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Review

Strategies for design of improved biocatalysts for industrial applications

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H I G H L I G H T S

- Overview of enzyme bio-prospecting.
- Recent trends in enzyme technologies for biocatalyst improvement.
- Directed evolution and Immobilisation as tools for enzyme improvement.
- Computational strategies for novel biocatalyst design.

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A B S T R A C T

Biocatalysts are creating increasing interest among researchers due to their unique properties. Several enzymes are efficiently produced by microorganisms. However, the use of natural enzymes as biocatalysts is hindered by low catalytic efficiency and stability during various industrial processes. Many advanced enzyme technologies have been developed to reshape the existing natural enzymes to reduce these limitations and prospecting of novel enzymes. Frequently used enzyme technologies include protein engineering by directed evolution, immobilisation techniques, metagenomics etc. This review summarizes the recent and emerging advancements in the area of enzyme technologies for the development of novel biocatalysts and further discusses the future directions in this field.

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Contents

1. Introduction 1305
2. Bio-prospecting of enzymes 1305
   2.1. Bio-prospecting from extremophiles 1305
   2.2. Metagenomics for bio-prospecting 1306
      2.2.1. Function-based metagenomic screening 1306
      2.2.2. Sequence-based metagenomic screening 1306
3. Directed evolution 1306
   3.1. Selection methods 1306
   3.2. Directed evolution in industrial enzymes development 1307
   3.3. Site directed mutagenesis 1307
4. Micobial cell surface display 1307
5. Enzyme immobilisation 1308
   5.1. Adsorption 1308
   5.2. Covalent binding 1308
   5.3. Entrapment 1308

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5.4. Carrier free immobilisation. 1308
6. Nanotechnology enabled enhancement of enzyme activity 1308
6.1. Nanoparticles. 1308
6.2. Nanofibres 1308
6.3. Nanotube 1309
7. Computational tool for designing improved enzymes 1309
7.1. The prediction of protein structure 1309
7.2. *De novo* design of enzymes. 1309
8. Future directions 1311
9. Conclusion 1311
Acknowledgements 1311
References 1311

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1. Introduction

Enzymes are powerful biocatalysts and enhance the reaction rates of various biological and chemical processes. Recently there is a high requirement for the potent biocatalysts and are considered as eco-friendly alternatives for high value chemical synthesis. Majority of chemical synthesis now uses environmentally risky organic solvents and require high energy for the processes (Mackenzie et al., 2015). As microbial enzymes do not possess any environmentally hazardous nature, and thus provide "cleaner" solutions for the synthesis of bulk chemicals and compounds.

Large number of enzymes has been commercially used in several industries such as leather, textiles, food, paper, food, pharmaceuticals and detergent. Important industrial biocatalysts include lipase (biofuel and pharmaceutical industry), trypsin (leather industry), xylanase (paper and pulp industry), lipoxygenase (food industry), phytase (feed industry), cold active proteases and α-amylase (detergent industry), and hyaluronidase (a pharmaceutical enzyme) (Bhavsar et al., 2013; Plagemann et al., 2013; Yang et al., 2011; Khatri et al., 2016; Rebello et al., 2017; Ra[illegible]2017).

The major source of biocatalysts is microorganisms due to the advantage of easy laboratory culturing, natural abundance and rich diversity. Although biocatalysts catalyse a wide number of chemical reactions, they are developed towards the needs of their natural catalysis. Thus, they are not suitable for many of the important catalytic processes and other industrially relevant substrates. Enzymes should possess high activity as well as high specificity and enantio-selectivity towards range of different challenging substrates. In addition to that, they should be stable and withstand several harsh reaction conditions such as high temperature, strong acid, strong alkaline, high salinity, extreme pH, tolerate high substrate/product concentrations and solvent tolerance. The methodology for prospecting such stable enzymes is to prospect microorganisms from extreme habitats. The main difficulty is the propagation of potent microorganisms from extreme environment in laboratory conditions and most of the biocatalysts for industrial purpose are discovered through metagenomics (Yang et al., 2016). Thus, to accomplish all these qualities enzymes are nowadays tailor made by various enzyme engineering and stabilisation techniques for application in several fine chemical syntheses.

Modern bioengineering tools as well as advances in computational tools have revolutionised the field of enzyme engineering by designing different novel as well as improved enzymes suitable for different range of enzyme catalysed reactions during industrial processes. Biocatalysis is possibly to be the future of fine chemical production, and engineered and recombinant enzymes will be available in the future for most of the industrial and pharmaceutical processes. The immense advancement in this field has been made a reality by the fast development of different omics technologies, system biology, biochemistry, structural biology and other computational tools.

The intensifying interest for biocatalysts can be met in either by improving the catalytic power of the currently used enzymes or by bio-prospecting of novel enzymes. This review focuses on the current and emerging trends for enzyme technologies, and different strategies for creating novel enzyme biocatalysts.

2. Bio-prospecting of enzymes

Large number of enzymes possessing different activities is already discovered. But still it represents only a tiny fraction of the natural diversity available in nature. More than 99% of microbial diversity is also not possible to cultivate under laboratory conditions. Methods have been developed to prospect these extreme environmental niches and uncultivable microbes. The discovery of novel enzymes from such habitats would also involve the use of specialised techniques like microbial culture of uncultivable and high throughput screening methods for desired activities/properties (Wang et al., 2016).

2.1. Bio-prospecting from extremophiles

The genetic diversity of extreme environments of pH, temperature, alkalinity, salinity can be exploited to discover new and potent enzymes that are well suited for use in industrial processes and are relatively unexplored. The most important example is the discovery of biotechnologically important Taq DNA polymerase which is obtained from the extreme thermophilic bacterium *Thermus aquaticus* (Chien et al., 1976). The commercial market of this particular enzyme was about $500 million in 2009 (Adrio and Demain, 2014). Large number of extremophilic genomes has been sequenced by next-generation sequencing technologies and provides rich resources for prospecting of new enzymes with novel activities. The mechanism behind protein stability under extreme environmental conditions varies in different microbial species and level of adaption required for survival. For the acidophiles or alkaliphiles, the intracellular proteins are not tolerant at extreme pH since the intracellular pH is maintained at pH 5.0–6.0 and the cell membrane proteins are more tolerant to extreme pH. During industrial processes esterases has to function in solvent containing media and thus it should be solvent tolerant. The important functional characteristic that helps esterases to be stable in organic solvents include the presence of large number of negatively charged amino acid residues on their surface. This mechanism is also utilized by the halophiles to cope with high salt salinity, and hence, enzymes from such bacteria are expected to work in the presence of organic solvents. Similarly, cold active enzymes have tremendous applications in textile and detergent industry, fruit juice clarification, environmental bioremediation, etc., can be prospected from psychrophiles, which are normally adapted to function in extremely cold temperatures (Santiago et al., 2016). So detailed

knowledge about how the extremophilic biocatalysts could achieve their special properties and their structure function relationship is extremely important for developing novel enzymes with desirable properties and last decades witnessed tremendous developments in this area.

2.2. Metagenomics for bio-prospecting

Screening of biomolecules from environmental samples is done by metagenomic technique which mainly involves two basic approaches – function-based and sequence-based screening of metagenomic libraries (Escobar-Zepeda et al., 2015). These two methods of metagenomics are carried out by the construction of metagenomic libraries where fragmented genomic DNA is cloned in suitable expression vectors that let the expression of the required genes. Metagenomic libraries are constructed depending on the size of target gene using plasmids (15 kb), fosmids, cosmids (40 kb), or bacterial artificial chromosomes (40 kb) as expression vectors. For the identification of novel enzymes encoded by a single gene or a small sized operon, small sized insert clonal libraries are used, whereas large sized insert libraries are necessary for the segregation of large biosynthetic gene clusters, which encode for complex pathways containing multiple genes (Daniel, 2005). *Escherichia coli* are usually used heterologous host for the transformation of metagenomic clones obtained after library construction.

2.2.1. Function based metagenomic screening

In metagenomics, the commonly used function based strategies include enzyme activity detection (starch-iodine test for amylase, cellulase screening assay for cellulase etc.), phenotypic detect[illegible] and heterologous complementation of host strains and indu[illegible] gene expression. Dyes and substrates of target enzymes incorp[illegible]rated into the culture plate were used for the phenotypic chro[illegible]mogenic screening strategies employs, which pri[illegible]detects the presence of specific biocatalyst. Functional [illegible]ning [illegible]ables the identification of novel genes encoding no[illegible]nzyme[illegible]ithout knowing the gene sequence (Ferrer et al., 2009[illegible]

The presence of target gene for growth [illegible]der se[illegible]e condition is required for complemented host stra[illegible]or heterolo[illegible] complementation. Recently 10–13 restricti[illegible] with novel [illegible]stance genes were identified during the screening [illegible] 46000 clones of soil metagenomic library that [illegible]cifically sh[illegible]d resistance to β-lactam and aminoglycosi[illegible] antibiotic (Donato [illegible]l., 2010).

The main advantage [illegible]rect s[illegible]ning of metagenomic library is prior knowledge of s[illegible]nc[illegible] not required and we may get novel gene sequence with[illegible]y simil[illegible] to previously existing sequences. The [illegible]advan[illegible]e of [illegible]s screening is the chances for failure in [illegible]e exp[illegible]sion ma[illegible]ue to difficulties in promoter recognitio[illegible]ranslat[illegible]al inefficiency, misfolding of proteins, defective p[illegible]ra[illegible]ification of desired proteins etc. This can be re[illegible]d by using vectors capable of accommodating large insert size, [illegible] vectors with broad host range which allow expression in multi[illegible]hosts, use of rossetta *E. coli* strains which contains tRNA for rare amino acid codons (Perner et al., 2011).

The third type of functional screening is substrate induced gene expression screening which is a high through put screening which contains GFP as reporter gene. The system is based on the fact that the GFP expression is induced by the presence of a specific metabolite produced by the clones. The GFP is under the control of tightly regulated promoter. Researchers were successful in isolating hydrocarbon induced gene sequences from metagenomic library derived from ground water (Uchiyama et al., 2005).

2.2.2. Sequence based metagenomic screening

Sequence based approach is another widely used strategy for screening metagenomic clones. Here metagenomic clones of the target genes are screened using oligonucleotide primer or probes using the colony hybridisation technique. The target gene can be cloned in suitable expression vectors after amplifying by PCR with specific or degenerate primers. The main drawback of this technique is that the enzymes with known function and genomic sequence can only be identified which reduces the probability of isolating novel enzyme with entirely new sequences. Complexity of metagenomic community plays an important role in implementing the sequencing strategy where new sequencing strategy has to be used if the complexity of the community is high. More number of species is now being represented in metagenomic community after the initiation of new sequencing tec[illegible]e 454- pyrosequencing along with new computati[illegible]l tools [illegible] software's which easily analyse the bulk metag[illegible]ic sequen[illegible]formation (Brito and Alm, 2016).

3. Directed evolution

Directed evolutio[illegible] like [illegible]al evolu[illegible] but at relatively faster speed. Past fe[illegible]cades wit[illegible]ed t[illegible]endous impact of directed evolution. I[illegible]ted evolutio[illegible]om genome mutations are introduced a[illegible] the s[illegible]tion is base[illegible] on the desired function. The, major advantage of dir[illegible]d evolution is its tremendous pace compared [illegible]ural evolutio[illegible]hich takes millions and millions of yea[illegible]racewell and Arnol[illegible], 2009; Wang et al., 2012). Direction ev[illegible]ion compri[illegible] three main steps 1) Construction of mutant libr[illegible] 2) Screen[illegible]selection of mutants with improved function, and [illegible]olation o[illegible]mproved genes. So the main strategy for directed ev[illegible]nzymes is the creation of random mutations of [illegible]arting genes followed by screening and selection of target [illegible] with desirable phenotypes. This process is repeated sev[illegible] ro[illegible]s until the desired changes are observed. The introduc[illegible]ion of genetic diversity is the essential step in directed evolution. The directed mutation by single mutation strategy is highly [illegible]emanding with respect to the diverse number of gene mutants to be screened. For example an enzyme with 10 amino acid residue, the possible number of mutants getting is 2010. Among these mutants the chances of getting desired mutants is high. Most of the successful mutation strategies accumulate in substrate binding pockets and catalytic site. Severable mutation techniques are available now to introduce mutation in a gene such as targeted or random mutagenesis. The mutant gene is transformed into a bacterial cell with the help of a plasmid. The expression plasmid produces the mutant proteins. Chemical mutagenesis, random mutagenesis, saturation mutagenesis and DNA shuffling are major different mutagenesis techniques. Chemical mutagenesis use chemical agents such as ethyl methyl sulfonate, nitrous acid, etc.; use of different bacterial mutant or strains lacking DNA repair mechanisms (e.g. *E. coli* XL1-red) as hosts to express the heterologous enzymes in order to introduce mutations; error-prone PCR; DNA shuffling; site-saturation mutagenesis (which allows mutation of specific sites against all 20 possible amino acids); incremental truncation for the creation of hybrid enzymes (ITCHY), etc. More information on the methods used for generating sequence diversity may be obtained from the literature (Labrou, 2010). Although the availability of different strategies for introducing mutations are available, most studies in directed evolution are focussing on the use of error prone PCR for generation of genetic diversity due to the easy experimental procedure for the incorporation of mutations in target gene.

3.1. Selection methods

Directed evolution methods generate large number of mutant libraries. For the screening and selection of mutant libraries it is

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very important to have a robust and high-throughput method. In the last few decades, researchers have developed several methods for the screening of mutant libraries. Library screening methods involves agar plates and micro titre plates based screening strategy. Agar plate based rapid screening methods involve the direct correlation between growths of the host organism on selectiveagar plates for screening of enzyme function. Extracellular secretion of enzyme into the surrounding solid media of the agar plate containing particular substrate also allows the rapid screening of improved enzyme variants by the presence of halo or the presence of larger halo formation compared to the wild-type enzyme (Shim et al., 2004). Micro titre plate is the most widely used screening format for the large number of library screening, mainly because it resembles as a miniature cuvette system. The main advantage of micro titre plate based assay is that they have a broader dynamic range, which aids the detection of comparatively low improvements in the desired enzyme function compared to other assays like colony screening assays (Kelly et al., 2008). Most of the micro titre plate assays utilizes fluorogenic or chromogenic substrates, which are changed into spectroscopically different coloured products (Packer and Liu, 2015). Another minimizes screening strategy is the use of single bacterial host cells as femto-liter scale reaction compartments and analysis of single cells using flow cytometry (Varadarajan et al., 2005). Recently the mRNA display technique allows *in vitro* selection and directed evolution of different functional proteins from more than 1012 mutant libraries. Likewise, phage display and yeast display techniques have been developed and are effective for evolving enzymes (Chen et al., 2011).

3.2. Directed evolution in industrial enzymes development

Several researchers reported the use of directed evolutio[illegible]r enzyme improvement. The method was successfully emplo[illegible] for improving the temperature tolerance (Jochen[illegible] 2010), [illegible] engineer pH tolerance (Garcia-Ruiz et al., 201[illegible] ther[illegible] stabilit[illegible] (Zhou et al., 2015), enhance specific activi[illegible] Giger e[illegible] 2013), alteration of substrate specificity (Cheriyan e[illegible] 20[illegible] created naturally non-existing enzym[illegible]tivity ([illegible] et al., 2013). The field of directed evolution is ac[illegible] developi[illegible]d there are multiple excellent reviews av[illegible]or the me[illegible]s. Other successful examples includeimprovem[illegible]f catalytic activity of alcohol dehydrogenase wh[illegible] is 10-fold [illegible]her than the wild-type enzyme at low te[illegible]ratures for produ[illegible]n of enantiopure hexanediol from *Pyro[illegible]cus furi[illegible]s* (Machielsen et al., 2008). The glucose dehydrogena[illegible]cti[illegible] was improved by a factor of 13 using DNA shuffling me[illegible]or enh[illegible]ng the production of key intermediate[illegible] manu[illegible]re [illegible]torvastatin (Ma et al., 2010). The field [illegible]irect[illegible] evoluti[illegible] actively developing and there are mu[illegible]e exc[illegible]nt review[illegible] are available for the method (Packer a[illegible]iu[illegible]

3.3. Site directe[illegible]tagenesis

Site-directed mutagenesis is one of the tools implemented to construct novel proteins that serve as efficient biocatalysts to meet the industrial application. In site-directed mutagenesis an amino acid at specific site of a protein, whose structure and mechanism of action is known, is edited and the mutated protein is evaluated whether the modified protein performs better than the actual protein. Site-directed mutagenesis enhances specificity, stability, activity, solubility, and expression of the biocatalyst. Bioinformatics prediction of structure is important for site-directed mutagenesis, which includes single and combinational mutation. Single site-directed mutagenesis and multiple mutations have been recommended to accelerate and make simpler methods for mutagenesis (Winkler and Kao, 2014).

Site-directed mutagenesis study on α-galactosidase, a novel gene from deep sea bacteria *Bacillus megaterium*, improved the properties and increase of activity of the enzyme at molecular level. Several types of mutants of thermophilic archeal protein ST0452, isolated from *Sulfolobus tokodaii*, possessing glucosamine-1-phosphate (GlcN-1-P) AcTase activity and galactosamine-1-phosphate (GalN-1-P) AcTase activity, were constructed by site-directed mutagenesis. After analyses, the researchers identified that the amino acid His308 is essential for both GalN-1-P and GlcN-1-P AcTase activities, whereas Tyr311 and Asn331 are important only for the GalN-1-P AcTase activity (Zhang et al., 2015). In another study, Cys22 [illegible]ss glutathionine S-transferase from *Oryzasativa* was [illegible]laced w[illegible]alanine results in 2.2-fold increases of km value [illegible]tant compa[illegible] to wild type (Jo et al., 2012). Likewise, when the b[illegible]histidine [illegible]dues His275, His293, and His310 of α-a[illegible]ase fro[illegible] *subtil[illegible]* were replaced with aspartic acid via site[illegible]ected mutage[illegible]e kcat/km value of mutant increased b[illegible].7-fol[illegible]ompared [illegible]th that of the wild type (Yang et al., 2013).

4. Micobia[illegible]face display

Microbial cell surf[illegible] display accords peptides and proteins to be[illegible]ed on the su[illegible]e of microbial cells. This is generally a[illegible]eved by fusing the peptides/proteins of interest with the [illegible]horing mo[illegible] The protein to be displayed (passenger protein) c[illegible] be fused [illegible]o an anchoring motif (carrier protein) by N-[illegible]inal f[illegible]n, C-terminal fusion or sandwich fusion. (Lee et al., [illegible]he efficiency of display of proteins on microbial cell [illegible]rface solely depends on the features of carrier protein, passenger [illegible] and host cell, and fusion method. Applications mainly include live vaccine development, peptide library screening, bioconversion using whole cell biocatalyst and bio-adsorption. For instance, recombinants were prepared by genetically fusing the *Echinococcus granulosus* antigen EG95 on the surface proteins of a host cell. The sheep inoculated with recombinants showed good antibody response successfully reduced the infectivity during *in vitro* assay (Tan et al., 2012). In a similar study, the live oral vaccine against chicken coccidiosis using yeast *Saccharomyces cerevisiae* as host strain was developed, where *Eimeriatenella* EtMic2 protein used as a fusion protein for the first time (Sun et al., 2014). Recently, exosomes were engineered to develop nano shuttles utilizing cell surface display technology for multiple applications including targeted drug delivery and exosome mediated vaccine and therapy (Stickney et al., 2016).

The direct conversion of lignocellulosic materials to ethanol is made possible with the aid of recombinant yeast strains exhibiting the heterologous cellulolytic enzymes on the cell surface using the glycosylphosphatidylinositol inositol anchoring system. Usually, the cellulolytic activities of the typical cellulase-displaying yeast strains are inadequate for the hydrolysis of cellulose. However, a recent study by Inokuma et al. (2014) showed efficient utilization of cellulose by cellulase-displaying yeast strains by constructing novel gene cassettes for the same.

A combined strategy of cell-surface display technology has been utilized by researchers. By means of combined strategy of cell-surface display heterologous endoglucanase (EG) and cellobiohydrolase I (CBHI) were produced in a β-glucosidase displaying *S. cerevisiae* strain. In this study, strains EG-D-CBHI-D and EG-S-CBHI-S (with both enzymes displayed on the cell surface or with both enzymes secreted to the surrounding medium) showed higher ethanol production (2.9 and 2.6 g/L from 10 g/L phosphoric acid swollen cellulose, respectively), than strains EG-D-CBHI-S and EG-S-CBHI-D (with EG displayed on cell surface and CBHI secreted, or vice versa) (Liu et al., 2015).

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The hemicellulosic fraction in softwoods is mainly represented by mannans, which also serve as carbohydrate stores in large number of plants. However, mannans were not much utilized as sustainable resources for the development of sustainable biofuel. Through the immobilisation of different enzymes on the yeast cell walls by cell surface-display mechanics enables fermentation of mannans (Inokuma et al., 2014). Researchers have constructed yeast strains that co-display two enzymes - β-mannanase and β-mannosidase on the cell surface which successfully hydrolyzed 1,4-β-d-mannan and produced ethanol by incorporating the resulting mannose without adding any external enzymes (Ishii et al., 2016).

5. Enzyme immobilisation

Biocatalysts have several advantages like high selectivity, high turnover and efficiency etc., over industrial chemical catalysts. But several catalytic processes are hindered by the presence organic solvents, high temperature, lack proper storage conditions and stabilising agents etc. So the industrial and pharmaceutical applications of enzymes depend on the introduction of efficient and stable immobilisation techniques. The enzyme immobilisation effectively increases the stability, reusability of the enzymes and reduces the cost of the industrial processes and is environment friendly. Immobilized enzymes have ability to catalyze the reactions in wide environmental conditions. Several solid supports like xerogels, sand and clay are widely used for immobilisation in an eco-friendly manner (Homaei et al., 2013).

5.1. Adsorption

In this case the enzyme is attached to an inert material. This technique involves the attachment of enzyme to the surface of glass, alginate beads etc. Since adsorption is a physical process, the active site of the enzyme may be blocked by the immobilisation agent and this greatly reduces the activity of the immobilised enzyme. Adsorption of laccase on nano-porous gold (NPG) particles and [illegible] entrapment technique has been found to be more [illegible] as compared to surface adsorption (Sánchez-O[illegible] et al., 20[illegible]).

5.2. Covalent binding

Here the enzyme is bonded covalently to a support matrix or with the help of crosslinking agents that [illegible]ds the enzyme to matrix. This method is the most effective method and multipoint attachment of immobilised enzyme is p[illegible] and this increases the efficiency and stability of immobilised enzymes (Asgher et al., 2014).

5.3. Entrapment

Entrapment [illegible] simple adsorption technique in which enzyme is attached to basal support through forces such as ionic interactions, Vander Waals forces and hydrogen bonds; and has been reported as the best method for immobilisation. Entrapment is chosen over other techniques as this method is easy, cheap and produces are often stable. Hydrophobic and hydrophilic materials are used for enzyme entrapment. Hydrophobic materials are better as they can entrap more enzyme, higher degree of immobilisation and much activity is retained. The enzyme is usually trapped in insoluble beads (calcium alginate) or hydrophobic sol gels (propyletrimethoxysilane and tetra methoxysilane) (Asgher et al., 2012).

5.4. Carrier free immobilisation

Carrier free enzymes prepared by direct cross linking of enzyme preparations. The enzyme preparations can be crude or purified enzyme crystals and are named respectively as CLE (cross-linked [dissolved] enzyme) and CLEC (cross-linked enzyme crystals). Chemical cross-linking technique is used to prepare CLEs, in which enzymes have enhanced thermal stability. However CLEs are difficult to handle due to its gelatinous nature and apart from this enzyme in CLEs usually have less activity and lacks mechanical stability (Cao et al., 2003). Glutaraldehyde is the typically used cross-linking agent for the preparation of carrier free enzymes (Sheldon, 2011).

Cross-linked enzyme aggregates (CLEAs) were synthesized as an alternative to CLECs (Sheldon, 2007) and showed that aggregates of enzyme produced through precipitation [illegible] from enzyme solutions can be cross-linked effectively to form C[illegible] rather than using pure enzyme crystals (Cao et al., 2003). Enzyme aggregates can be precipitated from protein solutions by using ammonium sulfate, acetone or ethanol. [illegible] careful selection of precipitants and the conditions used for enzyme precipitation [illegible] is possible to modulate the properties of CLEAs. Since the enzyme crystallization part is eliminated in preparation of CLEAs, it is possible to perform the immobilisation [illegible] any [illegible]. Co-immobilisation of different enzymes is possible with CLEA[illegible] composites. CLEAs with magnetic pro[illegible] magnetic CL[illegible] (mCLEAs), can be prepared by cross linking in the presence of magnetic nanoparticles.

6. Nanotechnology enabled enhancement of enzyme activity

6.1. Nanoparticles

Nan[illegible]tic applications have evolved recently as easy, competent, and consistent methods for the immobilisation of [illegible]. The use of nanoparticles for immobilisation purposes is [illegible] more importance, as the nanoparticles have better adaptability. The nontoxic, biocompatible synthetic polymer poly (lactic-co-glycolic acid) (PLGA) has been regularly used as a support for enzyme immobilisation which provides better catalytic stability, viable uninterrupted operation, and effortless catalyst recycling; which in turn reduces the operational costs for various commercial processes. Enzyme immobilisation on nanoporous gold is very important for biocatalysis and biotransformation. Enzymes immobilized on chitosan coated magnetic nanoparticles by adsorption or covalent binding has been reported to improve their repeated reusability. Nano fibrous polymers have many advantages over other nanostructure supports (nanotubes, nanoparticles, and mesoporoussilica) due to their native high specific surface area, inter fibre porosity, low interference for mass transfer, easy management, and good mechanical strength (Andrade et al., 2010; Johnson et al., 2011). Recently novel magnetic nanoparticles coated with silica and gold were synthesized for immobilisation of α-amylase enzyme. The optimum temperature for the enzyme activity was enhanced from 60 °C for free enzyme to 80 °C for immobilized enzyme. The immobilized a-amylase maintained 60% of the enzyme activity even after recycling ten times (Singh et al., 2016). *Burkholderia cepacia* lipase (BCL) was immobilized on the phenyl-modified ordered mesoporous silica (Ph-OMMs) to obtain a novel immobilized lipase and was evaluated as a catalyst for the resolution of 1-phenylethanol, up to 50% conversion with more than 99% enantiomeric excess was obtained within 25 min, which is about 65-folds faster than that of the free lipase (Zheng et al., 2017).

6.2. Nanofibres

Nanofibres, widely used as support substances for enzyme immobilisation, own high specific surface area for high enzyme loading, fine porous structure allowing ready convenience to active

Table 1
List of nanomaterial for immobilisation.

Types	Material	Reference
Nanofibres	Poly surface nanofibres Poly styrene-co-maleic Anhydride Cellulose nanofibres	Verma et al. (2013)
Nanotubes	Carbon nanotubes, Peptides	Ji et al. (2010) Verma et al. (2013)
Nanosheets	Graphene oxide	Sun et al. (2014)
Nano particles	Chitosan, Poly lactic acid, Silver, Zinc Oxide	Chronopoulou et al. (2011)
Single enzyme nano particles	Porous composite, organic/ inorganic network	Kim et al., 2003

sites, low diffusion resistance, easy recoverability as well as potential applicability for continuous operations (Nair et al., 2007). Modified nanofibres with phospholipid moieties were used for CRL lipase immobilisation by adsorption method (Huang et al., 2003). The high enzyme loading property enhances lipase activity, thermal stability, higher km and lower Vmax for the immobilised lipases and then those for free lipase. Wang (2006) had studied effects of pH, temperature, and additive concentration on the adsorption capacity of polysulfone nanofibres. Poly (acrylonitrile-co-maleic acid) electrospun nanofibers were used for the immobilisation of lipase with their reactive carboxyl groups (Ye et al., 2006).

6.3. Nanotube

Carbon nanotubes (CNTs) have unique structural, mechanical, thermal and biocompatibility properties (Asuri et al., 2007); and have gained significant research attention in enzyme immobilisation and following application in biosensors. CNTs typically have a diameter ranging from an order of one to tens of nanometres and a length of up to several hundred micrometre for both single-walled carbon nanotubes and multi-walled carbon nanotubes (Lee et al., 2010). Single-walled carbon nanotubes (SWNTs) examined for biocatalytic performance (Rege et al., [illegible]3) showed improved enzyme activity in comparison to similar enzyme-containing composites without using SWNTs. The SWNTs with high specific surface area effectively adsorb and retain the enzyme molecules within the matrix without much loss while other forms of enzyme composites loss enzyme via leaching when they are placed in aqueous solution. Several nanomaterial used for enzyme immobilisation are listed in the Table 1.

7. Computational tool for designing improved enzymes

As discussed in the previous sections, the commercially available enzymes are usually not optimal for the desired industrial process. Therefore during last few decades several in*silico*, strategies have been matured to analyse for appropriate enzymes from the diverse natural pool (Damborsky and Brezovsky, 2009). However, natural enzymes rarely have the combined properties necessary for industrial chemical production such as high activity, high selectivity, and broad substrate specificity towards non-natural substrates, no inhibition by substrate or product, and a high stability in organic solvents and at high substrate or product concentrations. The advent of several computational tools has changed the scenario of engineering of proteins with a wide range of functions (Mak and Siegel, 2014). Most of the success is based on the fixed backbone approaches and that maintains the backbone conformations seen in the original high-resolution crystal structures and research focus on remodelling the side chains. Computational protein design tools uses two major approaches: a scoring function to assess how strong a peculiar amino acid sequence befitting a given scaffold and another search function that analyse the sequences, backbone and side chain conformations. Energy functions for protein-design often contain a combination of physically-based and knowledge based terms (Huang et al., 2016).

7.1. The prediction of protein structure

The modelling of protein structure is essential for further improvement of stability, activity, specificity, or selectivity. The experimentally determined structural [illegible]eral proteins are available in Protein Data Bank (Berman et al., 2[illegible]). The sequence of similarity of the newly discovered protein is sufficiently high the structure can also be modelled based on the sequence comparison to a similar protein sequence. At least 2[illegible] of sequence similarity is essential for the modelling of protein structure ([illegible]lan et al., 2012). Several homology modelling tools are available such as Swiss-Model (Arnold et al., 2[illegible]), Modeller (Marti-Renom et al., 2000) or Rosetta (Mari[illegible] et al., 2[illegible]). All these are based on the finding that during evolution structure has been more conserved than the amino acid sequence. Thus enzymes with similar sequence have a similar structure. Using these methods, structure models can be derived for majority of proteins.

7.2. De novo design of enzymes

De novo design of the enzyme is the design of a catalytic activity fro[illegible]ore as[illegible]ed by the insight of the configuration of the set of atoms [illegible]ereby specific aminoacid residues required for the stability of the transition state and further lowering of activation [illegible] stabilizing the transition state and thereby lowering the activation energy of the reaction to be catalysed. The *de novo* design of enzymes usually begins with the design of the active site. The active design needs a prior knowledge of the type of the enzyme catalysed reaction and its molecular mechanism and other interactions required for efficient catalysis. The technique involves the *in silico* modelling of active site using quantum-mechanic simulations and this will lead to correct positioning of residues in correct geometry to stabilise the transition state (Quin and Schmidt-Dannert, 2011). This particular design is called a theozyme (Kiss et al., 2013). Once the structural data for the target active site is available, a scaffold capable of accommodating the new catalytic design are selected from the Protein Data Bank (PDB) and these are used as the particular template for different molecular modelling tools like RosettaMatch or Gess (Smith et al., 2014). After the finishing of protein backbone template with newly designed active site, several favourable mutations are introduced at the active site to improve the favourable molecular interactions and stability of transition state.

Tremendous progress has been achieved in *de novo* design of biocatalysts catalysing a chemical reaction. Several computational tools have been designed by researchers to aid the designing and engineering of biocatalysts with desired catalytic properties aiming to improve the efficiency of the designed enzymes. Computational tools like METAL SEARCH (Klemba et al., 1995; Clarke and Yuan, 1995), DEZYMER (Hellinga and Richards, 1991), ORBIT (Dahiyat and Mayo, 1996), and ROSETTA (Zanghellini et al., 2006) have marked a breakthrough in *de novo* design of enzymes. DEZYMER is a molecular model building computer program that builds new ligand binding sites into a protein of known 3D structure by altering only the sequence and the side-chain structure of the protein, leaving the protein backbone folds intact by definition. This program enables computer-aided modelling of active sites with pre-defined geometric structures, providing a general method for the design of ligand-binding sites and enzyme active sites, which

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Table 2
Enzyme technologies for the improvement of biocatalysts.

Improvement method	Industrial enzyme	Recent example	Reference
Directed Evolution	Xylanase	Xyn A from *B. subtilis* has been engineered through directed evolution for high thermal stability and pH tolerance (pH 12 and Temperature 55 °C	Ruller et al. (2014)
		Engineered GH11 xylanase through directed evolution	Song et al. (2012)
Immobilisation	Lipase	A novel solvent tolerant lipase from *Pseudozyma* sps was immobilised on magnetic nanoparticles without loss in enzyme activity and prolonged shelf life	Alex et al. (2014)
	Protease	Proteases produced by solid state fermentation was immobilised on iron oxide nanoparticles	Yazid et al. (2017)
Metagenomic approach	Cellulase	Identification of novel cellulase genes from metagenomic library	Yang et al. (2016)
	β-Glucosidase	Identified novel thermotolerant β glucosidase from metagenomic library	[...]Pozo et al. (2012)
Site directed mutagenesis	α-Amylase	α-Amylase from *B. subtilis* was improved for pH stability by mutation in histidi[...] residue	Ya[...]l. (2013)
	β-Glucosidase	Improved the glucose sensitivity and stability of *Trichoderma reesei* β-glucosi[...]	Guo et [...]2016)
Molecular Modelling	Cellulase	Developed Stochartic cellulase automata based modelling approach t[...]cribe degradation of cellulosic material by a cellulase system at single m[...]e resolution	Eibinge[...]al. (2016)
Denovo design	Carbonic anhydrase	Denovo engineered desulfide bond in bacterial α type carbonic a[...]rase fo[...]rbon sequestration	[...]. (2016)
Surface display	Mannase Chitosanase	ManB, a mannanase from *Bacillus licheniformis* DSM13, a[...] CsnA[...]ase from *Bacillus subtilis* ATCC 23857 were fused to different anchoring motifs of [...]*tarum* for co[...] attachment to the cell surface for the developm[...]le cell biocata[...]	Nguyen et al. (2016)
Fusion enzymes		N-terminal fusion of a yeast homolog of SUM[...]otein-[...]could confer elevated optimal temperature and improved operational stability to [...]cose 3-epimerase. The Smt3-D-psicose 3-epimerase con[...] System showed relatively better cat[...]e efficiency, and improve[...] productivity	Patel et al. (2016)
		Two hybrid cellulases (BaCel5^{127} a[...]aCel5^{167}) from *Bisporaantennata* with replacement of the N-terminal (β[...]127 residues)[...]βα)$_4$ (167 residues)-barrel with the corresponding sequences [...]eEgl5A from [...]*romycesemersonii* were produced in Pichiapastoris and bioc[...]ally chara[...]zed and showed improved catalytic performance [...]ompared to wi[...]	Zheng et al. (2016)

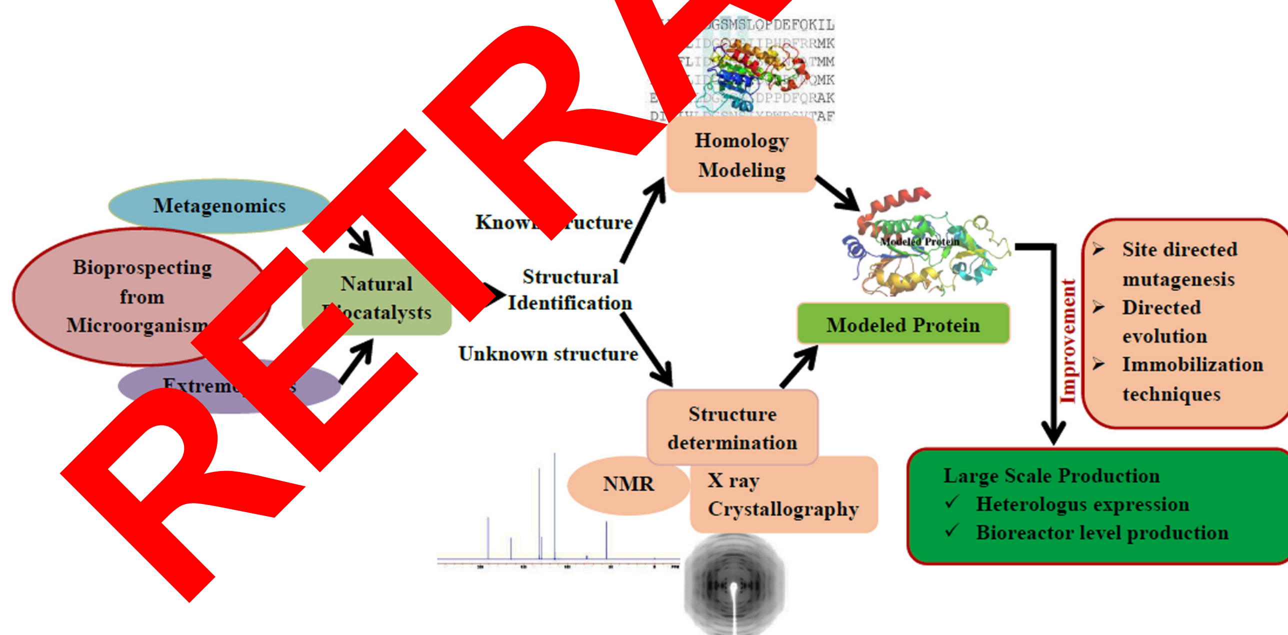

Fig. 1. Schematic outline of the technologies in enzyme improvement.

can then be tested experimentally. METAL SEARCH helps design tetrahedral coordinated metal binding sites in proteins of known structure. The major examples for *de novo* designed enzymes are the enzyme developed for the retro-aldol reaction, the Kemp elimination, and, more recently, the Diels-Alder reaction. (Rothlisberger et al., 2008; Kiss et al., 2011).

The advent of several molecular and physical technologies revolutionised the field of industrial biocatalysis and different strategies for enzyme improvement is summarised in the Table 2 with recent examples from different enzymes and schematically represented in the Fig. 1.

8. Future directions

Recently most of the enzyme industry is looking at biocatalysts as a cleaner agent to manufacture most of their products in eco-friendly processes. Consequently, the increased need for enzymes and industrial bioprocesses is driving a new era in enzyme technology, with the help of advances in modern biologyand chemical technology. Enzyme bioprospecting for novel activities from unexplored hot spots or extreme environments, computer aided *de novo* design of enzymes, molecular modelling, and developments in metabolic and genetic engineering is contributing significantly to the field of enzyme discovery. In addition to this, future technologies in metagenomics to explore the complete microbial population dynamics by integrating different approaches like culture methods, DNA and RNA analysis, proteome analysis, and metabolic profiling will help to explore undiscovered enzyme activities and artificial enzymes, hybrid biocatalysts, and enzyme-based nanoreactors being developed with the help of wealth of knowledge that has been generated from structural and functional studies of enzymes. Developments in structural chemistry and computational tool will aid in the design of active catalytic centers of novel biocatalysts that do not exist naturally or for further modification of the existing enzymes to meet the industrial requirements. Importantly, most of this techniques and tools are being commercialised and are accessible to public, which will enhance the developments in the field of industrial bioprocess.

9. Conclusion

Biocatalysts with novel and improved characteristics are [illegible]tially important to meet industrial demand. Currently there is [illegible]h demand for the potent biocatalysts and are considered as gree[illegible] alternatives for high value chemical synthesis. S[illegible] success[illegible] advances have been made to bioengineer the[illegible]ural [illegible]ocataly[illegible] through various technologies like site dire[illegible] muta[illegible]esis and protein engineering. But still there is a need [illegible]ev[illegible] and robust technologies to improve t[illegible]biocat[illegible]s to cater the needs of various industries. It is app[illegible]t that the g[illegible]th in development of enzymetechnology i[illegible] the dynam[illegible]hase and expected to bring about astonis[illegible]ng ou[illegible]es in nearest future.

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References

Adrio, J.L., Demain, A.L., 2014. Microbial enzymes: tools for biotechnological processes. Biomolecules 4 (1), 117–139.
Alex, D., Mathew, A., Sukumaran, R.K., 2014. Esterases immobilized on aminosilane modified magnetic nanoparticles as a catalyst for biotransformation reactions. Bioresour. Technol. 167, 547–550.
Andrade, L.H., Rebelo, L.P., Netto, C.G.C.M., Toma, H.E., 2010. Kinetic resolution of a drug precursor by *Burkholderia cepacia* lipase immobilised methodologies on superparamagnetic nanoparticles. J. Mol. Catal. B: Enzym. 66, 55–62.
Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.
Asgher, M., Iqbal, H.M., Irshad, M., 2012. Characterization of purified and xerogel immobilized novel lignin peroxidase produced from Trametes versicolor IBL-04 using solid state medium of corncobs. BMC Biotechnol. 12, 46.
Asgher, M., Shahid, M., Kamal, S., Iqbal, H.M.N., 2014. Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. J. Mol. Catal. B: Enzym. 101, 56–66.
Asuri, P., Bale, S.S., Pangule, R.C., Shah, D.A., Kane, R.S., Dordick, J.S., 2007. Structure, function, and stability of enzymes covalently attached to single-walled carbon nanotubes. Langmuir 23, 12318–12321.
Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., 2000. The Protein Data Bank. Nucleic Acids Res. 28, 235–242.
Bhavsar, K., Gujar, P., Shah, P., Kumar, V.R., Khire, J.M., 2013. Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by NCIM *Aspergillus niger* 563 under submerged fermentation condition. Appl. Microbiol. Biotechnol. 97, 673–679.
Brito, I.L., Alm, E.J., 2016. Tracking strains in the microbiome: insights from metagenomics and models. Front. Microbiol. 7, 712.
Cao, L., van Langen, L., Sheldon, R.A., 2003. Immobilized enzymes: carrier bound or carrier free? Curr. Opin. Biotechnol. 14, 387–394.
Chao, F.A., Morelli, A., Haugner, J.C., Churchfield, L., Hagmann, L.N., Shi, L., Masterson, L.R., Sarangi, R., Veglia, G., Seelig, [illegible]tructure and dynamics of a primordial catalytic fold generated by i[illegible]n. Nat. Chem. Biol. 9 (2), 81–83.
Chen, I., Dorr, B.M., Liu, D.R., 2011. A ge[illegible]trategy for th[illegible]olution of bond-forming enzymes using yeast display. P[illegible]atl. Acad. Sc[illegible].A 108, 11399–11404.
Cheriyan, M., Walters, M.J., Kang[illegible] Anzaldi, [illegible]one, [illegible]ierke, C.A., 2011. Directed evolution of a p[illegible]te ald[illegible]ase to[illegible] a long chain acyl substrate. Bioorg. Med. C[illegible]. 19, 64[illegible]453.
Chien, A., Edgar, D.B., T[illegible]M., [illegible]. Deoxyribo[illegible]ucleic acid polymerase from the extreme [illegible]rmo[illegible]*us aqua*[illegible] J. Bacteriol. 127 (3), 1550–1557.
Chronopoulou, L., [illegible]l, G., Sparag[illegible]ord[illegible]Lupi, S., Diociaiuti, M., Palocci, C., 2011. Struc[illegible]vity relations[illegible]*ndida rugosa* lipase immobilised on polylacti[illegible]n[illegible]rticles. Soft M[illegible] 7, 2653–2662.
Clarke, N.D., [illegible]an, S.M.[illegible] Metal search: a computer program that helps design tetrahedral metal-bin[illegible]ites. Proteins 23, 256–263.
Dahi[illegible]Mayo, S.L., 199[illegible]ein design automation. Protein Sci. 5, 895–903.
D[illegible]rsky, J., Brezovsky, J., [illegible]009. Computational tools for designing and engineering biocatalysts. Curr. Opin. Chem. Biol. 13 (1), 26–34.
[illegible]el, R., 2005. T[illegible]metagenomics of soil. Nat. Rev. Microbiol. 3, 470–478.
[illegible]ozo, M.V., Fe[illegible]dez-Arrojo, L., Gil-Martinez, J., Montesinos, A., Chernicova, T. [illegible]Nechitaylo[illegible], Waliszek, A., Tortajada, M., Rojas, A., Huws, S.A., Golyshina, [illegible]Newb[illegible].J., Polaina, J., Ferre, M., Golyshin, P.N., 2012. Microbial β-glu[illegible]rom cow rumen metagenome enhance the saccharification of lignocellulose in combination with commercial cellulase cocktail. Biotechnol. Biofuels 5, 1–13.
[illegible]., Noah, J.W., Hurt, D., 2012. Comparison of common homology modeling algorithms: application of user-defined alignments. Methods Mol. Biol. 857, 399–414.
Donato, J.J., Moe, L.A., Converse, B.J., Smart, K.D., Berklein, F.C., McManus, P.S., Handelsman, J., 2010. Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. Appl. Env. Microbiol. 76, 4396–4401.
Eibinger, M., Zahel, T., Ganner, T., Plank, H., Nidetzky, B., 2016. Cellular automata modeling depicts degradation of cellulosic material by a cellulase system with single-molecule resolution. Biotechnol. Biofuels 9, 56.
Escobar-Zepeda, A., Vera-Ponce deLeón, A., Sanchez-Flores, A., 2015. The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. Front. Genet. 6, 348.
Ferrer, M., Beloqui, A., Timmis, K.N., Golyshin, P.N., 2009. Metagenomics for mining new genetic resources of microbial communities. J. Mol. Microbiol. Biotechnol. 16, 109–123.
Garcia-Ruiz, E., Gonzalez-Perez, D., Ruiz-Dueñas, F.J., Martínez, A.T., Alcalde, M., 2012. Directed evolution of a temperature-, peroxide- and alkaline pH-tolerant versatile peroxidase. Biochem. J. 441 (1), 487–498.
Giger, L., Caner, S., Obexer, R., Kast, P., Baker, D., Ban, N., Hilvert, D., 2013. Evolution of a designed retroaldolase leads to complete active site remodeling. Nat. Chem. Biol. 8, 494–498.
Guo, B., Amano, Y., Nozaki, K., 2016. Improvements in glucose sensitivity and stability of *Trichoderma reesei* β-Glucosidase using site directed mutagenesis. PLoS One 11 (1), e0147301.
Hellinga, H.W., Richards, F.M., 1991. Construction of new ligand binding sites in proteins of known structure. I. Computer-aided modeling of sites with pre-defined geometry. J. Mol. Biol. 222, 763–785.
Homaei, A.A., Sariri, R., Vianello, F., Stevanato, R., 2013. Enzyme immobilization: an update. J. Chem. Biol. 6, 185–205.
Huang, P., Boyken, S.E., Baker, D., 2016. The coming of age of de novo protein design. Natural 537, 320–327.
Huang, S.H., Liao, M.H., Chen, D.H., 2003. Direct binding and characterization of lipase onto magnetic nanoparticles. Biotechnol. Prog. 19, 1095–1100.
Inokuma, K., Hasunuma, T., Kondo, A., 2014. Efficient yeast cell-surface display of exo- and endo-cellulase using the SED1 anchoring region and its original promoter. Biotechnol. Biofuels 7, 8.
Ishii, J., Okazaki, F., Djohan, A.C., Hara, K.Y., Asai-Nakashima, N., Teramura, H., Andriani, A., Tominaga, M., Wakai, S., Kahar, P., Yopi, Prasetya.B., Ogino Email, C., Kondo, A., 2016. From mannan to bioethanol: cell surface co-display of β-mannanase and β-mannosidase on yeast Saccharomyces cerevisiae. Biotechnol. Biofuels 9, 188.
Ji, P., Tan, H.S., Xu, X., Feng, W., 2010. Lipase covalently attached to multiwalled carbon nanotubes as an efficient catalyst in organic solvent. Aiche J. 56, 3005–3011.

Jo, B.H., Park, T.Y., Park, H.J., Yeon, Y.J., Yoo, Y.J., Cha, H.J., 2016. Engineering de novo disulfide bond in bacterial α-type carbonic anhydrase for thermostable carbon sequestration. Sci Rep. 6, 29322.

Jo, H.-J., Lee, J.-W., Noh, J.-S., Kong, K.-H., 2012. Site-directed mutagenesis of cysteine residues in Phi-class glutathione S-transferase F3 from *Oryza sativa*. Bull. Korean Chem. Soc. 33, 4169–4172.

Jochens, H., Aerts, D., Bornscheuer, U.T., 2010. Thermo-stabilization of an esterase by alignment-guided focused directed evolution. Protein Eng. Des. Sel. 23 (12), 903–909.

Johnson, P.A., Park, H.J., Driscoll, A.J., 2011. Enzyme nanoparticle fabrication: magnetic nanoparticle synthesis and enzyme immobilisation. Methods Mol. Biol. 679, 183–191.

Kelly, R.M., Leemhuis, H., Rozeboom, H.J., van Oosterwijk, N., Dijkstra, B.W., Dijkhuizen, L., 2008. Elimination of competing hydrolysis and coupling side reactions of a cyclodextrin glucanotransferase by directed evolution. Biochem. J. 413, 517–525.

Khatri, V., Hébert-Ouellet, Y., Meddeb-Mouelhi, F., Beauregard, M., 2016. Specific tracking of xylan using fluorescent-tagged carbohydrate-binding module 15 as molecular probe. Biotechnol. Biofuels 9, 74.

Kim, J., Grate, J.W., Wang, P., 2003. Nanostructures for enzyme stabilisation. Chem. Eng. Sci. 61, 1017–1026.

Kiss, G., Celebi-Olcum, N., Moretti, R., Baker, D., Houk, K.N., 2013. Computational enzyme design. Angew. Chem. Int. Ed. 52, 5700–5725.

Kiss, G., Johnson, S.A., Nosrati, G., Çelebi-Ölçüm, N., Kim, S., Paton, R., Houk, K.N., 2011. Computational design of new protein catalysts. In: Comba, P. (Ed.), Modeling of Molecular Properties. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Klemba, M., Gardner, K.H., Marino, S., Clarke, N.D., Regan, L., 1995. Novel metal-binding proteins by design. Nat. Struct. Biol. 2, 368–373.

Labrou, N.E., 2010. Random mutagenesis methods for in vitro directed enzyme evolution. Curr. Protein Pept. Sci. 11 (1), 91–100.

Lee, H.K., Lee, J.K., Kim, M.J., Lee, C.J., 2010. Immobilisation of lipase on single walled carbon nanotubes in ionic liquid. Bull. Kor. Chem. Soc. 31, 650–665.

Lee, S.Y., Choi, J.H., Xu, Z., 2003. Microbial cell-surface display. Trends Biotechnol. 21 (1), 45–52.

Liu, Z., Inokuma, K., Hasunuma, T., Kondo, A., 2015. Combined cell-surface display- and secretion-based strategies for production of cellulosic ethanol with *Saccharomyces cerevisiae*. Biotechnol. Biofuels 8, 162.

Ma, S.K., Gruber, J., Davis, C., Newman, L., Gray, D., Wang, A., Grate, J., Huisma[illegible] W., Sheldon, R.A., 2010. A green-by-design biocatalytic process for atorvas[illegible] intermediate. Green Chem. 12, 81–86.

Machielsen, R., Leferink, N.G., Hendriks, A., Brouns, S.J., Hennemann, H.[illegible] Daussmann, T., van der Oost, J., 2008. Laboratory evolution of *Pyrococc[illegible] furiosus* alcohol dehydrogenase to improve the produc[illegible] (2S,5S)-hexanediol at moderate temperatures. Extremophiles 12, [illegible]

Mackenzie, A.K., Naas, A.E., Kracun, S.K., Schückel, J., Fa[illegible] J.U., A[illegible]r, J.W., Willatsb, W.G.T., Eijsinka, V.G.H., Pope, P.B., 2015. A [illegible]accharid[illegible]lization locus from an uncultured bacteroidetes phyloty[illegible] adaptation and substrate versatility. Appl. Envir[illegible] Micro[illegible] 187–193.

Mak, W.S., Siegel, J.B., 2014. Computational en[illegible] design: [illegible]tioning from catalytic proteins to enzymes. Curr. Opin. S[illegible] Biol. 27, 87–[illegible]

Mariani, V., Kiefer, F., Schmidt, T., Haas, [illegible]e, T., 2011. A[illegible]ment of template based protein structure pred[illegible]ns i[illegible]9. Proteins 10, [illegible]7–58.

Marti-Renom, M.A., Stuart, A.C., Fiser, [illegible] Sanchez, R., [illegible] F., 2000. Comparative protein structure modeling of ge[illegible]nd genomes. A[illegible]ev. Biophys. Biomol. Struct. 29, 291–325.

Nair, S., Kim, J., Crawford, B., [illegible] S.H., 20[illegible] Improving bi[illegible]catalytic activity of enzyme-loaded nanofib[illegible]by dis[illegible]g entangled nanofiber structure. Biomacromolecules 8, 12[illegible]70.

Nguyen, H., Mathiesen, G., Stelze[illegible]ham, M.[illegible]czkowska, K., Mackenzie, A., Agger, J.W., Eijsin[illegible], Yam[illegible]M., Pe[illegible]uer, C.K., Haltrich, D., Nguyen, T., 2016. Disp[illegible]annana[illegible]itosanase on the cell surface of Lactobacillu[illegible]ntarur[illegible]wards th[illegible]opment of whole-cell biocatalysts. Microb. C[illegible]act. 15, 1[illegible]

Packer, M.S., [illegible]R., 2[illegible] for the directed evolution of proteins. Nat. Rev. Genet. [illegible]9–394.

Patel, S.N., Sharma, [illegible]ta, K., Singh, U., Kumar, V., Sangwan, R.S., Singh, S.P., 2016. Improved operat[illegible]stability of d-psicose 3-epimerase by a novel protein engineering strateg[illegible]d d-psicose production from fruit and vegetable residues. Bioresour. Te[illegible]hol. 216, 121–127.

Perner, M., Ilmberger, N., Köhler, H.U., Chow, J., Streit, W.R., 2011. Emerging fields in functional metagenomics and its industrial relevance: Overcoming limitations and redirecting the search for novel biocatalysts. In: de Bruijn, F.J. (Ed.), Handbook of Molecular Microbial Ecology II: Metagenomics in Different Habitats. John Wiley & Sons Inc, Hoboken, NJ, pp. 483–498.

Plagemann, I., Zelena, K., Arendt, P., Ringel, P.D., Krings, U., Berger, R.G., 2013. LOX (Psa)1, the first recombinant lipoxygenase from a basidiomycete fungus. J. Mol. Catal. B: Enzym. 87, 99–104.

Quin, M.B., Schmidt-Dannert, C., 2011. Engineering of biocatalysts: from evolution to creation. ACS Catal. 1 (9), 1017–1021.

Rai, A.K., Sanjukta, S., Chourasia, R., Bhat, I., Bhardwaj, P.K., Sahoo, D., 2017. Production of bioactive hydrolysate using protease, β-Glucosidase and α-Amylase of *Bacillus* spp. isolated from kinema. Bioresour. Technol. 235, 358–365.

Rebello, S., Jose, L., Sindhu, R., Aneesh, E.M., 2017. Molecular advancements in the development of thermostable phytases. Appl. Microbiol. Biotechnol. 101 (7), 2677–2689.

Rege, K., Raravikar, N.R., Kim, D.Y., Schadler, L.S., Ajayan, P.M., Dordick, J.S., 2003. Enzyme-polymer-single walled carbon nanotube composites as biocatalytic films. Nano Lett. 3, 829–832.

Rothlisberger, D., Khersonsky, O., Wollacott, A.M., et al., 2008. Kemp elimination catalysts by computational enzyme design. Nature 453, 190–195.

Ruller, R., Alponti, J., Deliberto, L.A., Zanphorlin, L.M., Machado, C.B., Ward, R.J., 2014. Concommitant adaptation of a GH11 xylanase by directed evolution to create an alkali-tolerant/thermophilic enzyme. Protein Eng. Des. Sel. 27 (8), 255–262.

Sánchez-Otero, M.G., Valerio-Alfaro, G., García-Galindo, H.S., Oliart-Ros, R.M., 2008. Immobilization in the presence of Triton X-100: modifications in activity and thermostability of *Geobacillus thermoleovorans* C[illegible]e. J. Ind. Microbiol. Biotechnol. 35 (12), 1687–1693.

Santiago, M., Ramírez-Sarmiento, C.A., Zamora[illegible], Parra, L[illegible]16. Discovery, molecular mechanisms, and industrial [illegible]ations of col[illegible]ive enzymes. Front. Microbiol. 7, 1408.

Sheldon, R.A., 2007. Enzyme immobiliz[illegible]—the [illegible] for optim[illegible] performance. Adv. Synth. Catal. 349, 1289–130[illegible]

Sheldon, R.A., 2011. Characteristi[illegible]tures and biote[illegible] applications of cross linked enzyme aggreg[illegible](CLEAs)[illegible]. Microb[illegible]iotechnol. 92, 467–477.

Shim, J.H., Kim, Y.W., Kim, [illegible]J., C[illegible] Park, J.H.[illegible] H., Kim, J.W., Kim, Y.R., Schaefer, T., Spend[illegible]T., Mo[illegible]W., Park[illegible], 2004. Improvement of cyclodextrin gluc[illegible]ansferase as [illegible]tis[illegible]g enzyme by error prone PCR. Protein Eng. D[illegible]7, 205–211.

Singh, V., Rakshi[illegible] Ra[illegible], Angmo, S., K[illegible]nal, S., Garg, P., Chung, J.H., Sandhir, R., Sangwan, [illegible]S., Sing[illegible], 2016. Metallic/bimetallic magnetic nanoparticle functionalization for im[illegible]ation of a-amylase for enhanced reusability in bio[illegible]processes. Bio[illegible] Technol. 214, 528–533.

Smit[illegible]D., Zanghellini, A., Grabs-[illegible]thlisberger, D., 2014. Computational design of [illegible]el enzymes withoutcofactors. Methods Mol. Biol. 1216, 197–210.

Son[illegible], Siguier, B., [illegible]on, C., Bozonnet, S., O'Donohue, M.J., 2012. Engineering [illegible]er biomass-d[illegible]ding ability into a GH11 xylanase using a directed e[illegible]ion strateg[illegible]otechnol. Biofuels 2012 (5), 3.

Stickne[illegible] Losac[illegible], McDevitt, S., Zhang, Z., Lu, B., 2016. Development of exoso[illegible]display technology in living human cells. Biochem. Biophys. Res. Commun. 472, 53–59.

[illegible] Wang, L., Wang, T., Zhang, J., Liu, Q., Chen, P., et al., 2014. Display of [illegible]nella EtMic2 protein on the surface of *Saccharomyces cerevisiae* as a potential oral vaccine against chicken coccidiosis. Vaccine 32, 1869–1876.

[illegible]an, J.L., Ueda, N., Heath, D., Mercer, A.A., Fleminga, S.B., 2012. Development of orf virus as a bifunctional recombinant vaccine: surface display of *Echinococcus granulosus* antigen EG95 by fusion to membrane structural proteins. Vaccine 30, 398–406.

Tracewell, C.A., Arnold, F.H., 2009. Directed enzyme evolution: Climbing fitness peaks one amino acid at a time. Curr. Opin. Chem. Biol. 13, 3–9.

Uchiyama, T., Abe, T., Ikemura, T., Watanabe, K., 2005. Substrate induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat. Biotechnol. 23, 88–93.

Varadarajan, N., Gam, J., Olsen, M.J., Georgiou, G., Iverson, B.L., 2005. Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity. Proc. Natl. Acad. Sci. U.S.A 102, 6855–6860.

Verma, M.L., Barrow, C.J., Puri, M., 2013. Nanobiotechnology as a novel paradigm for enzyme immobilisation and stabilisation with potential applications in biodiesel production. Appl. Microbiol. Biotechnol. 97, 23–39.

Wang, M., Si, T., Zhao, H., 2012. Biocatalyst development by directed evolution. Bioresour. Technol. 115, 117–125.

Wang, P., 2006. Nanoscale biocatalyst systems. Curr. Opin. Biotechnol. 17, 574–579.

Wang, G., Wang, Q., Lin, X., Ng, T.B., Yan, R., Lin, J., Ye, X., 2016. A novel cold-adapted and highly salt-tolerant esterase from Alkalibacterium sp. SL3 from the sediment of a soda lake. Sci. Rep. 6, 19494.

Winkler, J.D., Kao, K.C., 2014. Recent advances in the evolutionary engineering of industrial biocatalysts. Genomics 104 (6A), 406–411.

Yang, C., Xia, Y., Qu, H., Li, A., Liu, R., Wang, Y., Zhang, T., 2016. Discovery of new cellulases from the metagenome by a metagenomics-guided strategy. Biotechnol. Biofuels 9, 138.

Yang, H., Liu, L., Shin, H.D., Chen, R.R., Li, J., Du, G., 2013. Structure- based engineering of histidine residues in the catalytic domain of alpha-amylase From *Bacillus subtilis* for improved protein stability and catalytic efficiency under acidic conditions. J. Biotechnol. 164, 59–66.

Yang, H.Q., Liu, L., Li, J.H., Du, G.C., Chen, J., 2011. Heterologous expression, biochemical characterization, and overproduction of alkaline alpha amylase from *Bacillus alcalophilus* in *Bacillus subtilis*. Microb. Cell Fact. 10, 77.

Yazid, N.A., Barrena, R., Sánchez, A., 2017. The immobilisation of proteases produced by SSF onto functionalized magnetic nanoparticles: application in the hydrolysis of different protein sources. J. Mol. Catal. B: Enzym. http://dx.doi.org/10.1016/j.molcatb.2017.01.009.

Ye, P., Xu, Z.K., Wu, J., Innocent, C., Seta, P., 2006. Nanofibrous membranes containing reactive groups: electrospinning from poly(acrylonitrile- co-maleic acid) for lipase immobilisation. Macromolecules 39, 1041–1045.

RETRACTED

Zanghellini, A., Jiang, L., Wollacott, A.M., Cheng, G., Meiler, J., 2006. New algorithms and an in silico benchmark for computational enzyme design. Protein Sci. 15, 2785–2794.

Zhang, Z., Shimizu, Y., Kawarabayasi, Y., 2015. Characterization of the amino acid residues mediating the unique amino-sugar-1-phosphate acetyltransferase activity of the archaeal ST0452 protein. Extremophiles 19, 417–427.

Zheng, F., Huang, H., Wang, X., Tu, T., Liu, Q., Meng, K., Wang, Y., Su, X., Xie, X., Luo, H., 2016. Improvement of the catalytic performance of a *Bispora antennata* cellulase by replacing the N-terminal semi-barrel structure. *Bioresour. Technol.* 218, 279–85.

Zheng, M., Xiang, X., Wang, S., Shi, J., Deng, Q., Huang, F., Cong, R., 2017. Lipase immobilized in ordered mesoporous silica: a powerful biocatalyst for ultrafast kinetic resolution of racemic secondary alcohols. Process Biochem. 53, 102–108.

Zhou, C., Xue, Y., Ma, Y., 2015. Evaluation and directed evolution for thermostability improvement of a GH 13 thermostable α-glucosidase from *Thermus thermophilus* TC11. BMC Biotechnol. 15, 97.

RETRACTED