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1 **Solvent stable microbial lipases: Current understanding and biotechnological applications**

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12

13

14

15 **Abstract**

16 **Objective:** This review examines on our current understanding of microbial lipase solvent tolerance, with a specific

17 focus on the molecular strategies employed to improve lipase stability in a non-aqueous environment.

18 **Results:** It provides an overview of known solvent tolerant lipases and of approaches to improving solvent stability

19 such as; enhancing stabilising interactions, modification of residue flexibility and surface charge alteration. It shows

20 that judicious selection of lipase source supplemented by appropriate enzyme stabilisation, can lead to a wide

21 application spectrum for lipases.

22 **Conclusion:** Organic solvent stable lipases are, and will continue to be, versatile and adaptable biocatalytic

23 workhorses commonly employed for industrial applications in the food, pharmaceutical and green manufacturing

24 industries.

25

26 **Keywords:** industrial biocatalysis, lipase, lipase engineering, organic solvent stability, organic synthesis.

27

28 **1. Introduction**

29 The benefits of non-aqueous biocatalysis have strengthened the search for, and engineering of, solvent tolerant

30 enzymes. Non-aqueous reactions can assist the dissolution of hydrophobic compounds, drive reaction equilibria

31 from hydrolysis towards synthesis, have less undesirable side reactions (i.e. hydrolysis, polymerisation, racemisation)

32 and generally are unhampered by microbial contamination (Kumar et al. 2016). For biotransformation processes in

33 organic solvents to be more sustainable or "greener" than their chemical alternatives, they need to have higher

34 productivity, better selectivity and should involve less steps for the synthesis of the desired product (Tao &

35 Kazlauskas 2011, Wenda et al. 2011).

36 Lipases belong to the triacylglycerol ester hydrolase family (EC 3.1.1.3) and have long been of interest in non-

37 aqueous synthesis (Sharma and Kanwar 2014). A major advantage of bacterial and fungal lipases, compared to plant

38 and animal lipases, is their thermal and organic solvent tolerance. Microbial lipases are more widely used than yeast

39 or fungal lipases since they are often more thermostable and offer higher catalytic activities (Salihu and Alam 2015).

40 This review will focus on the characteristics of lipases that contribute to their stability in organic solvents and on

41 stability-enhancing modifications of lipases.

2. Stability of lipases in organic solvents: structural features and interfacial activation

The first lipase structures, from *Rhizomucor miehei* and human pancreas, were reported in the 1990s (Winkler et al. 1990; Derewenda et al. 1992). By October 2018, a search for 'lipase structure' in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) returned 273 hits. Structurally, all lipases fold in a similar fashion generating a characteristic α/β-hydrolase fold. This pattern contains parallel β-sheets surrounded by α-helices. This fold also gives rise to a catalytic triad composed of serine (Ser), histidine (His) and glutamate/aspartate (Glu/Asp) residues along with several oxyanion-stabilizing residues (Kazlauskas, 1994). This active site is conserved in most lipases irrespective of size (<1kDa to >60kDa).

A common characteristic of lipases is an increase in catalytic activity at a lipid water interface. This process, known as interfacial activation, involves a structural rearrangement of the lipase from an inactive to an active conformation. First identified in *M. miehei* lipase, the process is initiated by exposing a large hydrophobic area around the active site (Brady et al. 1990). The exposed hydrophobic area allows the anchoring of the lipase at the water/lipid interface thereby initiating catalysis. This resistance to denaturation at the water/lipid interface may account for lipase stability in organic solvents. The structural changes at the water/lipid interfaces typically involve a lid structure. The lid domain of lipases is amphipathic, having a hydrophobic and a hydrophilic side: in water the hydrophilic side of the lid faces the solvent while the hydrophobic side is directed towards the active site in a "closed" conformation. At the water/lipid interface the hydrophobic face becomes exposed allowing substrate access – the "open" conformation (Khan et al. 2017). This lid movement also changes the orientation of oxyanion-stabilizing residues to promote catalysis (Fischer et al. 2000). Lipase lid structures differ in terms of the position and number of the surface loops. Smaller lipases (e.g. *Rhizomucor miehei*) form the lid using a single α-helix while larger lipases (e.g. *Candida rugosa*) form the lid using two α-helices (see Figure One; Kazlauskas 1994b).

The activity of lipases in certain environments, e.g. in solvents, is dependent on the prevalence of the relevant conformation. Previous studies have shown that lid opening can be induced in an organic solvent (Maiangwa et al. 2017; Adlercreutz 2013). The efficiency of interfacial activation varies in different solvents (Abuin et al. 2007). Lipases show other changes in secondary structure in organic solvents. Lipase A from *Candida Antarctica,* for example, shows increased α-helical content following acetonitrile or acetone exposure and this change is

70 correlated with changes in hydrolytic activity (Yang et al. 2012). Significant research has focused on correlating
71 the conformational changes of lipases in organic solvents with catalytic activity (Benkovic and Hammes-Schiffer
72 2003). The structural integrity of lipases in solvents has been probed by X-ray crystallography, circular dichroism
73 (CD; Pelton and McLean 2000) and by nuclear magnetic resonance (NMR; Eppler et al. 2006; Kumar et al. 2014)
74 often coupled with modelling and molecular dynamics simulation (YooPark et al. 2013). It is worth noting,
75 however, that organic solvents can also cause denaturation and deactivation of lipases. Moreover, some solvents
76 can compete for substrate binding and thus act as lipase inhibitors (Dror et al. 2015; Grosch et al. 2017).
77 Conversely, Zaks and Klibanov (1984) noted the importance of water for lipase activity through formation of non-
78 covalent and hydrogen bonds with the enzyme. Water provides stability through a hydration shell, which protects it
79 from direct contact with destabilising solvent (Díaz-García and Valencia-González 1995; Halling 1997). Loss of
80 water molecules from the enzyme surface gives direct access to the solvent, thus disrupting its inter-, and intra-,
81 molecular structure and provides rationale for solvent stable lipases (Schulze and Klibanov 1991; Dror et al. 2015).
82

83 **3. Solvent Stable Lipase Sources**

84 Many solvent stable lipases have been reported (see Table 1). For example, *Pseudomonas aeruginosa* AAU2 lipase
85 is stable in organic solvents with a log P≥3.1 and even after 24 hours of incubation retains more than 70% of its
86 activity (Bose and Keharia 2013). Similarly, a lipase from *Streptomyces* sp. CS133 was stable in 25% (v/v) *n*-
87 Hexane (log P=3.5) and octane (log P=4.9; Mander et al. 2012) for 48 hours. In general, polar organic solvents are
88 harsher on lipases than non-polar solvents. Polar organic solvents can cause enzyme deactivation by hydrogen bond
89 disruption and by stripping the enzyme's protective hydration shell. However, a few lipases, for example a lipase
90 from *Bacillus sphaericus* MTCC 7542, were highly stable in both polar and non-polar organic solvents with a
91 residual activity of 80-95% in all solvents even after 12-hr of incubation (Tamilarasan and Kumar 2012). Some
92 lipases are stable in non-polar organic solvents (log P≥2), even after 7 days of incubation (e.g. *Stenotrophomonas*
93 *maltophilia* CGMCC 4254 lipase). *Acinetobacter radioresistens* CMC-1 and *Acinetobacter* EH 28 lipases have
94 higher stability and activity in 30% (v/v) *n*-Hexane, dimethyl sulfoxide (DMSO) and acetone than in 15% of the
95 same solvents, indicating solvent activation (Ahmed et al. 2010). A lipase from *Burkholderia ambifaria* YCJ01 was
96 noted to be stable for 60 days in a number of hydrophilic and hydrophobic solvents (25% v/v) and retained 100%
97 activity in 25% (v/v) ethanol and 80% activity in 25% (v/v) acetonitrile, even after 30 days (Yao et al. 2013).

98 TABLE 1 HERE

99

100 **4. Improving organic solvent stability of lipases: random mutation and rational approaches**

101 Exploiting our growing knowledge of lipase structure/function/stability relationships, in conjunction with protein
102 engineering, can improve the catalytic properties of lipases in solvents (Villeneuve et al. 2000). A random mutation
103 approach is useful when improving lipases when there is minimal structural information (Cobb et al. 2013). With
104 the advent of computational modelling and molecular dynamic simulations, solvent tolerance enhancement from
105 random mutation experiments can be understood at a structural level (Park, et al. 2013). In general, stabilising
106 mutations may be categorised as: those based on increasing the stabilising interactions of surface residues, those
107 reducing the flexibility of surface residues, and those changing the enzyme surface charge (see Table 2).

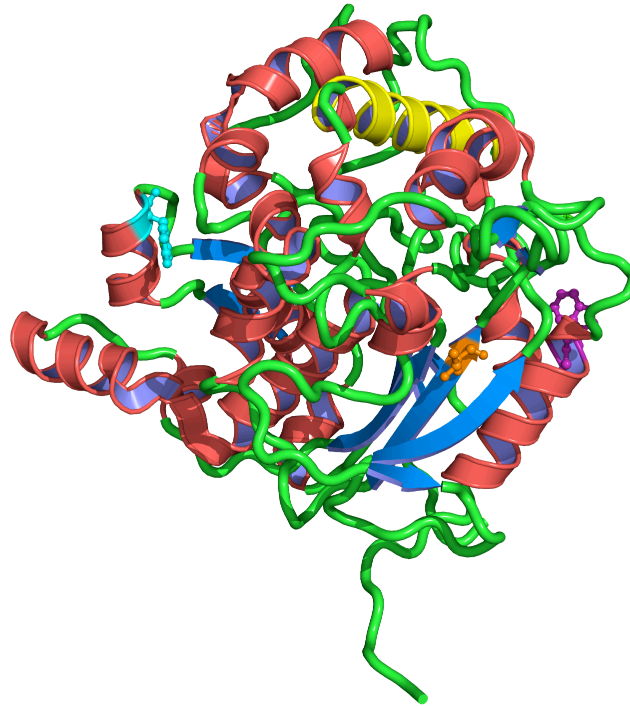
108

109 **Enhanced stabilising interactions**

110 The importance of stabilising interactions in protein stability has long been established as one of the '*rules of*
111 *thumb*' for protein engineering and the selection of target residues. Typical guiding principles include avoiding
112 changes to residues that contribute to stabilising interactions or residues involved in the formation of secondary
113 protein structure or those that might affect the formation of the active site (Yang et al. 2002). Based on the idea that
114 the action of a solvent (stabilising or destabilising) on the protein can be defined by the balance between its
115 preferential affinity for water or solvent (Timasheff 1993), many lipase engineering studies have involved mutation
116 of surface residues.

117 For example, a lipase variant from *Pseudomonas sp.* KWI-56, which was found to be 40% more stable in 80% (v/v)
118 DMSO than wild type, had only a single surface residue mutation (V304A; Nakano et al. 1998). Similarly, a
119 G157R mutation in *Pseudomonas aeruginosa* LST-03 lipase introduced additional bonds that promoted salt bridge
120 and H-bond formation. Another example showed that a single S194R mutation in *Pseudomonas aeruginosa* LST-
121 03 could introduce new hydrogen bonds that resulted in the lipase being more stable in several solvents (Kawata
122 and Ogino 2009, 2010). A methanol stable lipase variant from *Proteus mirabilis* was found to have mutations that
123 introduced new side chain interactions (L64I) and novel H-bond formation (A70T and R33T). Additionally, G202E,
124 K208N, G266S mutations are close to a Ca^{2+} binding site important for lipase stability. G266S introduced a new
125 interaction with water and a residue that coordinates Ca^{2+} (Korman et al. 2013). A methanol stable variant of

126 *Geobacillus stearothermophilus* Lipase T6 (see Figure One) was found to have mutations that enabled the
127 formation of hydrogen bonds with surface water (A269T, R374W) and a widened hydrogen bond network to
128 enable more direct contact with the Zn^{2+} coordinating residue (H86Y, Dror et al. 2014; Dror et al. 2015).



129

130 **Fig. 1** Methanol stable lipase variant H86Y/A269T/R374W from *Geobacillus stearothermophilus* T6 (PDB: 4X85)
131 (Gihaz et al. 2018). The lid residues, F177-A192, are shown in yellow. The mutated residues are highlighted as
132 H86Y (in cyan), A269T (in orange) and R374W (in purple). The image was generated using Pymol (DeLano 2018)

133

134 **Residue flexibility**

135 The flexibility of protein residues is often associated with enzyme stability (McAuley & Timson, 2016). It has been
136 suggested that the stability of lipases is due to their conformation being more rigid in organic solvents (Sharma &
137 Kanwar, 2014). This was supported by several studies including a DMSO-stable *Bacillus substilis* lipase variant
138 with an overall increased flexibility due to a single mutation to a more conformationally flexible residue (A269S),
139 resulting in a less stable variant than the more rigid wild-type residue (Yedavalli & Madhusudhana Rao, 2013). The
140 increase in rigidity associated with a network of intramolecular interactions, such as the extension of hydrogen
141 bonds on the lipase surface, can prevent solvent penetration (Dror et al. 2014, 2015) and prevent protein
142 denaturation (Reetz et al. 2006).

143 An increase in stabilising interactions has also been reported when targeting residue flexibility for improving
144 *Candida antarctica* lipase B (CalB) stability in methanol (Park, et al. 2013). In this case, all the stabilising
145 mutations were involved in the formation of additional hydrogen bonds with surface water (A8T, A92E, N97Q) or
146 had shorter hydrogen bond distances (T245S). By contrast, mutations (e.g. T244D) that reduced the number of
147 hydrogen bonds with water were linked to a reduced stability in methanol.
148 The flexibility of protein residues can be characterised using the B-factor value or Root Mean Square Deviation
149 (RMSD) value. The B-factor, or the Debye Waller value, is used in crystallography to rate the flexibility of a residue
150 in a protein structure: a higher value corresponds to greater flexibility (Reetz et al. 2006). This value represents the
151 degree of elastic scattering caused by positional disorder, or the thermal motion, of an atom and hence a higher
152 mobility of a protein residue is indicated by a high B-factor (Yuan et al. 2005). The selection of a residue based on
153 its B-factor is known as a B-factor iterative test (B-FIT, Illanes et al. 2012) and is commonly used to guide enzyme
154 thermostability enhancement (Wen et al. 2013; Kumar et al. 2014; Augustyniak et al. 2012). In this approach,
155 residues with a high B-factor score were selected as the target for mutation. Using this approach, Reetz and
156 colleagues (2010) successfully improved the stability of a lipase from *Bacillus subtilis* in polar organic solvents by
157 mutating residues with the highest B factors to enhance the rigidity of the lipase.
158 Additionally, molecular dynamics simulations (MDS) are used to understand the dynamic nature of lipases in
159 different environments. The relative flexibility of each individual residue in MDS is typically represented by Root
160 Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) values. RMSD is a measure of
161 deviation from the initial structure whereas RMSF is a measure of deviation from an average structure (Benson and
162 Daggett 2012). Employing this approach, Park and colleagues (2013) rationalised the selection of residues with high
163 RMSD as solvent affecting sites and the subsequent mutations (A8T, A92E, N97Q, T244D and T245S) enhanced
164 *Cal*B methanol stability and also had lower RMSD values in methanol.
165 Despite finding that greater lipase rigidity increases solvent stability, the decrease in enzyme flexibility observed in
166 organic solvents is thought to be related to the corresponding decline in catalytic activity. The more rigid structure of
167 LipA from *Candida antarctica* in ethyl esters is attributed to higher β-sheet content resulting in diminished catalytic
168 activity (Yang et al. 2012). Yagonia and colleagues (2015) incorporated such considerations when improving *Cal*B
169 lipase stability and activity in methanol. By considering the catalytic orientation of *Cal*B lipase, the structure was
170 divided into a solvent affecting region and substrate-binding region for flexibility modulation based on B-factor and

171 RMSD analysis. Mutations which increased rigidity in the solvent affecting region (A92E and T245S) showed
172 higher methanol stability, but were less catalytically active, than the variants with activity enhancing mutations
173 (V139E and A151D) and vice versa. The combination of both mutations resulted in mutants (V139E, A92E and
174 V139E, T245S) that were more active and stable in presence of methanol (Yagonia et al. 2015).
175
176 **Surface charge and polarity**
177 The effect of the polarity of organic solvent on surface charge has been correlated with lipase activity and stability
178 (Iyer and Ananthanarayan 2008; Chakravorty et al. 2012; Jain and Mishra 2015)). Solvents with low polarity cause
179 the dispersal of enzyme hydrophobic domains due to solvent penetration, resulting in enzyme inactivation (Ogino
180 and Ishikawa 2001). Conversely, the formation of a hydrated ion network, by charged amino acids, maintains the
181 stability of lipases in organic solvents by preventing protein aggregation via repulsing electrostatic charges (Jain and
182 Mishra 2015). In a set of random mutation studies by Kawata and colleagues (2009, 2010), *Pseudomonas*
183 *aeruginosa* LST-03 lipase variants selected for stability in solvents were noted to possess mutations of surface
184 residues (S164K, Y188F, L145H) that prevented penetration of the solvent into the protein. The increase in pI, due
185 to the mutations (S164K, S211R, G157R, S194R, D209N, L145H), repulsed the basic organic solvent molecules
186 through an ion repulsion interaction. In a comparable study, the mutation of lid residues to more hydrophobic
187 residues (F146L, I289T) changed the accessibility of solvents to the active site of a lipase from *Pseudomonas sp.*
188 KWI-56 (Nakano et al. 1998).
189 Yedavalli and collegues (2013) improved the stability of lipase (LipA) from *Bacillus substilis* in DMSO by
190 modifying the loop secondary structure of the enzyme. In this case, the stable mutants had a similar secondary
191 structure to the wild type, but with a more polar surface. Monsef Shokri and co-workers (2014) targeted a loop on
192 the protein surface to improve the stability of *Pseudomonas* sp. lipase in non-aqueous solvent, by using the strategy
193 of hydrophobic residue substitution (Arnold 1990). The variants that were more stable in hydrophilic organic
194 solvents had hydrophobic mutations at position 219 (N219A, N219I, N219L), resulting in increased lipase rigidity.
195
196 TABLE 2 HERE
197

198 **5. Selected applications of Organic Solvent Stable Lipases by industry**

199 Solvent compatible lipases are in use in the food, (bio)pharmaceutical and environmental industries (Ahmed et al.
200 2010). Selected applications in these industries are discussed below.

201

202 **Food industry; Flavours and Fragrances**

203 The world flavour and fragrance market was $22 billion (USD) in 2011 and has been increasing at a rate of 5.6%
204 annually (Badgujar et al. 2016). Although the isolation and extraction of flavour esters from natural sources is
205 expensive, consumers prefer products with a 'natural' label (Ahmed et al. 2010). Therefore, alternatives to chemical
206 synthesis of flavour esters, including alternative production technologies such as esterification by solvent stable
207 lipase, have gained attention (Matte et al. 2016 and see Table 3).

208

209 TABLE 3 HERE

210

211 **Pharmaceutical Industry: Regio- and Stereo-selective Resolution**

212 Lipases are widely used for the kinetic resolution of compounds (Xun et al. 2013). The regioselectivity of lipases
213 has been exploited for the resolution of racemic alcohols and kinetic resolution of racemic mixtures of compounds
214 such as flurbiprofen (*C. antartica* lipase Novozyme® 435) and N-hydroxymethyl vince lactam (*Mucor meihei* lipase;
215 Xun et al. 2013). Interestingly, lipase resolution has also been explored in the production of herbicides
216 (phenoxypropionate) by the resolution of 2-halopropionic acids and esterification of (*S*)-isomers in butanol and
217 hexane (Hasan et al. 2006).

218 Lipase regioselectivity has been used for the synthesis of compounds that are difficult to synthesize by chemical
219 methods (Miyazawa et al. 2014). Non-ionic and biodegradable sugar esters have extensive applications in detergents,
220 pharmaceutical and oral care products. Their conventional production, involving chemical sugar and fatty acid
221 esterification, is difficult due to poor regioselectivity and the low organic solvent solubility of sugars. This
222 esterification was achieved by immobilized lipase B from *Candida Antarctica* (Novozyme® 435) in DMSO and
223 acetone (1:10 v/v). This dual solvent environment has been used for the production of a xylose caproate ester with
224 64% yield (Abdulmalek et al. 2016). Various other precursor molecules required for the manufacturing of a range of
225 pharmaceutical and agrochemical products are currently synthesised by lipases in solvents (see Table 4).

226

227 TABLE 4 HERE

228

229 **Environmental applications: Biodegradable polymer synthesis and recycling**

230 In recent times, much attention has focused on polymer research due to their increased use in biomedical research,
231 food packaging and agricultural industries. However, the disposal of these polymers is a critical environmental issue,
232 and has led to the development of biodegradable polymers (e.g. polyesters) as an alternative to traditional plastics
233 (Banerjee et al. 2014). Solvent stable lipases have been widely used as catalysts for the synthesis of such
234 biodegradable polyesters (Barrera-rivera & Flores-carreón 2012 and see Table 5). The lipase from *Candida*
235 *antarctica* lipase B (CalB) is the most common catalyst used for polyester synthesis (Chen et al. 2008).

236 The production of biodegradable polyesters plays a significant role in a green environmental approach to packaging;
237 however, recycling is equally important from an environmental perspective. A wide range of aromatic and aliphatic
238 polyesters including poly(ethylene terephthalate), poly(butylene succinate), poly (β-caprolactone) and poly(lactic)
239 acids are used in the production of medical biomaterials such as surgical sutures and reinforcing plates (Kobayashi,
240 2010). Chemical recycling has limited applications due to its high-energy demand, both in terms of temperature and
241 pressure. However, degradation of biopolymers by enzymatic processes can occur with a lower energy requirement
242 and in milder conditions (see Table 5 for relevant examples). Moreover, chemical based recycling cleaves polymers
243 randomly, generating varying molecular weight oligomers while lipase catalyzed degradation involves cleaving the
244 amorphous regions of a polymer first, followed by its crystalline regions, consistently resulting in oligomers with
245 lower molecular weights (Banerjee et al. 2014). The lipase from *Candida antarctica* has been successfully utilised to
246 degrade poly(β-caprolactone; PCL) in dry toluene at 60°C. This 'one-pot degradation-polymerisation' reaction
247 successfully recycled poly(β-caprolactone; Kobayashi et al. 2000). Although lipases can hydrolyse poly(β-
248 caprolactone) in aqueous solution; the low solubility of hydrophobic PCL in water means that, solvents are generally
249 used for their degradation (Aris et al. 2016).

250

251 TABLE 5 HERE

252

6. Considerations in the development of organic solvent stable lipases for industrial application

Currently, industrial biocatalysis requires lipases for existing and emerging industries. New lipases can be delivered via biodiscovery or through improving existing lipase properties by protein engineering. Despite the increasing number and availability of organic solvent stable lipases there is a lack of translation of lab scale biocatalysis to industry scale. Optimising an enzyme for use in a given application is challenging as it is difficult to make the biocatalytic operational space (e.g. temperature, pH, pressure etc.) as wide as the chemical counterpart. Enzymes are prone to denaturation and deactivation under extreme processing conditions (Tufvesson et al. 2013; Ringborg and Woodley 2016). In reactions involving organic solvents there are several thermodynamic constraints including; interaction with the enzyme, substrate solubility and enzyme solubility (Grosch et al. 2017). This results in the need to understand the effective concentration of substrate available to the enzyme, competitive inhibition by the organic solvent, transition state stabilisation (Dutta Banik et al. 2016), as well as steric effects (Wang et al. 2016). In the past, biodiscovery and engineering of existing organic solvent lipases commenced without a defined target reaction; however, now these thermodynamic constraints are key drivers in the enzyme selection procedure for industrial scale biocatalysis (Ringborg and Woodley 2016). It is prudent to identify the organic solvent effects; such as solvation of substrate, inhibition by solvent molecule, and water activity, on catalysis (Sandoval et al. 2001; Kulschewski et al. 2013; Grosch et al. 2017).

7. Conclusion and Future Directions

Lipases continue to be an important biocatalyst in the food, pharmaceutical, and chemical industries (De Godoy Daiha et al. 2015). Previous lipase engineering efforts have demonstrated that, through engineering approaches, organic solvent stability can be achieved. The utility of lipases in biocatalysis depends on finding a balance between enzyme rigidity and catalytic activity for many applications. A recent study looked at filling lipase solvent tunnels with aromatic interactions to improve lipase stability in methanol, with the results demonstrating a stabilisation of 81-fold compared with wild-type (Gihaz et al. 2018). This rational approach could be extended to other lipases for stabilization in organic solvents.

The use of novel solvent systems such as ionic liquids or deep eutectic solvents appears to offer promising alternatives to traditional organic solvents. Recently, Brogan and colleagues (2018) reported that the combination of chemical modification and ionic liquids produced a highly robust glucosidase that displayed "solvent induced

substrate promiscuity” and activity at temperatures up to 137°C. Thus, through a synergistic combination of enzyme modification and solvent choice the biocatalytic capability of enzymes was enhanced. It would be of interest to explore whether such chemical modification might lead to similar stabilisation of lipases in solvents.. It is clear that the drive for more sustainable catalysis will provide an impetus for this field in the coming years.

8. Compliance with Ethical Standards

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors

Authorship: Conceived study (PP, YT, GKK, GTH, BJR), Performed research (PP, YT), Analyzed data (PP, YT), Contributed methods (GKK, GTH, BJR), Wrote the paper (PP, YT, GKK, GTH, BJR).

Data Availability: The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Table 1 Selected solvent stable lipases incubated in solvent at different times and temperatures.

Lipase Source	Stability test condition	Solvent % (v/v)	Activity	Solvent System	Reference
Pseudoalteromonas lipolytica SCSIO 04301	12 hours at room temperature	50%	>100% residual activity	ethanol, acetone, DMSO, *t*-butanol, hexane	(Su et al. 2016)
			>50% residual activity	acetonitrile, *t*-butanol, toluene, hexane	
Penicillum corylophilum	37°C after 1 hour		>100% residual activity	ethanol, acetone	(Romero et al. 2014)
			>50% residual activity	methanol, butanol and hexanol	
Streptomyces sp. OC119-7	30°C for 24 hours		>70% residual activity	methanol, ethanol, acetone	(Ayaz et al. 2014)

Idiomarina sp. W33	30°C for 12 days		>70% residual activity	toluene, cyclohexane, *n*-hexane, 1-decanol and isooctane	(Li et al. 2014)
Haloarcula sp. IG41	30°C for 2 and 5 days		>70% residual activity	toluene, cyclohexane, *n*-hexane, 1-decanol and isooctane	(Li & Yu 2014)
			>60% residual activity	chloroform and n-hexane	
Staphylococcus aureus ALA1	30 min at 37°C	25%	>90% residual activity	acetone, benzene, ethanol, methanol, 2-propanol and toluene	(Ben Bacha et al. 2016)
Bacillus sp.	30 min at 37°C		>80% relative activity	acetone, *t*-butanol	(Sivaramakrishnan & Incharoensakdi 2016)
			~100% relative activity	methanol and ethanol	
Staphylococcus	30 min at room		>120% residual	Diethyl ether, DMSO	(Kamarudin

epidermidis AT2	temperature		activity		et al. 2014)
			~100% residual activity	*n*-hexane, toluene, acetone	
Burkholderia cepacia RQ3	40°C for several days		half-life >10 days	isopropanol, ethanol and *n*-octane	(Xie et al. 2016)
			half-life of 6 days	DMSO	
Aneurinibacillus thermoaerophilus	30min of incubation		>60% relative activity	propyl acetate, *p*-xylene	(Masomian et al. 2013)
			>80% relative activity	toluene, benzene, 1-propanol	
			>100% relative activity	DMSO and methanol	
Xanthomonas oryzae	70°C for 24 hours	20%	Specific activity of >250 U/mg	heptane, hexane, methanol	(Mo et al. 2016)
Aureobasidium	30 min at 37°C	10%	>80% relative activity	methanol, acetonitrile, ethanol and	(Wongwatan

melanogenum	followed by 24 hours at 4°C			chloroform	apaiboon et al. 2016)
Pseudomonas sp. DMVR46	37°C for 4 hours	5%	>30% residual activity	ethanol, isopropanol, acetone	(Vrutika et al. 2014)

Table 2 Lipase engineering towards improved organic solvent stability. Studies are categorised based on type of mutation: random, surface or flexible residues. The lipase, its mutation(s), the solvent system (and logP value) are shown.

Lipase	Mutation(s)	Organic solvent (logP)	Wild type activity (Incubation time/half life)	Mutant activity (Incubation time)	Reference
Random mutation					
Pseudomonas sp. KWI-56	S26G, F146L, I289T, and V304A	DMSO (logP = -1.35)	60% residual activity (120min)	90% residual activity, (120min)	(Nakano et al. 1998)
Pseudomonas aeruginosa LST-03	S164K, T188F, S211R	Cyclohexane (logP = 3.44) n-Decane (logP = 6.25)	6.0 days half life	54.8 days half life	(Kawata and Ogino 2009)
	S155L, G157R, G177V, S194R, S202W, D209N	Cyclohexane (logP = 3.44)		41.1 days half life	
Pseudomonas aeruginosa LST-03	S155L	n-Octane (logP = 5.15)	6.6 days half life	31.6 days half life	(Kawata and Ogino 2010)
		DMSO (logP = -1.35)	6.9 days half life	17.4 days half life	
		n-Heptane (logP = 4.5)	9.5 days half life	>100 days half life	

		n-Hexane (logP = 3.44)	13.0 days half life	28.0 days half life	
		Cyclohexane (logP = 3.44)	8.0 days half life	15.8 days half life	
	S164K	DMSO (logP = -1.35)	6.9 days half life	14.3 days half life	
	S194R	n-Decane (logP = 6.25)	8.9 days half life	>100 days half life	
		n-Octane (logP = 5.15)	6.6 days half life	>100 days half life	
		n-Hexane (logP = 3.44)	13.0 days half life	>100 days half life	
		Cyclohexane (logP = 4.00)	6.0 days half life	44.3 days half life	
		n-Heptane (logP = 4.5)	9.5 days half life	33.5 days half life	
	D209N	n-Decane (logP = 6.25)	8.9 days half life	>100 days half life	
		n-Octane (logP = 5.15)	6.6 days half life	37.4 days half life	
		n-Hexane (logP = 3.44)	13.0 days half	>100 days half	

			life	life	
		Cyclohexane (logP = 4.00)	6.0 days half life	>100 days half life	
		n-Heptane (logP = 4.5)	9.5 days half life	>100 days half life	
		Toluene (logP = 2.73)	26.6 days half life	>100 days half life	
	G157R	n-Decane (logP = 6.25)	8.9 days half life	16.4 days half life	
		n-Octane (logP = 5.15)	6.6 days half life	25.4` days half life	
		n-Hexane (logP = 3.44)	13.0 days half life	28.8 days half life	
		Cyclohexane (logP = 4.00)	6.0 days half life	21.0 days half life	
		n-Heptane (logP = 4.5)	9.5 days half life	10.2 days half life	
	S211R	n-Octane (logP = 5.15)	6.6 days half life	18.7 days half life	
Proteus mirabilis Lipase	G181C/S238C/K208N/L64I/A70T/F225L/Q277L/G202E/G2	Methanol (logP = -0.74)	Inactivated (16 hrs)	80% residual activity (16 hrs)	(Korman et al. 2013)

	66S/D270N/N17S/I255F/R33T				
Surface residue properties					
Candida antarctica lipase B (CalB)	N97Q	Methanol (logP = -0.74)	~30% residual activity (72 hrs)	~50% residual activity (72 hrs)	(Park et al. 2012)
	N264Q			~50% residual activity (72 hrs)	
	D265E			~40% residual activity (72 hrs)	
	D223E			~5% residual activity (72 hrs)	
	N292Q			~20% residual activity (72 hrs)	
Bacillus subtilis lipase	I12L, W42L, A68S, P119S, L140F and Y139K	DMSO (logP = -1.35)	100% relative activity (5 min)	300% relative activity (5 min)	(Yedavalli and Madhusudhana Rao 2013)

Pseudomonas sp. lipases	N219A	DMF (logP = -1.51)	~25% residual activity (12 min)	~50% residual activity (12 min)	(Monsef Shokri et al. 2014)
		Methanol (logP = -0.74)	~10% residual activity (100 min)	~40% residual activity (100 min)	
		Ethanol (logP = -0.24)	~10% residual activity (10 min)	~40% residual activity (10 min)	
		n-Propanol (logP = 0.25)	Inactivated (5 min)	~30% residual activity (5 min)	
	N219I	DMF (logP = -1.51)	~25% residual activity (12 min)	~60% residual activity (12 min)	
		Methanol (logP = -0.74)	~10% residual activity (100 min)	~60% residual activity (100 min)	
		Ethanol (logP = -0.24)	~10% residual activity (10 min)	~50% residual activity (10 min)	
		n-Propanol (logP = 0.25)	Inactivated (5 min)	~30% residual activity (5 min)	
	N219L	DMF (logP = -1.51)	~25% residual activity (12 min)	~60% residual activity (12 min)	
		Methanol (logP = -0.74)	~10% residual	~60% residual	

			activity (100 min)	activity (100 min)	
		Ethanol (logP = -0.24)	~10% residual activity (10 min)	~60% residual activity (10 min)	
		n-Propanol (logP = 0.25)	Inactivated (5 min)	~60% residual activity (5 min)	
Geobacillus stearothermophilus T6 Lipase	A269T	Methanol (logP = -0.74)	2.9 min half life	77 min half life	(Dror et al. 2015) (Dror et al. 2014)
	Q185L			77 min half life	
	H86Y/A269T			116 min half life	
	Q185L/ A269T			231 min half life	
	H86Y/A269T/ R374W			347 min half life	
Residue flexibility					
Bacillus subtilis Lipase	M134D	Acetonitrile (logP = -0.34)	<30 mins half life	<300 mins half life	(Reetz et al. 2010)
		DMSO (logP = -1.35)	<10 hrs half life	<25 hrs half life	
		DMF (logP=-1.51)	<10 hrs half life	<25 hrs half life	
	M134D/I157M	Acetonitrile (logP = -0.34)	<30 mins half life	>300 mins half life	
		DMSO (logP = -1.35)	<10 hrs half life	<50 hrs half life	
		DMF (logP=-1.51)	<10 hrs half life	<25 hrs half life	

	M134D/I157M/ Y139C	Acetonitrile (logP = -0.34)	<30 mins half life	<600 mins half life	
		DMSO (logP = -1.35)	<10 hrs half life	>100 hrs half life	
		DMF (logP=-1.51)	<10 hrs half life	>100 hrs half life	
	M134D/I157M/ Y139C/K112D	Acetonitrile (logP = -0.34)	<30 mins half life	<900 mins half life	
		DMSO (logP = -1.35)	<10 hrs half life	>100 hrs half life	
		DMF (logP=-1.51)	<10 hrs half life	>100 hrs half life	
	M134D/I157M/Y139C/K112D /R33G	Acetonitrile (logP = -0.34)	<30 mins half life	>1500 mins half life	
		DMSO (logP = -1.35)	<10 hrs half life	>200 hrs half life	
		DMF (logP=-1.51)	<10 hrs half life	<200 hrs half life	
Candida antarctica lipase B (CalB)	A8T	Methanol (logP = -0.74)	35 hrs half life	52 hrs half life	(Park et al. 2013)
	A92E			63 hrs half life	
	N97Q			52 hrs half life	
	T244D			59 hrs half life	
Candida antarctica lipase B (CalB)	V139E	Methanol (logP = -0.74)	~20% residual activity (24 hrs)	~50% residual activity (24 hrs)	(Yagonia et al. 2015)
	A151D			~30% residual activity (24 hrs)	
	A92E			~50% residual	

				activity (24 hrs)	
	T245S			~50% residual activity (24 hrs)	
	V139E, A92E			~60% residual activity (24 hrs)	
	V139E, T245S			~60% residual activity (24 hrs)	

Table 3 Lipases commonly used in the food industry for the production of food flavours and aromas. The table shows lipase source, the product of the lipase catalysed reaction along with the solvent system employed for synthesis and typical yields.

Lipase Source	**Product (application)**	**Lipase state**	**Yield**	**Solvent system**	**Reference**
Aspergillus oryzae	*Cis*-3-hexen-1-yl-acetate (fresh/floral odour)	In dry mycelium	98%	60mM acetic acid and cis-3-hexen-1-ol	Kirdi et al. 2017
Pseudomonas cepacia	Cinnamyl Propionate (spicy floral flavour)	Immobilized on hydroxylpropyl methyl cellulose and polyvinyl alcohol	>90%	*Cinnamyl alcohol: vinyl propionate (1:2) with 1ml* *n*-hexane or and toluene (non-polar solvents)	Badgujar et al. 2016
			>50%, <80%	*Cinnamyl alcohol: vinyl propionate (1:2) with 1ml* acetone, dioxane (hydrophobic	

				solvents	
Thermomyces lanuginosus	Butyl butyrate (pineapple flavour)	Immobilised on immobeads 150	84%	butanol:butyric acid, 3:1	Matte et al. 2016
Bacillus aerius	Isoamyl acetate (pear/banana flavour)	Immobilised on silica gel matrix	68%	1:1 ratio of acetic acid and isoamyl alcohol	Narwal et al. 2016
Bacillus licheniformis	Ethyl lactate (fruity odour and fruity flavour)	Immobilized on magnetite particles	23%	(1:1) ethyl alcohol and lactic acid	Jain & Mishra, 2015
	Isobutyl acetate (pineapple flavour)		36%	isobutyl alcohol and acetic acid (1:1)	
Candida rugosa	Ethyl caprylate (flavour ester)	Immobilised on exfoliated graphene oxide	85%	ethanol and cyclo-octane (0.15:0.1M)	Patel et al. 2015
Pseudomonas	Ethyl	Immobilised	81%	Ethanol/butyric	Vrutika &

sp. DMRV46	butyrate (tropical fruit flavour)	on multi-walled carbon nanotubes		acid and n-heptane (0.15:0.2M)	Datta, 2015
Pseudomonas sp. DMVR46	Pentyl valerate (fruity aroma)	Immobilisation into AOT-organogels	88%	pentanol and valeric acid (1:1)	Vrutika et al. 2014
Bacillus safensis	Ethyl laurate (waxy odour and flavour)	Purified enzyme	80%	lauric acid and ethanol (1:1)	Kumar et al. 2014
Candida rugosa	Vitamin E succinate (food supplement)	Purified Enzyme	47%	1:5 (substrate: DMSO)	X. jun Jiang et al. 2013

Table 4 Pharmaceutical products synthesized by lipases in a solvent system. The table shows the product formed throughed the lipase catalysed reaction as well as its application, the lipase invovled and the mode of operation including the solvent system.

Product		Application	Lipase	Lipase state	Solvent system	Reference
(R)-Indano		Precursor of drugs (Sertraline, Indinavir, Irindalone, Rasagiline mesilate)	*Candida antarctica*	Immobilized on Cashew apple bagasse support	Toluene and diisopropyl ether	De Souza et al. 2016
Sugar Fatty Acid Ester	6-O-glucose tetradecanoate	Food, Pharmaceuticals, cosmetic, insecticidal, antimicrobial, oral care uses	*Candida antarctica* Sp-435	Immobilized lipase Novozym435	Dimethylformamide	Degn et al. 1999
	6-O-glucose octadecanoate		*Mucor meihei*	Immobilized from NOVO industries	Heptane	Oguntimein et al. 1993
	6-O-acetyl glucopyranoside		Porcine pancreatic lipase IIII	Free enzyme	Hexane	Sharma & Chattopadhyay 1993
	Dilauroyl maltose		*Candida antarctica*	Immobilized by Novo-Nordisk	Acetone and *n*-hexane	Jia et al. 2010

	Fructose Oleate		*Thermomyces lanuginosus* and *Pseudomonas fluorescens*	Immobilized on functionalized silica	*t*-butyl alcohol	Vescovi et al. 2017
Phytosterols		Cholesterol reduction, anti-viral and anti-inflammatory	*Candida rugosa*	Immobilized on macroporous acrylic resin	*n*-Hexane	Jiang et al. 2013
(R,S)-1-phenylethanol		cosmetics and the pharmaceutical industry	*Pseudomonas stutzeri*	Free enzyme	*n*-hexane	Cao et al. 2012

Table 5.2: Continued.

Product	Application	Lipase	Lipase state	Solvent system	Reference
Chiral mandelic acid and its derivates	Intermediates for pharmaceutical industry	*Burkholderia ambifaria*	Free enzyme	Diisopropyl ether	Yao et al. 2013
Ethyl oleate	Solvent for preparation of steroids; plasticizer	*Geobacillus stearothermophilus*	Immobilized on cellulosic nanogel	Ethanol and DMSO	Kumar et al. 2015
β-sitostanol ester	Decreasing cholesterol absorption	*Ophiostoma piceae*	Crude enzyme	Isooctane	Molina-Gutiérrez et al. 2016

Leutin Dipalmitate	Inhibition of Age-related macular degeneration (AMD); For high acuity vision; decrease UV-induced damage on skin	*Candida antarctica*	Immobilized on macroporous acrylic resin	Toluene	Wang et al. 2015

Table 5 Examples of the production and degradation of polymers by lipases. The source of the lipase, the state utilised and the substrate and solvent system employed are shown.

Action	Lipase Source	Lipase state	Substrate	Solvent system	Reference
Polyester synthesis	*Yarrowia lipolytica*	Immobilized on microporous resin	β-caprolactone	Heptane	Barrera-rivera & Flores-carreón 2012
	Candida antarctica	Immobilized on nanoclays	β-caprolactone	Dry toluene	Öztürk Düşkünkorur et al. 2014
	Candida antarctica	Immobilized from Novozyme	β-caprolactone and β-thiocaprolactone	Toluene	Duchiron et al. 2017
Polymer degradation	*Candida antarctica*	Immobilized on acrylic resin beads	Poly(β-caprolactone)	Toluene	Aris et al. 2016
	Bacillus	Free	Polyhydroxyalkano	Chlorofor	Kanmani

	subtilis	enzyme	ates (PHAs)	m	et al. 2016
	Lactobacillus plantarum	Free enzyme	Poly(β-caprolactone)	Chloroform	Khan et al. 2017