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Solvent Tolerance in Bacteria: Fulfilling the Promise of the Biotech Era?

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Abstract:	The challenge of sustainably producing highly valuable chemical compounds requires specialized microbial cell factories because many of these compounds can be toxic to microbial hosts. Therefore, solvent-tolerant bacteria are promising production hosts because of their intrinsic tolerance towards these compounds. Recent studies have helped to elucidate the molecular mechanisms involved in solvent tolerance. Advances in synthetic biological tools will enable further development of streamlined solvent tolerant production hosts and the transfer of solvent-tolerant traits to established industrial strains. In this review we outline challenges and opportunities to implement solvent tolerance in bacteria as a desired trait for industrial biotechnology.

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1 **Highlights**

2 Solvent-tolerant bacteria are promising platform cell factories for biobased production of a
3 plethora of high value aromatic compounds and biopolymer constituents.
4
5 Solvent-tolerance traits are advantageous for microbial platforms in biocatalysis of aromatic
6 compounds to overcome product and substrate toxicity.
7
8 Solvent-tolerant bacteria are well equipped for biocatalysis of high-value compounds in two-
9 phase biocatalysis systems, leading to significant improvement of production yields.
10
11 Synthetic construction and development of standardized genome editing tools, such as
12 SEVA, BioBricks, and CRISPR/Cas will enable rapid engineering and optimization of
13 solvent tolerant cell factories.
14
15 Genome streamlining is a promising strategy to solve host interference issues that often lead
16 to lower product yields.
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1 # Solvent Tolerance in Bacteria: Fulfilling the
2 # Promise of the Biotech Era?

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12
13

Abstract

The challenge of sustainably producing highly valuable chemical compounds requires specialized microbial cell factories because many of these compounds can be toxic to microbial hosts. Therefore, solvent-tolerant bacteria are promising production hosts because of their intrinsic tolerance towards these compounds. Recent studies have helped to elucidate the molecular mechanisms involved in solvent tolerance. Advances in synthetic biological tools will enable further development of streamlined solvent tolerant production hosts and the transfer of solvent-tolerant traits to established industrial strains. In this review we outline challenges and opportunities to implement solvent tolerance in bacteria as a desired trait for industrial biotechnology.

Solvent-tolerant bacteria are efficient biocatalysts

The transition of a fossil raw materials-based economy to a biobased economy is characterized by complex and ambitious systems innovations. Recent breakthrough developments in green chemistry and biotechnology are major drivers enabling production of biobased chemicals [1–4]. Today, in the new biotech era, increased demands for bio-based "green" chemicals and pharmaceuticals are met with rapid product development benefitting from years of research in the microbial physiology and metabolic engineering fields. Bio-based production of these compounds is becoming economically competitive with petrochemical based production. Both environmental considerations and the need to further improve the competitiveness of the chemicals industry promise to drive continued biotechnology developments and innovation in the production of biobased chemicals.

Biobased production of valuable chemicals and biopolymer compounds puts a challenge on the choice of microbial host strains [3–6]. Many of these chemicals have hydrocarbon-solvent properties and thus exhibit toxicity towards the microbial hosts [7,8].

38 Furthermore, the production of more complex biobased products, such as o-cresol and 3-
39 methylcatechcol, requires toxic solvent-like compounds as substrates or intermediates [9,10].
40 Therefore, solvent tolerance becomes an essential trait for microbial host in the biobased
41 production of valuable chemicals and biopolymer compounds. Several species of bacteria can
42 grow and survive in the presence of hydrocarbon solvents [11] and can therefore be identified
43 as promising and advantageous platforms for the production of such potentially toxic
44 compounds, or for bioremediation. These bacteria can efficiently withstand or degrade
45 various toxic solvent-like compounds [12,13]. Therefore, the application of solvent-tolerant
46 bacteria in the biocatalytic production of (new) chemical building blocks is rapidly increasing
47 [1–4]. Using these solvent-tolerant bacteria in biotechnological production processes,
48 however, requires a thorough understanding of the solvent tolerance mechanisms involved.
49 With recent advances in genome sequencing and omics studies of solvent-tolerant bacteria,
50 unique clusters of genes have been identified that confer solvent tolerance traits [14–18].
51 Better understanding these solvent tolerance traits in combination with modern synthetic
52 biology tools will enable further development of specialized biocatalysts, new applications,
53 and improved production processes of high value compounds [19–27]. In this review, we
54 discuss recent findings in solvent tolerance mechanisms and new advances in synthetic
55 biology tools that can help to design microbial hosts and processes in industrial productions
56 for a plethora of new and valuable compounds.

57 **Current understanding of solvent tolerance mechanisms**

58 Since the first discovery of solvent-tolerant bacterium *Pseudomonas putida* IH-2000
59 by Inoue and Horikoshi [12], the number of known solvent-tolerant strains has been rapidly
60 expanding. Despite this growing number of identified solvent-tolerant bacteria, the current
61 knowledge and understanding of solvent tolerance mechanisms has mostly been obtained
62 from studying various strains of *P. putida* [14,17,18]. But solvent tolerant traits are not

63 restricted to *P. putida*, as exemplified for instance by *Exiguobacterium* sp.,
64 *Pseudoalteromonas* sp., *Vibrio* sp., *Marinomonas* sp., *Paracoccus denitrificans*, and
65 *Halomonas* sp. [28–31]. The discovery of new solvent-tolerant strains and their unique
66 features may help to better understand the molecular and physiological mechanisms
67 underlying bacterial solvent tolerance.

68 Hydrocarbon solvents with a **log $P_{o/w}$ value** (see Glossary) in the range of 1-4 [Table
69 1] are toxic to microorganisms at very low concentrations because these solvents bind and
70 penetrate the cell membrane and severely affect cell permeability [32]. Solvents with log $P_{o/w}$
71 value lower than 1, like short-chain alkanols (C2-C4), exhibit toxicity in high concentrations.
72 Short-chain alkanols directly interact with the phospholipid headgroups, while longer-chain
73 alkanols (e.g. C8) accumulate within the lipid bilayer of the membrane, 'competing' with the
74 fatty acid acyl chains [33]. Solvent-invoked membrane damage inhibits various important
75 membrane functions, such as the permeability barrier function and the structural matrix
76 scaffold for many metabolic and enzymatic reactions [34]. Consequently, this membrane
77 damages leads to disrupted cellular metabolism, growth inhibition, and eventually, cell death
78 [11,33].

79 Tolerance to hydrocarbon solvents is a multifactorial trait. Bacterial cells employ
80 various strategies to change their physiology and gene expression to circumvent cellular
81 damage caused by these solvents [Figure 1]. Tolerance mechanisms have been more
82 extensively studied in Gram-negative bacteria than in Gram-positive bacteria, but similar
83 mechanisms have been observed for both groups [35,36].

84 *Membrane fluidity*

85 In the presence of a hydrocarbon solvent, tolerant Gram-negative bacteria respond by
86 changing their cell membrane composition towards saturated and trans-unsaturated fatty

87 acids [7,37]. The formation of trans-unsaturated fatty acids is catalyzed by a periplasmic,
88 haem-containing cis-trans isomerase (Cti) [38]. In *P. putida* DOT-T1E, Cti is constitutively
89 expressed at a constant level during log-growth and stationary-phase cells and moderately
90 upregulated in the presence of toluene [37]. Recently, a working model of Cti activity was
91 proposed by Eberlein and colleagues [39]: initially, Cti activity is regulated by the limited
92 accessibility to cis fatty acid under non-stressed condition due to membrane rigidity. The
93 membrane bilayer becomes more fluid upon interaction with hydrocarbon solvents, enabling
94 hydrophilic Cti to reach cis fatty acids and isomerize them into trans fatty acids. Saturated
95 and trans-unsaturated fatty acids increase membrane rigidity, exemplified by a higher phase-
96 transition temperature. This rigid membrane structure provides resistance to hydrocarbon
97 solvents by decreasing solvent influx and accumulation in the membrane. Similarly, Gram-
98 positive bacteria also shift their membrane composition towards a more rigid structure in the
99 presence of hydrocarbon solvents by a concentration-dependent decrease in the anteiso/iso
100 branched fatty acid ratio. This modification in branched fatty acid promotes a more compact
101 membrane structure, resulting in reduced accumulation of hydrocarbon solvents [38,40].

102 *Phospholipid headgroup species*

103 The phospholipid headgroup constituents found in *Pseudomonads* are phosphatidyl-
104 ethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Those phospholipid
105 headgroups, especially CL, appear to play an important role in aiding *Pseudomonads* in their
106 adaptation against hydrocarbon solvents [41]. Recently, an increase in CL-containing lipids
107 was reported in strains of *P. putida* S12 and *Pseudomonas taiwanensis* VLB120 grown in the
108 presence of n-butanol [30]. Accordingly, CL-containing lipids are important for the function
109 of the efflux pumps in *P. putida* DOT-T1E [37].

110 Recent metabolomic analyses of *P. putida* DOT-T1E showed that the intracellular
111 ornithine concentration increases in response to toluene exposure [42]. Ornithine-containing
112 lipids are known to play an important role in stabilizing the outer membrane and the negative
113 charge of **lipopolysaccharides (LPS)**, as well as in the stress response towards abiotic
114 conditions such as elevated temperature and acidic environment [43].

115 *Membrane vesicle formation*

116 The outer membrane vesicle (OMV) is a spherical compartment released from the
117 outer membrane of bacteria consisting phospholipids, LPS, and small amounts of outer
118 membrane proteins as a response to various stress condition encountered in the environment
119 [44]. Encapsulation of hydrocarbon solvents by the formation of membrane vesicles is an
120 effective defence mechanism in solvent-tolerant *P. putida* strains in the presence of toluene
121 [45]. By forming these membrane vesicles, the cells effectively discard toluene adhering to
122 the outer membrane. In *P. putida* DOT-T1E, the formation of outer membrane vesicles
123 contributes to a rapid and extreme rise in cell surface hydrophobicity, which prepares the
124 cells for **biofilm** formation as a protective response towards solvent induced stress [46,47].
125 Membrane vesicles also play a role in releasing lipids with lesser degrees of saturation
126 enabling rapid lipid turnover as a response to the presence of hydrocarbon solvents [47].

127 *RND efflux pumps and membrane proteins*

128 Adaptive cell membrane properties constitute a robust mechanism against toxic
129 hydrocarbon solvents. However, decreased membrane permeability does not necessarily
130 generate sufficient tolerance in the presence of hydrocarbon solvent [48]. Therefore, cells
131 need an effective mechanism to actively extrude accumulating toxic solvents.

132 In both Gram-positive and Gram-negative bacteria, the most important membrane
133 proteins in terms of solvent-tolerance are the resistance, nodulation, and division (RND)

134 efflux pumps [30,48–50]. The **RND efflux pumps** can extrude a broad range of compounds
135 with little chemical resemblance to each other. They are frequently associated with resistance
136 to a broad spectrum of antibiotics and heavy metals [49,51]. Some RND efflux pumps are
137 specifically induced by and only extrude hydrocarbon solvents and are not induced by, e.g.,
138 hydrophobic antibiotics. Illustrative examples are SrpABC from *P. putida* S12 and TtgDEF
139 from *P. putida* DOT-T1E [48,50]. Recent knowledge and advances in the field of these efflux
140 pumps, their role, control mechanisms, cross resistance with antibiotic and efflux properties
141 have recently been extensively reviewed [52,53].

142 Novel recent findings have pointed to differential expression of membrane porins and
143 other secretion systems in solvent-tolerant *Pseudomonads* exposed to solvents [14,15,54].
144 Unspecific outer membrane porins are downregulated in the presence of toluene to prevent
145 the influx of toluene [14,15,54]. A membrane protein OprH is found to be upregulated to
146 stabilize cell membrane and decrease the uptake of toluene [15,54]. Hence, alongside the
147 RND efflux pumps, other membrane proteins may play important roles in constituting solvent
148 tolerance.

149 *Molecular chaperones and general stress responses*

150 The presence of hydrocarbon solvents invokes similar stress responses in both Gram-
151 positive and Gram-negative bacteria [15,16,55]. In several bacterial species confronted with
152 hydrocarbon solvents, general stress response regulators such as the heat shock protein and
153 the cold shock protein are upregulated [15,16]. Other members of the general stress response
154 system may be induced by the presence of toluene, such as molecular chaperones, oxidative
155 stress response components, and other resistance proteins in Gram-negative *P. putida* DOT-
156 T1E and *P. putida* S12 as well as in Gram-positive *B. subtilis* [16,55]. Accordingly, the
157 Toluene repressed gene (*TrgI*) of *P. putida* S12 was found to control a large number of
158 protein modification and chaperone genes [18].

159 *Bioenergetics and redox balance*

160 Several studies in *P. putida* have indicated that in the presence of hydrocarbon
161 solvents, TCA cycle components are upregulated, the NAD(P)H regeneration rate is
162 increased and growth is reduced [14–16,18,56]. Differential expression of TCA cycle-related
163 proteins modulates the NAD(P)H concentration, and therefore the redox balance, throughout
164 the solvent stress [15]. Upregulation of the TCA cycle and concomitant increase of the
165 NAD(P)H regeneration rate enable the cells to cope with the energetic potential loss
166 connected with rapid solvent extrusion through the efflux pumps [15,56]. As a representative
167 illustration, the ATP content, cellular concentration of potassium and adenine nucleotides,
168 and the adenylate energy charge were all similar in cells of *P. putida* DOT-T1E grown in the
169 presence or absence of 1-decanol [46]. These findings reflected the efficient metabolic and
170 energetic adaptation of solvent-tolerant bacteria during their exposure to toxic hydrocarbon
171 solvents.

172 *Changes in cell morphology*

173 Both Gram-positive and Gram-negative bacteria exhibit changes in cell morphology
174 and in cell size as a response to the presence of hydrocarbon solvents [57–60]. For example,
175 decrease in cell size was observed in *P. aeruginosa* and *Enterobacter sp.* upon exposure to
176 hydrocarbon solvents [58,60]. However, conflicting observations were reported in *B.*
177 *lichineformis* S-86, *P. putida* P8, and *Enterobacter sp.* VKGH12, which have shown
178 increases in cell volume in the presence of hydrocarbon solvents [36,59]. Additionally, in the
179 presence of 0.6% 3-methylbutan-1-ol, *B. lichineformis* S-86 was reported to exhibit
180 filamentous growth [57]. By decreasing cell size, the cell surface-to-volume ratio increases,
181 contributing to a more efficient uptake of nutrient. With the decreased cell surface-to-volume
182 ratio, cell surface exposure is reduced, and solvent extrusion can be more effective.

183 **Applications of solvent-tolerant bacteria in biocatalysis of valuable compounds**

184 Employing bacteria for biocatalysis is currently a preferred method for industrial
185 synthesis of various biochemicals, pharmaceuticals, and enantiomerically pure intermediates.
186 Indeed, such synthesis routes require co-enzymes and co-factors and stepwise/multiple
187 enzymatic reactions that may be readily available within the microorganism of choice [33]. In
188 the bioproduction of industrial chemicals, the production process is often hampered by the
189 toxicity of the substrate or the product, which may severely affect the product yield [3,6].
190 Solvent-tolerant bacteria are favored for the biocatalytic production of many valuable
191 compounds, since they are far less prone to inhibition by toxic compounds, so the desired
192 yields can be better achieved. Valuable compounds that can be readily produced through the
193 use of solvent-tolerant bacteria include simple aromatic compounds such as phenol or p-
194 hydroxybenzoate, as well as more complicated compounds such as 2,5-furandicarboxylic
195 acid (FDCA), enantiomerically specific (S)-2-octanol, and pharmaceutically active 15β-
196 hydroxytestosterone (Table 2). Recently, the biobased production of the major building-block
197 chemical FDCA, a promising 'green' alternative to terephthalate in the production of
198 polyesters, from 5-hydroxymethyl-furfural (HMF) was achieved in the noted solvent-tolerant
199 strain *P. putida* S12 [Box 1]. Hence, solvent tolerance traits of microbial production strains
200 can enable the use of hydrocarbon solvents and solvent-like compounds as substrate and
201 intermediates for the production of high valuable compounds. In addition, the unique features
202 of solvent-tolerant bacteria allow tolerance towards broad range of potentially toxic
203 compounds and make them highly suitable for implementation in two-phase bioreactors
204 production set up [3,61]. The main challenges that arise in using solvent tolerant bacteria in
205 biocatalysis are maintaining product yield and system complexity [Box 2].

206 Solvent-tolerant bacteria are well suited for biocatalytic production in **two-phase**
207 **biocatalysis systems**, as reviewed previously [33]. These systems can significantly improve

208 production yield by reducing substrate and/or product toxicity [3,6]. The use of a
209 hydrocarbon solvent as the second phase has several advantages, including reduced reaction
210 inhibition, reduced toxicity towards the microbial host, and the prevention of product
211 hydrolysis [3]. Moreover, the second hydrocarbon phase acts as a simultaneous extraction
212 step, thus simplifying downstream processing and purification and increasing the yield of
213 poorly water-soluble products [62]. Hydrocarbon solvents having log $P_{o/w}$ values in the range
214 of 1 to 4 are considered suitable for product extraction and substrate reservoir in a two-phase
215 biocatalysis system, and solvent-tolerant bacteria can survive and exhibit biocatalytic activity
216 under these circumstances. Known bacterial **index values** have been extensively listed in
217 previous articles [11,63]. Predominantly Gram-negative bacteria have index values in the
218 ideal two-phase biocatalysis range from 1 to 4.

219 Several examples demonstrate increased product titer and optimized production of
220 valuable chemicals in a two-phase biocatalysis system [3,6,64]. Production of p-
221 hydroxystyrene in *P. putida* S12 was established by introducing the *pal* (L-phenylalanine/L-
222 tyrosine ammonia lyase) and *pdc* (p-coumaric acid decarboxylase) genes in combination with
223 inactivating the *fcs* gene [6]. A product titer of 4.5 mM with a yield of 6.7% (C-mol p-
224 hydroxystyrene/C-mol glucose) and maximum volumetric productivity of 0.4 mM h^{-1} was
225 initially achieved. However, due to the toxicity of p-hydroxystyrene, cell growth and
226 production was inhibited. Using decanol as a second phase, the toxicity of the product p-
227 hydroxystyrene was significantly reduced, which resulted in a p-hydroxystyrene titer of 147
228 mM (17.6 g l^{-1}), a fourfold increase compared to a standard fed-batch production. The
229 maximum volumetric productivity was also increased to 0.75 mM h^{-1}. Similarly, production
230 of p-hydroxystyrene from p-coumaric acid from corn cob hydrolysate using recombinant *E.*
231 *coli* and simultaneous extraction by n-hexane as the second phase clearly improved product
232 titer [64]. Another example is the bioproduction of vanillin from isoeugenol, which can be

233 inhibited by two major phenomena: the toxicity of isoeugenol and vanillin to the microbial
234 host, and the low solubility of isoeugenol in water [3]. The solvent-tolerant Gram-positive
235 bacterium *Brevibacillus agri* 13 can produce vanillin from 2 g l^{-1} isoeugenol with a yield of
236 7.6% (C-mol vanillin/C-mol isoeugenol) in a single-phase system. Using butyl acetate (30%
237 v/v) as a second-phase with 10 g l^{-1} isoeugenol increases the production yield to 17.2% with a
238 product titer of 1.7 g l^{-1} after 48 hours of fermentation. Here, the reduction of isoeugenol and
239 vanillin toxicity in combination with the simultaneous extraction of vanillin by the second
240 phase result in increased product formation.

241 **Synthetic biology and engineering towards advanced biocatalysts**

242 **Host interference** issues can be overcome by reducing the complexity of the genome
243 in the microbial chassis by genome streamlining [65]. **Genome streamlining** is widely used
244 in engineering industrial bacterial strains [66,67]. This approach has resulted in increased
245 biomass formation, reduced **doubling times**, increased product yield, and ultimately
246 optimized production systems [19]. Metabolic pathway optimization can resolve imbalances
247 in pathway fluxes and reduce accumulation of toxic intermediates to restore cellular fitness
248 [68,69]. Transferring solvent tolerant traits to a preferred industrial host strain is also a
249 plausible strategy [70]. In combination, these strategies comprise promising approaches to
250 exploit the solvent tolerance features of bacteria for producing a wide range of valuable
251 compounds with a high degree of predictability and robustness [Figure 2]. Existing and novel
252 tools for synthetic biology and the rapidly accumulating genome sequencing data of solvent
253 tolerant bacteria drive the opportunities to implement these strategies [Box 3].

254 *Pathway optimization and adaptation of enzyme expression*

255 Metabolic pathways can be optimized by characterizing enzyme expression,
256 identifying bottlenecking enzymes, and subsequently optimizing the expression and activity

257 of enzymes through modulation of transcription, translation, and specific enzyme
258 characteristics [68,69]. As an example, transcriptomics and proteomics studies of p-
259 hydroxybenzoate-producing *P. putida* S12 identified critical components of the tyrosine
260 degradation pathway [5,71]. Subsequent deletion of the *hpd* gene involved in p-
261 hydroxybenzoate degradation led to a 22% increase of p-hydroxybenzoate production. In
262 another case, by overproducing the pyruvate dehydrogenase subunit gene *acoA* or deleting
263 the glucose dehydrogenase gene *gcd* to overcome bottlenecking, production of
264 polyhydroxyalkanoate (PHA) in *P. putida* KT2440 was increased by 33% and 121%,
265 respectively [72].

266 In combination with rapidly emerging synthetic biology tools, pathway optimization
267 is a powerful strategy in designing optimized bacterial strains for application in industrial
268 biotechnology. The highest yield in microbial phenol production reported so far was achieved
269 by implementing pathway optimization on solvent-tolerant *P. taiwanensis* VLB120 [1]. To
270 optimize phenol production, catabolic routes toward aromatic compounds and shikimate
271 pathway intermediates are inactivated. This inactivation is accomplished by the deletion of
272 five genes—*pobA*, *hpd*, *quiC*, *quiC1*, and *quiC2*—along with the subsequent loss of the
273 megaplasmid pSTY. This process yields *P. taiwanensis* VLB120Δ5, which is unable to grow
274 on 4-hydroxybenzoate, tyrosine, and quinate. The introduction of a codon-optimized
275 tyrosine-phenol lyase (TPL) gene from *Pantoea agglomerans* facilitates tyrosine
276 transformation into phenol. Metabolic flux towards phenol production is further increased
277 using forward- and reverse-engineering from leads generated by previous mutagenesis of
278 phenol-producing *P. putida* S12 [73] and the addition of bottlenecking enzymes AroG and
279 TyrA. *P. taiwanensis* VLB120Δ5-TPL36 achieved the yield of 15.6% and 18.5% (C-mol/C-
280 mol) of phenol in minimal medium from glucose and glycerol, respectively, without
281 requiring additional complex nutrients.

282 Synthetic promoter libraries can optimize the expression of several modules in a
283 metabolic pathway [23]. Using synthetic promoters, the production of rhamnolipids in *P.*
284 *putida* KT2440 was significantly increased, reaching a yield of 40% rhamnolipids on sugar
285 [74,75]. These examples present further proof that pathway optimization is a highly
286 promising approach to resolving pathway flux imbalance and improving biomass and product
287 yield in solvent tolerant bacterial industrial host strains.

288 *Top-down strategies in genome streamlining*

289 Genome streamlining has been implemented in various industrial host strains such as
290 *E. coli* and *Streptomyces* species [66,67]. Top-down genome streamlining deletes from the
291 microbial chassis multiple genes or gene clusters that are predicted to be inessential for the
292 microbes, consume high amounts of energy, contribute to the degradation of products or
293 intermediates, or reduce metabolic flux towards the product of interest [66]. Alternatively, the
294 bottom-up strategy attempts to design a production chassis from scratch based on minimum
295 requirements for a functioning microbial chassis. The top-down strategy significantly
296 increased the biomass yield and the maximum specific rate for protein synthesis in the
297 streamlined hosts *P. putida* EM329 and *P. putida* EM383 compared to the parental strain *P.*
298 *putida* KT2440 [19,76]. One early example was *Pseudomonas arvilla* mt-2, described by
299 Murray and colleagues in 1972 as a fascinating strain of *Pseudomonas* able to grow on
300 benzoate, m-toluate (3-methylbenzoate) or p-toluate (4-methylbenzoate) as its sole carbon
301 source [77]. A derivative of this strain, *P. putida* KT2440, has been cured of the endogenous
302 megaplasmid pWW0 present in the parental strain *P. putida* mt-2. Since then, *P. putida*
303 KT2440 has proven to be a suitable host for gene cloning due to its deficiency in endogenous
304 DNA restriction, so it can efficiently receive **plasmid** DNA for gene cloning purposes [78].
305 *P. putida* KT2440 is a generally regarded as safe (GRAS) strain of *P. putida*. The genome of
306 *P. putida* KT2440 comprises of a 6,181,873 bp single circular chromosome [25].

307 In the process of optimizing *P. putida* KT2440 towards a robust industrial chassis, 11
308 chromosomal regions comprising 300 genes, including mobile elements, were found to be
309 responsible for genetic instability or massive energy spillage [19]. Together, these genes
310 comprise a 170 kb genome segment encoding two transposons (Tn7 and Tn4652), prophages,
311 two type I DNAses (*endA-1* and *endA-2*), an operon encoding type I DNA restriction-
312 modification system (hsdRMS operon), and the 69 kb complete flagellar operon. Mobile
313 elements play a significant role in the adaptation during solvent exposure, but mobile
314 elements are also responsible for genetic instability [79]. Removing all of these genes
315 resulted in a new optimized strain of *P. putida* EM42. To further diminish the probability of
316 genetic instability, *recA* was deleted, resulting in *P. putida* EM383. This streamlined *P.*
317 *putida* EM383 was shown to be superior to *P. putida* KT2440, as it exhibited a reduced lag
318 phase, increased biomass formation, and increased redox charge, leading to exceptional
319 tolerance against redox stress and ROS damage.

320 *Optimization of industrial host strains with solvent tolerance traits*

321 Improving tolerance against toxic compounds is an important step towards developing
322 a robust bacterial chassis for the industrial production of a wide range of valuable
323 compounds. Using a modular semisynthetic system, overexpression of heat shock proteins
324 GrpE, GroESL, and ClpB in *E. coli* generated a stress response that increased tolerance
325 towards ethanol, n-butanol, and other toxic compounds [80]. An engineered *E. coli* TG1-
326 derived strain expressing the solvent efflux pump srpABC from *P. putida* S12 was employed
327 for 1-naphthol production in a two-phase fermentation [70]. Although 1-naphtol production
328 did not reach the same levels as in *P. putida* S12, this result demonstrated the successful
329 transfer of the *Pseudomonas* solvent extrusion pump gene cluster, providing the engineered
330 *E. coli* strain with a genuine solvent-tolerant trait.

331 The introduction of multiple efflux pumps may promise further advantages, but
332 overexpression of efflux pumps may severely inhibit cell growth [81]. As demonstrated by
333 Turner and Dunlop, certain combinations of different efflux pumps can be highly toxic, even
334 at basal expression levels of the pump proteins. Another successful example of optimizing
335 solvent tolerance relates to bacterial fatty acid modification. Introducing cyclopropane fatty
336 acid synthase Cfa from the solvent-tolerant strain *Enterococcus faecalis* CM4A into *E. coli*
337 clearly increased tolerance towards to n-butanol [29]. Cfa activity to maintain the fluidity of
338 the cell membrane upon exposure to toxic hydrocarbon solvents. Further understanding of the
339 roles of and interplay between solvent tolerant mechanisms will enable the transfer of
340 solvent-tolerant traits into suitable industrial host strains.

341 **Concluding Remarks and Future Perspectives**
342

343 Increased insight into solvent tolerance mechanisms is an important basis for the
344 biotechnological production of challenging compounds. An increasingly wider variety of
345 compounds will be produced in microbial hosts due to the transition to a biobased economy.
346 However, biobased production of added-value compounds, many of which are aromatics, is
347 still challenging because of the inherently toxic nature of most of these compounds. Solvent-
348 tolerant strains indeed represent a promising solution to this problem. A deeper understanding
349 of the interplay in solvent tolerance mechanisms is still required to further increase the
350 applicability of solvent tolerant traits in industrial production (see Outstanding Questions).

351 With the help of modern synthetic biology tools, top-down genome streamlining of
352 solvent tolerant strains is essential to reduce host interference and increase production yields.
353 In this approach, the challenge is to identify minimal gene clusters required for solvent
354 tolerance and biosynthetic capacity that should not be disrupted. Implementing specific
355 synthetic biological tools like efficient gene editing for introducing heterologous genetic

356 feature or adjustable transcriptional regulators for pathway optimization will enable the rapid
357 generation of optimized production strains.

358 Transferring solvent-tolerance traits into existing industrial strains may be a
359 promising alternative strategy to optimize biobased production. The required synthetic
360 biology tools are already available for established industrial strains. The challenge in this
361 strategy is in obtaining the desired expression level of exogenous gene clusters in their new
362 hosts. Once again, this highlights the necessity for thorough analysis and understanding of
363 solvent tolerance mechanisms and the interplay of these mechanisms that orchestrate the
364 tolerance toward solvents.

365

366

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622 # Tables

623 **Table 1. Hydrocarbon solvents and their industrial relevance**

Hydrocarbon solvent	**Solvent class**	**Industrial relevance**	**$LogP_{o/w}$**	**Ref.**
acetone	ether	solvent in cosmetic, pharmaceutical, medical, and domestic uses	-0.24	-
ethyl acetate	ester	solvent in coating formulation for epoxies, urethanes, acrylics, and vinyls.	0.73	-
n-butanol	short chain alkanol	biofuel	0.88	[82]
phenol	aromatics	precursor for plastics	1.5	[62]
butyl acetate	ester	product co-solvent (vanillin)	1.78	[3]
benzene	aromatics	substrate for the production of 3-methylcatechol	2	[83]
toluene	aromatics	substrate for the production of 3-methylcatechol, o-cresol, & p-hydroxybenzoate	2.69	[9,83,84]

styrene	aromatics	substrate for the production of (S)-styrene oxide	2.9	[85]
1-octanol	long chain alkanol	product co-solvent (phenol)	3	[62]
ethylbenzene	aromatics	production of paints, varnishes, and lacquers	3.3	-
cyclohexane	cyclic alkane	precursor to nylon, adipic acid, caprolactam	3.4	-
m-xylene	aromatics	substrate for the production of 3-methylcatechol	3.46	[10]
n-hexane	alkane	extraction solvent for vegetable oil, cleaning agent	3.9	-
1-decanol	long chain alkanol	product co-solvent (p-hydroxystyrene)	4.57	[6]

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627 **Table 2. Biocatalysis using solvent tolerant bacteria**

Product	Biocatalyst	System	Challenge(s) in production process	Product titer (mM)	Yield ($Cmol_p/Cmol_s$)	Productivity	Comparison	Ref.
p-Hydroxybenzoate	*P. putida* S12 expressing *pal* gene from *Rhodosporium toruloides*	Fed batch whole-cell biocatalysis system	Toxic aromatic product	12.9	8.5%	0.168 (mmol h^{-1} $gCDW^{-1}$)	-	[5]
FDCA (2,5-furandicarboxylic acid)	*P. putida* S12 expressing hmfH gene from *Cupriavidus basilensis* HMF14	Fed batch whole-cell biocatalysis system	Toxic aromatic substrate	192.83	97%	0.096 ± 0.004 (mmol h^{-1} $gCDW^{-1}$)	-	[4,86]
Anthranilate	*P. putida* KT2440 expressing *trpDC* with futher optimization of anthranilate production pathway	Fed batch whole-cell biocatalysis system	Toxic aromatic product	11.23	3.6 ± 0.5%	N/A	1.83 mM, without further optimization of the anthranilate production pathway	[2]
(S)-2-Octanol	*P. putida* DSM 12264 expressing CYP154A8	Fed batch whole-cell biocatalysis system	Hydrocarbon solvent as product	15.7 (87% ee)	N/A	0.172 (mmol h^{-1} $gCDW^{-1}$)	2.2 mM, 58% ee in *E. coli* system expressing CYP154A8	[61]
3-Methylcathecol	*P. putida* DOT-T1E containing pWW0 plasmid from *P. putida* KT2440	Two phase batch whole-cell biocatalysis system with aliphatic alcohol as the second phase	Second-phase for product reservoir	70	N/A	4.83 (mM h^{-1})	3 mM, using the same strain without the two-phase system	[10]
Phenol	*P. taiwanensis* VLB120 with minimal genomic modification	Fed batch whole-cell biocatalysis system	Toxic product	3.62	18.5 ± 0.2%,	0.09 ± 0.00 (mM h^{-1})	1.5 mM in *P. putida* S12	[1]

	and expressing *tpl* gene from *Pantoea agglomerans*							
p-Hydroxystyrene	*P. putida* S12 expressing *pal* gene from *Rhodosporium toruloides* and *pdc* gene from *Lactobacillus plantarum*	Two phase fed batch whole-cell biocatalysis system with decanol as the second phase	Toxic product and second-phase for product reservoir	147	4.1%,	0.75 (mM h^{-1})	21 mM, using the same strain without the two phase system	[6]
Vanillin	*B. agri* 13	Two phase batch whole-cell biocatalysis system with butyl acetate as the second phase	Second phase for toxic product and substrate reservoir	11.17	27.8%	N/A	< 6.5 mM l^{-1}, on *Bacillus subtilis*, *Pseudomonas chlororaphis*, and recombinant *E. coli* without two-phase system	[3]
1-Naphthol	*E. coli* TG1 pBS(kan)TOM-Green expressing srpABC operon from *P. putida* S12	Two phase batch whole-cell biocatalysis system with lauryl acetate as the second phase	Toxic aromatic product	9.91	N/A	3.81 (mmol gCDW^{-1})	0.27 mM , without two-phase system	[70]

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Figure Legends

Figure 1. Current understanding on solvent tolerance mechanism of bacteria

The left panel represents the state of a bacterium upon the addition of solvent and before solvent tolerance mechanisms are induced, and the right panel represents the state of the bacterium after solvent tolerance mechanisms are induced. Gram-negative and Gram-positive bacteria employ similar strategies to compensate for solvent exposure. The increase in membrane compaction (1) is a consensus for solvent tolerance mechanism between Gram-positive and Gram-negative bacteria by using multiple strategies (1a & 1b). Resistance-nodulation-division (RND) efflux pumps (2) and general stress responses (3) play important roles in both groups. Several mechanisms are also unique to certain species, such as the increase of bioenergetics and NA(D)PH regeneration rate in *P. putida* (4), membrane vesicle formation in *P. putida* (1c), and filamentous growth in Gram-positive bacteria (5).

Figure 2. Synthetic biology and engineering towards advanced solvent-tolerant biocatalysts

Three optimization strategies for biocatalysis using solvent-tolerant bacteria are employed to solve problem of host interference, which can cause unpredictable yield of products. Pathway flux imbalance can be reduced by identifying bottlenecking enzymes and altering the expression level of the bioproduction pathway (red arrows). Genome streamlining can be applied to reduce the genome complexity of solvent tolerant strains (green arrow). Introducing solvent tolerance related genes into existing industrial strain is also proven to be a promising approach (blue arrow). Synthetic biology tools are useful in pathway optimization, altering the expression level of bioproduction, strain optimization, and conferring solvent-tolerant traits.

654 ## Glossary

655 **Biofilm:** A group of microbes attached to each other and onto a surface, protected by
656 extracellular polymeric substances like polysaccharides, DNA, and protein. The formation of
657 a biofilm is often signalled by quorum sensing molecules.

658 **Doubling time:** The rate of microbial growth in the exponential phase, measured as the
659 period of time required for a microbial population to double.

660 **Genome streamlining:** reduction of genome size to include only minimum requirements for
661 microbial survival and growth.

662 **Host interference:** A complication in a microbial host where it redirects its metabolism from
663 a product of interest towards maintenance energy or biomass growth, including product and
664 intermediate degradation.

665 **Index value:** the lowest log $P_{o/w}$ value among several hydrocarbon solvents tolerated
666 specifically by a bacterial strain; an indication of level of tolerance. In some cases, bacteria
667 with lower index values tolerate a wider range of hydrocarbon solvents.

668 **Lignocellulosic hydrolysates:** products obtained from degrading (i.e., hydrolysing) cellulose
669 through thermochemical treatment.

670 **Lipopolysaccharides (LPS):** negatively charged molecules that are found on the outer
671 membrane of Gram-negative bacteria to stabilize the structure of the cell membrane. They
672 consist of lipid A, an inner core, an outer core, and polysaccharide (O-antigen).

673 **Log $P_{o/w}$:** the logarithm of the ratio of a compound's concentration in the organic and water
674 phases after being dissolved in a mixture of 1-octanol and water. It describes the polarity of
675 an organic solvent or other compound. Hydrocarbon solvents with log $P_{o/w}$ values in the
676 range of 1-4 are known to exhibit toxicity towards the cell membrane, with higher toxicity
677 indicated by a lower value.

678 **Plasmid:** a unit of DNA separate from chromosomal DNA, typically circular double-
679 stranded DNA molecules that can replicate autonomously and may occur as single or
680 multiple copies in a single cell. Plasmids can be used to transfer and express genetic
681 information, mainly heterologous genes, into microbial chassis with increased copy number
682 to result in increased gene expression.

683 **RND efflux pumps:** A family of efflux pump proteins responsible for resistance, nodulation
684 and cell division (RND) activity, driven by proton-motive force (pH gradient).

685 **Two-phase biocatalysis system:** the addition of a second phase in biocatalysis as a reservoir
686 for substrates or as a simultaneous product extractant. Hydrocarbon solvents immiscible with
687 water are often used as such second phases in two-phase biocatalysis systems.

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Text Box 1

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The case of FDCA

691

692 A recent study identified and characterized a fully biobased enzymatic route for the
693 production of 2,5-furandicarboxylic acid (FDCA) directly from 5-hydroxymethylfurfural
694 (HMF) [86]. HMF, like furfural, is an intrinsically toxic furanic aldehyde occurring in
695 **lignocellulosic hydrolysates** [Figure I]. FDCA has been proclaimed by the USA Department
696 of Energy as one of 12 priority chemicals for the realization of a biobased green chemistry
697 industry [87]. It is regarded an important platform compound for the synthesis of a variety of
698 aromatic chemical building blocks, including as a biobased alternative for the monomer
699 terephthalic acid in polymeric polyethylene terephthalate (PET) [88–91]. Polymerisation of
700 ethylene glycol and FDCA yields polyethylene furanoate (PEF), which has improved barrier,
701 thermal and mechanical properties compared with PET [90].

702 Expression of a novel, specific HMF/furfural oxidoreductase from the soil bacterium
703 *Cupriavidus basilensis* in the solvent-tolerant industrial host bacterium *Pseudomonas putida*
704 enabled efficient high-yield production of FDCA from HMF [4]. In this process, *P. putida*
705 proved to be an efficient whole-cell biocatalyst. The company Corbion is currently
706 developing a cost-effective production route for FDCA, based on this novel enzymatic route
707 (http://www.corbion.com/bioplastics/products/fdca-for-pef).

708 **Figure I. Potential applications of FDCA, from lignocellulosic biomass to**
709 **biopolymers.**

710

711

712

713 **Text Box 2**

714 **Challenges in biocatalysis using solvent-tolerant bacteria**

715 The primary complication with using solvent-tolerant bacteria in industrial
716 biotechnology is the unpredictable product yield caused by host interference issues
717 [19,65,66]. Adapting native biocatalytic pathways often provokes imbalances in pathway
718 flux, the accumulation of toxic intermediates, and reduced cellular fitness, again causing
719 unpredictable product yields [69]. Genome streamlining reduces host interference, resulting
720 in increased biomass and growth rate, and subsequently leading to optimizing production
721 chassis [19]. Imbalances in pathway fluxes, bottlenecking enzymes, and accumulation of
722 toxic intermediates can be mitigated by optimizing metabolic pathways [5,62,69,71]. And the
723 development of synthetic biology tools is becoming crucial to support the implementation of
724 solvent-tolerant bacteria in biocatalysis [20,92].

725 Another advantage of solvent-tolerant bacteria is their easier implementation in two-
726 phase bioreactor systems. However, other challenges may arise in two-phase bioreactor
727 systems, such as increased system complexity, problems with waste disposal, and the
728 hazardous risk of using flammable solvents [93]. By applying heat treatment or a continuous-
729 plate centrifuge, a solvent emulsion in an aqueous phase can be degraded or, preferably, be
730 avoided, resulting in a clear solvent that can be processed by further downstream treatment.
731 Distillation may be applied in the downstream process specifically to purify volatile product
732 from its volatile substrate. Schmid and colleagues developed a safe and efficient pilot-scale
733 two-phase bioreactor containing flammable solvent [93]. Finally, exogenously supplemented
734 glycerol provides effective protection and thus improves bacterial growth in a two-phase
735 bioreactor system [94].

736

737 **Text Box 3**

738 **Molecular synthetic tools for improving solvent tolerant process design and**
739 **application**

740 Synthetic molecular tools are crucial aspects for developing robust industrial bacterial
741 strains. BioBricks were developed as flexible exchangeable DNA fragments that can be
742 combined to fully synthesized a synthetic biology tools suitable for common industrial strains
743 like *E. coli* [92]. The Standard European Vector Architecture (SEVA) established a reliable
744 and efficient vector repository accompanied by a simple and user-friendly database mainly
745 implemented in solvent-tolerant *P. putida* and other industrial strains [20]. A plasmid system,
746 GeneGuard, was constructed to overcome the safety concerns including unwanted horizontal
747 gene transfer by host-mutual dependency, based on using SEVA plasmids [24].

748 Optimizing a robust bacterial chassis requires both precise genome editing tools and
749 the ability to incorporate new features into its genome. The CRISPR/Cas system has become
750 standard tool in editing bacterial genomes [95]. Using SEVA plasmids as its backbone, a
751 recombination event between free homologous DNA sequences, allowing an accurate
752 genome editing, was developed for a wide variety of Gram-negative bacteria [21].

753 Transposon tools for specific transgene introduction in combination with promoter
754 libraries for *P. putida* cell factories have been developed as an alternative to the use of multi
755 copy plasmids [22,23,96]. The developed mini-Tn5 vector offers the advantages of
756 maintaining introduced genes without selective pressure, construct stability, recurrent use of
757 the system, and introducing a relatively large DNA sequence [22]. The mini-Tn7 transposon
758 system can integrate with a high frequency in a specific location as a unidirectional single

759 copy of gene that is suitable in various studies for gene expression, characterization of certain
760 genes, and gene complementation [23]. Finally, a novel broad range system for the transfer
761 and expression of biosynthetic pathways (TREX) was developed using this transposon
762 system to include all functional elements that are essential for efficient introduction and
763 expression of pathway clusters in different bacteria [97].

1 **Outstanding Questions**

2 In native solvent tolerant strains, multiple efflux pumps operate simultaneously to prevent
3 accumulation of organic solvents. However, overexpression of multiple efflux pumps is
4 disadvantageous in engineered strains. How can expression levels be optimized for
5 combinations of pumps operating simultaneously?

6

7 With many omics data becoming available to date, genetic traits responsible for solvent
8 tolerance in different strains can be predicted. How to address the challenge of constructing a
9 solvent tolerance model operating in different species?

10

11 Initial efforts have been made to transfer solvent tolerant traits from solvent tolerant bacteria
12 to industrial production strains. How easy is it to extend the transfer to more industrial
13 strains? Moreover, will these engineered strains reach similar tolerance levels as the native
14 strains in combination with optimal production yields?

15

Figure_1

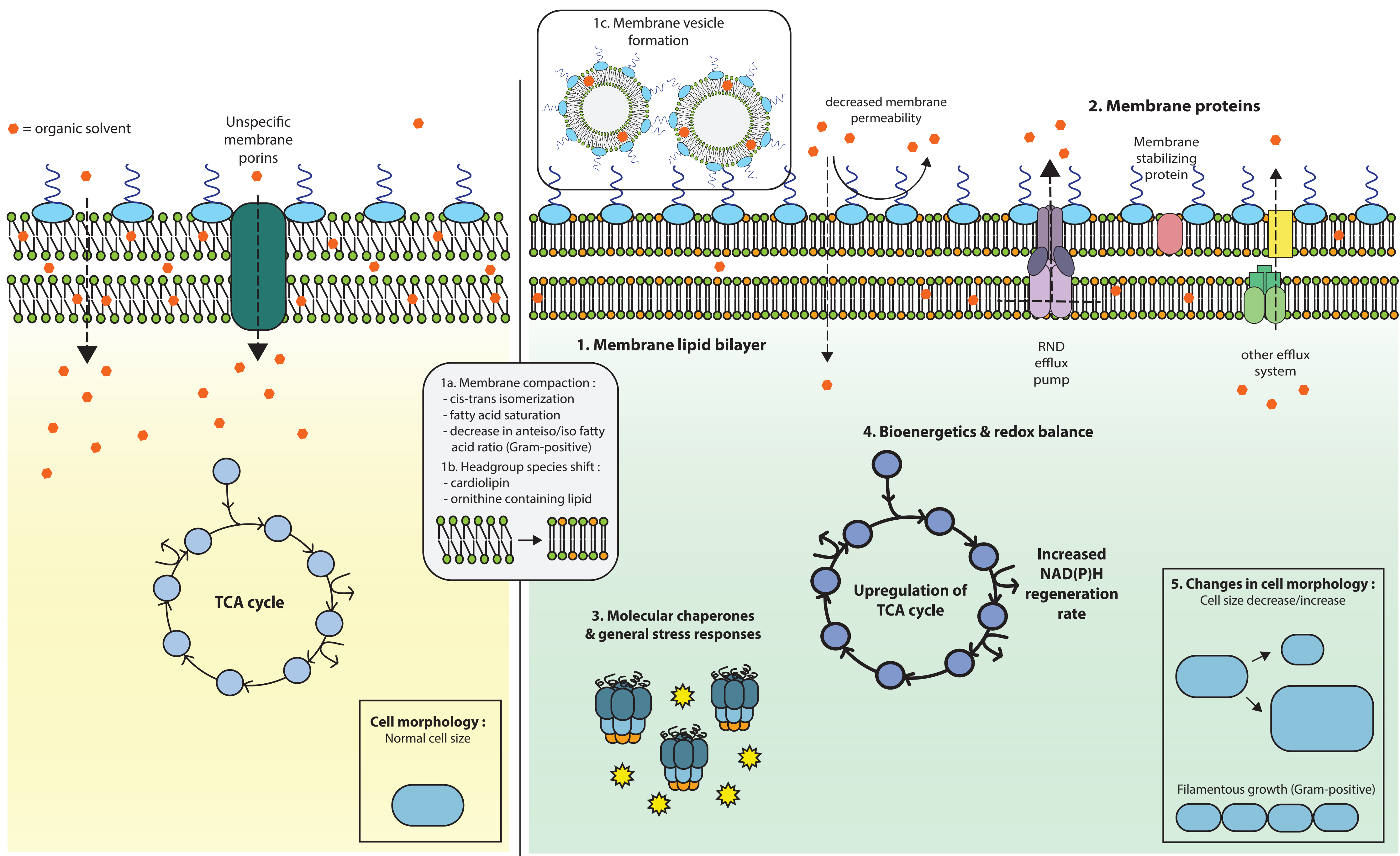
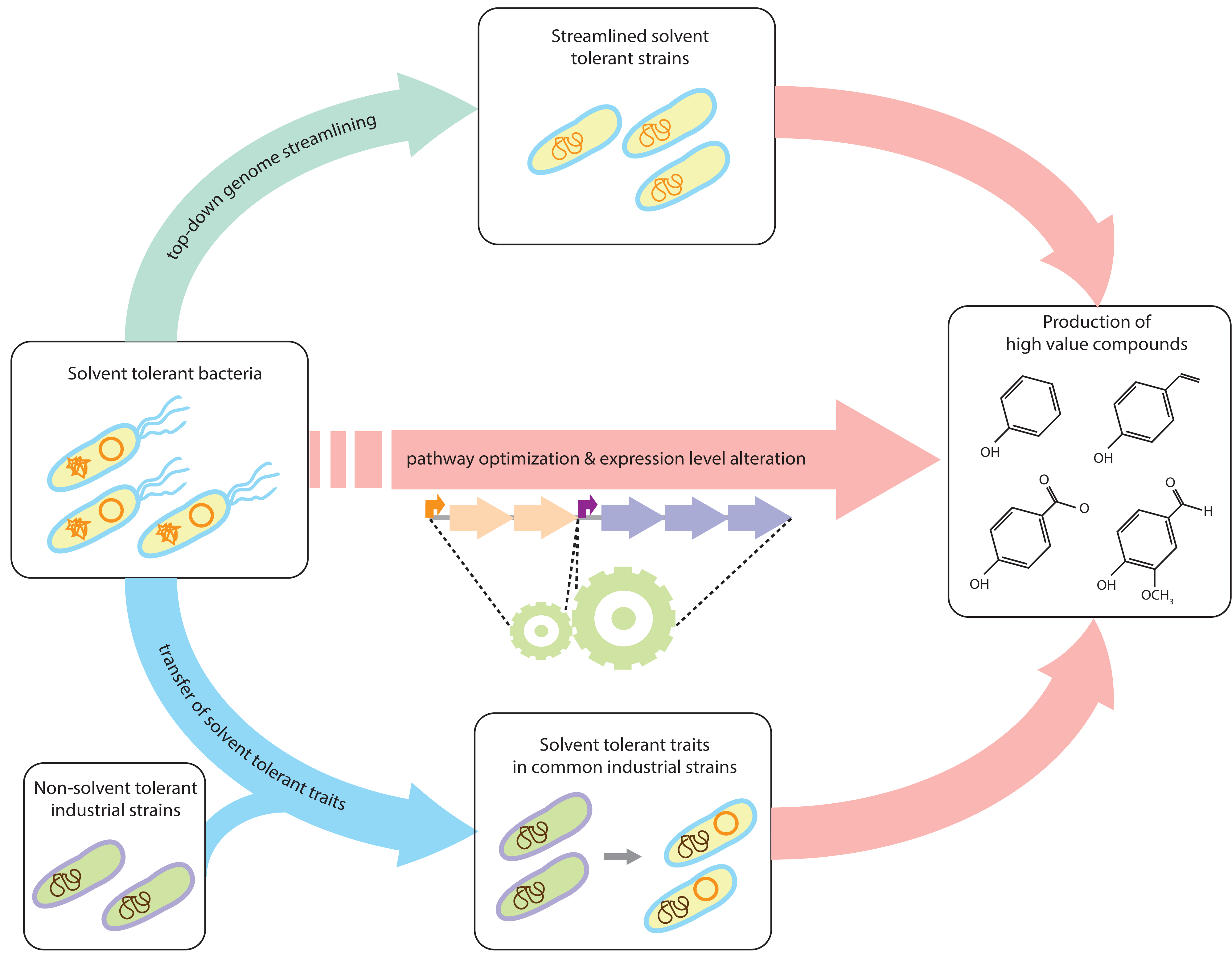


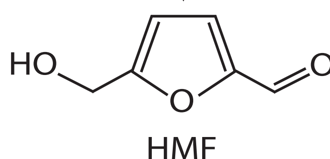
Figure_2



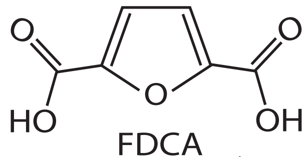
Figure_I

Lignocellulosic biomass

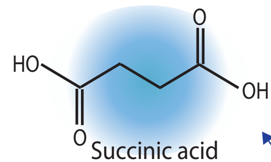
hydrolysis

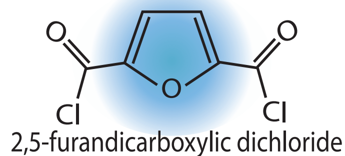


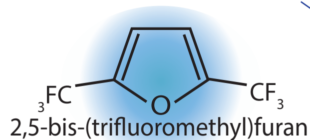
P. putida S12 expressing HmfH

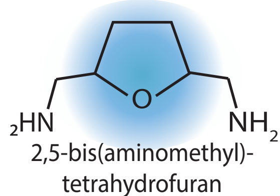


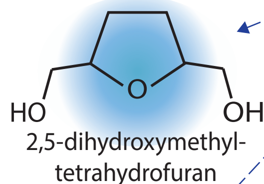
Derivatives of FDCA

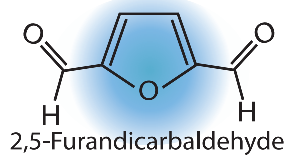


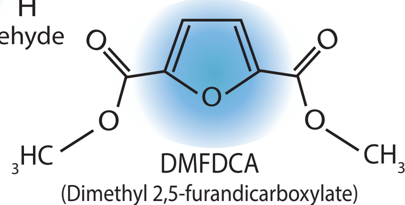


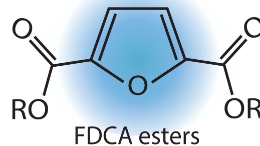






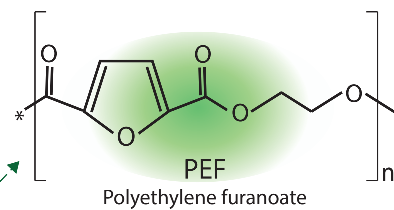


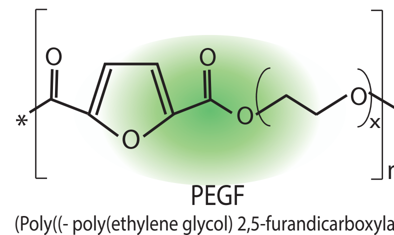


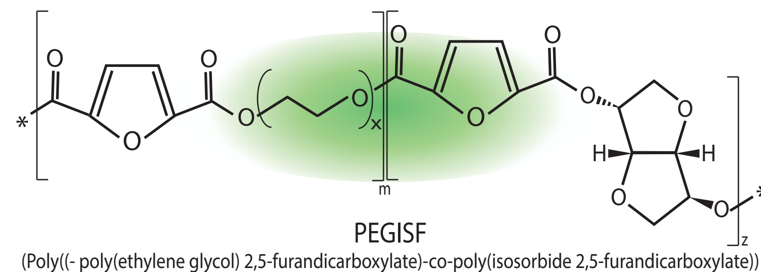


Polymers of FDCA

Polyesters







Polyamides