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Review

Solvent tolerant enzymes in extremophiles: Adaptations and applications

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ABSTRACT

Non-aqueous enzymology has always drawn attention due to the wide range of unique possibilities in biocatalysis. In general, the enzymes do not or insignificantly catalyze substrate in the presence of solvents. This is due to the interfering interactions of the solvents between enzyme and water molecules at the interface. Therefore, information about solvent-stable enzymes is scarce. Yet, solvent-stable enzymes prove quite valuable in the present day biotechnology. The enzymatic hydrolysis of the substrates in solvents synthesizes commercially valuable products, such as peptides, esters, and other transesterification products. Extremophiles, the most valuable yet not extensively explored candidates, can be an excellent source to investigate this avenue. Due to inherent structural attributes, many extremozymes can catalyze and maintain stability in organic solvents. In the present review, we aim to consolidate information about the solvent-stable enzymes from various extremophilic microorganisms. Further, it would be interesting to learn about the mechanism adapted by these microorganisms to sustain solvent stress. Various approaches to protein engineering are used to enhance catalytic flexibility and stability and broaden biocatalysis's prospects under non-aqueous conditions. It also describes strategies to achieve optimal immobilization with minimum inhibition of the catalysis. The proposed review would significantly aid our understanding of non-aqueous enzymology.

1. Introduction

Biocatalysts are widely used for a range of applications in many biotechnological industries. In general, enzymes are a good choice in industrial bioprocesses due to their biodegradability, cost-effectiveness, and generation of non-toxic by-products. The enzymes must have high catalysis and stability across various unfavorable conditions, including extremes of temperatures, pH, surfactants, inhibitors, chelators, and solvents. Non-aqueous enzymology deals with solvent-stable enzymes. The advantages of non-aqueous biocatalysis have propelled the search for solvent-tolerant organisms and enzymes. Non-aqueous reactions enhance the solubility of the hydrophobic substrates, shift the reaction equilibrium from hydrolysis to synthesis, and reduce side reactions, such as hydrolysis, polymerization, and racemization, besides reducing the risk of microbial contamination [1]. The biotransformation reactions in organic solvents must be productive, highly selective, and require only a few steps to synthesize the desired product [2,3]. However, the most significant problem of biocatalysis in organic solvents is the non-native nature of organic solvents for enzymatic functions [4,5]. Enzymes are folded according to their function in the aqueous solutions, while their surrounding residues commonly interact with water molecules. Thus, non-aqueous biocatalysis encounters several significant challenges for the large-scale applications, such as high cost of the enzyme, protein fragility, and exceedingly low enzyme activity [6]. The effects of organic solvents on enzymes are mainly manifested in five ways; conformational changes within enzymes, loss of enzyme-bound water essential for activity, competitive inhibition by organic solvent molecules, changes in substrate solubility, and stabilization of charge transition states. Compared to non-polar solvents, polar organic solvents penetrate enzymes more profoundly and can disrupt secondary and tertiary structures [7]. Besides, polar solvents can easily strip off essential water molecules, adversely affecting the structure and function of the protein.

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The water activity (aw), indicative of water content around the enzyme molecules, significantly affects catalytic activity in the organic solvent [4,8,9]. Moreover, the catalytic reaction shifts towards synthesis in organic solvents in one or two-phase systems. As per the log *P* values, the organic solvents are classified as polar or non-polar. The polar organic solvents are identified with a log P value of 2, while the non-polar solvents have log P values >4 [10,11]. However, this constraint can be addressed by several strategies, such as immobilization, chemical modification, and protein engineering [12]. Alternatively, the naturally evolved solvent tolerant enzymes can be explored for the wider applications in non-aqueous biocatalysis. It is preferred to protect and patent any innovative idea. A recent report suggests that China, India, and Brazil are the leaders in immobilization and patenting of lipases [13]. The solvent-stable lipase variants with improved thermal, pH, and solvent stabilities were patented and approved by the European Patent office, EP1590452B1 [14]. Similarly, a process to stabilize lipases was also granted US patent (publication number 20110287994), where the factors, such as preferred ratio of acid to amines, non-ionic interactions and solvents usage were optimized to stabilize the lipases for efficient use as an antimicrobial enzyme [15]. In this review, the solvent tolerant enzymes of the extremophilic microorganisms, their adaptive features, and strategies to improve catalysis and stability are illustrated with the updated information.

2. Historical developments

The chemical basis of the interactions of proteins with organic solvents was first investigated and reported long back [16]. In an aqueous solution, the structure of an enzyme gets denatured even by methanol and ethanol [17]. The protein β-lactoglobulin A forms complexes with toluene, α,α,α-trifluorotoluene and hexafluorobenzene. Equilibrium solubility studies suggest the two binding strategies. Stronger covalent bonds create a single localized hydrophobic region within the protein monomer, while multiple weaker bonds are established elsewhere in the protein to ensure a stable configuration. Strong binding sites bind two molecules of toluene or α,α,α-trifluorotoluene, or one molecule of hexafluorobenzene. [18]. Enzymes with higher degrees of cross-linked disulphide bonds are relatively less susceptible under solvent stress [19]. Non-aqueous solvents reduce the hydrogen exchange rate of accessible protons. However, high solvent concentrations change the equilibrium concentration of accessible protons and increase the hydrogen exchange rate [20]. Phosphatases have been reported to catalyze the formation of phosphomonoesters, the reverse reaction of inorganic phosphates at high concentrations in the solvent with the corresponding alcohols [21]. The glycosidase catalysis can be considered among the earlier known transfer reactions [22]. At low water activity, the equilibrium of the reaction shifts from hydrolysis to condensation. For instance, the peptide bonds are created by proteinases in non-aqueous solvents, and the reaction can be highly specific, as the resynthesis of single hydrolysed bond in ribonuclease S [23]. The immobilization of solvent-stable enzymes was earlier reported by Mosbach et al. [24]. The non-polar enzyme substrates with low water solubility are usually utilized efficiently by the enzymes located at the oil/water interface in a biphasic system. Phospholipases, laccase, and 3-hydroxysteroid dehydrogenase are examples of such interface oriented enzymes [25]. Besides regular substrate binding sites, certain enzymes contain super substrate binding sites that control the attachment and orientation of the enzyme in the matrix (micelle, membrane) [26]. Various enzyme engineering strategies, such as medium and biocatalyst engineering, to improve the performance of the solvent-stable enzymes are reported earlier [27]. Polyphenol oxidase was immobilized on chitin using non-covalent interactions [28], while nanoparticles [29] and carbon nanotubes [30] were employed to immobilize *Candida antarctica* lipase B for efficient performance. In recent years, newer approaches such as, ionic liquids (ILs) and deep eutectic solvents (DESs) are being explored at commercial scale. Deep eutectic solvents (DESs), sometimes known as "green solvents," have shown promise as an alternative to ionic liquids and traditional organic solvents (ILs). DESs are created when two or more components interact differently via H-bonds. MClx, including $FeCl_2$, $AlCl_3$, $ZnCl_2$, $CuCl_2$, AgCl, and others, and quaternary ammonium salts create type I DESs; metal chloride hydrates and quaternary ammonium salts form type II DESs; choline chlorides and various kinds of HBDs form type III DESs; and type IV DESs are formed by salts of transition metals and urea. Low vapor pressure, good substrate solubility, and thermal stability are among the DESs' inherent qualities that support their commercial viability [31]. As a recent approach, the deep eutectic solvents (DESs) are emerging alternates to the organic solvents, where the amount of hydroxyl and the length of the carbon chain represent advantageous features for the activities of β-galactosidase and *Candida antarctica* lipase B [32].

3. Microbial adaptations for the solvent stress

It is important to understand the adaptations at the physiological and molecular levels in various microorganisms against different stresses. Solvent stress, in general, is lethal to microorganisms. However, certain microorganisms survive and grow at high concentrations of solvents. The cell membrane is the immediate target for the solvents (Fig. 1). The binding of the solvents to the cell membrane leads to cell lysis and cell death [33]. The halophilic microorganisms, such as *Halomonas* sp., *Haloarcula vallismortis*, *Haloarcula* species OHF-1 [34], *Haloarcula* species OHF-2 [35], *Haloarcula argentinensis* [35], *Pseudomonas putida strain IH-2000* [36], *Halococcus*, *Natronococcus species*, *Halogeometricum borinquense JCM 10706T*, *Halorubrum saccharovorum JCM 8865T*, *Halobacterium salinarum*, *JCM 8978T*, *Halococcus morrhuae JCM 8876T*, *Haloferax volcanii JCM 8879T*, *Natrialba asiatica JCM 9576T*, *Natrinemapelli rubrum JCM 10476T*, *Halomicrobium mukohataei JCM 9738T* [34], *Rhodococcus* species [37], have been investigated for their solvent tolerance [34]. Several mechanisms for solvent tolerance in bacteria have been proposed, including (a) changes in the composition of membrane fatty acids and phospholipid head groups, (b) the formation of vesicles loaded with toxic compounds, and (c) energy-dependent active efflux pumps discovered in the resistance-nodulation-cell division (RND) family, which export toxic organic solvents into the external medium (Fig. 1). Among these mechanisms, changes in the phospholipid profile and solvent extrusion have received much attention [38]. The most significant change in phospholipids is an increase in membrane melting temperature caused by rapid cis-to-trans isomerization of unsaturated fatty acids and modifications to phospholipid head groups [39]. Cell aggregation and biofilm formation in *R. erythropolis* DCL14 is apparently due to the increased cell surface hydrophobicity in the presence of organic solvents [40]. The bacterial aggregation was complemented with the up-regulation of the stress-associated genes, namely *sigF3* and *prmA*. Desiccation, heat, and osmotic stress induce *SigF3* to encode a stress associated sigma factors, whereas *prmA*, a constituent of the propane monooxygenase operon usually gets up-regulated during the solvent exposure and starvation [41].

4. Solvent stable enzymes from extremophiles

The solvent-tolerant enzymes possess multiple potential for various commercial applications and thus attract considerable attention worldwide. In general, the solvent-tolerant enzymes are purified using salt precipitation method or molecular cut off filters, followed by suitable column chromatography techniques, such as ion exchange chromatography and/or size exclusion chromatography and/or hydrophobic interaction chromatography. The selection of the method depends upon optimum fold purification and percentage enzyme yield. Certain key solvent-tolerant enzymes of the extremophilic microorganisms are discussed.

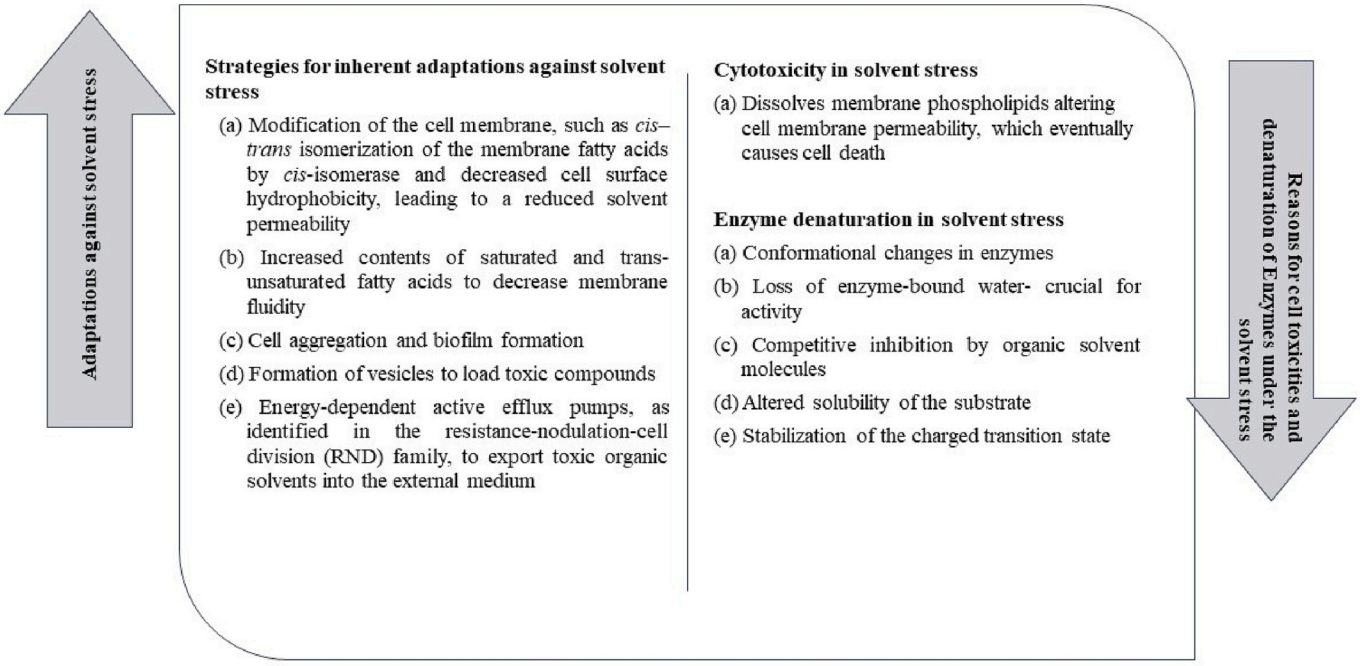

Fig. 1. Possible adaptations inherently exhibited by the solvent tolerant microorganisms at cellular and molecular level.

4.1. Proteases

Proteases have focused attention worldwide due to their versatile applications in varied fields such as pharmaceuticals, clinical synthesis, food production and processing, fermentation, and organic synthesis. Proteases, approximately 60 % of the total sale of enzymes, are the significant group with numerous industrial applications. In addition, the proteases are indispensable biocatalysts with potential applications in detergent, textile, food, meat tenderization, pharmaceuticals, and cleaning of the ultrafiltration membrane. However, proteases, in general, display reduced activities in organic solvents because of partial denaturation, reduced flexibility, and limited diffusion of the substrates.

Specific proteases possess significant stability under varied non-aqueous conditions. The solvent-tolerant alkaline proteases are investigated from the haloalkaliphilic bacteria, actinobacteria, and archaea of the various saline habitats of coastal Gujarat, India [42–45]. A protease from the haloalkaliphilic archeon, *Natrialba magadii* requires a high salt concentration (1.5 M NaCl) to exhibit stability in the organic solvents [46]. An alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1 was investigated for organic solvent tolerance [47]. A protease of the organic solvent-tolerant *Pseudomonas aeruginosa* PST-1 possesses specificity and conformational stability in methanol [48]. The conformational transitions of polyamino acids formed in methanol were closely related to the secondary structure of the enzyme.

A protease from an organic solvent-tolerant *Bacillus cereus* WQ9-2 was purified and characterized for its biochemical properties with particular reference to solvent stability [49]. A recombinant elastase (a metalloprotease) displayed stability in the hydrophilic organic solvents; DMSO, methanol, ethanol, and 1-propano l [50]. The recombinant enzyme was stable in 50 % methanol, probably due to the alteration in the secondary structures. Another alkaline protease from a marine *Bacillus tequilensis* P15 was significantly stable over a wide range of organic solvents of log P ranging from 8.8 to 1.249 [51]. The protease also displayed high compatibility for detergent additives, dehairing of the hide, and gelatin hydrolysis from used photographic film to recover silver. The crystal structure elucidated the protein-solvent interaction of a protease of *Pseudomonas aeruginosa* strain K [52]. The catalysis and stability of a native, its recombinant counterpart, and a metagenomic alkaline protease is recently reported as a function of various organic solvents under the influence of pH, temperature, and salt concentrations [53]. Among the three forms of proteases, the metagenomically derived protease was relatively more sensitive against solvents.

4.2. Amylases

Amylases, with 25–33 % of the world market of industrial enzymes, are significant due to their broad spectrum of applications in starch saccharification, food, textile, brewing, and baking. An organic solvent-tolerant α- amylase from *Haloarcula* sp. strain S-1 was effective in the bioremediation of carbohydrate-polluted sale marshes and solvent-contaminated industrial wastewaters [54]. Further, an organic solvent tolerant α-amylase from a moderately halophilic *Nesterenkonia* sp. strain F was purified and characterized. The enzyme was highly active over a wide range of NaCl concentrations with stability in methanol, ethanol, and DMSO [55]. Thermally stable amylases with significant catalysis and stability in organic solvents have been described from the thermophilic and haloalkaliphiic bacteria and actinobacteria [44,56–58]. The catalysis of an amylase of haloalkaliphilic bacteria under the influence of organic solvent was investigated as a function of pH, temperature, and salt concentrations [59].

4.3. Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are hydrolases that catalyze the hydrolysis of triaclyglycerols into fatty acid, partial acylglycerols, and glycerol at oil–water interfaces. Lipases catalyze reactions such as esterification, transesterification, stereospecific hydrolysis of the racemic esters, and organic synthesis under a water-restricted environment [60,61]. For the stability of the lipase, polar (water-miscible) solvents cause destabilization as compared to their non-polar counterparts [62]. A recently described salt- and solvent-tolerant alkaline lipase of *Bacillus* sp. DM9K3 was highly sensitive to hydrophilic organic solvents, ethanol, and acetone [63]. The adverse effect on lipase was arguably because the hydrophilic organic solvents removed the water molecules from the enzyme surface [64]. Further, a cold-active lipase from a psychrotolerant strain, *Pseudomonas pallerioniana* GBPI-508 showed decreased stability in the organic solvents of low polarity [65]. More than 75 % of the residual activity was apparent

with methanol, ethanol and acetone, while the activity was further reduced in other solvents. For instance, the residual activity was reduced to 24 % in toluene [65].

Further, a positive effect of the hydrophobic solvents on lipase activity is reported [62]. An alkaline lipase from *Streptomyces* sp. CS268 is stable in the solvents of log *P* values equal to or greater than −0.23. More importantly, benzene (log P 2) and octane (log P 4.9) significantly enhanced the lipase activity [62], a trend that contradicts the findings of lipase from *Aneurinibacillus thermoaerophilus* [66]. Among the tested organic solvents, toluene (log P 2.5) enhanced the lipase activity by 2 folds. Further, a marginal reduction in the lipase activity was evident in DMSO (log P −1.35) as opposed to a drastic decrease in the activity by 90% in acetonitrile (log P −0.34) [66].

A salt-tolerant lipase of *Marinobacter lipolyticus* SM19 functions in hydrophilic and hydrophobic solvents [67]. Methanol enhanced the activity to 120 %. A similar trend was observed with halo- and thermostolerant lipase from *Bacillus* sp., where the enzymes retained above 70 % of its activity in the polar solvents of log $P \leq 2$ such as acetone, butanol, benzene, acetonitrile, DMSO, ethyl acetate and ethanol at concentrations as high as 75 % for 7 days [67]. A lipase from *Alkalibacillus salilacus* was considerably stable in hydrophobic organic solvents even at 50 % *v*/v concentration [68]. Interestingly, alkanes, except dodecane, enhanced the lipase activity in cyclohexane up to 190 % [68]. Halophilic microorganisms generally produce enzymes as part of their adaptation strategies to high salt conditions. Since salt reduces water activity, the enzymes from the halophiles are considered valuable biocatalysts in aqueous–organic media [42,69].

An extracellular salt-tolerant, cold active and organic solvent stable lipase was purified and investigated from *Bacillus licheniformis* KM12 [70]. The KM12 lipase was highly stable in different organic solvents for many days, especially in polar organic solvents. The lipase activity was enhanced by 25 % (*v*/v) in the non-polar hydrophobic solvents (log $P >$ 2.0), such as n-hexane, n-heptane, and toluene; however, the organic solvents at 50 % (v/v) reduced the activity. The enzyme was also stable in polar organic solvents; DMSO, acetonitrile, isopropanol, methanol, ethanol, and glycerol in the concentration range of 25 %-50 % (*v*/v) [70]. An organic solvent-tolerant and cold-adapted lipase from *Psychrobacter* sp. ZY124 from Arctic habitat has been described [71]. This report demonstrates the enhancement of the activity in the hydrophobic organic solvents (log $P \geq 3$). Notably, the lipase activity was enhanced by 2 folds even at solvent concentrations as high as 70 % (*v*/v). While most lipases are adversely affected by hydrophilic solvents, the Lipase ZC12 was highly tolerant. The ZC12 Lipase was stable and maintained high activity in 10–50 % of the hydrophilic organic solvents [71]. A similar trend was reported in a cold-active lipase (SML) from *Stenotrophomonas maltophilia* CGMCC 4254 SML, which retained 81–94 % of its original activity in 20 % (v/v) of the organic solvents of low polarities (log $P < 0$) at 30 °C for 24 h [72]. The lipase powder didn't form clumps even in the neat solvent after incubation for 7 days. The residual activity of SML was reduced by 50 % in 100 % methanol, acetone, and acetonitrile increased.

A thermally stable organic solvent-tolerant lipase from *Acinetobacter baylyi* was highly capable of functioning in many organic solvents, especially benzene and isoamyl alcohol, with the inhibition by decane, hexane, acetonitrile, and short-chain alcohols [73]. The findings corroborate that the hydrophobic solvents of high log *P* values cause hindrance to the efficient interaction between the enzymes and substrates. A thermostable lipase of *Aneurinibacillus thermoaerophilus* strain HZ was highly stable in water-miscible solvents of low log P values [66].

Further, an alkalitolerant and thermostable lipase from a thermophilic *Anoxybacillus flavithermus* showed no significant correlation between the stability of HBB 134 lipase and the solubility of the organic solvents in water [74]. The high thermal stability of the enzymes is usually positively correlated with their strength in organic solvents. Thermostable lipases are easily inactivated in water-miscible solvents than in water-immiscible solvents [1].

4.4. Esterase

Esterases (EC. 3.1.1.1) hydrolyze the ester bonds in short-chain aliphatic or aromatic esters and are inducible. Structurally, they belong to the members of the serine hydrolase families. They possess a consensus amino acid sequence (Gly – X – Ser – X – Gly) with an active catalytic triad (Ser – Asp/Glu – His) and the embedded nucleophilic serine residue in the active site [71]. Unlike lipases, esterases lack interfacial activation. They not only hydrolyze the ester bonds, but are also involved in the acidolysis, alcoholysis, esterification, and transesterification reactions. Due to these properties, esterases are significant in the food, cosmetic, pharmaceutical, chemical, and detergent industries [75]. Many novel microbial salt-tolerant esterases have been investigated and functionally characterized in recent decades. The majority of these microbial sources are from marine and soil environments [76–81].

The hydrophilic and hydrophobic solvents significantly affect the esterase from thermo- and halotolerant *Bacillus cereus* strain AGP-03 [82]. The AGP-03 esterase retains 70 % activity with high stability for 7 days in the presence of these solvents [82]. However, the hydrophilic solvents inhibit the esterase activity. The enzyme activity is adversely affected by the hydrophilic organic solvents, ethanol, methanol, butanol, acetone, and acetonitrile. A cold-active esterase from marine bacteria *Alcanivorax dieselolei* B-5(T) was highly stable in isopropanol, acetone, and N-butyl alcohol at a high concentration of 70 % (*v*/v). With regard to other solvents, such as methanol, DMSO, and ethanol, the esterase was marginally inhibited at low concentrations of the solvent. However, the activity was severely reduced at the solvent concentrations of 50–70 % (v/v) [83].

An extracellular esterase from a halotolerant, *Salimicrobium* sp. LY19 is considerably stable in hydrophobic organic solvents, with the retention of above 80 % of the activity in glycerol, DMSO, benzene, n-hexane or isooctane. Interestingly, isooctane even acts as an inducer for the esterase. However, when exposed to hydrophilic solvents, acetonitrile, ethanol, and acetone, the esterase activity and stability were drastically reduced [84]. This trend corresponds to a cold-adapted, alkalistable, and highly salt-tolerant esterase from *Bacillus licheniformis*, where the activity was enhanced by n-hexane, n-heptane, isooctane, n-dodecane, n-tetradecane and n-hexadecane at 30 % (*v*/v). On the contrary, the Est700 esterase was inhibited by most polar organic solvents (log $P < 2$), except isopropanol [71].

More recently, miscible organic solvents, methanol, ethanol, acetone, DMSO, propan-2-ol, acetonitrile, and DMF were reported to adversely affect the esterase of *Geobacillus thermodenitrificans* NG80-2 [85]. Among the immiscible solvents, chloroform and n-butanol adversely affect the esterase; while, n-hexane at 30 % concentration activates the EstGtA3 [85]. A novel cold-adapted and highly salt-tolerant esterase from *Alkalibacterium* species SL3 was found sensitive against all tested solvents. While it was strongly inhibited by butanol, isobutanol, isoamyl, acetone and chloroform at 20 % (*v*/v), methanol, ethanol, propanol, n-hexane, glycerol, and acetonitrile marginally affected the enzyme [86].

On the contrary, an esterase from a thermoalkaliphilic halotolerant *Rhodococcus* sp. LKE-028 was stable in many water-miscible organic solvents; DMSO, benzene, toluene, methanol, ethyl alcohol, acetone, isoamyl alcohol. In DMSO and benzene, LKE-028 esterase retained over 100 % relative activities even after 10 days [87]. Further, another novel cold-active and salt-tolerant esterase from *Zunongwangia profunda* exhibited good stability against DMSO, ethylene glycol, ethanol, and isopropanol. However, methanol, acetonitrile, and n-propanol at 50 % (*v*/v) significantly inhibited the activity [88].

However, certain studies suggest that there is no correlation between the esterase activity and the polarity of the additives [89]. Biochemical and structural analysis of the cold-active and salt-tolerant esterase from a marine bacterium, *Thalassospira* sp. revealed that hexane, toluene, and benzene at 10–30 % promoted activity. The polar organic solvents,

methanol, acetonitrile, ethanol, and acetone at above 10 % concentrations, inactivated the activity. On the other hand, the enzyme was activated by DMSO, glycerol, and diethyl ether. This finding corresponds to the earlier conclusions of the esterase stability in organic solvents [90,91]. A comparative summary on various solvent-tolerant enzymes is provided in Table 1.

5. Protein engineering approaches to improve solvent stability

Due to the advancements in molecular biology and elucidation of molecular structures, the understanding of the structure and function of the enzymes has increased manifolds (Fig. 2). The genetic information, in conjunction with the prospects of protein engineering, has improved the catalytic properties and stability of many enzymes (Fig. 3), particularly the catalysis by the lipases under the non-aqueous conditions [61,108,109].

5.1. Directed evolution

Directed evolution has emerged as a powerful approach to enzymatic features for various unique applications, such as the production of chemicals and pharmaceuticals. The Nobel Prize in Chemistry for 2018 was awarded to Francis Arnold, one of the key scientists to evolve this concept. Several studies in recent years on amino acid shuffling through directed evolution have yielded significant improvements in the catalysis and stability in organic solvents [110–113]. For instance, it has been established that the hydrogen bonds formed by polar or charged amino acids in loop regions are crucial for enzyme stability in hydrophilic organic solvents [4,114,115]. The evolved variant (DhaA80) of the dehalogenase, DhaA, catalyzing four hydrophobic/polar substitutions, has twice as much resistance against the polar organic solvent dimethyl sulfoxide (DMSO) [114]. Further, the resistance against DOX, TFE, and DMSO is improved by the surface-involved substitutions to charged and polar amino acids. Such a surface substitution might advocate that introducing charged residues could lead to the formation of new salt bridges that stabilize the enzyme in the presence of organic co-solvents [4]. Thus, directed evolution is a proven powerful approach to enhancing the functionality and stability of the enzymes in organic solvents.

5.2. Site saturation mutagenesis (SSM)

Site saturation mutagenesis (SSM), is a random mutagenesis approach of protein engineering through which a single or set of codons is substituted [116]. It is achieved by site-directed mutagenesis with a randomized codon in the primers or by artificial gene synthesis through a mixture of synthetic nucleotides [117].

The Site Saturation Mutagenesis (SSM) libraries of a gene, with a natural diversity at each amino acid position, provide an effective strategy for the stability landscape and thus highlighting the sequence-to-function relationships [118,119]. Furthermore, exploring different interaction possibilities appears to be a prerequisite to discover general design principles [120] addressing the interactions between the enzyme and organic solvents [121,122]. A study explicated how the B-Fit method improved solvent stability of *Bacillus subtilis* lipase. Accordingly, it involved saturation mutagenesis iteratively at sites displaying high B-factors available from X-ray data. The B-Fit method based on ISM advocated that the residues with high B-factors had high flexibility, and that appropriate mutations at those precise sites enhanced rigidity and stability. In a follow up studies involving the molecular dynamics revealed that the altered residues on the surface of the mutant enzymes, increased hydrogen bonds and salt bridges might have enhanced stability of at elevated temperatures. Moreover, it altered aggregation tendency and interactions with other biomolecules, which might have been the reason for improved solvent stability too [123]. The aqueous ionic liquids are preferred solvents advocating green industrial processes in the present day biotechnology based industries but it generally leads to reduction in the enzyme activity. A recent study used the concept to improve stabilities of proteins in presence of aqueous ionic liquids. The in vitro and in silico approaches were employed to predict preferred substitution sites and surface charge modifications to improve enzyme resistance towards aqueous ionic liquids by creating site saturation mutagenesis library of *Bacillus subtilis* Lipase A (BsLipA). The researchers reported almost 4 folds improvement in the gain-in-precision values by combining experimental properties with the rigidity theory which suggested the possible predictions of structural weak spots [124].

Some earlier efforts with a hyperthermophilic bacteria, *Thermotoga maritima*, a moderate thermophile, *Agrobacterium tumefaciens*, and a mesophilic bacteria *Cellvibriogilvus* have successfully improved thermal stability, kinetic properties, and solvent tolerance of the cellulose utilizing enzymes using protein engineering through gene shuffling and chimeric genes [125–128].

Protein engineering in the present context includes three significant approaches: directed evolution, rational design, and semi-rational approach. The selection of the parameters depends upon the availability of the structural details of the molecules and the trends observed during the high throughput screening strategies [129,130]. For the rational design of the enzyme, in-depth knowledge about the structure and function relationship is inevitable. The structural details of the solvent tolerant enzymes have been generally elucidated by the X-ray crystallography. For instance, increasing rigidity of the enzyme by introducing salt bridges and disulphide bonds may improve stability in solvents [131].

On the contrary, the directed evolution approach does not require detailed structural information about the enzymes. The directed evolution involves two methods, DNA shuffling and error-prone polymerase chain reaction with the primary objective of creating a pool of the genetic heterogeneity of the molecule. While the semi-rational approach involves the combination of both, i.e., directed evolution and rational design, wherein the site-directed mutagenesis is conducted based on computational, algorithmic predictions [132]. In general, the mutations may either increase and stabilize the interactions or reduce the flexibility of the surface residues, thus changing the enzyme surface charge.

5.3. Enhanced stabilizing interactions

The enhanced stabilizing interactions are considered as one of the key strategies to improve the performance of an enzyme. The approach aims to avoid any changes in the amino acid residues, which are either responsible for stabilizing the interactions or involved in forming a secondary structure or enzyme active site [133]. In comparison, it advocates imparting mutations in the surface amino acid residues, which stabilizes the interactions by balancing its affinity for water and solvents. Recently, a recombinant solvent-tolerant elastase was crystallized to elucidate its structure to understand the inherent solvent stability. Based on the molecular simulation, the amino acid residues located on the surface area and the disulfide bridge in Cys-30 to Cys-58 were related to the stability of the enzyme in organic solvents [52]. Similarly, the solvent tolerance of a metalloprotease was improved by directed evolution [134]. The mutants T46Y, H224 F, and H224Y of the metalloprotease enhanced the half-life of the enzyme by 2–3 folds in acetonitrile and acetone, compared to its native enzyme. The T46Y/H224 F and T46Y/H224Y mutants in combination significantly enhanced the stability and catalysis due to the extensive non-covalent bonding network, leading to a highly compact structure.

5.4. Surface charge and polarity

The surface charges and polarity affect the catalysis and stability in the non-aqueous environment [135–137]. Specifically, the solvents of low polarity denature the hydrophobic domains of the enzyme due to

Table 1
A comparative profile of the solvent tolerant enzymes from Extremophilic Bacteria and Archaea.

Enzyme (organisms or source)	Molecular weight (kDa)	Temperature optima (°C)	pH optima	Solvent stability	Reference
Protease (*Natrialba magadii*)	–	–	8	Stable in 15 % (*v*/v) glycerol, DMSO, DMF	[46]
Protease (*Streptomyces clavuligerus* Mit-1)	–	–	–	Enhanced production, efficient catalysis and stability in xylene, acetone and butanol	[47]
Protease (*Pseudomonas aeruginosa* PST-01)	–	–	–	Solvent tolerant strain; Conformational stability of protease in methanol	[48]
Protease (*Bacillus cereus* WQ9-2)	37	50	8	Solvent stability; Inhibition by EDTA and 1,10-phenanthroline; Peptide synthesis in 50 % dimethylsulfoxide	[49]
Elastase (Recombinant enzyme expressed using *E. coli* KRX/pCon2 (3))	65	40	6	Stable in Methanol; the CD Spectroscopy revealed a reduction in the α-helix content and an increase in β-sheets in the presence of solvents	[50]
Protease (*Bacillus tequilensis* P15)	–	50	8	Stable up to 75 % (v/v) n-hexadecane, 1-dodecanol, iso-octane, cyclohexane, xylene, toluene, benzene, chloroform and DCM	[51]
Amylase (*Haloarcula* sp. strain S-1)	70	50	7	Stable in chloroform; Catalytic inhibition by ethyl alcohol and acetone	[54]
Amylase (*Nesterenkonia* sp. strain F)	57	45	6.5	Stable in benzene, chloroform, toluene, and cyclohexane	[55]
Amylase (Thermophilic *Bacillus* species TSSC-3)	25	80	7	Stable in toluene, benzene, diethyl ether, and acetone; Stable secondary structure probed by the CD Spectroscopy	[56]
Amylase (thermophilic actinobacterium, *Laceyella sacchari* TSI-2)	31	70	7	Stable in the range of temperatures, pH, surfactants, and solvents	[57]
Amylase (Halophilic *Bacillus agaradhaerens* Mi-10-6)	–	60	10	Solvent-tolerant organism; Amylase stability in 30 % (*v*/v) dodecane, decane, heptane, n-hexane, methanol, and propanol	[59]
Lipase (Alkaliphilic *Bacillus* sp. DM9K3)	35	50	9	Stable in cyclooctane and benzene	[63]
Lipase (Alkaliphilic *Pseudomonas* sp. DMVR46)	32	37	8.5	Enzyme stability in isooctane, cyclohexane and n-hexane; Enzyme used for the production of flavor ester- pentyl valerate in the presence of organic solvents	[65]
Lipase (Psychrotolerant *Pseudomonas palleroniana*)	Two active bands of 50 and 54	40	Constant between 7 and 12	Cold adaptive; Solvent stable; except 25 % (*v*/v) toluene and dichloromethane	[66]
Lipase (*Aneurinibacillus thermoaerophilus* strain HZ)	50	65	7	Enhanced catalysis in DMSO, methanol, n-tetradecane and n-hexadecane	[67]
Lipase (*Marinobacter lipolyticus* SM19)	45.3	80	7	Stable in 30 % (*v*/v) DMSO, *N*,*N*-dimethyl formamide, methanol, acetonitrile, ethanol, diethyl ether, acetone, 1-propanol, 2-propanol; Used to produce Eicosapentaenoic Acid (EPA)	[68]
Lipase (*Alkalibacillus salilacus*)	–	40	8	Stable in hexadecane, dodecane, nonane, decanol, heptane, hexane, cyclohexane, octanol, chloroform, cyclohexanol, hexanone, butanol, ethanol, acetone and acetonitrile	[69]
Lipase (Halophilic *Bacillus licheniformis* KM12)	33	35	8	Stable in N-hexane, N-heptane, toluene, DEE, 2-propanol, ethanol, methanol, glycerol, DMSO	[70]
Esterase (Alkaliphilic *Bacillus licheniformis*)	25	30	8	Stable in 30 % (*v*/v) glycerol, DMSO, dimethylformamide, isopropanol, methanol, acetonitrile, ethanol, tetrahydrofuran, n-butanol, isoamyl alcohol, n-amyl alcohol, n-propanol, benzene, chloroform, toluene, xylene, n-hexane, n-heptane, isooctane, n-dodecane, n-tetradecane, and n-hexadecane	[71]
Esterase (Halophilic *Bacillus* species)	35	45	8	Improved Catalysis and stability in DMSO	[76]
Esterase (Metagenomic clone)	–	50	7	Function-based metagenomics; Stable in 30 % dimethylsulfoxide, ρ-xylene, hexane, heptane, and octane	[77]
Esterase (Halophilic *Janibacter* sp. R02)	–	80	9	Thermal and salt stability; first member of a new lipase family (Family XVII)	[79]
Esterase (Halotolerant thermoalkaliphilic *Erythrobacter seohaensis* SW-135)	29.5	60	10.5	Stable in 5–15 % (*v*/v) acetone, acetonitrile, ethanol, *N*, *N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), glycerol, isopropanol, and methanol	[80]
Esterase (Halotolerant *Enterobacter cloacae*)	26	40	9	Stable in 10–30 % (*v*/v) acetone, methanol, dimethyl sulphoxide (DMSO), isopropanol, ethanol and ethylene glycol	[81]
Esterase (Thermohalo tolerant *Bacillus cereus* AGP-03)	41	55	8.5	Salt-enzyme; Improved catalysis and stability in benzene	[82]
Esterase (*Alcanivorax dieselolei* B-5 (T))	45.1	20	8.5	Cold adaptive; a distinct catalytic triad (Ser^{211}-Trp^{353}-Gln^{385}) and a motif (Gly^{209}-X-Ser^{211}-X-Gly^{213}); Stable in a range of solvents	[83]
Esterase (*Geobacillus thermodenitrificans* NG80–2)	–	60	8	Improved catalysis and stability in n-hexane	[82]
Esterase (*Alkalibacterium* sp. SL3)	24	30	8	Stable in methanol, ethanol and propanol	[85]
Esterase (Halophilic *Zunongwangia profunda*)	30	30	8	Stable in 50 % (v/v) in ethanol, isopropanol, DMSO, and ethylene glycol	[88]
Esterase (Marine bacterium *Thalassospira* sp.)	–	45	8.5	Cold adaptive enzyme; structurally-high methionine content and reduced hydrogen bonds and ion pairing	[89]
thermostable alkalophilic cellulase from *Bacillus vallismortis*	80	65	7	Enzyme activity was enhanced in the presence of organic solvents (30 %) n-dodecane, iso-octane, n-decane, xylene, toluene, n-haxane, n-butanol, and cyclohexane, after prolonged incubation	[92]
Aminopeptidase from the hyperthermophillic bacterium, *Aquifex aeolicus*	54	80	8–8.5	Highly resistant to organic solvents such as methanol, ethanol, tetrahydrofuran, dimethyl sulfoxide, acetone, acetonitrile, dimethyl formamide, 1-propanol, 2-propanol, and dioxane.	[93]
		80	4.6	High activity in ethanol, methanol, DMF, and DMSO	[94]

(*continued on next page*)

Table 1 (*continued*)

Enzyme (organisms or source)	Molecular weight (kDa)	Temperature optima (°C)	pH optima	Solvent stability	Reference
Thermostable, pH-stable, and organic solvent-tolerant laccase from *Bacillus pumilus* W3					
Halostable β-Endoglucanase from a Marine Bacterium *Photobacterium panuliri*		40	4	Remarkable stability in different organic solvents (50 %, *v/v*), and activity increased nearly 1.5-fold in presence of butanol, isopropanol, petroleum ether, benzene, acetone, and n-hexane.	[95]
Thermostable tannase from *Bacillus subtilis* PAB2		40	5	Polar and protic solvents like glycerol, isopropanol, ethanol, methanol and isoamyl alcohol were found to increase the enzyme activity	[96]
Thermostable glucoamylase from a halophilic isolate, Halolactibacillus sp.		70	8	High stability in the presence of hydrophobic organic solvents	[97]
Thermostable amylopullulanase from *Thermococcus kodakarensis*	125	>100	5.5	Enzyme is active in the presence 20 % and 50 % (v/v) ethanol and isopropanol	[98]
Psychrophilic phthalate esterase from *Sphingomonas glacialis*	32.67	40 °C	9	Tolerance to polar organic solvents, including alcohols and DMSO	[99]
Chitinase from *Paenibacillus timonensis*	70.16	80 °C	4.5	The stability shown by ChiA-Pt70 in the presence of DMF (195 %), chloroform (177 %), DMSO (162 %), n-hexane (155 %), cyclohexane (131 %), and iso-propanol (120 %)	[100]
Cellulase from *Bacillus subtilis*	51.4	45	8	Enhanced activity in presence of acetone, hexane, benzene, acetonitrile and isopropanol	[101]
Amylopullulanase from the haloarchaeon, *Halorubrum* sp. strain Ha25	-NA-	50	7.5	Enzyme was more stable in the presence of non-polar organic solvents than polar solvents	[102]
Laccase from *Bacillus licheniformis* ATCC 9945a	-NA-	90	5–8	Stable in presence of acetonitrile and methanol	[103]
Cholesterol oxidase from *Bacillus* species	55	40	7.5	Stable in methanol, isopropanol and Tween80	[104]
Chitinase, MtCh509, from *Microbulbifer thermotolerans*	60	55	5	Enhanced activity in presence of Benzene, DMSO, hexane, isoamyl alcohol, isopropyl alcohol, and toluene (10–20 %, *v/v*)	[105]
β-Glucosidase, OaBGL84, from *Olleya aquimaris*	84	40	6	Stability until 50 % (*v/v*) benzene, n-hexane, or toluene	[106]
Keratinase from *P. aerugenosa*	-NA-	40	8	Stable in various solvents with efficient feather degradation ability	[107]

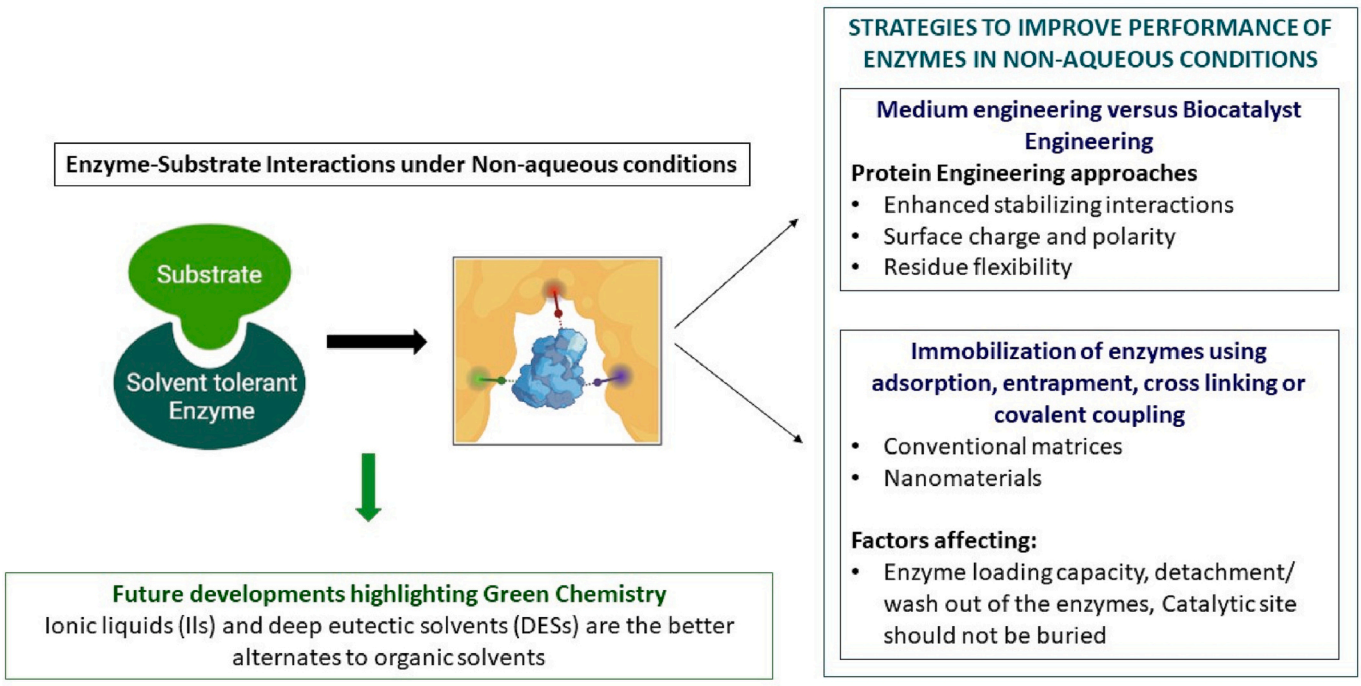

Fig. 2. Catalysis and stability of an enzyme in the organic solvents.

the increased penetration of the solvent, leading to enzyme inactivation [90]. On the contrary, the charged amino acids form the hydrated ion networks and maintain the stability of the enzyme preventing abrupt aggregation [137].

5.5. *Residue flexibility*

The stability and conformational integrity of an enzyme in solvent or other extreme conditions depend on the flexibility of the amino acid

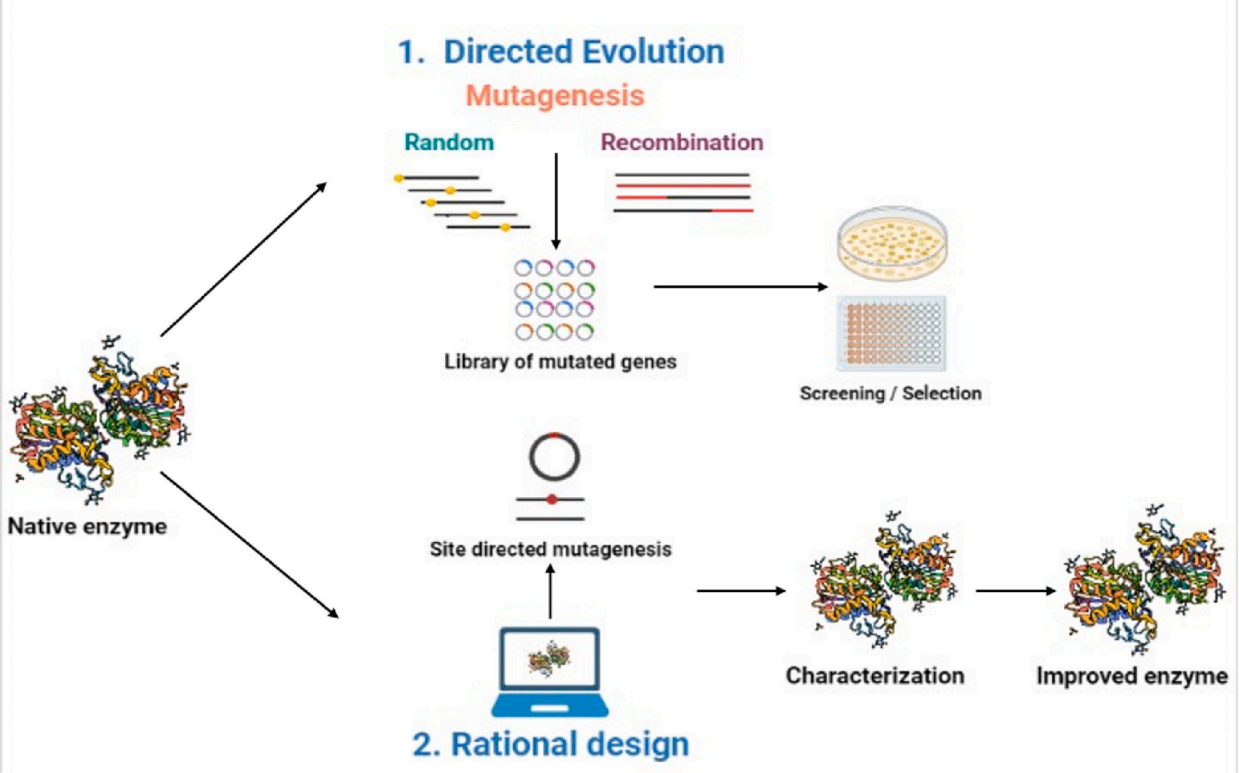

Fig. 3. Schematic diagram explaining protein engineering of solvent tolerant enzymes.

residues [138]. For instance, conformational rigidity appears as the major attribute for the solvent tolerance of lipases [139]. The increased intramolecular interactions and rigid confirmation preventing the entry of the solvents are the major reasons for solvent tolerance in lipases [140,141].

Besides, alterations in the dynamics of the enzymes in various solvents can be deduced by the molecular dynamic simulations. It involves analysis of the flexibility of each residue by Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) values. Notably, deviations in the initial structural attributes are represented by RMSD, whereas deviations from the average structure are represented by RMSF [142]. Molecular modelling was used to predict the putative active site and solvent tolerance of a haloarchaeal transaminase. The study represents new dimensions in the biocatalysis of extremophilic microorganisms [143].

6. Immobilization of the enzymes to improve stability and reusability

Applications of soluble enzymes are restricted due to the catalytic efficiency, substrate stability in solvents, high cost, and non-reusability [58,144,145]. Many industrial processes involve extensive use of solvents, and thus the enzymes should function and maintain stability under the non-aqueous conditions. However, since it is not always possible to obtain the microbial enzymes with enhanced solvent stability, immobilization of the solvent-stable enzymes may provide a viable alternative [130,146]. Immobilization of cells and enzymes is one of the most effective and economically feasible strategies for the biotechnological processes relevant to biotransformation, medical diagnostics, biodiesel, pharmaceutical science, and food industries [147–149]. In organic solvents, the immobilized enzymes are in contact with the substrates soluble in the aqueous phase but not with the molecules available in the organic phase [150]. Numerous attempts based on chemical modifications and protein engineering have improved the activity and stability of the enzymes in the organic solvents [151,152]. Mesoporous materials, metal oxides, carbon nanotubes, nanoparticles, graphene polymers, and hybrids are used for immobilization. Selecting the substrate and immobilization techniques are the most critical aspects of immobilization. The immobilization ultimately regulates the enzyme efficiency [153,154]. The immobilization protocols are broadly classified into two groups based on the nature of the interconnection between the enzyme and the immobilization matrix (Fig. 4). Among, the physical methods are hydroelectric interactions, van der Waals forces, affinity binding, hydrogen bonds, and mechanical incorporation within the support matrix [155]. While covalent bonding, such as amide, ether, carbamate, and thioether bonds [155], and crosslinking [156] are involved in chemical interactions.

6.1. Immobilization of the solvent-tolerant enzymes on conventional supports

The naturally occurring organic polymers; Ca-Alginate, Chitosan and chitin, Collagen, and DEAE Cellulose have been widely used to immobilize the solvent-tolerant enzymes for the production of the regioselective and enantioselective compounds suitable for biomedical applications [149,157]. Ca-alginate beads with alginate-polyacrylamide gels enhanced enzyme activity and reusability [158,159]. Moreover, DEAE-cellulose or modified cellulose has a longer storage capacity. Immobilization with ionic-cellulose film activated by gluteraldehyde provided huge formability and flexibility [160,161]. At the same time, collagen is a suitable support matrix for catalase immobilization with reusability for 26 cycles [162]. Chitosan appears most suitable for immobilization as chitosan-coated enzymes have a low leaching effect as compared to other support. Besides, the physical and ionic interactions of the chitosan with the solvent-tolerant enzymes lead to greater entrapment [163,164].

For solvent-tolerant enzymes, chitosan is a highly suitable matrix for immobilization. Immobilized proteases and α-amylases showed the highest yields, close to 90 %; however, for the lipase, the efficiency and stabilization were around 50 % compared to the native enzymes [165].

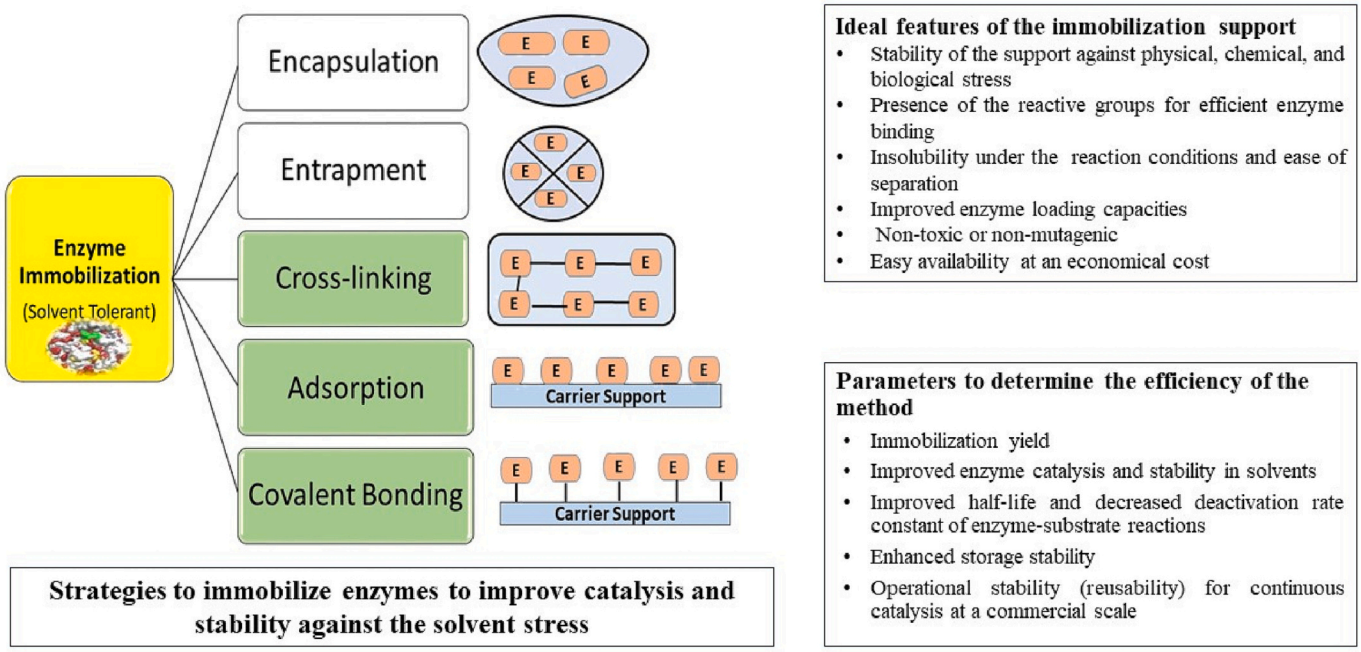

Fig. 4. Schematic diagram explaining various strategies to immobilize solvent stable enzymes.

Immobilization of a protease shifted the pH optima from 10.0 to 8.0 and temperature optima from 37 to 60 °C. For an α-amylase, the range of pH and temperature were 5.0–10.0 and 37–70 °C, respectively. While for lipase, the optimum pH shift was from 8.0 to 9.0 [166]. K_m and Vmax of the immobilized protease were 0.015 mg/mL and 11.1–35.7 mol/mL/min, and for the α-amylase, the corresponding values were 0.8 mg/mL and 2186 mol/mL/min [167]. Immobilizing lipase by noncovalent adsorption was useful in non-aqueous systems [168,169]. Lipase and protease were immobilized on the starch-chitin support via covalent binding for improved reusability, storage, and stabilities against temperatures, pHs and solvents. The immobilized enzymes were efficiently used, proving economic viability as a detergent additive for improvement [170].

The changes in the microenvironment of the gel matrix at various temperatures, leading to enhanced conformational flexibility, could be one of the reasons for the improved functionality [171,172]. More recently, organic solvent-tolerant cross-Linked protease aggregate (CLEAs) was investigated [173]. The protease retained significant activities with enhanced stability in 25 % (*V*/V) of acetonitrile, ethanol, and benzene. In a recent study, the lipases and peroxidases were immobilized on the disaccharides and polysaccharides decorated tannic acid functionalized magnetic cross-linked enzyme aggregates for improved stability and reusability. In this system, glutaraldehyde served as a cross-linker, while the amino groups of lysine, sulfhydryl groups of cysteine, phenolic groups of tyrosine, or imidazol group of histidine arguably played a key role in the enzyme immobilization [174]. The cross-linked lipases were reportedly immobilized over hyaluronic acid-coated magnetic nanoparticle functionalized graphene oxide composites (lipase-GO-MNPs-CLEAs) for improved performance under extreme conditions [175]. The cross-linked isocitrate dehydrogenase was immobilized on hyaluronic acid functionalized magnetite nanoparticles with bovine serum albumin as an additive (IDH/BSA/HA/MNPs-CLEAs). The immobilization was confirmed and structurally elucidated by XRD, SEM, and VSM [176].

6.2. *Immobilization of the solvent-tolerant extremozymes on nanomaterials*

Nanomaterials are excellent support to immobilize a range of catalysts to achieve continuous catalysis at an economically viable commercial level [177]. Different immobilization strategies yield variable outcomes. For instance, a comparative account on *Rhizomucor miehei* lipase immobilized on magnetite nanoparticles via absorption or covalent coupling (APTES or MPTS) was evaluated. It was revealed that the physical adsorption binding provided higher surface loading and low catalytic activity. On the other hand, the covalent coupling yielded good catalytic activity with poor surface loading [178]. A solvent tolerant esterase from *Pseudozyma* sp. NII 08165 was immobilized on APTES activated magnetite nanoparticles by cross-linking with glutaraldehyde. The immobilization of lipase improved its shelf-life and stability for effective applications in biotransformation reactions for the production of ethyl acetate and biodiesel [179]. Further, a solvent-tolerant lipase from extremophile immobilized on dialdehyde-coated magnetite nanoparticles led to improved physicochemical parameters [180]. Similarly, a lipase of *Thermomyces lanuginosus* immobilized on polydopamine functionalized magnetic nanoparticles displayed highly efficient loading capacity and immobilization yield [181]. An extremely cold-adapted lipase of *Candida antarctica* was immobilized on the graphene oxide-magnetite nanoparticles. The use of hyaluronic acid during the immobilization further improved the solvent stability and reusability of the enzyme [182]. A novel haloalkaliphilic lipase of *Alkalispirillium* species NM-R002 was successfully immobilized on floricil functionalized with glutaraldehyde leading to the binding efficiency and immobilization yield of 95 % and 97 %, respectively. The stability against salts, surfactants, temperatures, and various solvents was significantly improved in haloalkaliphilic lipase on immobilization for the esterification of levulinic acid with n-butanol [183]. In another study, the lipase was immobilized on the magnetite nanoparticles, with dialdehyde starch used as a cross-linker. The storage stability and reusability of lipase was enhanced with dialdehyde starch than gluraldehyde [180]. A lipase from *Candida rugosa* immobilized on epoxy-activated cloisite yielded improved stability against temperature, pH, and solvents. The system remained functional for 9 successive cycles for the esterification and transesterification process [184]. In yet another study, *Candida rugosa* lipase was immobilized on a heterofunctional support, tosylated cloisite, to efficiently produce biodiesel from the waste frying oil [185]. Similarly, the *Candida rugosa* lipase was also immobilized by the interfacial adsorption method using the two synthetic layered doubled hydroxides

for improved temperature and storage stability [186]. Lipases were also encapsulated in ZIF-8 by the one-pot facile self-assembly method for improved solvent stability for greater commercial avenues. The immobilization was further elucidated by FTIR, XRD and SEM studies. The immobilized lipase exhibited 250 % enhanced activity with almost 2 folds improved thermostability than the native enzyme. It was reused efficiently for 6 cycles to aid to its economic potentials [187]. More recently, acetylcholinesterase (AChE) was immobilized by covalent bonding on silica-coated magnetite nanoparticles to improve its solvent tolerance. The immobilized acetylcholine was used to detect carbamate pesticides in traces, even at 0.01 μM [188]. An alkaline protease from alkaliphilic *Bacillus* species NPST-AK15 was immobilized over hollow core-mesoporous shell silica (HCMSS) nanospheres leading to remarkable improvement in the solvent stability. An immobilized alkaliphilic protease was successfully used as a detergent supplement in the lab scale studies [189]. In another study, amino-functionalized silica-coated magnetic nanoparticles with core-shell structure were constructed to immobilize laccase for improved temperature and storage stabilities, and tolerance to organic solvents and metal ions. It was used repeatedly to degrade chlorophenols in the effluents of wastewater treatment plants [190]. In yet another instance, xylanase, and lichenase were immobilized on the peptide inspired magnetic nanocomposites with high immobilization yield, catalytic efficiency, and improved solvent and operational stability [191]. The hybrid matrices, such as chitosan-coated magnetite nanoparticles were used to immobilize *Candida antarctica* lipase with an immobilization yield of 84 %. The immobilized lipase catalyzed the reaction more efficiently under alkaline pH as compared to the soluble lipase [192]. The immobilization of β-glucosidase on SiO_2 and ZIF-8 revealed that Nano SiO_2 was comparatively a better option; however, the enzyme immobilized on ZIF-8 exhibited anti-protease attributes [193]. An amylase immobilized on cloisite 30B, and activated cloisite 30B with tosyl chloride and epichlorohydrin displayed improved stability at higher temperatures and alkaline pH [194]. The advanced strategies are also suitable for immobilizing more than one enzyme on a single nanomaterial to enhance application prospects. For instance, the α-amylase of *B. subtilis* and lipase of *C. rugosa* was adsorbed together on the surface of the modified Na-sepiolite. BET, XRD, and SEM probed the immobilization. The immobilized enzymes system was reused for 10 successive cycles [195]. The solvent-tolerant enzymes with special emphasis on their immobilization, and possible applications have been described in Table 2.

7. Applications of the solvent-stable enzymes

Solvent-tolerant bacteria and solvent-stable enzymes have unique applications in Biotechnology. For instance, solvent-tolerant bacteria can be effectively used to treat and manage solvent-polluted sites. A phosphotriesterase-loaded membrane was reported to degrade the paraoxon-ethyl pesticide in vegetative water coming from olive mill. The stability of the immobilized enzyme was enhanced 4 folds compared to the free enzyme [196]. The peroxidase-like artificial enzyme immobilized on Fe_3O_4-doped MnO_2 microspheres was used to detect glucose with high accuracy and sensitivity [197]. In another study, the cross-linked isocitrate dehydrogenase, immobilized on BSA-coated hyaluronic acid functionalized magnetite nanoparticles, was used to determine magnesium in the drinking water and other samples [176]. Further, solvent-stable enzymes have traditionally been used in bioconversion in a two-phase system [198]. The solvent stable lipases are used in food industries to improve fragrance and flavor. The solvent stable lipases are effective in the extraction and purification of the flavours esters from natural sources, a process which otherwise is highly tedious [199–201]. These unique prospects are also explored in pharmaceuticals to achieve the kinetic resolution of the compounds. Recently, a hybrid metal-organic framework, the zeolitic imidazole framework-8 (ZIF-8) was used to immobilize *Candida rugosa* lipase for enantioselective hydrolysis of racemic naproxen methyl ester [202]. The

Table 2
A comparative profile of the immobilized solvent-tolerant enzymes from Extremophilic Bacteria and Archaea.

Enzyme	Matrices used	Additional remarks	Reference
Lipase (Sigma-Aldrich, India)	carboxylic acid-modified silica nanoparticles	Production of mono and diglycerides production; Operational stability: 11 cycles	[168]
Lipase (Candida cylindracea)	Zeolite type Y	Immobilization efficiency: 33 %; hydrolysis of palm oil in a two-phase reaction system; Isooctane as organic medium and lecithin as surfactant	[169]
Elastase (Recombinant enzyme expressed using *E. coli* KRX/pCon2(3))	Cross-linked enzyme aggregates (CLEAs) in presence of glutaraldehyde	Improved solvent tolerance in 25 % (v/v) acetonitrile, ethanol, and benzene; Enhanced thermal and pH stability with better recovery	[173]
Esterase (*Pseudozyma* sp. NII 08165)	Glutaraldehyde linked 3-amino-propyl triethoxy silane coated magnetite nanoparticles	Biocatalysis of ethyl acetate synthesis and biodiesel production; Improved shelf life of the enzymes in a range of conditions	[179]
Lipase (Jiangsu Yiming Biological Co., Ltd., China)	Dialdehyde starch linked magnetite nanoparticles	Improved stability and reusability	[180]
Lipase (Thermomyces lanuginosus)	Magnetic nanoparticles coated with polydopamine	Enhanced stability in a range of solvents; elevated temperatures and extremes pH; improved enzyme loading efficiency	[181]
Lipase (Candida antarctica)	Glutaraldehyde cross-linked graphene oxide coated magnetite nanoparticles	Improved stability at the broad range of temperatures, pH and organic solvents; enhanced reusability	[182]
Lipase (*Alkalispirillum* sp. NM-ROO2)	Florisil functionalized with glutaraldehyde	Stability in a range of solvents and surfactants; used in the esterification of levulinic acid with alcohols; operational stability: 9 cycles	[183]
Acetylcholinesterase	Magnetic mesoporous silica	Enhanced solvent stability compared to free enzyme; used to detect pesticides with improved sensitivity	[188]
Alkaline protease (*Bacillus* sp. NPST-AK15)	Hollow core-mesoporous shell silica nanospheres	Improved stability in amyl alcohol, ethanol, benzene, acetone, isopropanol, chloroform, and butanol; efficiently used as a detergent additive; operational stability: 12 cycles	[189]
Laccase (Trametes versicolor)	Amino-functionalized	Improved stability against a broad	[190]

(*continued on next page*)

Table 2 (*continued*)

Enzyme	Matrices used	Additional remarks	Reference
	magnetite nanoparticles	range of temperatures, metal ions, and organic solvents; used in the degradation of chlorophenols in water and wastewater	

amylases were also immobilized over bovine serum albumin-coated ZIF-8 for improved reusability (20 cycles) and stability at higher temperatures and a broad pH range [203]. An α-amylase and a glucoamylase were simultaneously immobilized on a combi-metal organic framework for improved hydrolysis of corn, rice, wheat, and potato starch [204]. The amylase immobilized within ZIF-67 was proposed for continuous biotransformation (catalysis), biosensing, and biomedicine application [205]. Lipase regioselectivity is used to synthesize compounds that are difficult to make chemically [206]. Sugar esters that are non-ionic and biodegradable have a lot of potential in detergents, pharmaceuticals, and oral care products. The fatty acid ethyl ester (FAAE) from the babassu oil was produced using the *Rhizomucor miehei* lipase immobilized on the APTES-coated magnetite nanoparticles [207]. Polymers have recently received much attention due to their increased applications in the biomedical field, food packaging, and agricultural industries. However, because the disposal of these polymers is a critical environmental issue, the development of biodegradable polymers as an alternative to traditional plastics has occurred [208]. Such biodegradable polyesters are synthesized using solvent-stable lipases [209,210]. Besides, several laccase-based commercial products are used in the textile, food, and paper industry. These enzymes are frequently used in the textile industry for bleaching [211]. The solvent-tolerant lipases are also used in transesterification reactions to produce biodiesel [212–215]. An immobilized lipase on the tannic acid functionalized magnetite nanoparticles (TA-MNPs-lipase) and the cross-linked lipase aggregates immobilized with starch and tannic acid functionalized magnetite nanoparticles (TA-MNPs-CLEAs-starch) were efficiently used for the production of biodiesel [216].

8. Conclusion and prospects

The non-aqueous enzymology is highly significant and an emerging field for environment-friendly and economically viable operations. Exploring enzymes from extremophilic microorganisms is attractive due to their inherent structural and functional stability under multiple adverse reaction conditions. The microorganisms exhibit adaptations at various physiological levels for their survival against solvent stress. Similarly, the solvent-stable enzymes and proteins also reveal unique adaptations at structural and molecular level. Besides, protein engineering and enzyme immobilization are also suitable approaches to improve catalysis and stability in solvents [58,61]. The protein engineering approaches emphasize forming hydrogen bonds by polar or charged amino acids in loop regions of the enzyme molecules to maintain stability [61]. On the other hand, immobilization of enzymes on organic, inorganic, or hybrid matrices may improve strength by hiding the sensitive regions from the solvents [58]. Further, the advancements in molecular biology, bioinformatics, and molecular simulation tools have facilitated the development and creation of unique properties in biocatalysts. The function and sequence-based information may foster protein engineering efforts shortly. The prospect of immobilizing more than one solvent-stable enzyme on a single career is useful for multiple commercial applications. On a similar note, the recent developments in genome editing and CRISPR-Cas9 Technology may further revolutionize molecular evolution approaches. The ionic liquids (ILs) and deep eutectic solvents (DESs) are alternatives to the organic solvents. The solvent tolerant extremophilic microorganisms can be isolated in laboratory to investigate the factors for improving the solvent stability of the commercially valuable enzymes using conventional and newer immobilization approaches. Besides, molecular routes such as gene shuffling can also be considered. The expansion of the search and application of enzymes able to function under non-aqueous conditions would provide unique opportunities, such as reverse synthesis. Investigations and advancements would lead to further insight into the interactions of the enzymes with the solvents and avenues of unique applications.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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