindings between enzymes and solid matrix and thereby impart greater stability to covalently bound enzymes. This method covalently immobilizes enzyme through activation of carboxylic groups and amino groups, and chemisorption. Ispas et al. [283] discovered highly stable and hyperactive bio-catalysts through covalent binding of enzymes. A recent study conducted by Terrasan et al. [284] utilized glutaraldehyde and dextran to stabilize β-xylosidase (BXYL I and BXYL II) purified from *Penicillium janczewskii*. The immobilized enzymes showed thermal stability by a factor of 12 and 33 for BXYL I and BXYL II, respectively. Covalent bonding also has some drawbacks. It is a complex phenomenon requiring longer incubation time, and enzyme immobilized by covalent bonding is poorly reproducible and sometimes does not ensure enzyme purity level.

Entrapment is a strategy where an enzyme is entrapped within a polymeric network using gel-matrices such as polyacrylamide and calcium alginate. This technique reduces the enzyme leaching and denaturation, enhances the enzyme stability across wide pH range, and improves thermal and storage stability along with better kinetic parameters. Cellulase and β-glucosidase enzymes immobilized in calcium alginate beads demonstrated a threefold increment in its thermostability [285]. Enzymes immobilized by entrapment through the use of electrospun nanofibers and virgin materials manifest wide range of applications such as in biosensors, bio-fuel industry, biomedicine, and chemistry [286, 287]. *Burkholderia cepacia* lipase immobilized by encapsulating within a K-carrageenan has been reported to maintain 82% transesterification activity after five cycles and is highly thermostable [288]. Nevertheless, this immobilization technique is thwarted by several factors such as low enzyme loading capacity, enzyme leaching, and polymerization leading to mass transfer resistance.

Cross-linking enzyme aggregate (CLEA) and cross-linking enzyme crystal (CLEC) are the two different approaches employed in cross-linking enzyme immobilization. A cross-linking agent such as glutaraldehyde is used in both the techniques in order to cross link enzyme molecules on the reactive site. The immobilization through CLEC requires the formation of crystals and the immobilized enzymes are stable, whereas CLEA is an improved version of CLEC production and has an ability to function in aqueous solution [279]. The enzyme immobilized using this method maintains enzymatic catalytic properties. The advantages of cross-linking immobilization involve minimal enzyme leakage, and safe and easy use of stabilizing agents for enzyme micro-environment adjustment. It also has some pitfalls such as severe enzyme modifications resulting in the loss of enzyme activity.

Enzymes developed through nanotechnology demonstrate better activity and stability. Such enzymes can be applied in food processing industries to improve flavor, better nutritional value, and enhance health benefits. An epitome is the hydrolysis of olive oil using nano-silicon dioxide particles deriving greater stability, efficient activity, and reusability [289]. Polymer-assisted magnetic nanoparticles (MNP) immobilized keratinase derived from *B. subtilis* showed concomitant enhancement in thermostability, storage, and recyclability; thereby paving the way to diverse prospect of biotechnological applications [258]. Mukhopadhyay et al. [290] reported the enhancement in activity, half-life, and stability of purified laccase from *Escherichia coli* AKL2 by 4-, 42-, and 36-fold, respectively, when supplemented with cu_2O nanoparticles. Laccases are used in many industries such as wastewater treatment, bio-remediation, removal of synthetic dyes, discoloration of wine, pectin gelation, food processing, and paper and pulp processing [291].

Lithography is a technology used in various sectors to create micro/nanostructure. Currently employed lithographic techniques in industry include nanoimprint lithography, dip-pen nanolithography, and ion-beam lithography [292, 293]. Enzyme lithography is an eco-friendly appropriate technique to fabricate nanostructure biomaterials at suitable temperature and pH that could be beneficial for tissue engineering, and bio-sensing [294]. The delivery of proteolytic enzyme on a thin film of bovine serum albumin (BSA) using enzyme lithography was first introduced in 2003 [295]. Lockhart et al. [296] reported the immobilization of three different enzymes namely galactosidase, glucose oxidase, and horseradish peroxidase using electron-beam lithography and observed 39% more retention of enzyme bio-activity after 30 days in comparison to free enzyme devices. In addition, the enzymes also exhibited fivefold chromogenic output. The use of enzyme lithography technique is in its infancy and the need to restrict enzyme mobility is one of the crucial restrictions in using this technique [297].

Nanotechnology has led to the development of nanomaterials that could imitate the natural enzyme characteristics such as size, enzyme activity, and efficiency. Such nanomaterials are referred as "nanozymes" [298]. Natural enzymes have certain physiological limitations while delivering catalytic functions. Unlike this, nanozymes promote persistent functional activity with biological substrates and products even in harsh environmental conditions. The production of nanozymes is economical and are more robust for wide range of applications like environmental remediation, biosensor development, stem cell growth, immunoassays, disease diagnosis and therapy, and oxidative stress prevention [299–301]. Cyclodextrins, porphyrins, polymers, supramolecules, and metal complexes have been extensively explored as an alternative to natural enzymes. Based on their function during the catalytic reaction, nanozymes are classified as anti-oxidants and pro-oxidants. Gao et al. [302] reported iron oxide (Fe_3O_4) nanoparticles expressing peroxidase like activity. Similarly, Korsvik et al. [303] stated that cerium oxide nanoparticles (CeNPs) offered improved superoxide dismutase (SOD) mimetic activity in comparison to the native CuZn. SOD mimics are used as anti-oxidation and anti-inflammatory agents, neuroprotection, and stem cell growth enhancement. Nanoceria has an ability to remove reactive oxygen species and thus is extensively used in biology and biomedical science. Iron oxide nanoparticles are used instead of horseradish peroxidase (HRP) in bio-analytical assay like enzyme-linked immunosorbance assays (ELISA) [302].

There has been tremendous growth in the development and application of nanozymes for the last 10 years. However, the field has certain limitations. Natural enzymes have well-defined tertiary structures, whereas owing to the variation in shape and size, nanozymes lack uniformity (except fullerene-based nanozymes) [304]. Gold nano-particles and peroxidase mimicking enzymes can be used to mimic glucose oxidase and HRP. Nevertheless, there is a room to maximize their efficiency. Unlike biological enzymes, nanozymes show less selectivity towards their substrates. Compared to natural enzymes and organic catalysts, the catalytic activity of nanozymes is still poorer. Protein enzymes undergo bio-conjugation using their cysteine and lysine side chain, nonetheless densely capped nanozymes lack such activity [305]. Natural enzymes perform together as enzyme clusters and are multi-functional. To the contrary, a functional nanozyme lacks the synergic effect of combined multiple enzyme-like properties [306]. Similarly, the toxic nature of nanozymes is another concern that is receiving considerable attention. This needs to be addressed by "safe by design" approach before translating nanozymes for fundamental applications in enzymology, material science, animal biotechnology, vaccine and adjuvants development, drug discovery, and nanomedicine [307, 308].

Conclusion and Future Perspectives

Enzyme industry accounts as one of the prime industries of the world. There is always a need for the discovery of enzymes with new and improved activities in the global market. The enormous pool of the microbial diversity delivers splendid amount of potential enzymes that have unequaled advantages in a broad spectrum of industries such as food processing, feed industry, polymer and textile industry, paper and pulp industry, detergent industry, fine chemicals and pharmaceutical industry, therapeutic sectors, and bio-fuel industry. From biotechnological approach, the biochemical attributes of enzymes such as thermostability, pH stability, multi-functionality, high specificity, and biodegradability are considered as significant aspects applicable in various bioprocesses. Such characteristics facilitate enzyme-assisted processes in industry at accelerated rate with enhanced yield, better quality, and economical and innocuous environmental effects. As naturally available enzymes lack the above-mentioned enzymatic characteristics necessary for bio-catalytic phenomenon, such enzymes should undergo further tailoring or redesign process to complement the catalytic properties. The recent advances in metagenomics screening, microbial genome mining, extremophiles diversity tapping techniques, protein engineering, extensive and efficient expression systems, and high-throughput sequencing technology have enabled the discovery and exploitation of diverse new enzymes from microbial and other extreme environmental sources, thereby advancing the catalytic traits, and broadening the enzymatic capacities. Yet, more rigorous application-oriented research study is of utmost concern for the effective manipulation of their full biotechnological potential. The possibilities of prospecting diversity and distribution of microbial enzymes in their natural sources could offer intense lens for exploring the impact of these enzymes in their natural habitat. The advancement in techniques such as molecular characterization, crystallography, enzyme modulation using bioinformatics tools, and algorithms (for the analysis of sequence–function relationship to generate diverse and systematic libraries) coupled with omics approaches and improvement in synthetic biology and chemical screening could further underpin and augment our basic understanding of the microbial ecology, enzymes, their evolution, and inherent relevance in various industrial sectors and human therapeutics. One should have strong mechanistic knowledge on the structure–function analysis and dynamics–function relationship to accelerate the use of bioinformatics and algorithm techniques for computational enzyme modulation. This could possibly aid in addressing questions associated with optimization of synthetic enzymes catalytic performance. Last but not the least, in light of the advanced research studies and enhanced understanding related to microbial diversity, their origin, and role in the environment, it is rational to anticipate the discovery of much exciting and intriguing microbial enzymes with crucial insights in regulation, disposition, and functional principles while facilitating their use in industries and human medicines.

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